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# Regulation of Na,K-Atpase activity by tyrosine phosphorylation in lens cells.

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# REGULATION OF Na,K-ATPASE ACTIVITY BY TYROSINE PHOSPHORYLATION IN LENS CELLS

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A Dissertation Approved on

November 25, 2003

By the following Dissertation Committee:

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Dissertation Director

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**DEDICATION**

This dissertation is dedicated to my parents

Mr. Vedran Bozulic

And

Mrs. Nedijeljka Bozulic

Who have instilled in me the qualities essential for a lifetime of success and the capacity to prevail over life's many obstacles

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**ABSTRACT**

REGULATION OF Na,K-ATPASE ACTIVITY BY TYROSINE PHOSPHORYLATION IN LENS CELLS

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Na,K-ATPase is essential for the regulation of cytoplasmic $Na^+$ and $K^+$ levels in lens cells. Insufficient Na,K-ATPase activity is associated with cataract formation. Based on earlier studies in which Src-tyrosine kinase inhibitors were found to suppress Na,K-ATPase activity changes that occur in response to either thrombin, endothelin, or dopamine, I hypothesize that regulation of Na,K-ATPase activity might occur through phosphorylation of the Na,K-ATPase α1 (catalytic) subunit by Src-family tyrosine kinases. Here, I tested the influence of Src-kinase family members on tyrosine phosphorylation and Na,K-ATPase activity in membrane material isolated from porcine lens epithelium. Western blot studies indicated the expression of Src-kinase family members, Lyn, Fyn, Src, and Lck in lens cells. When membrane material was incubated in

ATP- containing solution containing partially purified Lyn kinase, Na,K-ATPase activity was reduced by ~38%. Similarly, Fyn kinase inhibited Na,K-ATPase activity by 25%. In contrast, Src kinase increased Na,K-ATPase activity by 30%. Fes kinase, a non-Src-Family member did not change Na,K-ATPase activity. Lyn caused tyrosine phosphorylation of multiple protein bands. Immunoprecipitation and Western blot analysis showed Lyn treatment causes an increase in density of a 100 kDa phosphotyrosine band immunopositive for Na,K-ATPase α1 polypeptide. Incubation with protein tyrosine phosphatase 1B (PTB-1B) reversed the Lyn-dependent tyrosine phosphorylation and the reduction of Na,K-ATPase activity.

Na,K-ATPase α1-catalytic subunit is abundant in both lens epithelium and fibers. Lens epithelium exhibits high Na,K-ATPase activity while Na,K-ATPase activity in the fibers is low. Lens fiber cells show an abundance of Na,K-ATPase α1, however, Na,K-ATPase activity is low. Western blot analysis revealed tyrosine phosphorylation of multiple membrane proteins in both lens cell types. When membrane material was subjected to immunoprecipitation using an antibody directed against Na,K-ATPase α1, a co-localized phosphotyrosine band was detected in lens fibers but not

epithelium. Incubation with PTP-1B caused a ~50% increase of Na,K-ATPase activity in fiber membrane material. In contrast, Na,K-ATPase activity in lens epithelium membrane material was not significantly altered by PTP-1B treatment. While endogenous PTP-1B was detected in both cell types, endogenous tyrosine phosphatase activity was low in both epithelium and fiber membrane material. Taken together, PTP-1B and Src-family kinases may be important mechanisms for regulating Na,K-ATPase activity in lens cells.

**TABLE OF CONTENTS**

PAGE

## LIST OF TABLES

PAGE

TABLE

## LIST OF FIGURES

PAGE

FIGURE

# CHAPTER I

# GENERAL INTRODUCTION

## Lens Structure

The lens of the eye is located between the aqueous humor and the vitreous body. Light enters the eye and passes successively through the cornea, aqueous humor, lens, vitreous body, and the light-sensitive retina (**Figure 1**). The lens must be transparent for proper passage of light to reach cells in the retina (Harding, 1991).

The lens is held in place within the eye by zonular fibers and its contents and shape are maintained by the lens capsule. The lens is composed of two cell types; a single layer of epithelial cells on the anterior surface of the lens capsule, and fiber cells which make up the lens cortex. These cells are important in maintaining fluid and electrolyte balance. The epithelium terminally differentiates and elongates at the equatorial zone to form fiber cells (**Figure 2**). As these cells elongate they lose vital organelles such as cell nuclei, mitochondria, and rough endoplasmic reticulum. Continuous differentiation of

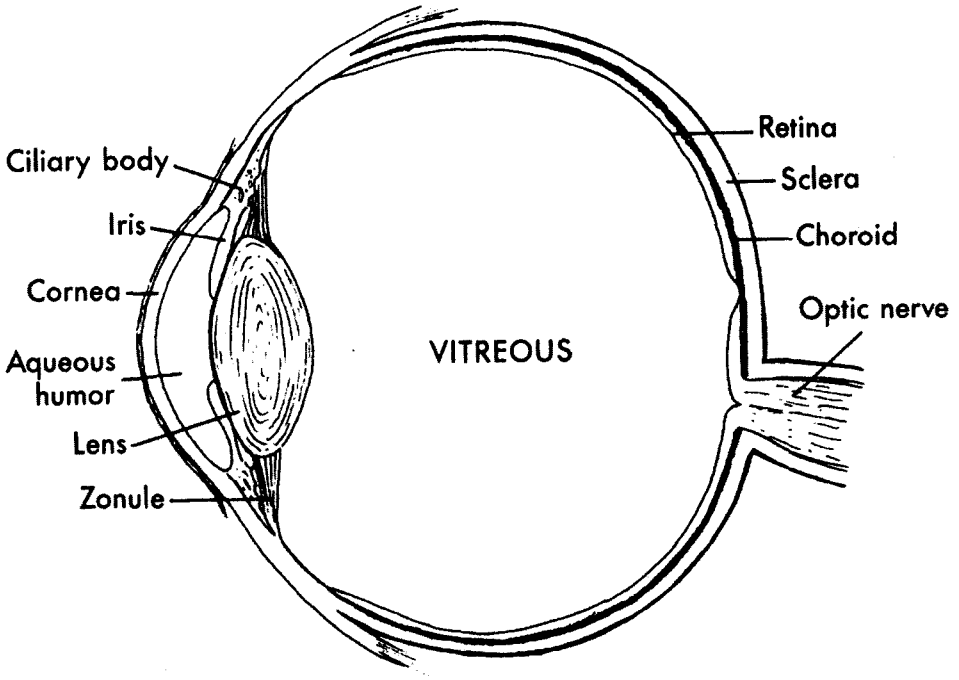


**Figure 1. Diagrammatic section of the eye.** When light enters the eye, it first passes through the cornea, the aqueous humor, and then the lens and vitreous humor. Light reaches the light-sensing structure of the eye called the retina. Light contacts rod and cone cells in the retina and produces a series of complex chemical reactions creating electrical impulses in retinal ganglion cells. These electrical impulses in the optic nerve are carried to the occipital lobe of the brain where visual signals are interpreted. Hart, WM. Adler's Physiology of the Eye. 1992 Mosby-Year Book, Inc.

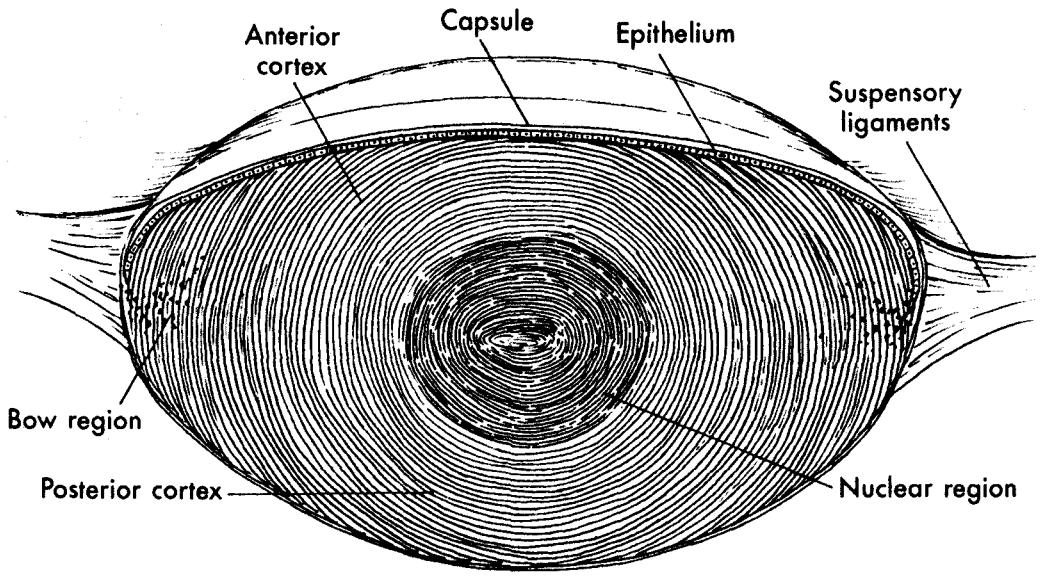


**Figure 2. The lens structure.** The lens is held in place by suspensory ligaments or zonule fibers running from the ciliary body to the capsule. Contraction and relaxation of the zonular fibers changes the shape of the lens and allows for visual acuity called accommodation. The lens is made up of a monolayer of epithelial cells on the lens anterior surface and fiber cells which make up the lens bulk. The epithelial cells continually divide in the equatorial region of the lens and give rise to epithelial cells that differentiate into fiber cells. The newly formed fiber cells are displaced towards the lens center form the nuclear region. Hart, WM. Adler's Physiology of the Eye. 1992 Mosby-Year Book, Inc.

the lens epithelium into fibers results in the displacement of older cells towards the center of the lens, whereas newer metabolically active tissue is found in the peripheral regions. Thus, the lens nucleus is made up of fiber cells dating from the earliest stages of development (Harding, 1991).

Lens fiber cells are organized in a densely packed interdigitated arrangement with numerous gap junctions for important metabolic cooperation. Young differentiating fiber cells are rich in ribosomes and rough endoplasmic reticulum allowing for continual synthesis of unique lens proteins called crystallins (Forrester *et al.*, 1996). Tightly packed crystallins make up more than 90% of the total water-soluble proteins of the lens and are maintained at concentrations greater than 400mg/ml. These proteins have the necessary refractive properties required for lens transparency (Jaffe and Horwitz, 1992). The arrangement of fiber cells and the single layer of epithelium have a cytoplasmic space with characteristic high $K^+$ and low $Na^+$ concentrations sustained by Na,K-ATPases in both cell types (Kobayashi *et al.*, 1983).

**Na,K-ATPase**

The Na,K-ATPase is a ubiquitous active transport protein complex found in the plasma membrane of all animal cells. The catalytically active Na,K-ATPase is composed of a 112 kDa catalytic α-subunit and a 35 kDa β-subunit and appears to exist as an α2β2 tetramer (Askari, 1987). The catalytic α-subunit is composed of ten membrane-spanning domains with both N- and C-termini located on the cytoplasmic side of the membrane. The β-subunit is composed of a single transmembrane domain with a short N-terminus exposed to the cytoplasm (**Figure 3**). The catalytic α-subunit functional domains include the N-terminal tail, important in sodium translocation; conformational transitions are carried out by cooperation between cytoplasmic loops M2 and M3 with cytoplasmic loops M4 and M5; cation occlusion is carried out by amino acid residues E327 in M4, S775 and E779 in M5, and D804 and D808 in M6; catalytic phosphorylation occurs at D369. Sensitivity to ouabain, a potent inhibitor of the Na,K-ATPase (Wallick and Schwartz, 1988), has been directed to all extracellular loops of the catalytic subunit. The cytoplasmic N-terminus of the β-subunit is important in obtaining

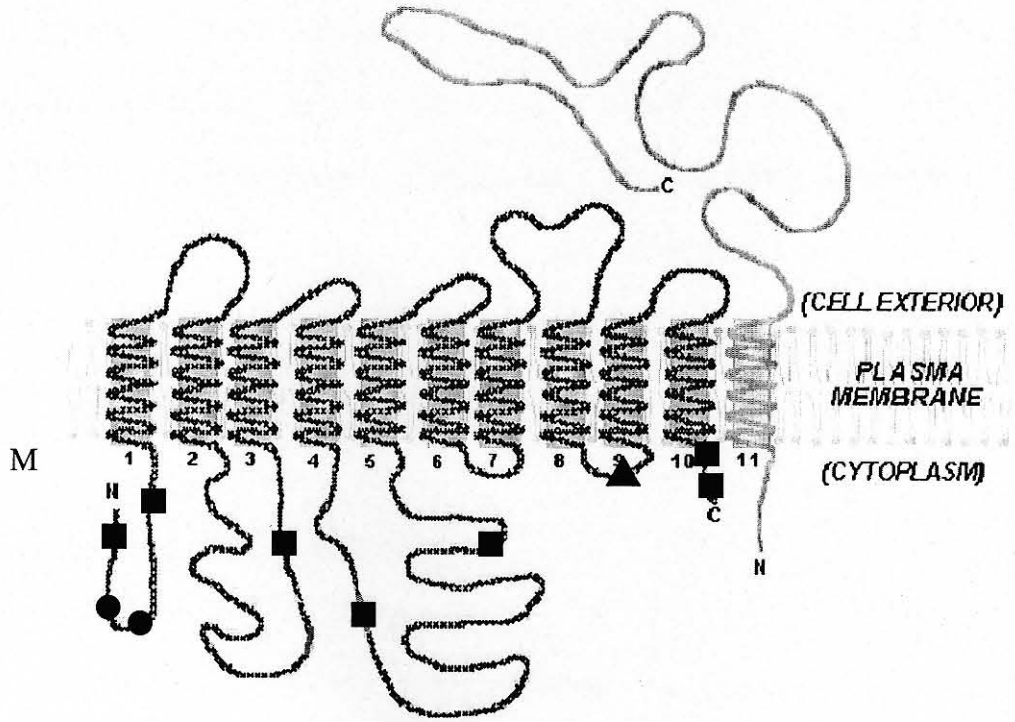


**Figure 3. Model of Na,K-ATPase subunit topography.** The Na,K-ATPase model illustrates the 10 transmembrane domains of the catalytic α-subunit. Both the $NH_2$ and COOH termini are located in the cytosol. PKC (●) and PKA(▲) phosphorylation sites are shown. Potential tyrosine phosphorylation sites (■) based on Src sequence recognition are also shown. The β-subunit $NH_2$ termini is located in the cytosol, spans the membrane once, contains 3 disulfide bridges, and may have potential glycosylation sites. (Figure from Whikehart et al., (1997)). Molecular Vision, 3(3):1-7.

correct Na,K-ATPase protein conformation. In **Figure 3**, important Na,K-ATPase PKC and PKA serine/threonine phosphorylation sites that have been shown to alter Na,K-ATPase activity are displayed as circles and triangles, respectively. Tyrosine phosphorylation sites that potentially could be important in regulating Na,K-ATPase activity are represented as boxes.

## Na,K-ATPase isoforms

Differences in Na,K-ATPase activity in different tissues may partly be explained by the presence of various isoforms of the Na,K-ATPase, which include α1, α2, α3, α4, β1, β2, and β3. The α1-β1 complex is ubiquitously expressed and may function as the housekeeping Na,K-ATPase in cells (Blanco and Mercer, 1998). The α2-β and α3-β complexes are expressed in excitable tissues such as the heart and brain (Geering, 1997). Studies using a baculovirus expression system showed that the apparent affinities for $Na^+$ and $K^+$ differ depending on the composition of the isoform complex (Blanco and Mercer, 1998). Although functional differences are not completely understood, it is apparent that α3 isoforms differ slightly from α1 and α2 in their cation

activation, while α1 and α2 differ in catalytic turnover, $K^+$ deocclusion, and ATP affinity (Geering, 1997). These latter variations appear to occur through interactions between the N-terminus and the rest of the protein. Although the physiological role of β isoforms has not been elucidated, β isoforms may be responsible for the differences in apparent potassium affinities between various isozymes (Geering, 1997).

## Na,K-ATPase function

The Na,K-ATPase complex couples the hydrolysis of one molecule of ATP to the outward translocation of three sodium ions and inward translocation of two potassium ions against their steep electrochemical gradients (**Figure 4**). Thus, Na,K-ATPase maintains the normally high potassium (140 mM) and low sodium (5-15 mM) (**Table 1**) intracellular concentrations of animal cells (Blostein, 1999). This difference in sodium concentration is the driving force for secondary active transport proteins important in transporting hydrogen ions, calcium, glucose, amino acids, and biogenic amines against their concentration gradients (**Figure 5**)(Bertorello *et al.*, 1991). The Na,K-ATPase is

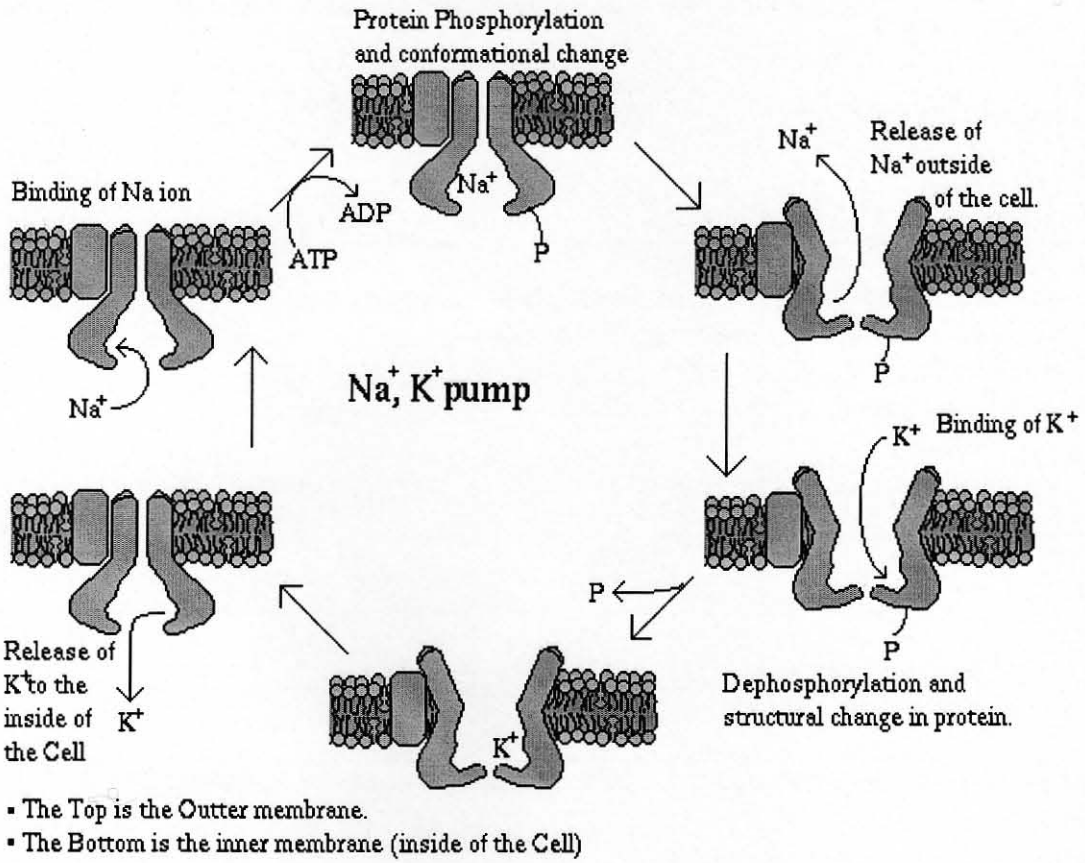


**Figure 4. Schematic Model of the Na,K-ATPase in Operation as a Cation Pump.** The binding of 3 $Na^+$ ions and the cytoplasmic phosphorylation of the α-subunit by ATP induces a conformational change in the protein that results in the translocation of the 3 $Na^+$ ions across the plasma membrane. The binding of 2 $K^+$ ions on the external surface and a dephosphorylation event revert the Na,K-ATPase to its original conformation and the transfer of the 2 $K^+$ ions across the membrane into the cytosol. http://www.cbc.umn.edu/~mwd/cell_www/chapter2/Na-Kpump.html

**Table 1: Typical ion concentrations in the lens.**

| Component | Intracellular Concentration (mEq/kg lens water) | Extracellular Concentration (mEq/kg lens water) | Cortical Cataract Intracellular Concentration (mEq/kg lens water) |
|---|---|---|---|
| $Na^+$ | 5-15 | 145 | 30-80 |
| $K^+$ | 140 | 5-15 | 80-100 |

| Component | Normal Lens (%) | Cataractous Lens (%) |
|---|---|---|
| $H_2O$ | 63 | 70+ |

(Duncan, G. and A. R. Bushell (1975). Ion analyses of human cataractous lenses. *Exp Eye Res.* **20**(3): 223-230)

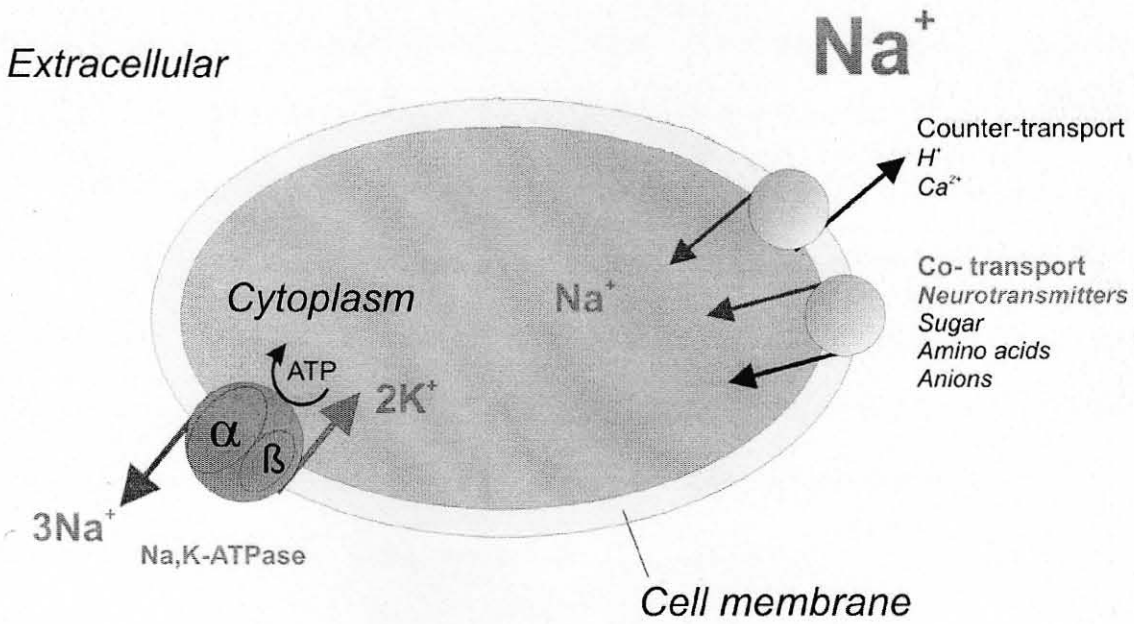


**Figure 5. Cellular function of Na,K-ATPase.** The Na,K-ATPase controls the intracellular solute concentrations thereby regulating osmotic forces that allow the cell to swell or shrink. The steep concentration gradient created by the Na,K-ATPase complex is harnessed by other membrane proteins for essential cellular functions such as membrane potential changes, uptake of neurotransmitters, amino acids, sugars, and extrusion of $Ca^{2+}$ and $H^+$ ions. The Na,K-ATPase is instrumental in regulating heat production and intracellular pH. http://www.mpibpfrankfurt.mpg.de/schwarz/Cellularfunctions.html

also involved in the regulation of resting membrane potential in animal cells. The electrochemical gradient for $K^+$ is maintained and finely tuned by the Na,K-ATPase and $K^+$ channels, allowing a cell to maintain a characteristic negative membrane potential. The maintenance of membrane potential and transport systems are imperative for the survival of all animal cells. In the lens, Na,K-ATPase regulates cytoplasmic electrolyte concentrations vital for lens transparency (Delamere et al., 1998).

**Lens Na,K-ATPase activity**

Insufficient Na,K-ATPase activity in the lens results in ionic imbalance that may lead to lens opacification or cataract formation. Cortical opacification in the human lens has been correlated with changes in sodium and potassium concentrations (Duncan and Bushell, 1975). Delamere and Dean (1993) analyzed the distribution and activity of Na,K-ATPase in rabbit lenses to determine whether reduced Na,K-ATPase expression in lens fibers is responsible for low activity. An immunoblot of rabbit membrane preparation showed the presence of Na,K-ATPase α1 throughout the lens including the nucleus (data not shown). The lens epithelium was responsible for more than 50% of

the total activity in the lens. The Na,K-ATPase specific activity in the fiber cell mass was significantly lower than that of the epithelium (Delamere and Dean, 1993). Na,K-ATPase activity was not detected within the lens nucleus. Thus, the two lens cell types have a similar abundance of Na,K-ATPase α1 protein but very different Na,K-ATPase activity. These data suggest there could be regulatory mechanisms for Na,K-ATPase activity in lens cells that suppress activity in fiber cells.

**Regulation of Na,K-ATPase activity**

Various Na,K-ATPase regulatory mechanisms have been described, such as transcriptional and translational control of Na,K-ATPase synthesis, degradation rate, and translocation of Na,K-ATPase from active and inactive pools (Horisberger, 1994). Changes in Na,K-ATPase activity in lens cells may be caused by posttranslational modifications of Na,K-ATPase α1 protein. Na,K-ATPase activity may be low due to oxidative posttranslational modification. The loss of organelles necessary for protein synthesis in the lens fiber mass suggests that Na,K-ATPase α1 protein turnover may be quite low. In cultured human lens epithelium HLE-B3

cells, studies using methionine incorporation showed that continuous Na,K-ATPase protein synthesis was required for normal Na,K-ATPase activity. When protein synthesis was inhibited, the Na,K-ATPase α1 protein abundance remained the same but Na,K-ATPase activity was reduced (Cui et al., 2002). A slow rate of turnover in lens fibers may leave the Na,K-ATPase polypeptide vulnerable to oxidative modification, which in turn may contribute, to low Na,K-ATPase activity.

Oxidative modification is known to inhibit $Ca^{2+}$-ATPase in rabbit lens cells (Borchman *et al.*, 1989). Oxidative modification of histidine residues is one of the posttranslational changes that occur with old age. Modification of cysteine residues is the major posttranslational change that occurs in the aging lens. In kidney, studies have shown that oxidation of Na,K-ATPase by hydrogen peroxide resulted in a 50% inhibition of Na,K-ATPase activity (Boldyrev and Kurella, 1996). Oxidation by hydrogen peroxide was shown to target SH-groups of the Na,K-ATPase (van Iwarden et al., 1992). In the aging lens, a concurrent increase in oxidized methionine to methionine sulfoxide also occurs (Garner and Spector, 1980). The distribution of oxidized Na,K-ATPase within the two lens cell types is not known and difficult to analyze. In

contrast, phosphorylation of Na,K-ATPase has been widely studied and is easier to analyze.

Studies on the effects of phosphorylation of the Na,K-ATPase by PKA and PKC have resulted in a wide range of response patterns in different tissue types (**Tables 2,3**) (Therien and Blostein, 2000). However, PKA and PKC phosphorylation at specific Ser/Thr residues have never fully accounted for basal levels of phosphorylation when either the Na,K-ATPase Ser/Thr phosphorylation sites on the Na,K-ATPase protein were removed or with the addition of specific PKA or PKC inhibitors, suggesting alternative phosphorylation of tyrosine residues (Beguin *et al.*, 1994).

It is possible to build the argument that alternative regulation of Na,K-ATPase may involve tyrosine phosphorylation by protein tyrosine kinases (PTK). PTKs serve an important role in signaling pathways that regulate growth and differentiation (Isakov and Biesinger, 2000). Several years ago it was shown that activation of tyrosine kinases in the kidney may be responsible for the modulation of Na,K-ATPase activity (Feraille *et al.*, 1997). More recent studies suggest a role for PTKs in thrombin-induced inhibition of Na,K-ATPase activity in porcine lens, since

**Table 2. Summary of Na,K-ATPase regulation by PKC**

| Tissue/Cell | Effectors* | Effect*† |
|---|---|---|
| Nerve (diabetic rat) | PMA, dioctanoylglycerol | ↑ |
| Hepatocytes (rat) | PMA, mezerein | ↑ |
| Pancreatic acinar cells (guinea pig) | TPA | ↑ |
| Erythrocytes (hypertensive human) | TPA | ↑ |
| Brain (rat) | (ET-18-$OCH_3$, BM 41.440) | (↓) |
| Tracheal smooth muscle (rabbit) | PDB | ↑ |
| Kidney PCT (rat) | OAG, PDB | ↓ |
| Nerve (diabetic rabbit) | DOG, PMA | ↑ |
| Oocytes (frog) | PMA | ↓ |
| Ileal smooth muscle (guinea pig) | PDB, PDA, PMA | ↑ |
| Aorta (rabbit) | PDB, endothelin | ↑ |
| Rectal gland (shark) | Purified PKC | ↓ |
| Kidney cortex (rat) | Purified PKC | ↓ |
| Sciatic nerve (diabetic mouse) | (H7) | (↓) |
| L 1210 cells (mouse leukemia) | PMA | ↑ |
| Kidney PCT (rat) | OAG (short-term) | ↑ |
| | OAG (long-term) | ↓ |
| MDCK cells (dog) | OAG, DOG, PMA | ↓ |
| Ciliary epithelium (rabbit) | PMA | ↓ |
| OK cells (opossum) | PDB | ↓ |
| Skeletal muscle (rat) | PMA | ↑ |
| Cultured ciliary epithelial (human) | PDB | ↑ |
| Skeletal muscle cells (rat) | PMA, insulin | ↑ |
| Brain (rat) | PDB, serotonin | ↓ |
| Kidney PCT (rat) | PDB (+ oxygen) | ↑ |
| | PDB (− oxygen) | ↓ |

(Therien, A.G., and Blostein, R. (2000). Mechanisms of sodium pump regulation. *American Journal of Physiology.* **279**:C541-C566)

**Table 3. Summary of Na,K-ATPase regulation by PKA**

| Tissue/Cell | Effectors* | Effect*† |
|---|---|---|
| Brown adipose tissue (rat) | cAMP, adrenergic agents | ↑ |
| Liver (rat) | (Chlorpropamide, phenformin) | (↑) |
| Skin (frog) | cAMP, oxytocin | ↑ |
| Reconstituted renal enzyme (human) | cAMP | ↓ |
| Thyroid (guinea pig) | cAMP | ↑ |
| Colon (rat) | cAMP, bisacodyl | ↓ |
| Tail artery (rat and pig) | cAMP, isoproterenol | ↑ |
| Swiss 3T3 cells | BrcAMP | ↑ |
| Brain (rat) | cAMP, PKA | ↓ |
| Sperm (hamster) | cAMP, PKA | ↓ |
| Kidney CCT, cTAL (rabbit) | db-cAMP, isoproterenol, vasopressin | ↓ |
| Kidney medulla (rat) | db-cAMP, forskolin, IBMX, isoproterenol | ↓ |
| Kidney cortex (rat) | db-cAMP, forskolin, IBMX, isoproterenol | ↑ |
| Macrophage (mouse) | cAMP, IBMX | ↑ |
| Hepatocytes (rats) | db-cAMP | ↑ |
| Rectal gland (shark) | cAMP | ↑ |
| Urethral smooth muscle (guine. pig) | cAMP, PGE, forskolin, IBMX | ↑ |
| Diaphragm muscle fibers (rat) | db-cAMP, theophylline, aminophylline | ↑ |
| Retinal pigment epithelium (frog) | cAMP | ↑ |
| Submandibular gland (dog) | db-cAMP | ↑ |
| Pancreatic islets (rat) | db-cAMP, theophylline, caffeine | ↓ |
| Rectal gland (shark) | Purified PKA | ↓ |
| Kidney cortex (rat) | Purified PKA | ↓ |
| Ciliary epithelium (rabbit) | db-cAMP | ↓ |
| Rectal gland (shark) | db-cAMP, theophylline | ↑ |
| MDCK cells (dog) | db-cAMP, PGE | ↑ |

(Therien, A.G., and Blostein, R. (2000). Mechanisms of sodium pump regulation. *American Journal of Physiology*. **279**:C541-C566)

tyrosine kinase inhibitors, genistein and herbimycin suppress the inhibition (Okafor *et al.*, 1999).

**Src-Kinase Family**

There are at least nine members of the Src tyrosine kinase family: Src, Yes, Fgr, Fyn, Lck, Lyn, Hck, Blk, and Yrk. All of the Src-Family members share a common domain structure (**Figure 6**). All Src-Family members have an N-terminal sequence important in anchoring the protein to the cell membrane, a Src-homology 3 domain important in binding to proline-rich regions of proteins, a Src-homology 2 domain that binds to tyrosine phosphorylated sequences, and a SH2-CD linker domain that binds intramolecularly to SH3 and associates with the catalytic domain (Thomas and Brugge, 1997). The catalytic domain of the kinase is divided into two lobes. The activation loop participates in regulation and is found between the two catalytic lobes; the C-terminal tail when phosphorylated binds to the SH2 domain. Some non-Src-family tyrosine kinases, like Fes kinase, lack the SH3 domain but have another domain in its place to regulate activity (Thomas and Brugge, 1997).

Studies examining thrombin activation show increased levels of activated Src in human proximal tubular cells

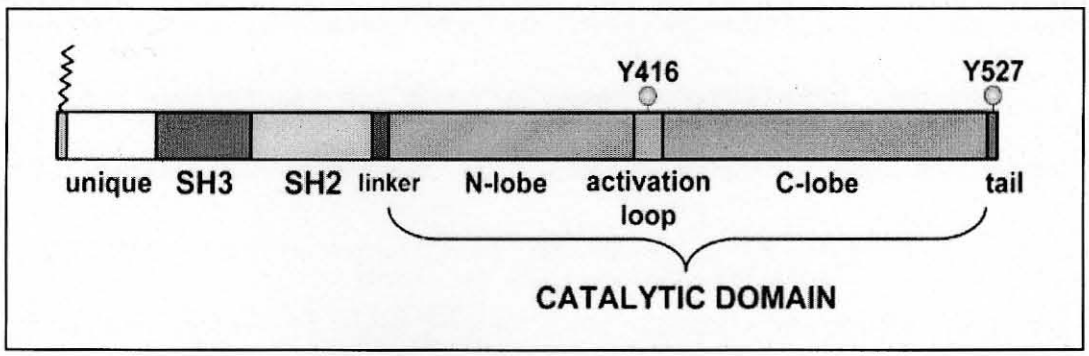


**Figure 6. The Src-family members share a common structure.** The Src-family tyrosine kinases share an N-terminal sequence important in anchoring the protein to the cell membrane. The kinases share a unique domain with unknown function. The SH2 and SH3 domains bind to phosphorylated tyrosine sequences and proline-rich ligands, respectively. Each kinase contains a catalytic domain (CD) with the necessary enzymatic activity. The SH2-CD linker binds to SH3 and associates with CD. The activation loop is thought to be important in regulation of kinase activity. When the C-terminus is tyrosine phosphorylated, it binds to the SH2 domain. http://www.hxms.com/chime/Fig2.JPG

(Grandaliano *et al.*, 2000) and increased kinase activity of Src and Fyn in transformed human fibroblast cells (Chen *et al.*, 1994). Thrombin-induced inhibition of ouabain-sensitive rubidium uptake in lens epithelium is suppressed in the presence of Src kinase inhibitors genistein and herbimycin A. In the following study, the argument is developed that phosphorylation of Na,K-ATPase α1 protein at tyrosine residues by Src-kinase family members may be an important posttranslational modifications responsible for the regulation of Na,K-ATPase activity in lens cells.

## Specific Aims

**Specific Aim I:** Test the hypothesis that the Na,K-ATPase catalytic subunit can be tyrosine phosphorylated.

Questions to be answered:

1. Is endogenous tyrosine phosphorylation detectable in lens epithelium and lens fibers?
2. Can Lyn kinase tyrosine phosphorylate the Na,K-ATPase in lens cells?
3. Can tyrosine phosphorylation in the intact lens be altered by tyrosine kinase inhibitors?

**Specific Aim II:** Test the hypothesis that tyrosine phosphorylation causes a change in Na,K-ATPase activity.

Questions to be answered:

1. Does Lyn kinase-induced tyrosine phosphorylation *in vitro* alter Na,K-ATPase activity?
2. Is Na,K-ATPase activity in the intact lens altered by tyrosine kinase inhibitors?
3. Do different Src and non-Src-family tyrosine kinases have similar effects on Na,K-ATPase activity?

**Specific Aim III:** To test the hypothesis that tyrosine phosphatase treatment causes a change in Na,K-ATPase activity.

Questions to be answered:

1. Does PTP-1B treatment alter the phosphorylation status of lens epithelium and lens fiber cell proteins?
2. Does PTP-1B treatment alter Na,K-ATPase activity in lens epithelium and restore activity in lens fiber?
3. Does PTP-1B alter the Na,K-ATPase phosphorylation state and activity in Lyn-treated lens epithelium membrane preparation?

## CHAPTER II

## THE INFLUENCE OF LYN KINASE ON NA,K-ATPASE IN PORCINE LENS EPITHELIUM

### INTRODUCTION

Na,K-ATPase is a ubiquitous active ion transport protein complex found in the plasma membrane of all animal cells. The functional Na,K-ATPase comprises a 112 kDa catalytic α-subunit together with a non-catalytic 35 kDa β-subunit and appears to exist as an α2β2 tetramer (Askari, 1987). Na,K-ATPase couples the hydrolysis of one molecule of ATP to the outward translocation of three sodium ions and inward translocation of two potassium ions against their steep electrochemical gradients. Thus, Na,K-ATPase maintains the normally high potassium and low sodium concentrations in the cytoplasm of animal cells (Blostein, 1999). In the lens, regulation of cytoplasmic electrolyte concentration is vital for transparency. The majority of human cataractous lenses display an abnormally high sodium content and the degree of lens opacification is more severe in lenses with higher sodium values

(Duncan and Bushell 1975). For normal lens function, it has been argued that Na,K-ATPase activity must be high in the monolayer of epithelium that covers the anterior lens surface (Mathias et al., 1997). Fiber cells, which make up the bulk of the lens, do not lack Na,K-ATPase polypeptides but have a lower Na,K-ATPase activity than the epithelium (Delamere and Dean, 1993). Na,K-ATPase-mediated ion transport by the epithelium appears to play a major role in regulating electrolyte composition for the entire lens. Regulation of Na,K-ATPase activity and function in lens epithelium has not been widely studied.

Regulation of Na,K-ATPase function can occur through several different protein kinase-mediated mechanisms. Studies on the effects of phosphorylation by PKA and PKC on Na,K-ATPase function reveal a wide range of response patterns in different tissue types, some stimulatory, some inhibitory (Therien and Blostein, 2000). The activation of tyrosine kinases appears to stimulate Na,K-ATPase in the intact kidney proximal tubule, as judged by an increase of ouabain-sensitive $^{86}Rb$ uptake (Feraille et al., 1997; Narkar et al., 2002). The proximal tubule response may involve recruitment of tyrosine-phosphorylated Na,K-ATPase protein from the cytosol to the plasma membrane (Narkar et al., 2002). In rat skeletal muscle, insulin causes tyrosine

phosphorylation of Na,K-ATPase α1 and α2 protein (Chibalin et al., 2001) and stimulates Na,K-ATPase α1 and α2 translocation to the plasma membrane (Al-Khalili et al., 2003). In rat astrocytes, tyrosine kinase activation by insulin causes an increase of Na,K-ATPase activity associated with a selective increase in the synthesis of Na,K-ATPase α1 protein (Matsuda et al., 1993). In other tissues, however, tyrosine kinase activation appears to inhibit Na,K-ATPase function. In cultured rabbit nonpigmented ciliary epithelium, tyrosine kinase inhibitors were found to prevent the inhibitory action of dopamine and D-1 agonists on Na,K-ATPase-mediated ion transport (Nakai et al., 1999). In the intact organ cultured lens, previous studies show genistein reduces the inhibitory effects of endothelin (ET-1) on ouabain-sensitive $^{86}Rb$ uptake (Okafor and Delamere 2001). Also in the lens, thrombin-induced inhibition of Na,K-ATPase-mediated active ion transport is suppressed by herbimycin A, an inhibitor of Src-family tyrosine kinases (Okafor et al., 1999). Inhibition of Na,K-ATPase function occurs in parallel with increased tyrosine phosphorylation of multiple membrane proteins in the epithelium of thrombin-treated lenses. Non-receptor tyrosine kinases including Src-family kinases, are

activated in thrombin-treated tissues (Fox, 1996). In platelets, the Src-family kinase Lyn plays a key role in the response to thrombin (Hirao et al., 1997). When platelets from Lyn-deficient mice are challenged with thrombin, normal platelet aggregation, thromboxane A2 production and ADP secretion fail to occur (Cho et al., 2002). In the present study, experiments were conducted to test whether a change of intrinsic Na,K-ATPase activity is detectable following Lyn kinase-dependent tyrosine phosphorylation of isolated, partially purified lens epithelium membrane preparation. Lyn treatment caused a partial inhibition of Na,K-ATPase activity associated with tyrosine phosphorylation of multiple membrane proteins including the Na,K-ATPase α1 catalytic subunit.

## MATERIALS AND METHODS

Tris-base, glycine, hepes, sucrose, 2-mercaptoethanol, bromophenol blue, EGTA, EDTA, sodium chloride, triton X-100, tween-20, phenylmethylsulfonyl fluoride (PMSF), leupeptin, antipain, pepstatin A, aprotinin, sodium orthovanadate, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), ammonium persulfate, tetramethyl-ethylenediamine (TEMED), ammonium bicarbonte, histidine, potassium chloride,

magnesium chloride, adenosine-5'-triphosphate, ouabain, ferrous sulfate, alamethacine, thrombin, herbimycin, dimethyl sulfoxide (DMSO), sodium phosphate, trichloro acetic acid (TCA), bovine serum albumin (BSA), and dry milk were purchased from Sigma Chemical Company (St. Louis, MO). Acetic acid, methanol, hydrochloric acid, sodium hydroxide, Kodak film, and ammonium molybdate were purchased from Fisher Scientific (Pittsburgh, PA). Nitrocellulose membranes, filter paper, pre-stained low molecular weight markers, bio-safe Coomassie blue, IPG strips, and goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bio-Rad (Richmond, CA). The chemiluminescent detection substrate and bicinchoninic acid (BCA) protein detection assay kit were purchased from Pierce Chemical (Rockford, IL). Lyn kinase, anti-Lyn monoclonal antibody, polyclonal phosphotyrosine antibody, monoclonal phosphotyrosine antibody, and monoclonal antibodies against Na,K-ATPase α1 were all purchased from Upstate Biotechnology (Lake Placid, NY). HRP-conjugated phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY).

***Tissues***

Porcine eyes and kidneys were obtained from the Swift Meat Packing Company (Louisville, KY). The lens was isolated by dissecting open the posterior of the eye, cutting the suspensory ligaments, and transferring the lens to a petri dish. The lens capsule and attached monolayer of epithelial cells covering the anterior portion of the lens was removed and snap-frozen in liquid nitrogen. Membrane preparations from 40-50 lenses was pooled. Kidneys were bisected and the dark red outer medulla was isolated and snap-frozen in liquid nitrogen (Dean, Delamere et al. 1996). Membrane preparations from 2-3 kidneys was pooled.

***Membrane Preparation***

Membrane preparations containing plasma membranes as well as intracellular membranes were obtained following methodology described by Okafor et al. (1999). Previously frozen samples of lens capsule-epithelium and kidney outer medulla were homogenized in ice-cold homogenization buffer A (150 mM sucrose, 4 mM EGTA, 5 mM Hepes, 800 μM dithiothreitol (DTT), pH 7.4) in the presence of protease inhibitors (100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 2

μg/mL aprotinin) using a glass homogenizer. The homogenate was centrifuged at 115,000g for 60 minutes at 4°C. To remove extrinsic proteins, the membrane pellet was then resuspended in homogenization buffer A containing 600 mM KCl and subjected to centrifugation once again at 115,000g for 60 minutes at 4°C (Dean, Delamere et al. 1996). The membrane pellet was resuspended in homogenization buffer A and subjected to centrifugation a final time at 115,000g for 60 minutes at 4°C. The final pellet containing epithelium or kidney membrane preparation was resuspended in buffer A and the protein content measured using the BCA protein assay kit (Pierce, Rockford, IL).

***Lyn-dependent phosphorylation and Na,K-ATPase activity measurement***

Lens epithelium or kidney membrane preparation was incubated in kinase buffer containing 1 mM EGTA, 10 mM Tris pH 7.2, 20 mM $MgCl_2$, 1 mM ATP, 0.2 mM sodium orthovanadate, 10 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM PMSF, 5 mM DTT, and Lyn kinase (0.08 units/μg protein) (Upstate Biotechnology, Lake Placid, NY) for 20-30 minutes at 30°C. Treated material was then used for western blot analysis, immunoprecipitation or Na,K-ATPase activity measurements. Sodium orthovanadate, an inhibitor of Na,K-

ATPase activity, was removed prior to Na,K-ATPase activity measurements. To remove sodium orthovanadate the membrane preparation was centrifuged at 100,000g for 3 minutes. The membrane pellet was resuspended two times in 100 μl of centrifugation buffer (10 mM Tris pH 7.2, 5 mM DTT, 10%(w/v) glycerol) and centrifuged at 100,000g for 3 minutes. The final pellet was resuspended in ~100 μl of Na,K-ATPase buffer and assayed immediately for Na,K-ATPase activity.

In some experiments, Lyn kinase-treated membrane preparation was subsequently incubated with protein tyrosine phosphatase 1B (PTP-1B) (0.5 U/μl) (Upstate Biotechnology, Lake Placid, NY) for 30 minutes at 37°C in PTP-1B assay buffer (25 mM Hepes, 50 mM NaCl, 5 mM DTT, 2.5 mM EDTA, 100 μg/ml bovine serum albumin, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 1mM PMSF, pH 7.2). To remove buffer constituents, the PTP-1B treated membrane preparation was resuspended once in 200 μl of centrifugation buffer and centrifuged at 100,000g for 3 minutes. The final pellet was resuspended in Na,K-ATPase assay buffer then assayed immediately for Na,K-ATPase activity.

Na,K-ATPase activity was determined as described by Okafor et al., (1999). Aliquots of Lyn kinase-treated and untreated epithelium membrane preparation (~100 μg) or

kidney membrane preparation (~25 μg) were added to Na,K-ATPase buffer (100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1mM EGTA, pH 7.4). Ouabain, a specific inhibitor of Na,K-ATPase (Wallick and Schwartz, 1988) , was added to half the sample aliquots to a final concentration of 1 mM. Samples were preincubated for 15 minutes at 37°C with gentle agitation. ATP hydrolysis was initiated by the addition of ATP to a final concentration of 1 mM. The ATP hydrolysis reaction was carried out for 45 minutes at 37°C with gentle agitation. The reaction was stopped with the addition of 15% ice-cold trichloroacetic acid. ATP hydrolysis was quantified using a colorimetric method that measured the amount of inorganic phosphate released in each reaction sample (Okafor et al., 1999). Less than 20% of the available substrate ATP was hydrolyzed. The difference in ATP hydrolysis in the presence and absence of ouabain was a measurement of Na,K-ATPase activity. The data are presented as nanomoles phosphate released per milligram protein per minute.

Since Na,K-ATPase activity was measured in samples of lens epithelium membrane preparation that had been treated with buffer containing 0.2 mM sodium orthovanadate, then washed, separate studies were conducted to confirm Na,K-ATPase activity was not inhibited by residual vanadate. Na,K-ATPase activity was 9.7 ± 0.4 nmoles Pi/mg

protein/minute (mean ± SE; n = 5) in vanadate-treated samples, which was not significantly different from the activity of 10.2 ± 0.6 measured in control samples lacking exogenous vanadate.

### *$^{86}$Rb uptake*

Ouabain-sensitive $^{86}$Rb uptake by the intact lens was used as an index of Na,K-ATPase-mediated active sodium-potassium transport. Intact lenses were bathed at 37°C in Krebs solution with the composition (in mM): 119 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$ and 5.5 glucose at pH 7.4. It was assumed that the Na,K-ATPase mechanism transports $^{86}$Rb similarly to potassium. Lenses were preincubated for 10 minutes in Krebs solution containing test agents, and then $^{86}$Rb (~0.1 μCi/ml) was added. Half of the lenses in each group also received 1 mM ouabain, added simultaneously with the $^{86}$Rb. The $^{86}$Rb uptake period was 30 minutes. Previous studies show during this time, $^{86}$Rb uptake is linear. After the 30-minute $^{86}$Rb uptake period, each lens was placed in a large volume of ice-cold nonradioactive Krebs solution for 2 minutes to wash $^{86}$Rb from extracellular space. After this, the lenses were weighed, lyophilized and reweighed to determine water

content. The dried tissue was digested in 30% nitric acid and radioactivity in the acid digest was measured by scintillation counting. Based on the specific activity of $^{86}Rb$ in the medium, the data were calculated as nmoles potassium ($^{86}Rb$) accumulated/g lens water/minute.

***Western blot analysis***

Membrane preparation was solubilized with Laemmli sample dilution buffer and proteins separated on a 7.5% gel by SDS-PAGE at 40 mA for 2 hours using the Laemmli buffer system (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose sheets at 30 V for 16 hours. The nitrocellulose membranes were blocked for 1hr with 5% dry milk in TTBS (30 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.4). For immunodetection of Na,K-ATPase α1, Lyn kinase, or tyrosine phosphoproteins, the nitrocellulose membranes were incubated at room temperature for 60 minutes with either a monoclonal antibody directed against Na,K-ATPase α1 (Sigma, St. Louis, MO), Lyn kinase (Upstate Biotechnology, Lake Placid, NY), or anti-phosphotyrosine antibody PY20 (PY) (Transduction Lab, Lexington, KY) conjugated to horseradish peroxidase. Nitrocellulose membranes probed for Na,K-ATPase α1 and Lyn kinase were

washed with TTBS twice for 15 minutes and then three times for 5 minutes before being incubated for 60 minutes with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Nitrocellulose membranes probed for PY20 were washed with TTBS twice for 15 minutes and then four times for 5 minutes at room temperature, then visualized with chemiluminescence substrate (Pierce, Rockford, IL). Nitrocellulose membranes were exposed to x-ray film.

***Immunoprecipitation***

Following a methodology modified from a technique described by Khundmiri & Lederer (2002). lens epithelium (500 μg) and kidney membrane preparation (200 μg) was solubilized in sufficient immunoprecipitation buffer (10 μM deoxycholate, 100 mM D-mannitol, 5 mM Tris pH 7.6, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin A) to bring the final protein concentration to 2 or 0.8 μg/μl respectively. The membrane preparation was mixed on a rotating wheel at 4°C for 3 hours. The insoluble material was then pelleted by centrifugation at 8000g for 15 minutes at 4°C. The supernatant (250 μl) was removed then pre-cleared with 20 μg of mouse IgG and 50 μl of immobilized protein G (ImmunoPure, Pierce, Rockford, IL)

for 15 hours on a rotating wheel at 4°C. The membrane preparation mixture was then centrifuged at 1000g for 3 minutes at 4°C. The supernatant was removed and pre-cleared once again with rabbit IgG and 50 μl of immobilized protein A (ImmunoPure, Pierce, Rockford, IL) for 3 hours on a rotating wheel at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and 10 μg of polyclonal antibody directed against Na,K-ATPase α1 polypeptide (RDI, Flanders, NJ) was added. The membrane preparation mixture was incubated on a rotating wheel for 15 hr at 4°C. After this, 50 μl of immobilized protein A (ImmunoPure, Pierce, Rockford, IL) was added and mixed for an additional 3 hr on a rotating wheel at 4°C. The mixture was then washed with 200 μl of PBS pH 7.4, then centrifuged at 1000g for 3 minutes at 4°C. The wash procedure was repeated two more times then immunoprecipitated Na,K-ATPase α1 polypeptide was dissociated from the protein A and antibody mixture by incubating in 45 μl of Laemmli sample dilution buffer for 20 minutes at 65°C. The samples were centrifuged at 4000g for 5 minutes. The supernatant was then subjected to SDS-PAGE followed by western blot analysis. In some experiments, a different immunoprecipitating antibody was used. The immunoprecipitation was carried out using 2 μg of monoclonal antibody directed against tyrosine

phosphoproteins (PY99) (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

In some experiments, Lyn kinase-treated membrane preparation was incubated with PTP-1B (100 mU/μl) for 30 minutes at 37°C in PTP-1B assay buffer. The Lyn-PTP-1B-treated membrane preparation was subjected to immunoprecipitation followed by SDS-PAGE. Resolved proteins were analyzed for tyrosine phosphoproteins by western blot.

***In-gel protein digestion for MALDI analysis***

Protein bands from Coomasie Blue stained gels were excised and incubated with 50 mM $NH_4HCO_3$ at room temperature for 15 minutes. Gel pieces were then incubated with an equal volume of acetonitrile (ACN) for an additional 15 minutes. Gel pieces were then dried by Speedvac and then swelled with 20 mM DTT/0.1 M $NH_4HCO_3$ for 45 minutes at 56°C. The gel pieces were then chilled, the DTT solution removed, and 55 mM iodoacetamide/0.1 M $NH_4HCO_3$ added and incubated at room temperature for 30 minutes. The iodoacetamide solution was removed and the gel pieces washed and incubated with 50 mM $NH_4HCO_3$ for 15 minutes followed by the addition of an equal volume of ACN. The gel pieces were then dried by speedvac and the digestion buffer (20 ng/l

Promega modified trypsin in 50 mM $NH_4HCO_3$) was then added and incubated overnight at 37°C. Samples were then used for MALDI analysis.

***Statistical Analysis***

Student's t-test was used for statistical analysis.

## RESULTS

**Figure 7** illustrates tyrosine phosphorylation of membrane proteins in the intact lens. Membrane preparation isolated from lenses exposed to thrombin exhibited a marked increase in the intensity of several tyrosine phosphoprotein bands (**Figure 7**). The phosphorylation increase was abolished by herbimycin, a recognized inhibitor of Src-family tyrosine kinases. Among the many tyrosine phosphoprotein bands, a 100 kDa band appeared to co-migrate with Na,K-ATPase α1 subunit. In some studies the 100 kDa band was excised, subjected to in-gel tryptic digestion, and analyzed using MALDI-TOF mass spectrometry. Database search analysis resulted in the identification of peptides covering ~20% of the Na,K-ATPase α1 protein sequence corresponding to approximately 16 peptide matches for each analyzed sample (**Tables 4,5**). Similar to findings reported

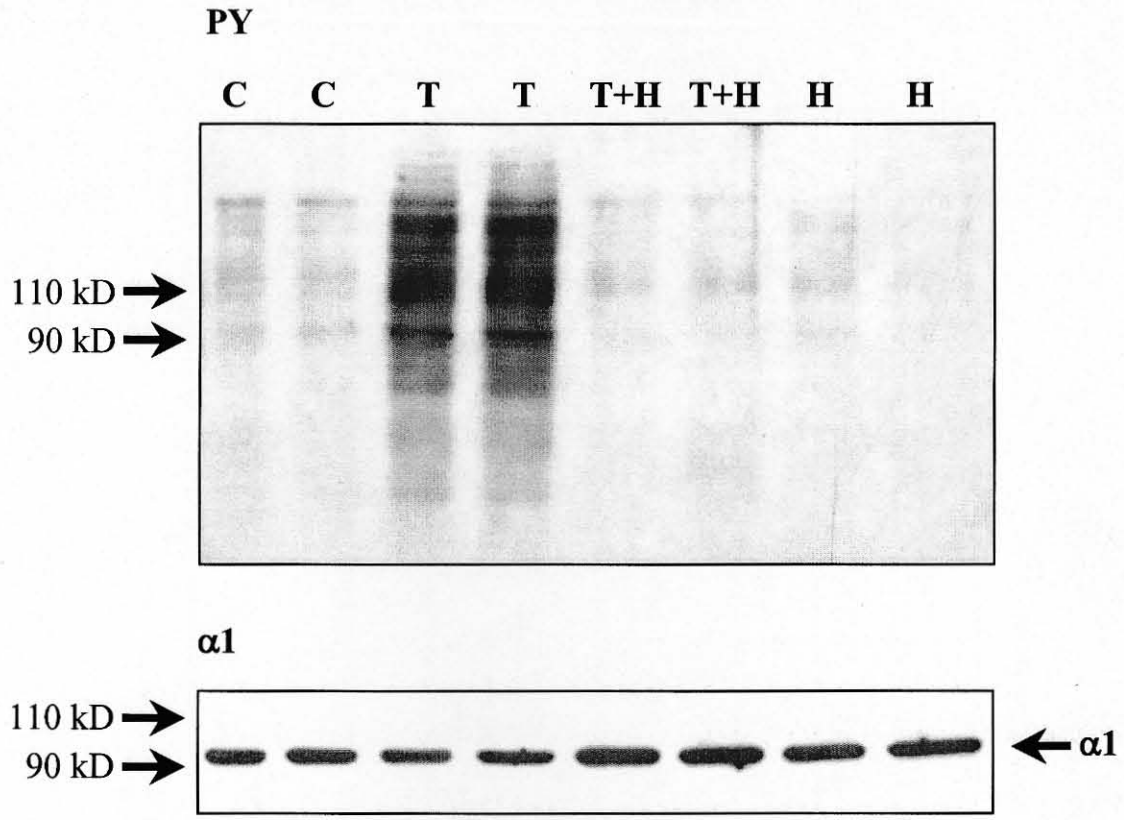


**Figure 7. The influence of thrombin on tyrosine phosphorylation of lens epithelium membrane proteins.** Intact lenses were exposed to 2 units/ml thrombin (T) or vehicle (Control; C) or exposed to thrombin in the presence of 7.5 μM herbimycin A (T + H) or herbimycin A alone (H) for 45 minutes and then used as a source of membrane preparation for western blot to probe for phosphotyrosine (PY) residues. The phosphotyrosine blot (upper) was stripped and reprobed for Na,K-ATPase α1 (lower).

**Table 4: Database Search Results from MALDI-TOF Analysis of Trypsin-Digested Lens Epithelium Membrane Proteins.**

**MS-Fit Search Results**
**(UCSF Mass Spectrometry Facility)**

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**Database searched: SwissProt.8.21.2003**
**Full Molecular weight range selects 132648 entries.**
**Full pI range selects 132648 entries.**
**Full Species search selects 132648 entries.**
**Combined molecular weight, pI and species searches select 132648 entries.**
**Parameters used in search:**

| Min.# Peptides to Match | Peptide Mass Tolerance (+/-) | Peptide Masses are | Digest Used | Max. # Missed Cleavage | Cysteines modified by |
|---|---|---|---|---|---|
| 4 | 50.0 ppm | monoisotopic | Trypsin | 1 | Carbamidomethylation |

| Rank | MOWSE Score | #(%) Masses Matched | Protein MW (Da)/pI | Species | Protein Name |
|---|---|---|---|---|---|
| 1 | 2.087e+07 | 17/63 (26%) | 112682/5.4 | PIG | Sodium Pump Na,K-ATPase α1 |
| 2 | 7.773e+06 | 17/63 (26%) | 112659/5.4 | SHEEP | Sodium Pump Na,K-ATPase α1 |
| 3 | 7.660e+06 | 16/63 (25%) | 113055/5.3 | RAT | Sodium Pump Na,K-ATPase α1 |

**ProteinProspector (Ver 3.4.1) from ExPASy Proteomics tools at http://ca.expasy.org**
**For matching α1 peptide fragments on the primary structure of α1 protein refer to figure 10 in the Appendix**

**Table 5: Na,K-ATPase Peptides Identified by ProteinProspector Assigned to Matched Masses**

**MS-Fit Search Results**
**(UCSF Mass Spectrometry Facility)**

17/63 matches (26%). 112682 Da, pI+ 5.4. Acc. # P05024. PIG. Sodium/potassium transporting ATPase alpha-chain.

The matched peptides cover **22% (223/1020AA's)** of the protein.

| M/z submitted | MH+ matched | Delta ppm | Start | End | Peptide Sequence |
|---|---|---|---|---|---|
| 739.4415 | 739.4653 | -32 | 501 | 506 | (R)HLLVMK(G) |
| 887.4865 | 887.5103 | -27 | 486 | 492 | (K)YQLSIHK(N) |
| 927.4926 | 927.5165 | -26 | 1010 | 1017 | (R)RPGGWVEK(E) |
| 978.5134 | 978.5373 | -24 | 254 | 262 | (R)GIVVTTGDR(T) |
| 1087.4968 | 1087.5206 | -22 | 35 | 43 | (K)KEVSMDDHK(L) |
| 1134.5822 | 1134.6060 | -21 | 682 | 689 | (K)YHTEIVFAR(T) |
| 1209.5924 | 1209.6162 | -20 | 939 | 948 | (R)RNSVFQQGMK(N) |
| 1235.6986 | 1235.7224 | -19 | 646 | 656 | (R)LNIPVSQVNPR(D) |
| 1267.7071 | 1267.7309 | -19 | 161 | 171 | (K)NMVPQQALVIR(N) |
| 1429.6837 | 1429.7076 | -17 | 10 | 22 | (K)YEPAAVSEHGDKK(K) |
| 1583.8671 | 1583.8910 | -15 | 429 | 442 | (R)AVFQANQENLPILK(R) |
| 1751.8478 | 1751.8717 | -14 | 534 | 549 | (K)DAFQNAYLELGGLGER(V) |
| 1818.9152 | 1818.9390 | -13 | 73 | 89 | (R)DGPNALTPPPTTPEWVK(F) |
| 1828.9166 | 1828.9405 | -13 | 628 | 645 | (K)GVGIISEGNETVEDIAAR(L) |
| 1923.9096 | 1923.9333 | -12 | 358 | 375 | (K)NLEAVETLGSTSTICSDK(T) |
| 2316.0083 | 2316.0321 | -10 | 892 | 909 | (R)WINDVEDSYGQQWTYEQR(K) |
| 24.63.1951 | 2463.2190 | -9.7 | 742 | 764 | (K)QAADMILLDDNFASIVTGVEEGR(L) |

earlier (Cicmil et al., 2000), exposure of the intact lens to thrombin was observed to cause a 35% decrease in the rate of ouabain-sensitive potassium ($^{86}Rb$) uptake to 19.5 ± 1.8 nmoles/g lens water/minute from a control value of 30.1 ± 1.4 (mean ± SE: n = 6 lenses; significant $p < 0.01$) while in the presence of herbimycin + thrombin no reduction in the rate was apparent (35.3 ± 1.0) (**Figure 8**).

Thrombin induces the activation of non-receptor tyrosine kinases (Li et al., 1994; Kutz et al., 1998; Cicmil, Thomas et al. 2000) including Lyn, a Src tyrosine kinase family member (Hirao et al., 1997; Cho et al., 2002). Lyn kinase is expressed in lens tissue. When lens epithelium membrane preparation was isolated and used for western blot analysis, two immunopositive bands corresponding to the two known isoforms of Lyn kinase were detected (**Figure 9**) although the results do not signify the degree to which Lyn is activated. To determine the effects of tyrosine kinase-mediated phosphorylation on lens epithelium membrane proteins *in vitro*, isolated lens epithelium membrane preparation was incubated with active, partially-purified Lyn kinase (0.08 units Lyn/$\mu$g membrane protein) in ATP-containing kinase reaction buffer. After this, tyrosine phosphorylation and Na,K-ATPase activity

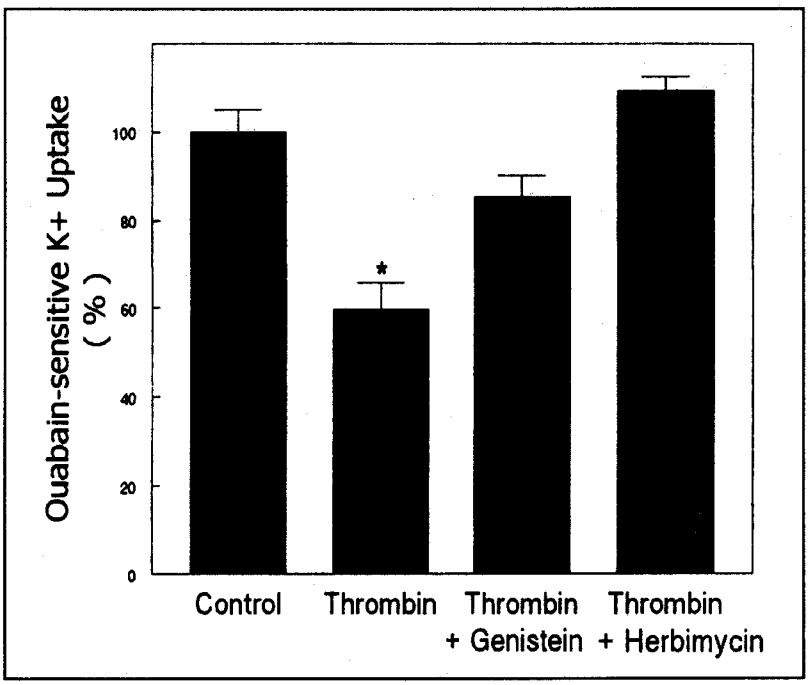



**Figure. 8. The influence of genistein and herbimycin on thrombin-induced inhibition of Na,K-ATPase activity in intact lenses.** Lenses were preincubated in the presence of either genistein (150 μM) or herbimycin (15 μM) or thrombin (1 unit/ml) or genistein + thrombin or herbimycin + thrombin. $^{86}Rb^{+}$ was added for 30 minutes. Half the lenses received ouabain (1 mM final). The values are shown as mean ± SE (vertical bar) of data from 6 to 11 lenses. * indicates a significant difference from control ($P<0.01$). (Okafor *et al.*, IOVS(1999)40(9) p.2036)

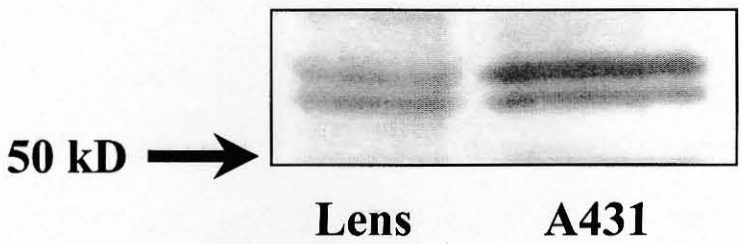



**Figure 9. Detection of Lyn kinase in lens epithelium membrane preparation.** Lens epithelium membrane preparation (50 µg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against Lyn kinase. A431 non-stimulated cell lysate (20 µg) was used as a positive control for Lyn kinase expression.

were examined. Western blot analysis revealed a marked increase in several phosphotyrosine protein bands (**Figure 10**). A smaller increase of phosphotyrosine band intensity observed in the presence of ATP but absence of added Lyn may signify the activity of endogenous Lyn and other tyrosine kinases. No increase was observed in the presence of Lyn and absence of ATP (**Figure 11**). Na,K-ATPase activity was reduced significantly in lens epithelium material subjected to Lyn pretreatment (**Table 6**). To compare the effects of Lyn on a different tissue, Na,K-ATPase activity was also measured in membrane preparation isolated from kidney medulla. Lyn pretreatment was found to cause a ~20% reduction of Na,K-ATPase activity measured in kidney membrane preparation (**Table 6**).

Studies were conducted to determine whether tyrosine phosphorylation of the Na,K-ATPase α1 polypeptide occurs. Na,K-ATPase α1 protein was first isolated from lens epithelium membrane samples by immunoprecipitation using a polyclonal antibody directed against the Na,K-ATPase α1 subunit and then the immunoprecipitated Na,K-ATPase α1 protein was subjected to treatment with Lyn kinase. The Lyn-treated immunoprecipitates were resolved by SDS-PAGE and subjected to western blot analysis (**Figure 12**). A

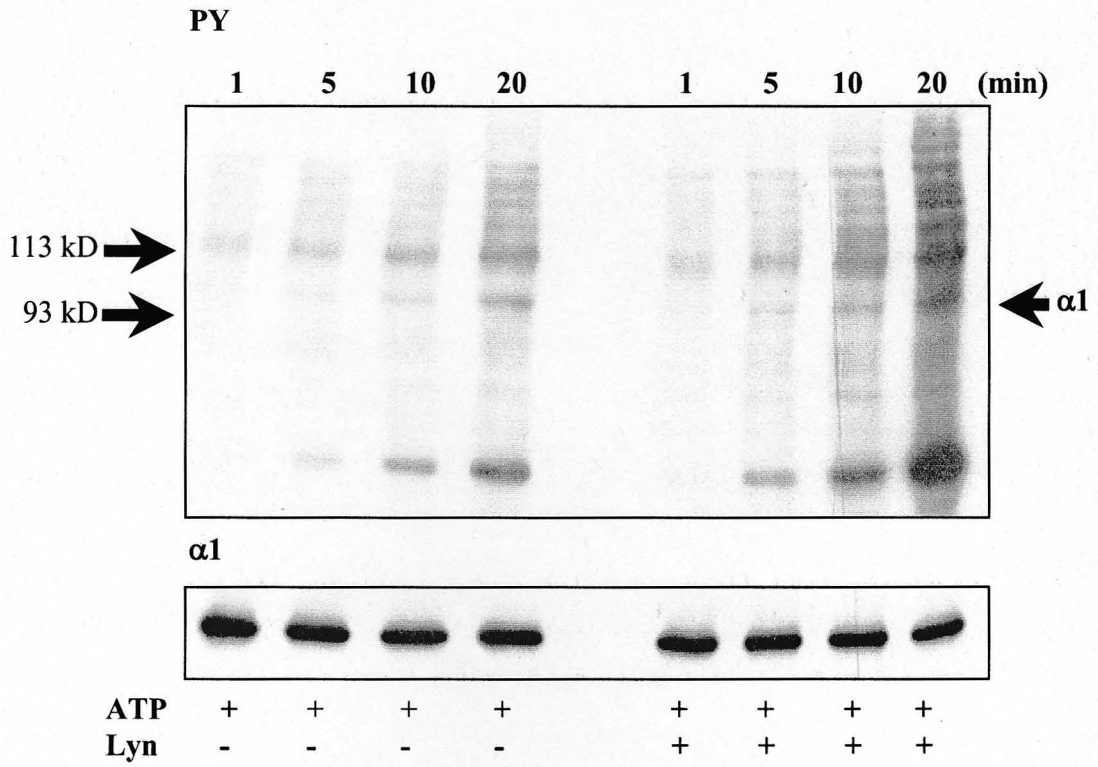



**Figure 10. The influence of Lyn kinase and ATP on lens epithelium membrane preparation.** Lens epithelium membrane preparation (25 μg) was incubated for 1, 5, 10 or 20 minutes with or without partially purified Lyn kinase (2 Units) in ATP-containing buffer. The treated membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against phosphotyrosine (PY) residues. The right hand arrow indicates the location of Na,K-ATPase α1. The phosphotyrosine western blot (upper) was stripped and reprobed for Na,K-ATPase α1 (lower).

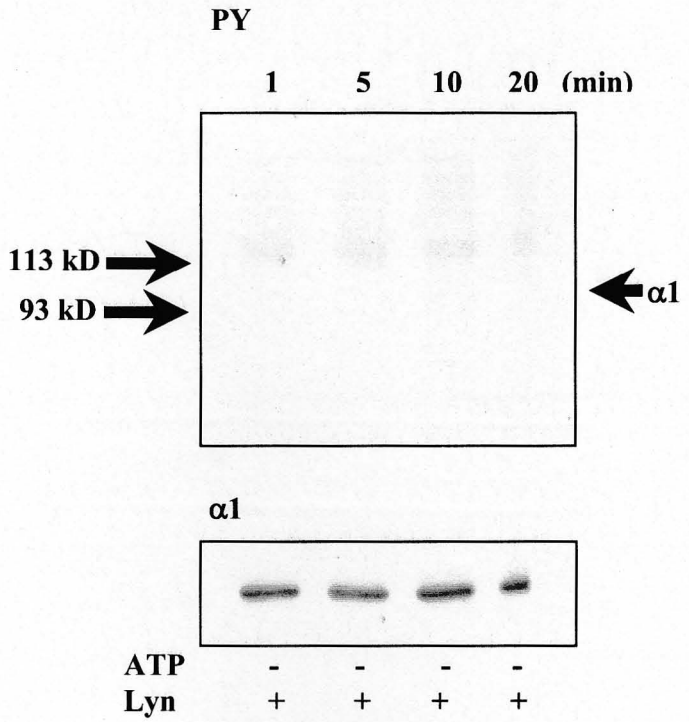



**Figure 11. The influence of Lyn alone on lens epithelium membrane preparation.** Lens epithelium membrane preparation (25 μg) was incubated for 1, 5, 10 or 20 minutes in the presence of ATP and absence of Lyn. The treated membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against phosphotyrosine (PY) residues. The right hand arrow indicates the location of Na,K-ATPase α1. The phosphotyrosine western blot (upper) was stripped and reprobed for Na,K-ATPase α1 (lower).

**TABLE 6. The influence of Lyn kinase on Na,K-ATPase activity.**

| | Na, K-ATPase activity following Lyn kinase treatment |
|---|---|
| | (% of control) |
| Lens Epithelium (+ATP -Lyn) | 110.9 ± 7.7 |
| Lens Epithelium (+ATP +Lyn) | *62.3 ± 3.1 |
| Kidney Medulla (+ATP +Lyn) | *82.2 ± 6.6 |

The data are presented as the mean ± SE of results obtained from 25 measurements made using 6 different pools of Lyn-treated and non Lyn-treated membrane preparation (n = 6; lens epithelium) or from 33 measurements made using 8 different pools of Lyn-treated and non Lyn-treated membrane preparation (n = 8; kidney medulla). Control Na,K-ATPase specific activity was 9.1 ± 1.7 and 230.8 ± 13.8 nmoles $P_i$/mg protein/minute, respectively, in lens epithelium and kidney medulla preparations. Data are shown as mean ± SE. * indicates a significant difference from control ($P < 0.01$).

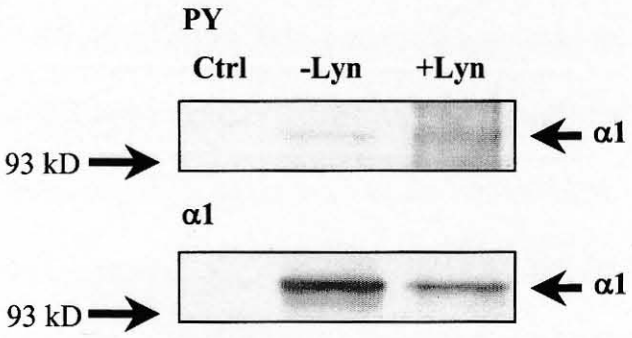



**Figure 12. The influence of Lyn kinase on Na,K-ATPase α1 subunit isolated by immunoprecipitation from lens epithelium membrane preparation.** Na,K-ATPase α1 subunit polypeptide was isolated by immunoprecipitation from lens epithelium membrane preparation and then was incubated in the presence (+Lyn) or absence (-Lyn) of partially purified Lyn kinase (50 Units) in ATP-containing buffer for 20 minutes. Immunoprecipitates were subjected to SDS-PAGE and probed for phosphotyrosine (PY) proteins (upper). The phosphotyrosine blot was then stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (lower). In the control, the immmunoprecipating antibody was omitted.

dense 100 kDa tyrosine phosphoprotein band was observed in Lyn-treated Na,K-ATPase α1 immunoprecipitates. The 100 kDa tyrosine phosphoprotein band was undetectable in Na,K-ATPase α1 immunoprecipitates that were not treated with Lyn. For technical reasons, Na,-K-ATPase activity could not be determined reliably in the immunoprecipitates.

To examine the effects of Lyn on Na,K-ATPase in the non-solubilized membrane, lens epithelium membrane preparation was treated first with Lyn before Na,K-ATPase α1 protein was isolated by immunoprecipitation. Western blot analysis revealed a 100 kDa phosphotyrosine band in immunoprecipitates isolated from lens epithelium membrane preparation that had been subjected to Lyn treatment (**Figure 13A**). A similar result was observed in membrane preparation isolated from porcine kidney medulla, a non-lens tissue where the major Na,K-ATPase isoform is α1 (**Figure 13B**). Phosphotyrosine bands were not detectable in immunoprecipitates isolated from either lens or kidney membrane preparation that was not treated with Lyn.

The results suggest Na,K-ATPase α1 polypeptide is subject to tyrosine phosphorylation by Lyn. To confirm this idea, studies were conducted to test whether Na,K-ATPase α1 protein could be immunoprecipitated from Lyn-treated lens

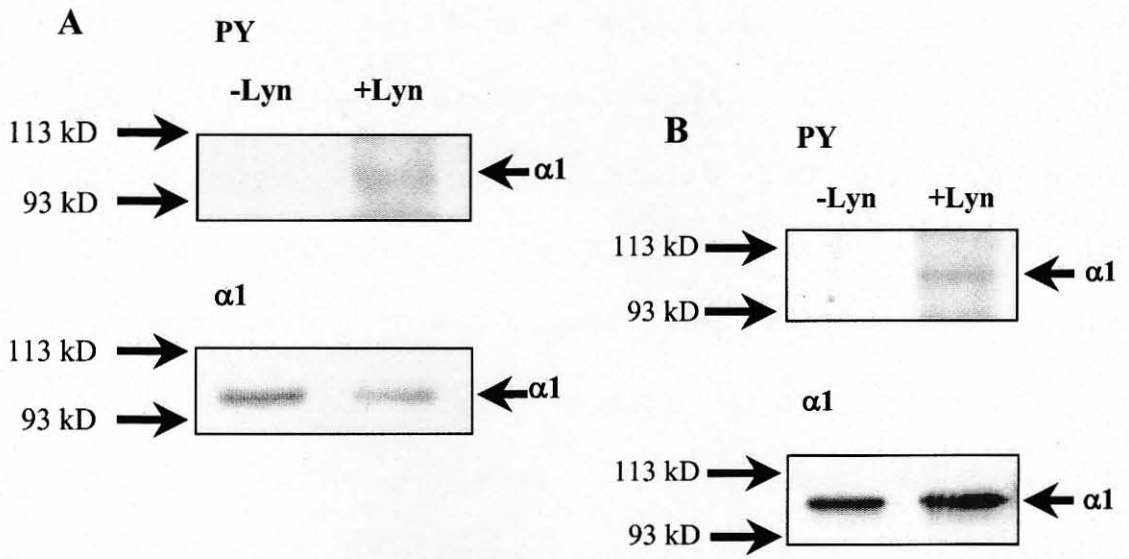



**Figure 13. Immunoprecipitation of Na,K-ATPase α1 subunit from Lyn kinase-treated lens epithelium and kidney membrane preparation.** Lens epithelium membrane preparation (Panel A) or kidney membrane preparation (Panel B) was incubated in the presence (+Lyn) or absence (-Lyn) of partially purified Lyn kinase in ATP-containing buffer for 20 minutes. Treated membrane preparation was immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase α1. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal antibody directed against phosphotyrosine (PY) residues (upper). The phosphotyrosine blot was then stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (lower).

membrane preparation using a monoclonal antibody directed against tyrosine phosphoproteins. A 100 kDa phosphotyrosine band immunopositive for Na,K-ATPase α1 protein was observed in immunoprecipitates isolated from Lyn-treated lens membrane preparation (**Figure 14**). Neither Na,K-ATPase α1 nor phosphotyrosine bands were detected in immunoprecipitates isolated from non-Lyn treated lens epithelium membrane preparation or control samples where the immunoprecipitating antibody was substituted for mouse IgG.

In some studies, lens epithelium membrane preparation was first treated with Lyn kinase and then subjected to tyrosine phosphatase treatment with PTP-1B. Treated membrane preparation was then immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase α1. A 100 kDa phosphotyrosine band was observed in the Na,K-ATPase α1 immunoprecipitate obtained from Lyn kinase-treated lens epithelium membrane preparation but not from membrane preparation that had subsequently been treated with PTP-1B (**Figure 15**). Na,K-ATPase activity measured in Lyn-treated lens epithelium membrane preparation increased from 7.1 ± 1.7 nmoles $P_i$/mg protein/minute to 12.3 ± 2.4 by subsequent PTP-1B treatment (**Figure 16**). This represents a 39.5 ± 14%

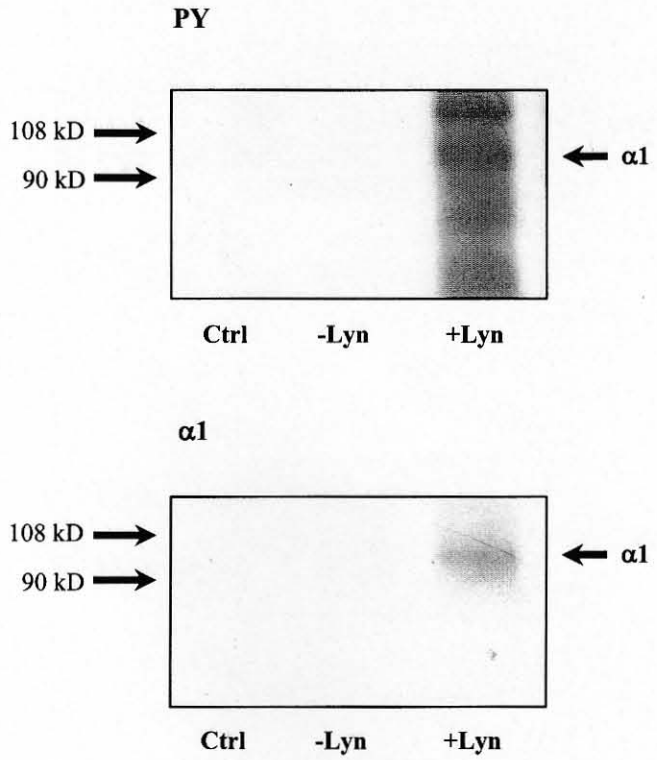



Figure 14. Immunoprecipitation of Na,K-ATPase α1 from Lyn kinase-treated lens epithelium using an antibody directed against phosphotyrosine residues. Lens epithelium membrane preparation was incubated in the presence (+Lyn) or absence (-Lyn) of partially purified Lyn kinase in ATP-containing buffer for 20 minutes. Treated membrane preparation was immunoprecipitated with a monoclonal antibody directed against tyrosine phosphoproteins. In the control, the immunoprecipitating antibody was omitted and mouse IgG was substituted. Immunoblots were probed for tyrosine phosphoproteins (PY) (upper). The blot was then stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (lower).

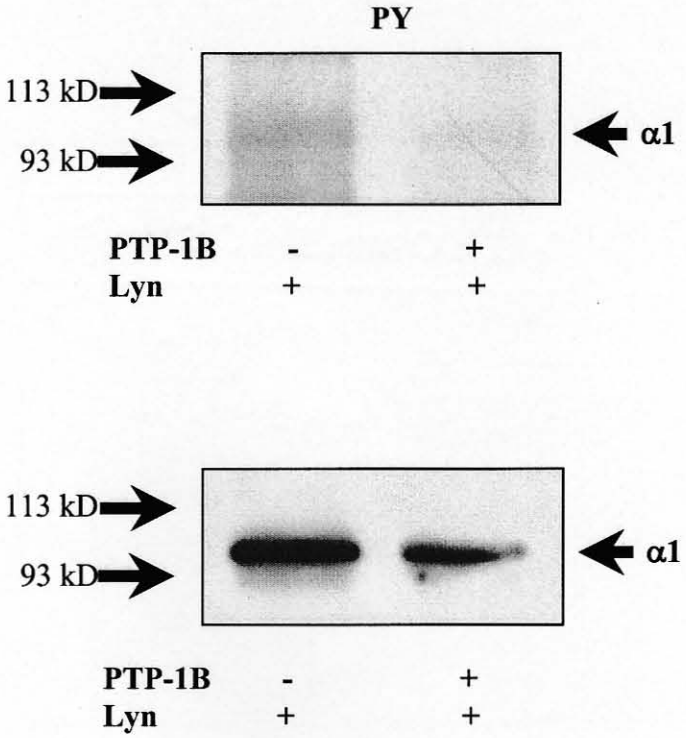



**Figure. 15. The influence of PTP-1B on Lyn kinase-treated lens epithelium membrane preparation.** Lens epithelium membrane preparation was incubated with partially purified Lyn kinase in ATP containing buffer for 20 minutes. Membrane preparation was then pelleted and incubated in the presence or absence of PTP-1B (40 Units) in reaction buffer for 20 minutes. Treated membrane preparation was immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase α1. Immunoprecipitated samples were subjected to SDS-PAGE and western blot and probed for tyrosine phosphoproteins (PY) (upper). The phosphotyrosine blot was stripped and reprobed with an antibody directed against Na,K-ATPase α1 (lower).

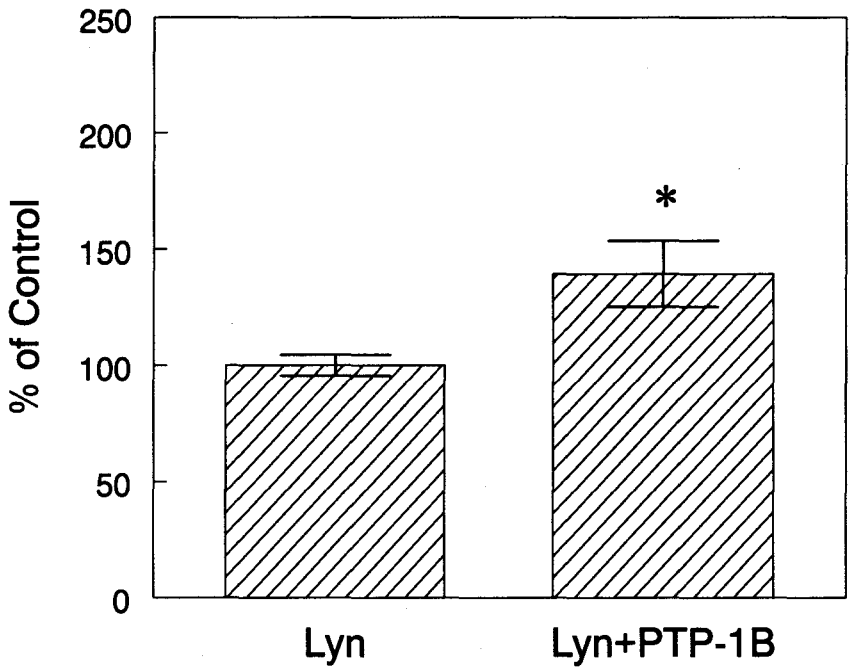



**Figure 16. Na,K-ATPase activity measured in Lyn-treated lens epithelium membrane preparation subsequently treatment with PTP-1B.** Lens epithelium membrane preparation was incubated in the presence of Lyn in ATP-containing buffer for 20 minutes. The Lyn-treated material was washed and treated with PTP-1B for 20 minutes. The PTP-1B treated material was washed and Na,K-ATPase activity measured. The data are shown as mean ± SE (*vertical bar*) of results from 5-6 independent determinations. *Indicates a significant difference from control ($P<0.05$).

increase in Na,K-ATPase activity (data as mean ± SE; n = 5; $p < 0.05$). In comparison, PTP-1B failed to change Na,K-ATPase activity in lens epithelium membrane preparation that was not pretreated with Lyn (**Figure 19B**).

## DISCUSSION

The results suggest tyrosine phosphorylation of isolated, partially-purified lens epithelium membrane preparation can partially inhibit Na,K-ATPase activity. Lyn treatment caused Na,K-ATPase inhibition and PTP-1B reversed the effect. The findings add to previous evidence for an association between tyrosine phosphorylation and inhibition of Na,K-ATPase activity in the intact lens (Li et al., 1994, Mathias et al., 1997). The Na,K-ATPase inhibition response does not appear unique to lens cells because similar findings were observed in kidney medulla. Since multiple membrane proteins, including the Na,K-ATPase α1 catalytic subunit polypeptide, were subject to tyrosine phosphorylation by Lyn, it is not possible to specify the extent to which the observed change of Na,K-ATPase activity depended on tyrosine phosphorylation of a specific protein.

Na,K-ATPase α1 is the main isoform expressed in porcine lens cells and neither Na,K-ATPase α2 or α3 isoforms are detectable by western blot (Delamere et al., 1996). Immunoprecipitation using antibodies directed against Na,K-ATPase α1 and against phosphotyrosine residues confirmed tyrosine phosphorylation of Na,K-ATPase α1 polypeptide in Lyn-treated membrane preparation. It was also demonstrated that Na,K-ATPase α1 polypeptide could be isolated from lens epithelium first by immunoprecipitation and then subjected to Lyn treatment to elicit tyrosine phosphorylation detectable by western blot. Furthermore, recombinant protein tyrosine phosphatase PTP-1B was found to cause reversal of Lyn-induced tyrosine phosphorylation. Taken together, the results suggest Lyn causes tyrosine phosphorylation of Na,K-ATPase α1 polypeptide.

Exogenous Lyn was used in this study as a means of causing tyrosine phosphorylation in isolated lens epithelium membrane preparation. Endogenous Lyn kinase was detected in lens epithelium, but the western blot results do not provide information on its activation state. Lyn kinase, a member of the Src-family of tyrosine kinases, is a membrane-associated non-receptor tyrosine kinase that also is expressed in myeloid and B lymphoid hematopoietic

cells (DeFranco et al., 1998). Lyn has also been detected in human endometrium and brain endothelium where it is thought to play an important role in human reproduction and blood-brain-barrier development respectively (Achen et al., 1995; Couchman et al., 1997). It is also expressed in intestinal crypt cells (Siyanova et al., 1994). Alternative splicing of the Lyn gene results in the expression of Lyn A (56 kDa) and Lyn B (53 kDa) forms of Lyn (Stanley et al., 1991). Except for a 20 amino acid deletion, Lyn B is identical to Lyn A (Yi et al., 1991). Lyn kinase is thought to be involved in signal transduction mechanisms following activation of B-cell antigen receptors, FcE high affinity receptors (Hibbs et al., 1995), and interleukin-3 receptors (Torigoe et al., 1992). Lyn kinase phosphorylates many substrates including phosphoinositol-3 kinase (PI3-K), ras GTPase activating protein (GAP), and mitogen-activating protein kinase (MAPK) (Corey et al., 1993).

Several studies have shown that the Na,K-ATPase α1 subunit is phosphorylated by PKC and PKA on serine and threonine residues (Bertorello et al., 1991; Chibalin et al., 1992). However, the existence of additional phosphorylation sites was suspected because neither the deletion of the known Na,K-ATPase α1 subunit serine and

threonine sites nor treatment with PKC or PKC inhibitors was able to fully suppress residual levels of Na,K-ATPase α1 phosphorylation (Beguin et al., 1994). This fits with the notion that Na,K-ATPase α1 can be subject to tyrosine phosphorylation. To examine a possible tyrosine phosphorylation site, Feraille et al. (1997) analyzed Na,K-ATPase pump activity in opossum kidney (OK) cells transfected with mutant Na,K-ATPase α1 in which tyrosine-10 was substituted either by alanine or glutamate. Insulin-induced stimulation of Na,K-ATPase function was suppressed in cells expressing the Y10 substitutions. Consistent with the idea of phosphorylation of the Na,K-ATPase α1 subunit at Y10 in the presence of a Src-family tyrosine kinase, this region of the Na,K-ATPase α1 protein exhibits a Src kinase consensus phosphorylation sequence composed of multiple acidic residues (Zhou et al., 1995). Interestingly, the gastric $H^+$,$K^+$-ATPase proton pump is also subject to phosphorylation at Y10 (Togawa et al., 1995).

Lyn kinase-induced inhibition of Na,K-ATPase activity in isolated, partially-purified lens epithelium and kidney medulla membrane preparation observed in the present study differs from the response to insulin and other agonists in intact astrocytes, proximal tubule and skeletal muscle

where tyrosine phosphorylation is associated with stimulation of Na,K-ATPase function (Matsuda et al., 1993; Feraille et al., 1997; Feraille et al., 1999; Narkar et al., 2002). This may reflect differences in the cascade of events triggered by insulin and partially-purified Lyn, differences in the response of intact cells where changes in Na,K-ATPase synthesis or recruitment to the plasma membrane may occur (Matsuda et al., 1993; Feraille et al., 1999; Narkar et al., 2002), differences in cell-specific regulatory mechanisms, or differences in Na,K-ATPase α1 isoform characteristics. In the porcine lens, the Na,K-ATPase α1 isoform is predominant although long term changes in Na,K-ATPase activity might occur through the upregulation of the α2 subunit in response to alteration of cytoplasmic ion balance (Delamere et al., 1996).

The lens epithelium is specialized for active sodium-potassium transport. Na,K-ATPase-mediated ion transport by the epithelial monolayer is essential for maintenance of electrolyte homeostasis in the mass fiber cells that constitute the bulk of the lens (Mathias et al., 1997). The results of the present study suggest changes in the activity of Lyn or other tyrosine kinases could lead to modulation of Na,K-ATPase function in lens epithelium.

Modulation of Na,K-ATPase activity as the result of Lyn kinase activation has not previously been reported and while there is strong evidence from several different cell types indicating the susceptibility of Na,K-ATPase to tyrosine phosphorylation, the identity of the tyrosine kinases that influence Na,K-ATPase in intact tissues is not known. (Feraille et al., 1999; Yingst et al., 2000; Okafor and Delamere, 2001) In gastric mucosa there is evidence suggesting that plasma membrane $H^+,K^+$-ATPase is subject to tyrosine phosphorylation (Kanagawa et al., 1999) and chromatographic separation of deteregent-solubilized membrane preparation revealed an endogenous Src-family kinase at ~60 kDa (Kanagawa et al., 2000). Src-family kinases are known to influence other ion transporters. In platelets for example, phosphorylation of plasma membranes by $pp60^{Src}$ kinase resulted in significant inhibition of calcium ATPase activity that correlated with the degree of PMCA tyrosine phosphorylation (Dean et al., 1997). In mouse erythrocytes, activation of Src-family tyrosine kinases appears to modify $K^+$-$Cl^-$ cotransporter function (De Franceschi et al., 2001). In the lens, Lyn kinase is likely to be just one of several non-receptor tyrosine kinases and it is possible that other tyrosine kinases also influence Na,K-ATPase activity. The present experiments

did not permit us to identify the tyrosine kinases activated by thrombin.

Several studies have identified Src-family tyrosine kinases in the lens. It has been suggested that non-receptor tyrosine kinases play an essential role in differentiation with inhibition of Src-family tyrosine kinases acting as one of the events required for lens epithelial cells to withdraw from the cell cycle and commence differentiation toward the lens fiber cell phenotype (Walker et al., 2002). When the intact lens is maintained in organ culture, inhibition of Src-family tyrosine kinases with PP1 appears to prevent opacification (Zhou and Menko, 2002).

In summary, the results of the present study suggest Na,K-ATPase activity in lens epithelium is susceptible to modulation by tyrosine phosphorylation. The significance of modulating Na,K-ATPase activity in lens epithelium remains to be determined. It has been proposed that spatial localization of high Na,K-ATPase activity to specific regions of the lens surface is essential in order to support circulation of electrical currents that work via electroosmosis to speed solute movement through the tortuous extracellular space between the tightly packed lens cells (Mathias et al., 1997). Although Na,K-ATPase

protein is abundant in all lens cells (Delamere and Dean, 1993; Dean et al., 1996), Na,K-ATPase activity is higher at the epithelium than the fibers and highest in epithelium at the equator of the lens (Gao et al., 2000; Candia and Zamudio, 2002; Tamiya et al., 2003). In order to establish the circulating currents, there may be a need for mechanisms that modulate Na,K-ATPase activity to produce unequal activity in different parts of the lens.

## CHAPTER III

## THE INFLUENCE OF PROTEIN TYROSINE PHOSPHATASE-1B ON Na,K-ATPase ACTIVITY IN LENS

### INTRODUCTION

Na,K-ATPase is an active transport protein complex present in all animal cells. The functionally active Na,K-ATPase heterodimer is composed of a 112 kDa catalytic α-subunit and a 35 kDa non-catalytic β-subunit (Askari, 1987). The Na,K-ATPase complex couples the hydrolysis of ATP to the outward translocation of sodium ions and inward translocation of potassium ions against their electrochemical gradients. Thus, the normally high intracellular potassium and low intracellular sodium concentrations are maintained by Na,K-ATPase (Blaustein and Lederer, 1999). In the eye lens, as in other tissues, Na,K-ATPase-mediated regulation of cytoplasmic ion composition serves indirectly to preserve osmotic equilibrium (Davson, 1990). Inhibition of lens Na,K-ATPase activity leads to cell swelling, and this in turn can lead to calcium accumulation and loss of optical transparency

(Hightower and Farnum, 1985). Interestingly, the degree of cortical opacification in human cataract has been correlated with high lens sodium content (Duncan and Bushell, 1975).

In the lens, Na,K-ATPase activity is distributed unequally. Fiber cells, which are highly differentiated, make up almost the total mass of the lens. Fiber cells display lower Na,K-ATPase specific activity than the epithelium monolayer, non-differentiated cells that cover just the anterior surface (Delamere and Dean, 1993). In spite of their very different Na,K-ATPase activities, the two lens cell types have a similar abundance of Na,K-ATPase catalytic ($\alpha$) subunit protein (Delamere and Dean, 1993). This suggests there could be regulatory mechanisms that modify Na,K-ATPase activity.

In several tissues it is known that Na,K-ATPase activity can be modulated as a result of serine/threonine phosphorylation of the Na,K-ATPase $\alpha$ subunit polypeptide (Therien and Blostein, 2000). More recently, activation of tyrosine kinases has also been shown to alter Na,K-ATPase activity. Stimulation of Na,K-ATPase activity by insulin has been shown to require the phosphorylation of Y10 in kidney tubule cells (Feraille et al., 1999). In the lens, thrombin-induced inhibition of Na,K-ATPase-mediated active

ion transport is suppressed in the presence of herbimycin A, a Src-family tyrosine kinase inhibitor (Okafor et al., 1999). In a recent study, the Src-family Lyn was shown to be capable of inducing tyrosine phosphorylation of Na,K-ATPase α1 subunit in porcine lens epithelium. The Lyn response was associated with inhibition of Na,K-ATPase activity (Bozulic et al., 2003).

In most cells, tyrosine phosphorylation is reversible. Regulation of tyrosine phosphorylation occurs through the opposing activities of tyrosine kinases and protein tyrosine phosphatases (PTPases) that become activated in response to a variety of cellular signals (Blanquet and Croquet, 1995a). Thus, a decreased level of protein tyrosine phosphorylation in differentiating HL-60 cells is observed following PTPase activation (Frank and Sartorelli, 1986). PTPases have a variety of roles. For example, PTPases have been implicated in neuronal differentiation (den Hertog et al., 1993) as well as differentiation of the adult bovine lens (Blanquet and Croquet, 1995b). Insulin-induced maturation of Xenopus oocytes is inhibited in the presence of PTP-1B, a non-transmembrane tyrosine phosphatase (Tonks et al., 1990). PTP-1B is believed to be localized within the endoplasmic reticulum of most cells through a C-terminal 35-amino acid protein sequence

(Frangioni et al., 1992). In platelets, PTP-1B is shuttled to the cytosol following cleavage of the 35-amino acid sequence by a calcium-dependent calpain protease (Frangioni et al., 1992).

In the present study, we examined the influence of PTP-1B on Na,K-ATPase activity. PTP-1B treatment caused an increase of Na,K-ATPase activity in fiber membrane preparation but not epithelium membrane preparation. The PTP-1B response was consistent with endogenous tyrosine phosphorylation of Na,K-ATPase α1 subunit protein observed in porcine lens fibers.

## MATERIALS AND METHODS

All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO). Nitrocellulose membranes, filter paper, pre-stained low molecular weight markers, bio-safe Coomassie blue, IPG strips, and goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bio-Rad (Richmond, CA). The chemiluminescent detection substrate and bicinchoninic acid (BCA) protein detection assay kit were purchased from Pierce Chemical (Rockford, IL). PTP-1B tyrosine phosphatase, anti-PTP-1B monoclonal antibody, and

monoclonal antibodies against Na,K-ATPase α1 were all purchased from Upstate Biotechnology (Lake Placid, NY). HRP-conjugated phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY).

***Tissues***

Porcine eyes were kindly donated by the Swift Meat Packing Company (Louisville, KY). The lens was isolated by dissecting open the posterior of the eye and cutting the zonules. The lens capsule and attached anterior monolayer of epithelial cells was removed from the fiber mass and snap-frozen in liquid nitrogen. The use of animal tissues in this study conformed to the ARVO statement on the Use of Animals in Ophthalmology and Vision Research.

***Membrane Preparation***

Membrane preparations were obtained using the methodology described by Okafor et al. (1999). Previously frozen samples of lens capsule-epithelium or lens cortex were homogenized using a glass homogenizer in ice-cold homogenization buffer A (150 mM sucrose, 4 mM EGTA, 5 mM Hepes, 800 μM dithiothreitol (DTT), 1 mM sodium orthovanadate, pH 7.4) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL antipain,

10 μg/mL leupeptin, 10 μg/mL pepstatin, 2 μg/mL aprotinin). The homogenate was subjected to centrifugation at 115,000g for 60 minutes at 4°C. To remove extrinsic proteins, the membrane pellet was resuspended in ice-cold homogenization buffer A containing 600 mM KCl then subjected to centrifugation once again at 115,000g for 60 minutes at 4°C (Dean et al., 1996). The resulting membrane pellet was resuspended in ice-cold homogenization buffer A and subjected to centrifugation a final time at 115,000g for 60 minutes at 4°C. The final pellet containing epithelium or fiber membrane preparation was resuspended in buffer A and the protein content measured using the BCA protein assay kit (Bio-Rad, Richmond, CA).

***PTB-1B treatment and Na,K-ATPase activity measurement***

In specified experiments, membrane preparation was incubated with recombinant protein tyrosine phosphatase 1B (PTP-1B) (100 mUnits/μl) (Upstate Biotechnology, Lake Placid, NY) at 37°C in phosphatase buffer (25 mM Hepes, 50 mM NaCl, 5 mM DTT, 2.5 mM EDTA, 100 μg/ml bovine serum albumin (BSA), 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 1mM PMSF, pH 7.2). Treated material was then used for western blot analysis or Na,K-ATPase activity measurements.

To remove phosphatase buffer constituents prior to Na,K-ATPase activity measurements the membrane preparation was centrifuged at 100,000g for 3 minutes. The membrane pellet was resuspended three times in 100 $\mu$l of centrifugation buffer (10 mM Tris pH 7.2, 5 mM DTT, 10%(w/v) glycerol) and centrifuged at 100,000g for 3 minutes. The final pellet was resuspended in ~100 $\mu$l of Na,K-ATPase buffer and assayed immediately for Na,K-ATPase activity.

Na,K-ATPase activity was measured following methodology described by Okafor et al. (1999). Aliquots of PTP-1B treated and untreated membrane preparation (~100 $\mu$g) were added to Na,K-ATPase buffer (100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1mM EGTA, pH 7.4). Ouabain, a specific inhibitor of Na,K-ATPase (Wallick and Schwartz, 1988), was added to half the samples (final concentration 1 mM) which then were preincubated for 15 minutes at 37°C with gentle agitation. ATP hydrolysis was initiated by the addition of ATP (final concentration 1 mM). After 45 minutes at 37°C, the ATP hydrolysis reaction was stopped by the addition of 15% ice-cold trichloroacetic acid. ATP hydrolysis was quantified using a colorimetric method to measure the amount of inorganic phosphate released. Na,K-ATPase activity was calculated as the difference between ATP hydrolysis in the

presence and absence of ouabain. The data are presented as nanomoles phosphate released per mg protein per minute.

***Western Blot analysis***

Lens epithelium or fiber membrane preparation was solubilized and proteins separated on a 7.5% gel by SDS-PAGE at 40 mA for 2 hours using the Laemmli buffer system (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose at 30 V for 16 hours. Nitrocellulose membranes were blocked 60 minutes with 5% dry milk in TTBS (30 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.4). To detect immunoreactive Na,K-ATPase α1, PTP-1B, or tyrosine phosphoproteins, the nitrocellulose membranes were incubated for 60 minutes at room temperature with either a monoclonal antibody directed against Na,K-ATPase α1 (Sigma, St. Louis, MO), a polyclonal antibody directed against PTP-1B (Upstate Biotechnology, Lake Placid, NY), or a monoclonal anti-phosphotyrosine antibody (PY20) (Transduction Lab, Lexington, KY) conjugated to horseradish peroxidase. After this, nitrocellulose membranes probed for Na,K-ATPase α1 and PTP-1B were washed with TTBS two times for 15 minutes followed by three times for 5 minutes before being incubated for 60 minutes with a horseradish

peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Nitrocellulose membranes probed with PY20 were washed with TTBS two times for 15 minutes and then four times for 5 minutes at room temperature. To visualize the results, the nitrocellulose membranes were treated with chemiluminescence substrate for 1 minute (Pierce, Rockford, IL) then exposed to x-ray film.

***Immunoprecipitation***

Lens epithelium or fiber membrane preparation (500 μg) was solubilized in immunoprecipitation buffer (10 μM deoxycholate, 100 mM D-mannitol, 5 mM Tris pH 7.6, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin A) at a final protein concentration of 2 μg/μl. The solubilized membrane preparation was mixed for 3 hours on a rotating wheel at 4°C. After this, the insoluble material was pelleted by centrifugation at 10000g for 15 minutes at 4°C. The supernatant (250 μl) was removed and pre-cleared for 15 hr on a rotating wheel at 4°C with 20 μg of mouse IgG and 50 uL of immobilized protein G (ImmunoPure; Pierce, Rockford, IL). The resulting mixture was then centrifuged at 1000g for 3 minutes at 4°C, then the supernatant was removed and pre-cleared once again with 20 μg of rabbit IgG and 50 uL of immobilized protein A

(ImmunoPure; Pierce, Rockford, IL) for 3 hours on a rotating wheel at 4°C. The supernatant was then transferred to a clean microcentrifuge tube and 10 μg of polyclonal antibody directed against Na,K-ATPase α1 polypeptide (Upstate Biotechnology, Lake Placid, NY) was added and the mixture was placed on a rotating wheel for 15 hours at 4°C. Next, 50 μl of immobilized protein A (ImmunoPure, Pierce, Rockford, IL) was added and mixed for an additional 3 hours on a rotating wheel at 4°C. The mixture was then washed three times with 200 μL of phosphate buffered saline (PBS) at pH 7.4, centrifuging each time at 1000g for 3 minutes at 4°C. Immunoprecipitated Na,K-ATPase α1 polypeptide was dissociated from the Protein A-antibody mixture by incubation with 45 μL of Laemmli buffer for 20 minutes at 65°C (Laemmli, 1970). Finally, the samples were centrifuged at 4000g for 5 minutes and the supernatant subjected to SDS-PAGE followed by western blot analysis.

## RESULTS

Studies were carried out to examine tyrosine phosphorylation of membrane protein isolated from lens epithelium and fiber cells. Samples were resolved by SDS-PAGE and subjected to western blot analysis for tyrosine

phosphoproteins. Multiple phosphotyrosine protein bands were detected in both lens epithelium and fiber cell membrane preparation. However, distinct differences in band pattern and band intensities were observed between the two tissues (**Figure 17**).

Na,K-ATPase α1 is the only α isoform detectable in porcine lens (Delamere et al., 1996). To examine Na,K-ATPase, lens membrane preparation was subjected to immunoprecipitation using a polyclonal antibody directed against the Na,K-ATPase α1 subunit. Immunoprecipitated samples were subjected to SDS-PAGE then western blot analysis for phosphotyrosine proteins. A 100 kDa phosphotyrosine band was detected in samples isolated from fiber membrane preparation (**Figure 18A**). The phosphotyrosine band was not detected in epithelium membrane preparation (**Figure 18B**) nor in negative controls in which membrane preparation was omitted from the immunoprecipitation procedure.

Endogenous Na,K-ATPase α1 protein tyrosine phosphorylation is evident in lens fibers. To study the possible effect of tyrosine dephosphorylation on Na,K-ATPase activity, membrane preparation was incubated with PTP-1B for 30 minutes then washed to remove the phosphatase

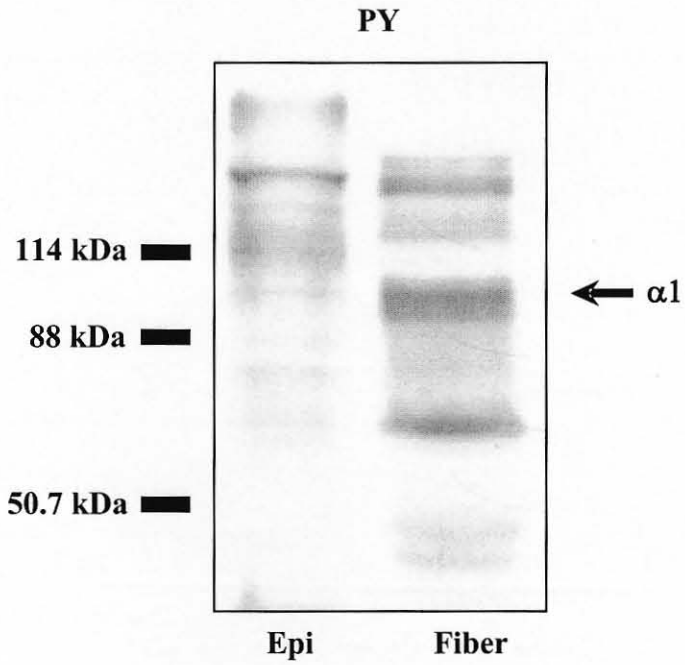



**Figure 17. Endogenous tyrosine phosphorylation of lens cell membrane preparation.** Panel A: phosphotyrosine (PY) western blot. Lens epithelium and fiber membrane preparation (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against tyrosine phosphoproteins (PY). The right hand arrow indicates the predicted position of Na,K-ATPase α1 protein.

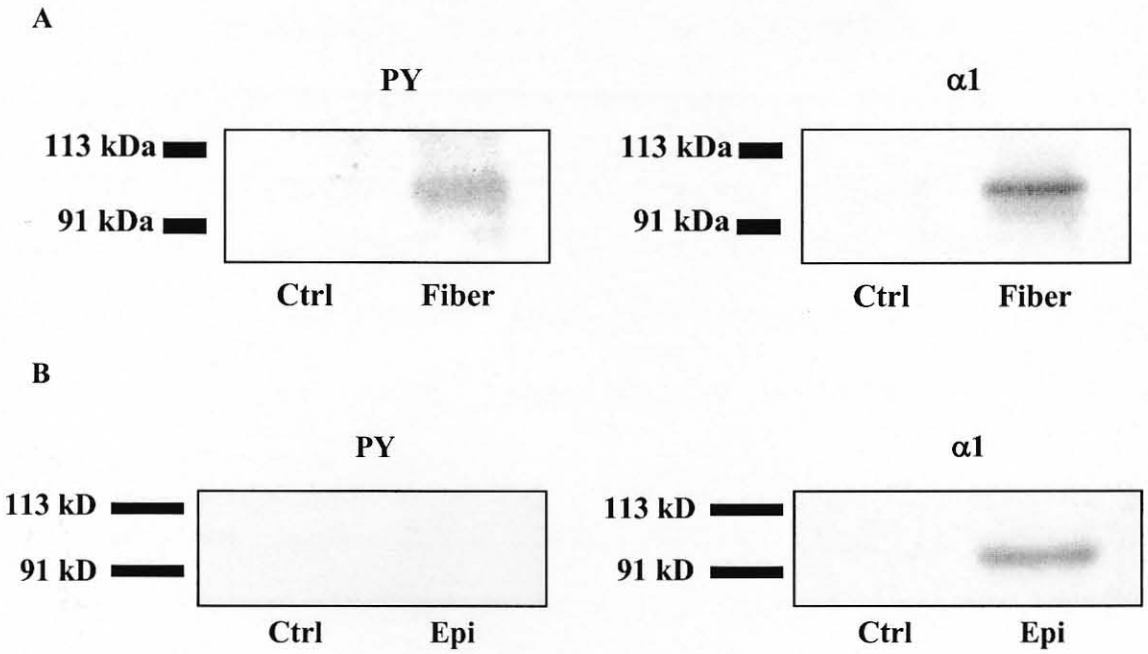



**Figure 18. Immunoprecipitation of Na,K-ATPase α1 subunit from lens fiber (Panel A) and epithelium (Panel B) membrane preparation.** Membrane preparation was immunoprecipitated with a polyclonal antibody directed against Na,K-ATPase α1. Immunoprecipitated samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against tyrosine phosphoproteins (PY) (left). The phosphotyrosine blot was stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (right). In control samples, the immmunoprecipitating antibody was present in the absence of epithelium membrane preparation.

buffer constituents prior to measurement of ouabain-sensitive ATP hydrolysis as described in Methods. Na,K-ATPase activity was increased by ~50% in fiber membrane preparation subjected to PTP-1B treatment (**Figure 19A**). Na,K-ATPase activity in lens epithelium membrane preparation was not significantly altered by PTB-1B treatment (**Figure 19B**).

Studies were conducted to confirm PTP-1B is capable of dephosphorylating membrane proteins in both lens epithelium and fibers. Lens epithelium and fiber membrane preparation were incubated with recombinant PTP-1B then subjected to SDS-PAGE and immunoblotted with a monoclonal antibody directed against phosphotyrosine residues (**Figure 20**). PTP-1B treatment resulted in reduction in the intensity of most phosphotyrosine bands. Time course studies showed significant dephosphorylation was achieved within 10 minutes (**Figure 21**).

Western blot analysis suggests PTP-1B is expressed in lens cells. Lens epithelium and fiber cell lysates were isolated and subjected to western blot analysis. A dense immunopositive band was detected in lens fiber cell lysates (**Figure 22**). A similar PTP-1B immunopositive band was detected in the 3T3 cell lysate positve control. An immunopositive PTP-1B band was detected in lens epithelium

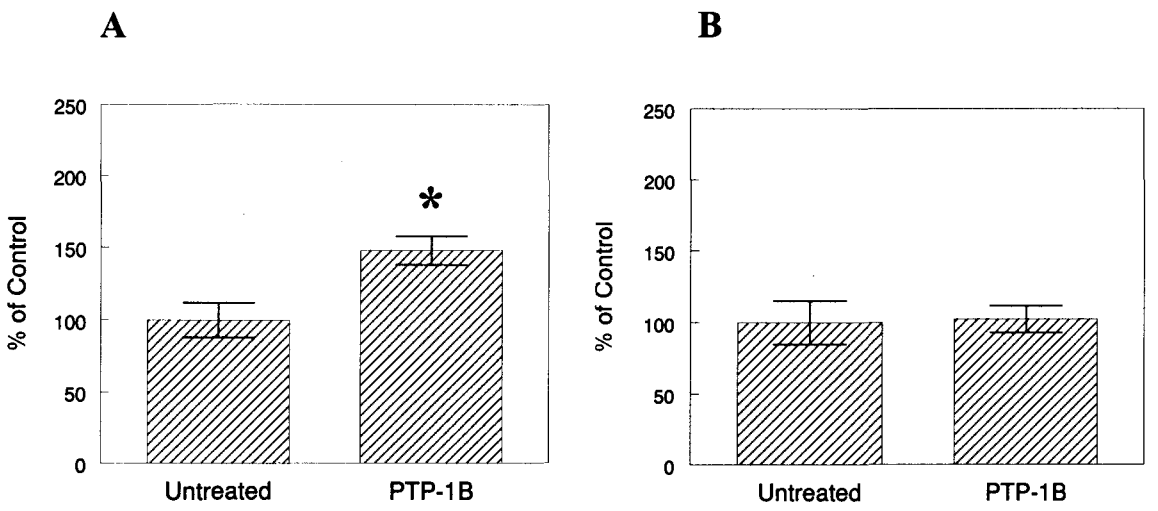



**Figure 19. Na,K-ATPase activity (ouabain-sensitive ATP-hydrolysis) measured in isolated fiber membrane preparation (Panel A) or epithelium material (Panel B)** previously subjected to incubation for 10 minutes in the presence or absence (untreated) of recombinant PTP-1B (100 mU/μl). The data are shown as mean ± SE (*vertical bar*) of results from 40 measurements using 8 different batches of membrane preparation or 8 measurements from two different batches of epithelium membrane preparation. *Indicates a significant difference from untreated ($P<0.01$). Control Na,K-ATPase activity in fiber and epithelium membrane preparation was 2.5 ± 1.1 and 9.1 ± 2.5 nmoles phosphate released/mg protein/minute, respectively.

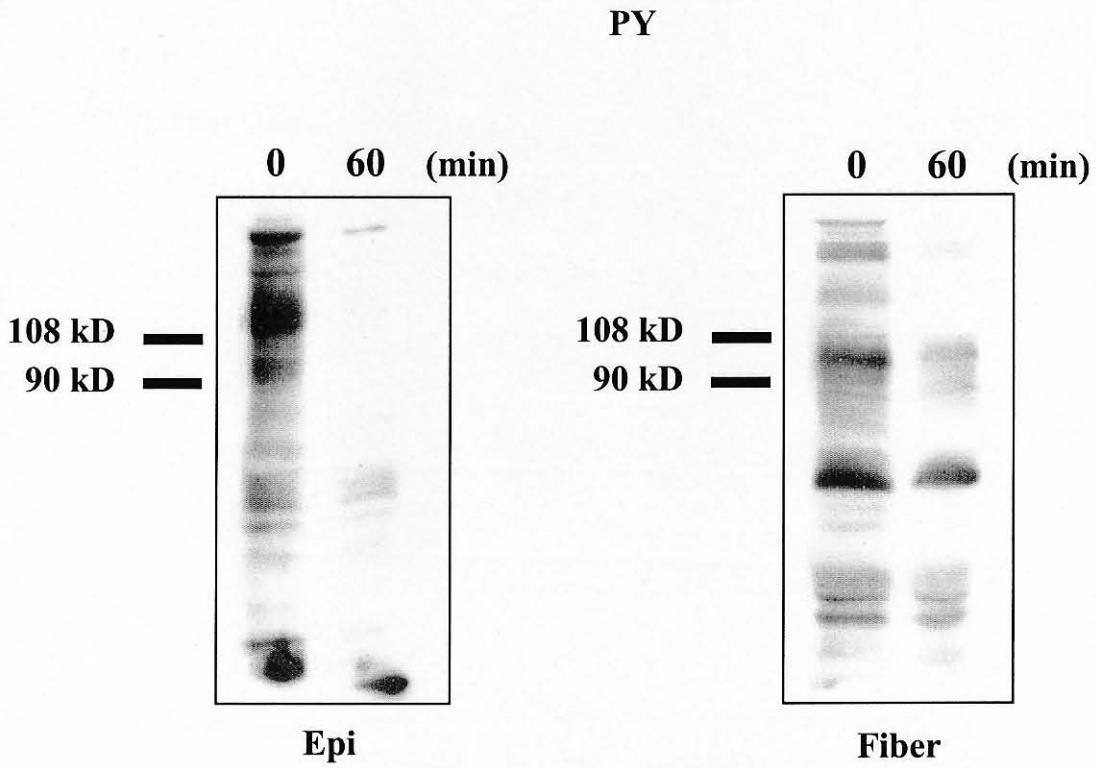



**Figure 20. Tyrosine dephosphorylation by PTP-1B.** Lens fiber or epithelium membrane preparation (50 μg) was incubated with recombinant PTP-1B for 60 minutes. Phosphotyrosine residues (PY) were then detected by western blot analysis.

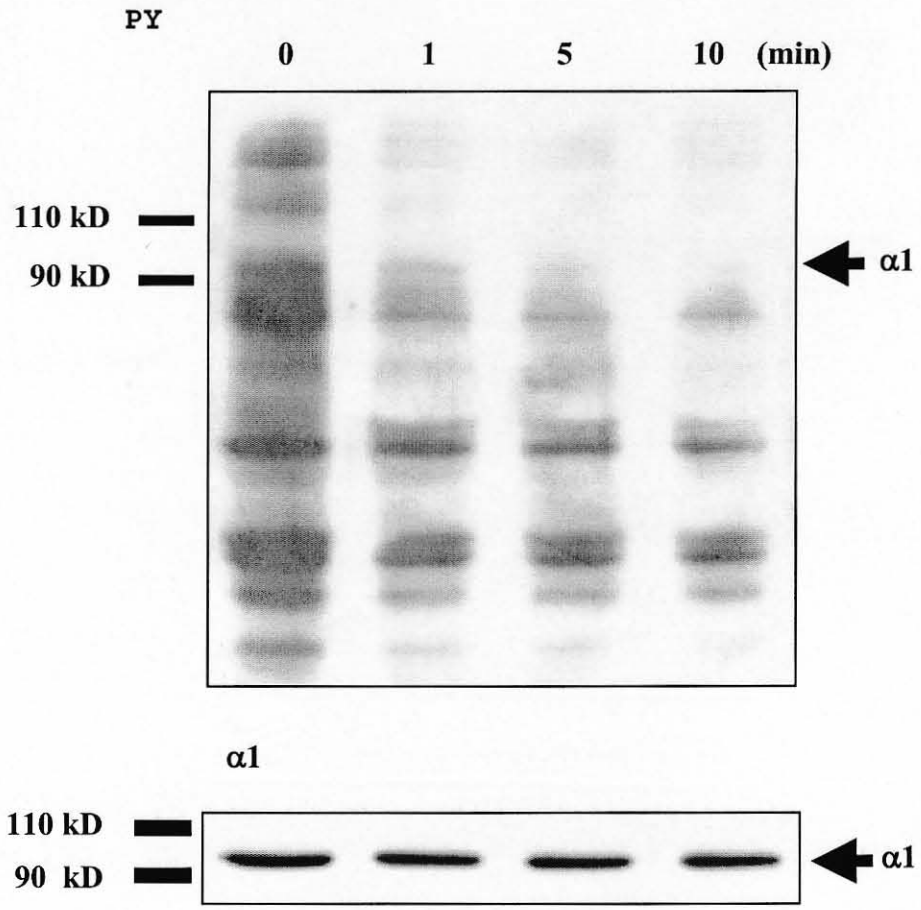



**Figure 21. Time course for tyrosine dephosphorylation by PTP-1B.** Lens fiber membrane preparation (50 µg) was incubated with recombinant PTP-1B (100 mUnits/µl) for 0-10 minutes. Phosphotyrosine residues (PY) were detected by western blot analysis (upper) then the blot was stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (lower). The right hand arrow indicates the predicted location of Na,K-ATPase α1.

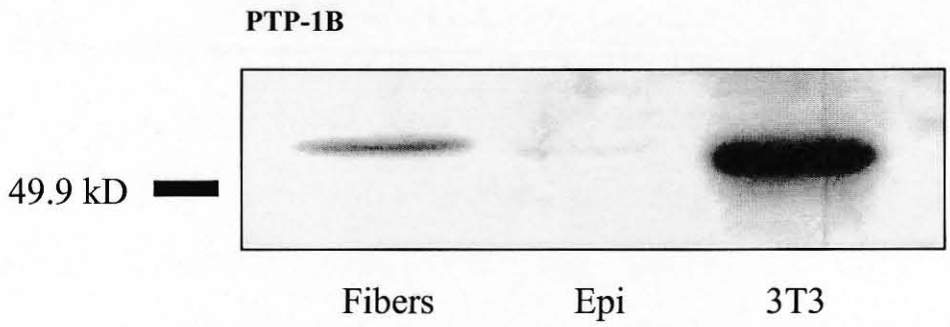



**Figure 22. Detection of PTP-1B in lens cell lysates.** Lens epithelium and fiber cell lysate samples (100 µg) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody directed against PTP-1B. 3T3 cell lysate (20 µg) was used as a positive control for PTP-1B expression.

cell lysate but the band intensity was considerably less than the band intensity in fibers.

To examine the influence of endogenous tyrosine phosphatases on the phosphorylation state of lens membrane proteins, fiber and epithelium membrane preparation was incubated in phosphatase buffer without added exogenous PTP-1B. Under these conditions there was only a slight reduction of phosphotyrosine band intensity (**Figure 23A, B**).

## DISCUSSION

Fibers and epithelium responded differently to PTP-1B. PTP-1B treatment of lens fiber membranes increased Na,K-ATPase activity by almost 50%. In contrast, PTP-1B treatment did not alter Na,K-ATPase activity in lens epithelium membrane preparation. Several tyrosine phosphorylated membrane protein bands were observed in both lens fiber and epithelial cells. In immunoprecipitation studies, endogenous tyrosine phosphorylation of a 100 kDa band immunopositive for Na,K-ATPase α1 protein was observed in lens fibers but not epithelium. Taken together, these results support the idea that a reduction in the endogenous tyrosine phosphorylation status of Na,K-ATPase α1 polypeptide may increase Na,K-ATPase activity in lens

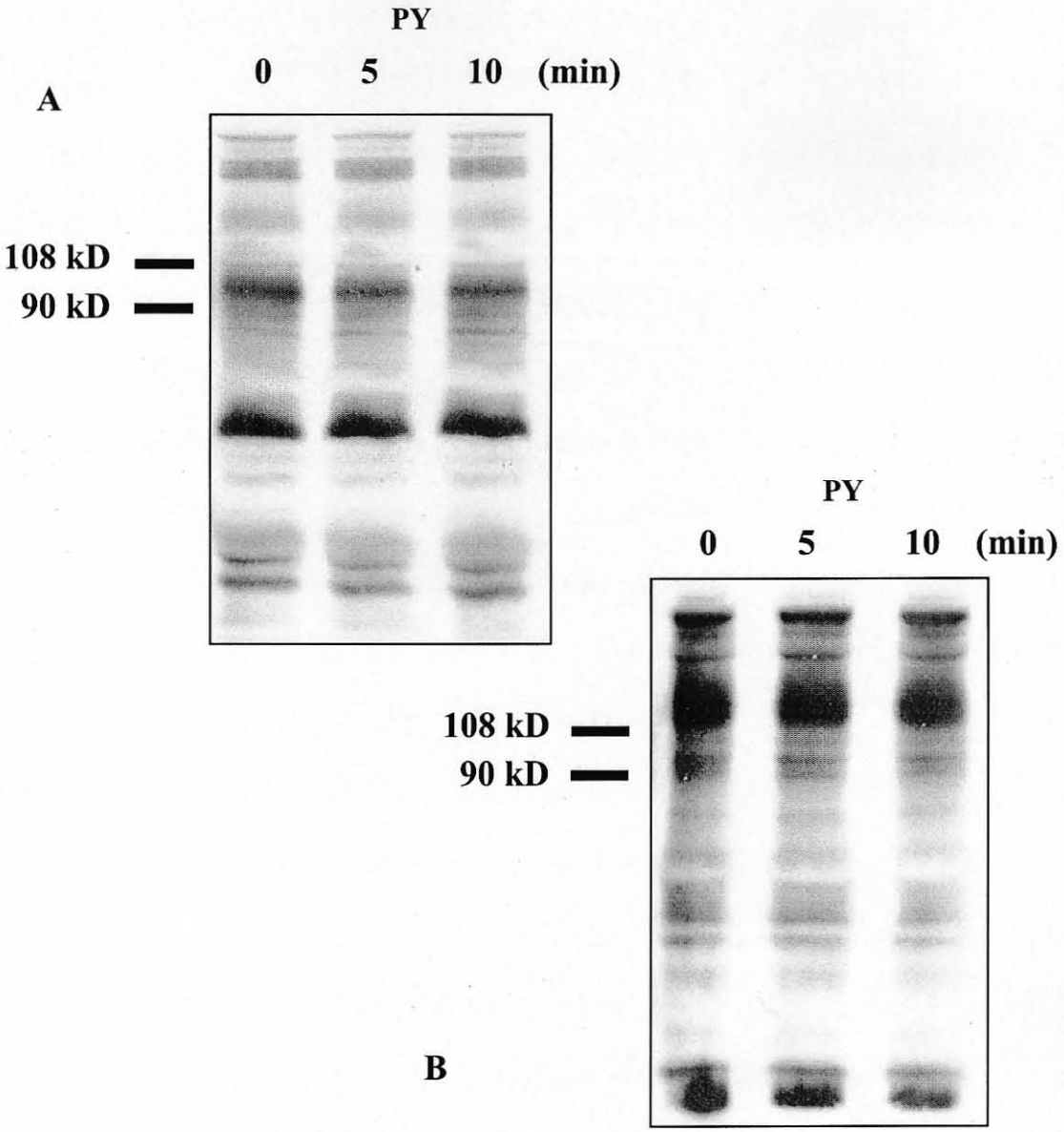



**Figure 23. Tyrosine dephosphorylation by endogenous phosphatases.** Lens fiber (A) or epithelium (B) membrane preparation (50 $\mu$g) was incubated in phosphatase buffer for 0-10 minutes. Membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against tyrosine phosphoproteins (PY).

fibers. Under normal circumstances, Na,K-ATPase activity is low in lens fibers. The ability of PTP-1B to alter fiber Na,K-ATPase activity may signify a mechanism for the lens to boost Na,K-ATPase activity in the cortex if additional ion transporting capacity is required.

Tyrosine phosphorylation of the Na,K-ATPase α1 subunit could be one of several mechanisms that contribute to the low Na,K-ATPase activity observed in lens fibers. In cultured human lens epithelium HLE-B3 cells, methionine incorporation studies showed that continuous Na,K-ATPase protein synthesis was necessary for normal Na,K-ATPase activity. When protein synthesis was inhibited, the Na,K-ATPase α1 protein abundance remained the same but Na,K-ATPase activity was reduced (Cui et al., 2002). A slow rate of protein turnover in lens fibers may leave the Na,K-ATPase polypeptide vulnerable to modifications that contribute to low Na,K-ATPase activity.

PTP-1B is a non-transmembrane tyrosine phosphatase originally discovered in human placenta (Tonks et al., 1988). It is expressed in a wide variety of tissues. As judged by immunoblot intensity, PTP-1B protein was more abundant in fibers than epithelium. The reason for this difference is unclear. The lens epithelium may express different tyrosine phosphatases. It has been reported

elsewhere that the abundance of tyrosine phosphatase protein does not necessarily reflect tyrosine phosphatase activity since the enzyme requires activation (Blanquet and Croquet, 1995a). Different mechanisms for PTP-1B activation have been proposed. Phosphorylation of PTP-1B at S50 by CLK1 or CLK2 (cdc2-like kinases) is required for PTP-1B enzymatic activation when both CLK1 or CLK2 are co-expressed with PTP-1B in HEK 293 cells (Moeslein et al., 1999). Tyrosine phosphorylation of PTP-1B at Y66 after binding to EGFR results in a 3-fold increase in PTP catalytic activity (Liu and Chernoff, 1997). Other evidence suggests that PTP-1B activity is determined by its association with the insulin receptor and Grb2 in pig skeletal muscle (Suryawan and Davis, 2003). The results of the present study suggest lens PTP-1B may exist in a non-active state since neither epithelium nor fiber cell membrane preparation exhibited marked tyrosine phosphatase activity as judged by lack of dephosphorylation of endogenous membrane protein bands.

Modulation of Na,K-ATPase activity by PTP-1B has not been previously reported. In the lens, regulation of Na,K-ATPase may be necessary for fiber cell differentiation. As the epithelium differentiates to become a fiber, the cell elongates fifty fold. Because Na,K-ATPase exports three $Na^+$

ions in exchange for two $K^+$ ions, it effects a net cation export. Partial inhibition of Na,K-ATPase activity in lens fibers would tend to promote cell swelling. It has been proposed earlier that a cell volume increase is required for fiber elongation (Beebe et al., 1990; Parmelee and Beebe, 1988). Other evidence suggests inhibition of Src-family tyrosine kinases is essential for epithelial cell withdrawal from the cell cycle and initiation of cell differentiation (Walker et al., 2002). Opacification appears to be prevented in whole lenses treated with PP1, a Src-family tyrosine kinase inhibitor (Zhou and Menko, 2002).

Protein phosphorylation and dephosphorylation in eukaryotic cells is a vital mechanism for regulating function. Na,K-ATPase activity in a variety of tissues is modulated by PKA and PKC phosphorylation events (Therien and Blostein, 2000). Tyrosine kinase activation in kidney appears to alter Na,K-ATPase activity (Feraille et al., 1997). Ion channels are also subject to phosphorylation. In rat ventricular myocytes, Kwak et al.(1996) showed that $K^+$ channel inhibition could be brought about either by phosphorylation of a tyrosine residue or by dephoshorylation of serine/threonine residues. The influences of kinases and phosphatases are counterbalanced. Thus, activation of the same $K^+$ channel occurs as the result

of phosphorylation of serine/threonine residues or by dephosphorylation of a phosphorylated tyrosine residue. Tyrosine phosphorylation of gastric $H^+$, $K^+$-ATPase appears to be reversed by a novel membrane-bound tyrosine phosphatase (Togawa et al., 1995). Previous studies in lens epithelium have shown that tyrosine kinase inhibitors genistein and herbimycin suppress Na,K-ATPase activity changes that occur in response to thrombin or endothelin (Okafor et al., 1999; Okafor and Delamere, 2001). Moreover, tyrosine phosphorylation of Na,K-ATPase α1 catalytic subunit by Lyn, a Src-family tyrosine kinase, is associated with a ~40% reduction of Na,K-ATPase activity in lens epithelium (Bozulic et al., 2003). In the same study, PTP-1B was found to reverse the Lyn-induced Na,K-ATPase α1 tyrosine phosphorylation response and also reverse the change of Na,K-ATPase activity. In kidney it has been shown that angiotensin II inhibition of Na,K-ATPase activity might require PTPase activation (Yingst et al., 2000). The current study suggests that activation of PTP-1B or other endogenous tyrosine phosphatases could possibly result in changes of Na,K-ATPase function in lens fibers. Tyrosine dephosphorylation and phosphorylation may be a mechanism for regulation of Na,K-ATPase activity in the lens.

# CHAPTER IV

# THE INFLUENCE OF SRC-FAMILY TYROSINE KINASES ON NA,K-ATPASE ACTVIITY IN LENS EPITHELIUM

## INTRODUCTION

Na,K-ATPase is an active ion transport protein complex ubiquitously expressed in the plasma membrane of animal cells. Acting as a pump, Na,K-ATPase couples the hydrolysis of ATP to the outward translocation of three $Na^+$ and inward translocation of two $K^+$ against their steep electrochemical gradients. Regulation of cytoplasmic electrolyte concentration is imperative for maintaining the transparency of the lens. It has been reported that Na,K-ATPase activity in the monolayer of epithelium covering the anterior lens surface must be high for normal lens function (Mathias et al., 1997). Fiber cells do not lack Na,K-ATPase polypeptides but exhibit a lower Na,K-ATPase activity than the epithelium (Delamere and Dean, 1993). In the lens epithelium, regulation of Na,K-ATPase activity and function has not been widely studied.

Na,K-ATPase function can be regulated through several different protein kinase-mediated mechanisms. PKA and PKC have a range of effects on Na,K-ATPase function, eliciting inhibitory and stimulatory response patterns in different tissue and cell types (Therien and Blostein, 2000). Regulation of Na,K-ATPase activity in the lens may be regulated in part by a mechanism that blocks tyrosine kinase activation. Genistein, a tyrosine kinase inhibitor, was shown to decrease the inhibitory effects of endothelin (ET-1) on ouabain-sensitive $^{86}Rb$ uptake in the intact porcine lens (Okafor et al., 2001). Also in the lens, thrombin-induced inhibition of Na,K-ATPase-mediated active ion transport was suppressed by a Src-family tyrosine kinase inhibitor, herbimycin A (Okafor et al., 1999). Inhibition of Na,K-ATPase function and increased tyrosine phosphorylation of multiple epithelium membrane proteins occur concomitantly in thrombin treated lenses.

In a recent study, the Src-family kinase, Lyn, was shown to induce tyrosine phosphorylation of the Na,K-ATPase α1 subunit in porcine lens epithelium (Bozulic et al., 2003). The Lyn response was associated with a ~40% inhibition of Na,K-ATPase activity. In the same study, Lyn tyrosine phosphorylation of the Na,K-ATPase α1 subunit and activity response was reversed with protein tyrosine

phosphatase (PTP-1B) treatment (Bozulic et al., 2003). We also show endogenous tyrosine phosphorylation of the Na,K-ATPase α1 subunit was detected in lens fibers and not epithelium. The low endogenous Na,K-ATPase activity associated with lens fibers was partially restored with PTP-1B treatment (Bozulic et al., Submitted).

In other tissues, tyrosine phosphorylation has been reported to alter Na,K-ATPase activity. Tyrosine kinase activation has been reported to stimulate Na,K-ATPase as judged by an increase of ouabain-sensitive $^{86}Rb$ uptake in the intact kidney proximal tubule (Feraille et al., 1997; Narkar et al., 2002). In rat astrocytes, tyrosine kinase activation by insulin elicits an increase of Na,K-ATPase activity and an increase in the synthesis of Na,K-ATPase α1 protein (Matsuda et al., 1993). In other tissues, however, tyrosine kinase activation appears to inhibit Na,K-ATPase function. Genistein was found to suppress the inhibitory action of dopamine and D-1 agonists on Na,K-ATPase-mediated ion transport in cultured rabbit nonpigmented ciliary epithelium (Nakai et al., 1999). Studies in other tissues suggest that Na,K-ATPase activity can be stimulatory or inhibitory. Presently, there is no explanation why tyrosine kinases apparently inhibit Na,K-ATPase activity in some cell types but cause stimulation of activity in others.

In the present study, experiments were conducted to test whether Src and non-Src-family of tyrosine kinases could alter Na,K-ATPase activities in lens cells. Lyn and Fyn tyrosine kinase treatment of lens epithelium material is shown to significantly inhibit Na,K-ATPase activity. In contrast, Src tyrosine kinase treatment of lens epithelium membrane preparation caused a significant increase in Na,K-ATPase activity. No significant change in Na,K-ATPase activity was observed after treatment with Fes, a non Src-family kinase.

## MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless specified. Lyn kinase, Src kinase, Fes kinase, Lck kinase, Fyn kinase, anti-Src family monoclonal antibodies, polyclonal phosphotyrosine antibody, monoclonal phosphotyrosine antibody, and monoclonal antibodies against Na,K-ATPase α1 were all purchased from Upstate Biotechnology (Lake Placid, NY). HRP-conjugated phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY).

***Tissues***

Porcine eyes were kindly donated by the Swift Meat Packing Company (Louisville, KY). The posterior of the eye was dissected and the lens removed by cutting the suspensory ligaments. The lens was transferred to filter paper where the capsule was removed. The lens tissue was then snap-frozen in liquid nitrogen. Material from 40-50 lenses was pooled.

***Membrane Preparation***

Lens membrane preparation was prepared following methodology described by Okafor et al. (1999). Frozen lens capsule-epithelium samples were homogenized in ice-cold homogenization buffer A (150 mM sucrose, 4 mM EGTA, 5 mM Hepes, 800 μM dithiothreitol (DTT), pH 7.4) in the presence of protease inhibitors (100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 2 μg/mL aprotinin) using a glass homogenizer. The cell homogenate was then centrifuged at 115,000g for 60 minutes at 4°C. The membrane pellet was then resuspended in homogenization buffer A containing 600 mM KCl. The resuspended membrane pellet was then subjected to centrifugation once again at 115,000g for 60 minutes at 4°C to remove extrinsic proteins (Dean et al., 1996). The

membrane pellet was resuspended in homogenization buffer A a final time and subjected to centrifugation at 115,000g for 60 minutes at 4°C. The final pellet containing plasma membranes as well as intracellular membranes was resuspended in buffer A. The protein content was then measured using the BCA protein assay kit (Pierce, Rockford, IL).

***Tyrosine phosphorylation and Na,K-ATPase activity measurement***

Lens epithelium membrane preparation was incubated in kinase reaction buffer containing 1 mM EGTA, 10 mM Tris pH 7.2, 20 mM $MgCl_2$, 1 mM ATP, 0.2 mM sodium orthovanadate, 10 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM PMSF, 5 mM DTT, and Src, Lyn, Lck, Fyn or Fes kinases (0.08 units/μg protein) for 20-30 minutes at 30°C. Treated epithelium membrane preparation was then used for western blot analysis and Na,K-ATPase activity measurements. Sodium orthovanadate was removed prior to Na,K-ATPase activity measurements, to prevent Na,K-ATPase inhibition. Sodium orthovanadate was removed from the membrane preparation by centrifugation at 100,000g for 3 minutes. The membrane pellet was resuspended twice in 100 μl of centrifugation buffer (10 mM Tris pH 7.2, 5 mM DTT, 10%(w/v)

glycerol) and centrifuged at 100,000g for 3 minutes. The final pellet was resuspended in ~100 $\mu$l of Na,K-ATPase buffer and assayed immediately for Na,K-ATPase activity.

Na,K-ATPase activity was determined following a methodology described by Okafor et al., (1999). Aliquots of kinase-treated and untreated epithelium membrane preparation (~100 $\mu$g) were incubated in Na,K-ATPase buffer (100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1mM EGTA, pH 7.4). Ouabain, a specific inhibitor of Na,K-ATPase (Wallick and Schwartz, 1988), was added to half the samples to a final concentration of 1 mM. Samples were then preincubated with gentle agitation for 15 minutes at 37°C. ATP was added to a final concentration of 1 mM to initiate ATP hydrolysis. The ATP hydrolysis reaction was carried out with gentle agitation for 45 minutes at 37°C. The reaction was then stopped with the addition of 15% ice-cold trichloroacetic acid. ATP hydrolysis was then quantified. The amount of inorganic phosphate released in each reaction sample was measured using a colorimetric method (Okafor et al., 1999). Less than 20% of the available ATP was hydrolyzed. The difference in ATP hydrolysis in the presence and absence of ouabain was a measurement of Na,K-ATPase activity. These data are presented as nanomoles phosphate released per milligram protein per minute.

Separate studies were conducted to confirm Na,K-ATPase activity was not inhibited by residual vanadate. Na,K-ATPase activity was 9.7 ± 0.4 nmoles Pi/mg protein/minute (mean ± SE; n = 5) in vanadate-treated samples, which was not significantly different from the activity of 10.2 ± 0.6 measured in control samples (Bozulic et al., 2003).

***Western Blot analysis***

Membrane preparation was solubilized in Laemmli sample dilution buffer (Laemmli, 1970). Proteins were separated on a 7.5% gel by SDS-PAGE at 40 mA for 2 hours. Proteins were electrophoretically transferred to nitrocellulose membranes at 30 V for 16 hours. The nitrocellulose membranes were blocked for 1hr with 5% dry milk in TTBS (30 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.4). Na,K-ATPase α1, Src-family kinases, and tyrosine phosphoproteins were detected by incubating the nitrocellulose membranes at room temperature for 60 minutes with either a monoclonal antibody directed against Na,K-ATPase α1 (Sigma, St. Louis, MO), Src-family kinases (Upstate Biotechnology, Lake Placid, NY), or anti-phosphotyrosine antibody PY20 (PY) (Transduction Lab, Lexington, KY) conjugated to horseradish peroxidase. Nitrocellulose membranes probed for Na,K-

ATPase α1 and Src-family kinases were washed with TTBS two times for 15 minutes and then three times for 5 minutes before being incubated for 60 minutes with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Nitrocellulose membranes probed for PY were washed with TTBS two times for 15 minutes and then four times for 5 minutes at room temperature, the blots were visualized with chemiluminescence substrate (Pierce, Rockford, IL). Nitrocellulose membranes were exposed to x-ray film. In some experiments, nitrocellulose membranes were exposed to x-ray film and quantified using Kodak 1D image-analysis software (Kodak; Rochester, NY). The ratio of 100kDa phosphotyrosine (PY) band intensity to 100 kDa Na,K-ATPase α1 band intensity was determined in Src and Fyn treated epithelium membrane preparation. All quantified blots were within the linear range of chemiluminescence reponse.

***Statistical Analysis***

Student's t-test was used for statistical analysis.

**RESULTS**

Studies were conducted to compare the ability of different Src-family tyrosine kinases to change Na,K-ATPase activity and cause tyrosine phosphorylation of lens

epithelium membrane proteins. Isolated lens epithelium membrane preparation was incubated for a period of 20 minutes in ATP-containing kinase reaction buffer with either Src, Fyn, Lyn, Lck, or Fes, a non-Src family member. The treated samples were subjected to western blot analysis of tyrosine phosphorylated proteins (**Figure 24**). A marked increase in several phosphotyrosine protein bands was observed. The band patterns and intensities of many of the protein bands appear to be distinct for each tested tyrosine kinase. This may suggest that distinct tyrosine phosphorylation sites exist in epithelium membrane proteins that were treated with a specific tyrosine kinase. The black box in **Figure 24** represents the position of a shared 100kDa phosphotyrosine band immunopositive for Na,K-ATPase α1. As described earlier, tyrosine phosphorylation of Na,K-ATPase appears as a major PY band at 100kDa (Bozulic et al., in press). Phosphorylation of the 100 kDa band appeared as early as 1 minute, with maximal tyrosine phosphorylation reached at 15 minutes in Src-family treated samples (**Figure 25A,B,C,D**). An increase in the intensity of the 100 kDa phosphotyrosine band was also observed in lens epithelium membrane preparation treated with Fes tyrosine kinase, a non-Src family member (**Figure 26**). Tyrosine

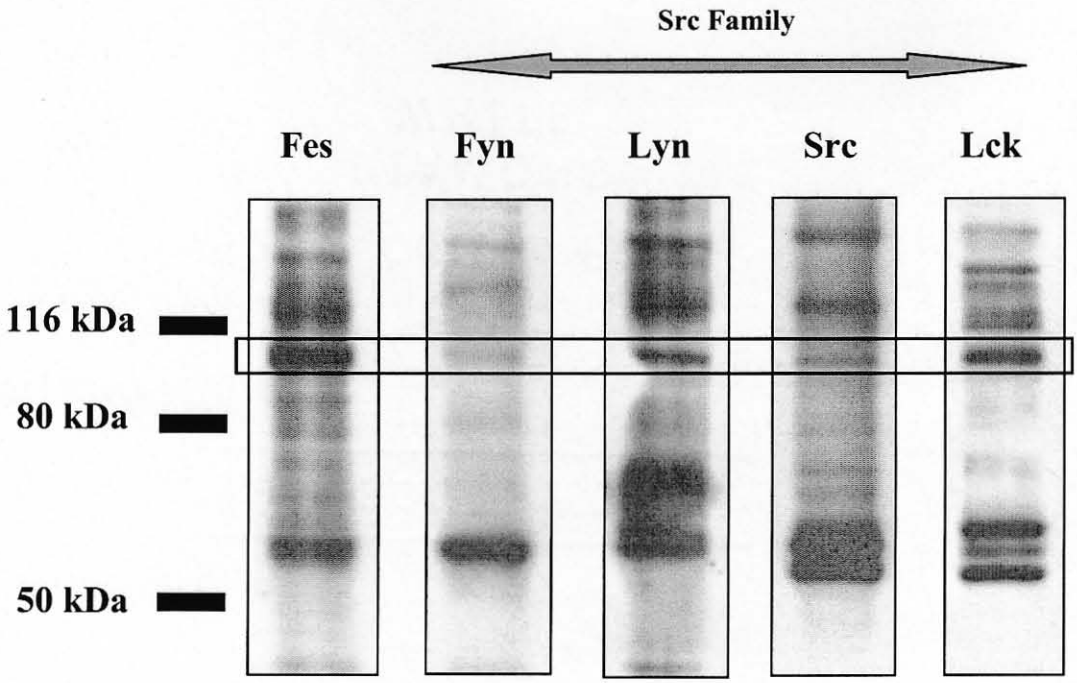



**Figure 24. The influence of non-receptor tyrosine kinases on lens epithelium membrane preparation.** Phosphotyrosine Western blot. Lens epithelium membrane preparation (25 μg) was incubated with Fes, Fyn, Lyn, Src or Lck kinase (2 Units) in ATP-containing buffer for 0-10 minutes. Tyrosine kinase-treated membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against phosphotyrosine residues (PY). The box indicates the location of Na,K-ATPase α1.

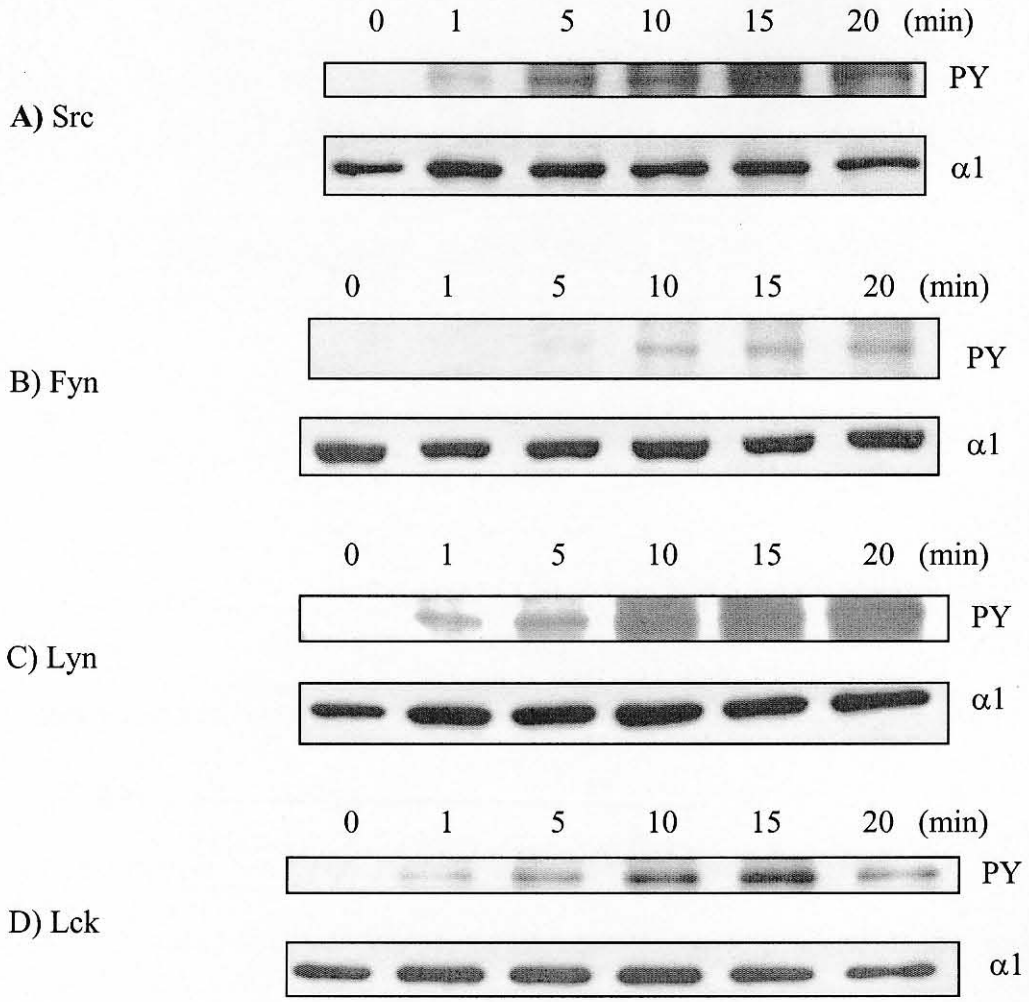



**Figure 25. Src family kinase-induced tyrosine phosphorylation of a putative 100kDa band immunopositive for Na,K-ATPase α1 polypeptide.** Panels A-D: Time course for tyrosine phosphorylation of protein at ~100 kDa. Lens epithelium membrane preparation (25 (μg) was incubated with Src, Fyn, Lyn, or Lck kinase (2 Units) in ATP-containing buffer for 0-20 min. The phosphotyrosine Western blot (PY) (upper) was stripped and reprobed for Na,K-ATPase α1 (lower).

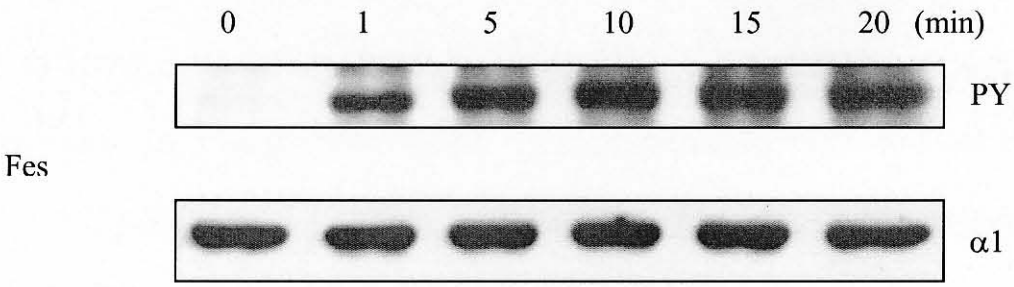


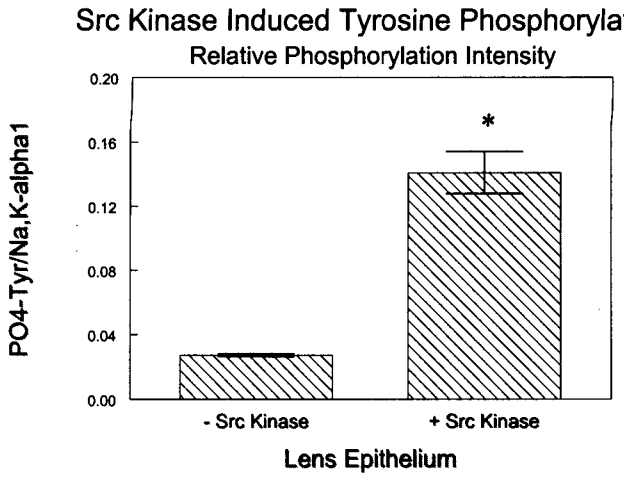

**Figure 26. Non-Src family kinase-induced tyrosine phosphorylation of a putative 100kDa band immunopositive for Na,K-ATPase α1 polypeptide.** Time course for tyrosine phosphorylation of protein at ~100 kDa. Lens epithelium membrane preparation (25 g) was incubated with Fes kinase (2 Units) in ATP-containing buffer for 0-20 min. The phosphotyrosine Western blot (PY) (upper) was stripped and reprobed for Na,K-ATPase α1 (lower).

phosphorylation was detected within 1 minute. The phosphotyrosine blots were stripped and reprobed with an Na,K-ATPase α1 specific antibody to confirm that the increase in phosphotyrosine band intensity was not a result of differences in Na,K-ATPase α1 polypeptide abundance. The 100kDa Na,K-ATPase alpha1 band showed similar abundance throughout the time course.

The ratio of phosphotyrosine band intensity to Na,K-ATPase α1 band intensity was calculated for lens epithelium membrane preparation treated with and without Src or Fyn tyrosine kinases (**Figure 27A,B**). The ratio of phosphotyrosine band intensity to Na,K-ATPase α1 band intensity was significantly higher from Src or Fyn kinase-treated lens epithelium membrane samples.

Src-family and non-Src-family tyrosine kinases are expressed in lens tissue. In order to confirm the expression of tyrosine kinases used in this study, lens epithelium and fiber membrane preparation was isolated and used for western blot analysis. A single 60kDa band corresponding to the single known isoform of Src, Fyn, and Lck was detected in both lens epithelium and fibers. The relative abundance of each isoform appeared to be similar between lens fibers and lens epithelium (**Figure 28A,B,C**).

A) Src




B) Fyn

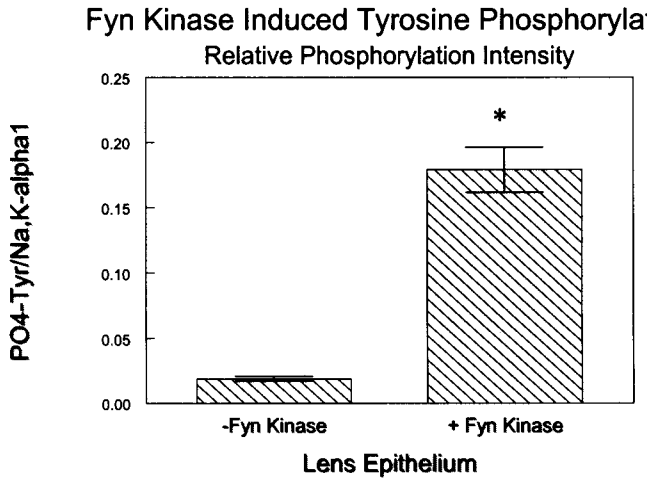



**Figure 27. Src and Fyn kinase-induced tyrosine phosphorylation of a putative 100kDa immunopositive Na,K-ATPase α1 polypeptide band.** Panel A-B: The ratio of the 100 kDa phosphotyrosine band intensity to Na,K-ATPase (1 band intensity determined in epithelium membrane samples treated with Src or Fyn kinases, respectively. The 0 (-kinase) and 10 minute (+kinase) time points were chosen for each analysis. Each bar represents the mean +/- S.E. of three independent determinations. * indicates a significant difference from control ($P < 0.01$).

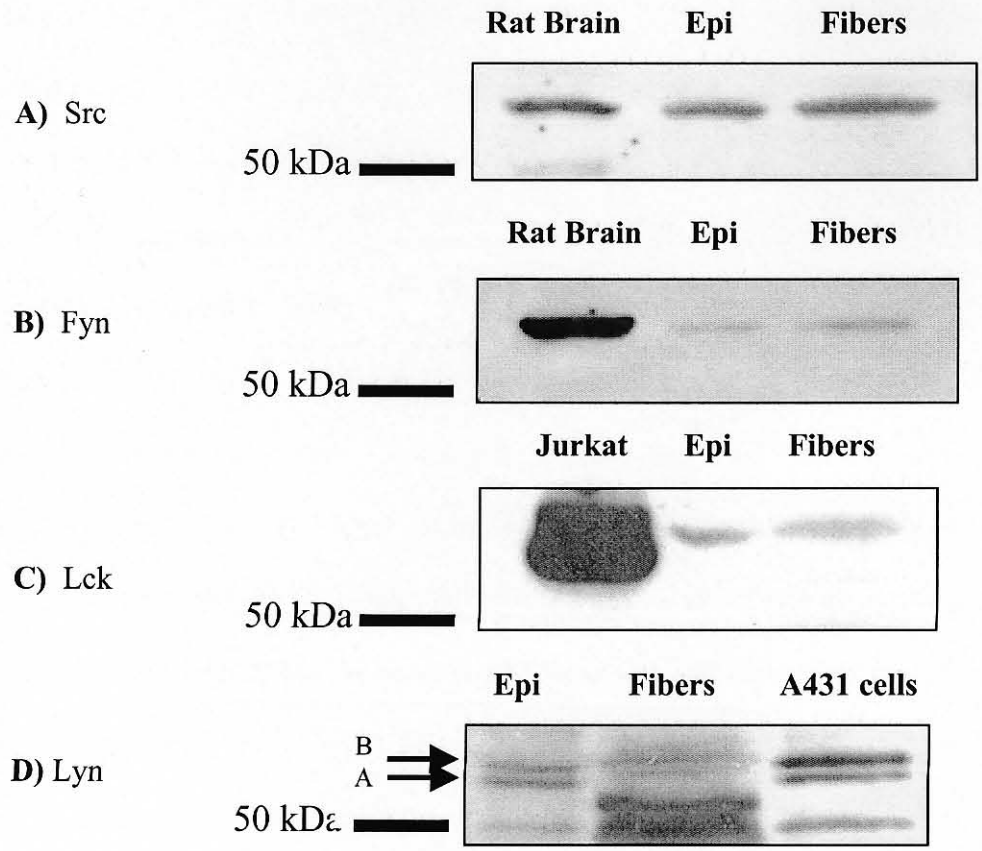



**Figure 28. Detection of Src-family kinases in lens epithelium and fiber membrane preparation.** Lens epithelium and fiber membrane preparation (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against Src, Fyn, Lck, or Lyn kinases. Rat brain microsomes (5 μg) were used as a positive control for A)Src and B)Fyn kinase expression. Jurkat cell lysate (20 μg) was used as a positive control for C)Lck kinase expression. A431 non-stimulated cell lysate (20 μg) was used as a positive control for D)Lyn kinase expression.

When lens tissue was probed for Lyn kinase, two immunopositive bands corresponding to the two known isoforms of Lyn kinase (A and B) were detected in lens epithelium while a single isoform (B) was detected in lens fibers (**Figure 28D**). The relative abundance of Lyn was greater in lens epithelium than in lens fibers, although the results do not signify the degree to which Lyn is activated. Fes, the non-Src-family tyrosine kinase, was also detected in lens epithelium and fibers (**Figure 29**). Fes kinase abundance appeared to be greater in lens fibers than in lens epithelium membrane preparation. The results for each of the detected tyrosine kinases do not signify the degree to which each particular tyrosine kinase is activated.

Src-family and non-Src-family tyrosine kinase treatment of membrane preparation appears to increase tyrosine phosphorylation of a 100 kDa Na,K-ATPase α1 immunopositive band as well as many membrane proteins. To test whether tyrosine kinase treatment is capable of modifying Na,K-ATPase activity, lens epithelium membrane preparation was incubated for 20 minutes with Lyn, Fyn, Src, or Fes tyrosine kinases in ATP-containing buffer. As described in Methods, kinase-treated membrane preparation was washed to remove exogenous tyrosine kinases and buffer

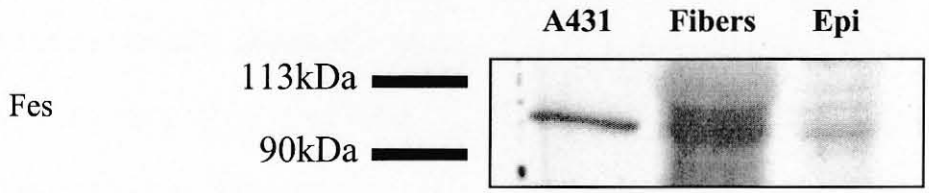



**Figure 29. Detection of Fes kinase, a non-Src tyrosine kinase, in lens epithelium and fiber cell membrane preparation.** Lens epithelium and fiber membrane preparation (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against Fes kinase. A431 non-stimulated cell lysate (20 μg) was used as a positive control for Fes kinase expression.

constituents. Na,K-ATPase activity was then measured in the treated samples. Na,K-ATPase activity was reduced by ~40% in lens epithelium preparation subjected to Lyn pretreatment (**Figure 30A**). Na,K-ATPase activity was also significantly reduced in lens epithelium membrane preparation subjected to Fyn pretreatment, but to a lesser degree (**Figure 30B**). In contrast, Na,K-ATPase activity increased by ~30% in lens epithelium material subjected to Src tyrosine kinase pretreatment (**Figure 30C**). No significant change in Na,K-ATPase activity was observed in lens epithelium membrane preparation subjected to Fes tyrosine kinase pretreatment (**Figure 31**).

## DISCUSSION

The results suggest tyrosine phosphorylation by different tyrosine kinases can either stimulate, inhibit, or not change Na,K-ATPase activity in lens epithelium membrane preparation. The Na,K-ATPase α1 subunit is the only Na,K-ATPase α isoform detectable in lens cells. Neither Na,K-ATPase α2 or α3 isoforms are observed by western blot in porcine lens cells (Delamere et al., 1996). Previous studies using Lyn kinase-treated lens epithelium

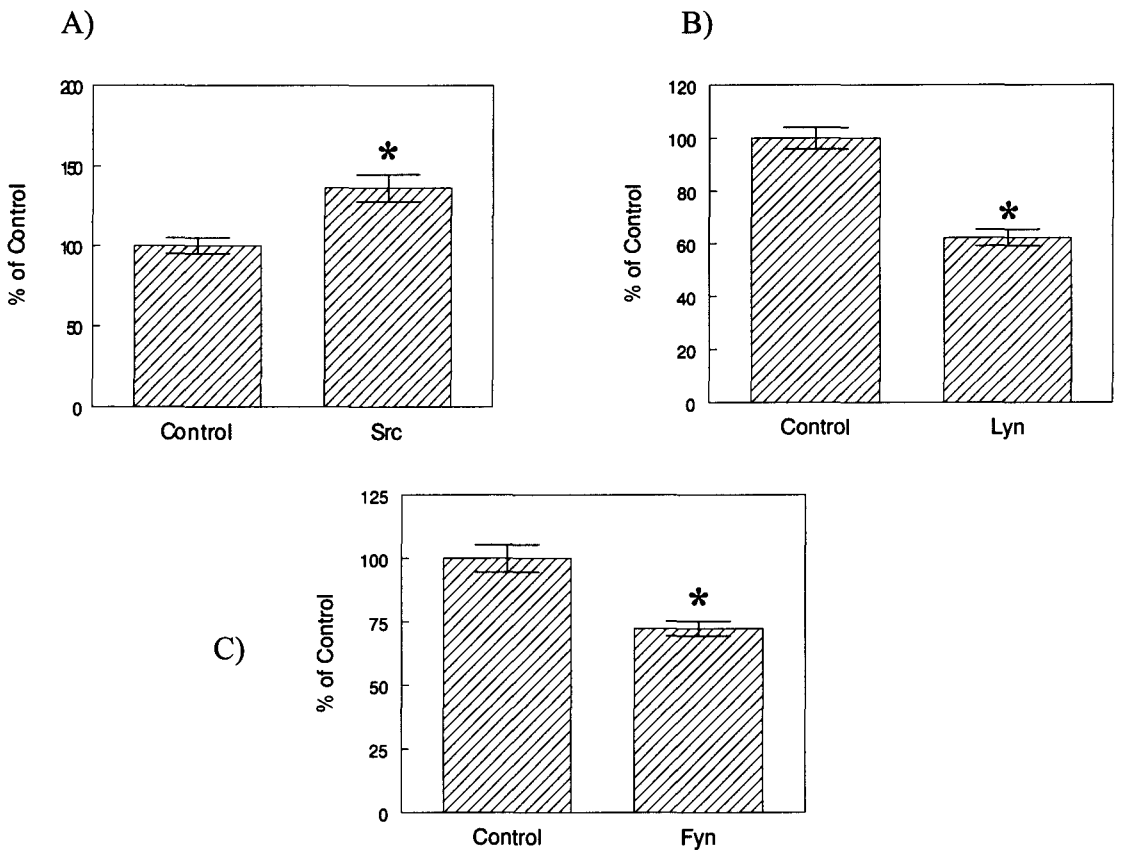



**Figure 30. Na,K-ATPase activity determined following Lyn, Src, or Fyn kinase treatment of epithelium membrane preparation.** Lens epithelium membrane preparation was incubated in the presence of A)Src, B)Lyn, or C)Fyn kinase in ATP-containing buffer for 20 min. Treated material was washed to remove buffer constituents. Na,K-ATPase activity was then measured and defined as the difference between ATP hydrolysis in the presence and absence of ouabain. The data are shown as mean ± SE (*vertical bar*) of results from 5-6 independent determinations. *Indicates a significant difference from control ($P<0.001$).

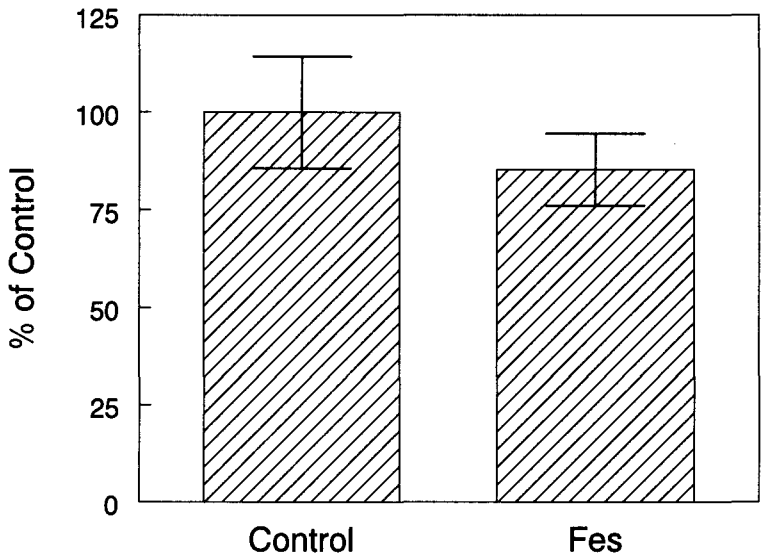



**Figure 31. Na,K-ATPase activity determined following Fes kinase treatment of epithelium membrane preparation.** Lens epithelium membrane preparation was incubated in the presence of Fes kinase in ATP-containing buffer for 20 minutes. Treated membrane preparation was washed to remove buffer constituents. Na,K-ATPase activity then determined and defined as the difference between ATP hydrolysis in the presence and absence of ouabain taken from 9 independent experiments from 2 kinase treated batches. The indicated values are shown as mean ± SE of 9 measurements.

membrane preparation showed that the Na,K-ATPase α1 subunit is a target for tyrosine phosphorylation along with other membrane proteins (Bozulic et al., 2003). Src, Fyn, Lck, Lyn and Fes tyrosine kinase treatment of lens epithelium membrane preparation was observed here to cause tyrosine phosphorylation of many membrane proteins including a ~100 kDa band that co-migrated on western blot with the Na,K-ATPase α1 subunit. Because several membrane proteins are tyrosine phosphorylated and other 100 kDa tyrosine kinase substrates are present in lens epithelium and cortex, the extent to which the observed change of Na,K-ATPase activity depended on the phosphorylation of Na,K-ATPase α1 protein itself is not known. However, it is clear that tyrosine phosphorylation events can change Na,K-ATPase activity.

Exogenous Src, Fyn, Lck, Lyn and Fes tyrosine kinases were used in this study to determine what effect phosphorylation by different tyrosine kinases might have on Na,K-ATPase activity. Endogenous detection of Fes tyrosine kinase appeared to be stronger in lens fiber cell membrane preparation, while lens epithelium displayed stronger detection of Lyn kinase. This may represent differences in cell-specific mechanisms and a more important role for Lyn in lens epithelium than lens fibers, though western blot

results do not provide information on their activation state.

Many studies on Lyn and Lck tyrosine kinases have focused on their function in myeloid B and lymphoid hematopoietic cells (Defranco et al., 1998; Corey and Anderson, 1999). Lyn kinase is important in signal transduction mechanisms following activation of B-cell antigen receptors, FcE high affinity receptors for IgE (Hibbs et al., 1995), and interleukin-3 receptors (Torigoe et al., 1992). Lck kinase binds to the cytoplasmic domain of CD4 and CD8 and participates in T-cell activation and development (Mahabeleshwar and Kundu, 2003). Lck also associates with cyclin-dependent kinase (cdc2) at the G2/M checkpoint of cell-cycle progression (Pathan et al., 1996).

Lyn and Lck tyrosine kinases have also been detected and studied in other tissues. For example, Lyn mRNA has been detected in the brain where it is thought to be important in blood-brain-barrier development (Achen et al., 1995). It has been detected in kidney glomerular endothelial cells as well (Takahashi et al., 1995). Lck mRNA has been detected in distinct regions of the mouse brain (Omri et al., 1996) and in mouse retinal neurons where it is believed to be important in retina development (Omri et al., 1998). Fyn and Src tyrosine kinases are

widely expressed. Fyn is expressed in T cells and brain while Src is expressed in platelets, brain, fibroblasts, and mammary (Corey and Anderson, 1999). Both Fyn and Src are required for G2 to M phase transition during cell-cycle progression (Roche et al., 1995). Fyn kinase, not Src, appears to be important in normal keratinocyte migration and squamous carcinoma invasion by disrupting hemidesmosomes during normal epithelial cell migration (Mariotti et al., 2001).

The Src-kinase-induced increase of lens Na,K-ATPase activity observed in the present study is similar to the response observed in proximal tubule cells where tyrosine phosphorylation and activation by insulin was associated with stimulation of Na,K-ATPase activity (Feraille et al., 1999). Src activation of Na,K-ATPase activity and Lyn or Fyn kinase-induced inhibition of lens Na,K-ATPase activity may reflect differences in Na,K-ATPase α1 subunit characteristics such as different tyrosine phosphorylation sites. It is interesting to note that Fes kinase, a non-Src-kinase member, also induced tyrosine phosphorylation of epithelium membrane proteins in this study but did not change Na,K-ATPase activity.

Taken together, the results may suggest a complex model in which stimulation by a particular kinase may

activate an unknown protein or complex of proteins that interact with the Na,K-ATPase differently to modulate Na,K-ATPase activity, in which case phosphorylation of the Na,K-ATPase α1 subunit may be less important. A more simplistic model is that the direct phosphorylation of Na,K-ATPase α1 subunit by a specific tyrosine kinase occurs at at least one of 9 tyrosine residues on the Na,K-ATPase α1 subunit or a kinase-specific mechanism that either inhibits, stimulates, or does not change activity of the phosphorylated Na,K-ATPase catalytic subunit.

There is evidence for an association between changes in tyrosine phosphorylation and changes of Na,K-ATPase activity in the intact lens. It was reported earlier that thrombin-induced inhibition of Na,K-ATPase-mediated active ion transport can be suppressed by the Src-family tyrosine kinase inhibitor herbimycin A (Okafor et al., 1999). Other investigators have demonstrated that thrombin may activate non-receptor tyrosine kinases including Lyn kinase in platelets (Cho et al., 2002; Hirao et al., 1997). In a recent study, the Na,K-ATPase α1 subunit was immunoprecipitated from Lyn-kinase-treated lens epithelium membrane preparation and shown to be tyrosine phosphorylated. The Lyn response was associated with

inhibition of Na,K-ATPase activity (Bozulic et al., 2003). In another recent study, endogenous tyrosine phosphorylation of the Na,K-ATPase α1 subunit was detected in lens fibers and not epithelium (Bozulic et al., 2003). The low endogenous Na,K-ATPase activity associated with lens fibers was partially restored with PTP-1B treatment (Bozulic et al., Submitted). Feraille et al. (1997) examined Na,K-ATPase pump activity in OK cells and found that substitution of Y10 in the Na,K-ATPase α1 subunit suppressed insulin-induced stimulation of Na,K-ATPase activity. Gastric H,K-ATPase is also susceptible to phosphorylation at Y10 (Togawa et al., 1995). These studies strongly suggest that the Na,K-ATPase α1 protein is itself tyrosine phosphorylated. It remains to be determined whether direct phosphorylation of the Na,K-ATPase α1 subunit is responsible for the Na,K-ATPase activity response.

Studies in other laboratories suggest tyrosine phosphorylation can bring about modulation of Na,K-ATPase activity but the identity of the tyrosine kinases that influence Na,K-ATPase in intact tissues is not known. Here we show that several different kinases can have different effects on Na,K-ATPase activity. In mouse erythrocytes,

activation of Src-family tyrosine kinases appears to modify $K^+$-$Cl^-$ cotransporter function (De Franceschi et al., 2001). In the lens, the Src-family kinases may likely be just a few of many non-receptor tyrosine kinases and it is possible that other tyrosine kinases also influence Na,K-ATPase activity.

In summary, the results of the present study suggest that different Src-family and non-Src-family tyrosine kinases are capable of either inhibiting or stimulating Na,K-ATPase activity. The Na,K-ATPase activity responses elicited by different tyrosine kinases might account for the diverse Na,K-ATPase activity responses observed in other tissues. Although the significance of modulating Na,K-ATPase activity remains to be determined, there may exist a need to modulate Na,K-ATPase activity to produce circulating currents in the lens (Mathias et al., 1997). The activation of different Src kinase family members may be necessary to stimulate or inhibit Na,K-ATPase activity in the lens to produce and regulate these circulating currents.

## Chapter V

## SUMMARY AND SIGNIFICANCE

In this work it has been shown that Na,K-ATPase activity can be regulated by tyrosine phosphorylation both *in vitro* and *in vivo*. However, no direct evidence was obtained indicating that direct phosphorylation of the Na,K-ATPase polypeptides is required. Nevertheless, direct tyrosine phosphorylation of the Na,K-ATPase in kidney proximal tubule cells has been shown to be required for the modulation of Na,K-ATPase activity in response to insulin. Feraille et al. (1997) analyzed Na,K-ATPase pump activity in opossum kidney cells transfected with mutant Na,K-ATPase α1 in which Y10 was substituted either by alanine or glutamate. Insulin-induced stimulation of Na,K-ATPase function was suppressed in cells expressing the Y10 substitutions. Phosphorylation of Na,K-ATPase α-subunit and modulation of Na,K-ATPase activity in kidney proximal tubule cells fits with the model presented in this study.

My results offer the first direct evidence that tyrosine phosphorylation can modulate Na,K-ATPase activity

in lens cells. Lyn kinase, a Src-kinase family member, induced the tyrosine phosphorylation of the Na,K-ATPase α1 protein and inhibited Na,K-ATPase activity. To reinforce the case for tyrosine phosphorylation, we showed the effect was reversed by PTP-1B treatment. The findings explain a previously observed association between tyrosine phosphorylation and inhibition of Na,K-ATPase activity in the intact lens (Okafor et al., 1999; Okafor and Delamere, 2001). Lyn kinase-induced inhibition of Na,K-ATPase activity in lens epithelium membrane preparations differs from the response to insulin and other agonists in proximal tubule cells where tyrosine phosphorylation is associated with stimulation of Na,K-ATPase function (Feraille et al., 1999). This may indicate differences in the signaling events triggered by insulin and partially-purified Lyn, differences in the response of intact cells where increased Na,K-ATPase expression in the plasma membrane may occur, or differences in cell-specific regulatory mechanisms, such as the activation of different tyrosine kinases as shown here. Different tyrosine kinases can increase or decrease Na,K-ATPase activity, thereby, providing a quick short-term mechanism for regulating Na,K-ATPase activity in cells (**Figure 32**).

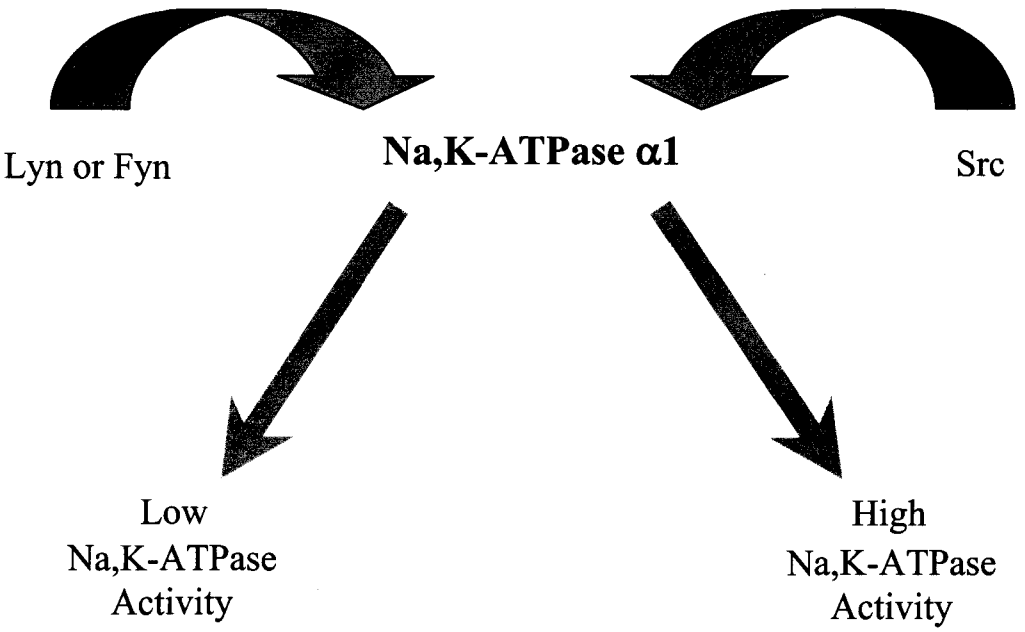



**Figure 32. Modulation of Na,K-ATPase activity by tyrosine phosphorylation.** Modulation of Na,K-ATPase activity through direct or indirect mechanisms can either inhibit Na,K-ATPase activity when subjected to Lyn or Fyn treatment or stimulate Na,K-ATPase activity when subjected to Src treatment. Direct tyrosine phosphorylation of the Na,K-ATPase α1 protein would require the presence of inhibitory or stimulatory sites, whereas, indirect mechanisms would require the interaction with other proteins.

The observation that different kinases may be involved in regulating Na,K-ATPase activity is supported by the results found in lens cells. Lens fibers display a higher degree of tyrosine phosphorylated Na,K-ATPase α1 protein than lens epithelium as well as lower Na,K-ATPase activity. The Na,K-ATPase α1 protein in lens fibers may be phosphoryated at inhibitory tyrosine residues. This idea is supported by results showing partial restoration of Na,K-ATPase activity when lens fiber membrane preparation was treated with PTP-1B. In lens epithelium, however, lower Na,K-ATPase α1 tyrosine phosphorylation is correlated with higher Na,K-ATPase activity. Another possibility is tyrosine phosphorylation at sites that do not alter the Na,K-ATPase structure or function, and therefore, no change in Na,K-ATPase activity. Different cell-specific regulatory mechanisms may therefore exist for lens fibers and epithelium.

Differences in Na,K-ATPase activities may occur as a result of differences in the observed tyrosine phosphorylation sites on the Na,K-ATPase α1 protein or on other membrane proteins. This idea is supported by the tyrosine phosphorylation pattern of PY bands elicited by Src, Fyn, Lck, Lyn, and Fes. Differences in the

phosphorylation profiles and in the intensities of many proteins suggest that there must be a difference in the recognized tyrosine phophorylation consensus sequences for each tested kinase. Others suggest that different concensus sequences do in fact exist for each of the Src-kinase family members. Recognition of this concensus sequence by a particular Src-kinase member may not soley depend on this sequence for binding (Corey and Anderson, 1999). It appears that tyrosine phosphorylation at a specific site in a protein does not actually depend on the presence of a concensus sequence but rather on the presence of a proline rich region recognized by the Src-kinases SH2 or SH3 domains (Corey and Anderson, 1999). In this manner differences in the level of phosphorylation would come about by weak binding to different proline rich regions and therefore different tyrosine residues. It is interesting to note that the catalytic Na,K-ATPase α1 protein has a proline rich region (Yudowski et al., 2000). The presence of a proline rich region on the Na,K-ATPase α1 protein may account for differences in the intensities of the observed 100 kDa phosphotyrosine/Na,K-ATPase α1 band for each kinase tested. However, it is not possible to determine whether the level of phosphorylation elicited by different tyrosine

kinases or differences in the levels of phosphorylation accounted for the observed changes in Na,K-ATPase activities.

Differences in Na,K-ATPase activity observed here may be a result of direct or indirect tyrosine phosphorylation. Lens epithelium treated with Lyn, Src, and Fes showed inhibition, activation, and no change in Na,K-ATPase activity, respectively. Direct tyrosine phosphorylation of the Na,K-ATPase α1 protein would require the presence of inhibitory, stimulatory, and perhaps tyrosine residues that do not alter Na,K-ATPase function when phosphorylated (**Figure 33A**). In an indirect manner, different tyrosine kinases can phosphorylate a specific substrate or substrates which in turn interact with the Na,K-ATPase α1 protein to modulate Na,K-ATPase activity **(Figure 33B)**. It has been shown that dopamine-induced activation of PKA in kidney and brain leads to the phosphorylation of the dopamine and cAMP-regulated phosphoprotein (DARPP-32) and the Na,K-ATPase (Aperia et al., 1991). DARPP-32 in turn becomes a potent inhibitor of protein phosphatase 1. Hence, phosphorylation of Na,K-ATPase and inhibition of protein phosphatase 1 help to keep Na,K-ATPase in an inactive phosphorylated state (Therien and Blostein, 2000).

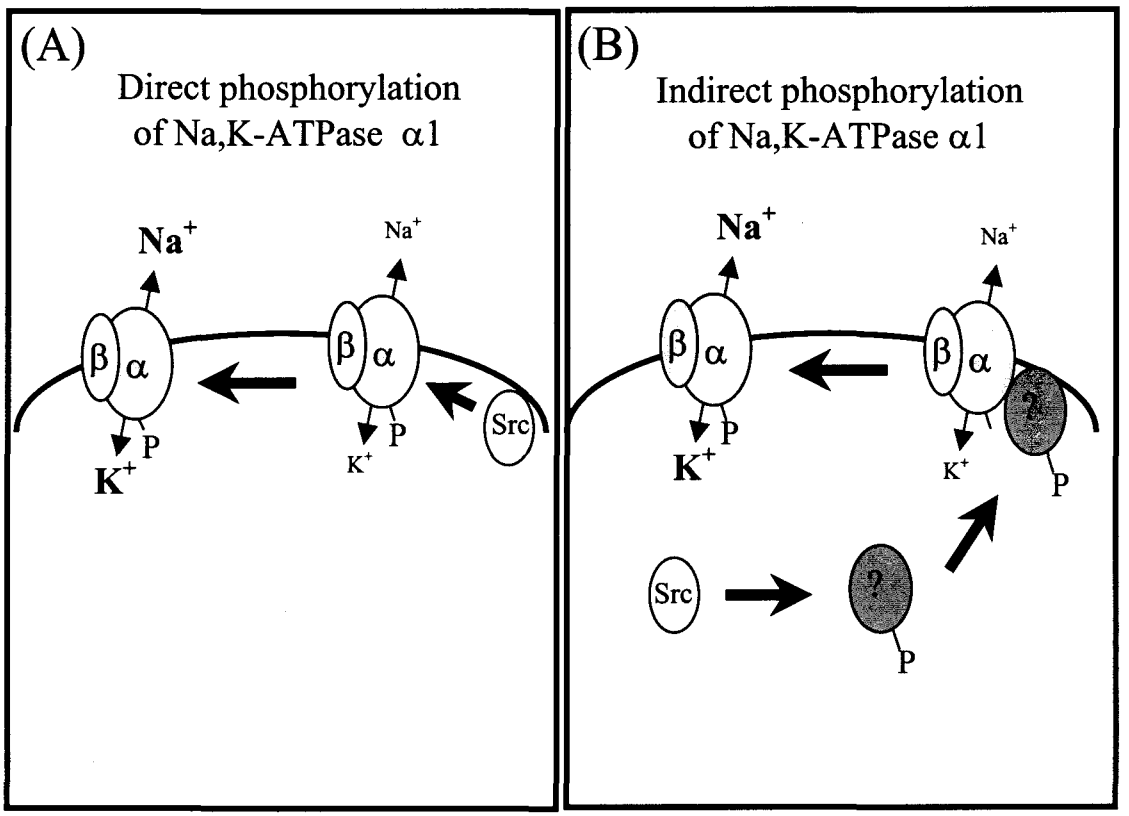



**Figure 33. Direct and indirect tyrosine phosphorylation of Na,K-ATPase α1 subunit.** Tyrosine phosphorylation of Na,K-ATPase α1 by Src-family kinases can modulate Na,K-ATPase activity either by direct phosphorylation (A) or indirect phosphorylation (B) via the phosphorylation of other proteins that subsequently interact with the Na,K-ATPase α1 subunit to modulate activity.

The significance of modulating Na,K-ATPase activity in lens epithelium remains to be determined. It has been proposed that spatial localization of high Na,K-ATPase activity to specific regions of the lens surface is essential in order to support circulation of electrical currents that work via electroosmosis to speed solute movement through the tortuous extracellular space between the tightly packed lens cells (**Figure 34**) (Mathias et al., 1997). These circulating currents may be important in delivering glucose and removing waste products in the lens. The innermost fiber cells have very low metabolic activity and are solely driven by anaerobic glycolysis (Mathias et al., 1997). The delivery of glucose and removal of waste products cannot simply be explained by simple diffusion. Glucose, with a diffusion coefficient of $10^{-6}$ $cm^2/s$, would take 4-8 days to reach the center of the lens (Mathias et al., 1997).

In order to establish the circulating currents, there may be a need for mechanisms that modulate Na,K-ATPase activity to produce unequal activity in different parts of the lens. Na,K-ATPase activity is suppressed in lens fibers and displays a high degree of Na,K-ATPase α1 tyrosine phosphorylation. The Lens epithelium displays low endogenous Na,K-ATPase α1 tyrosine phosphorylation and

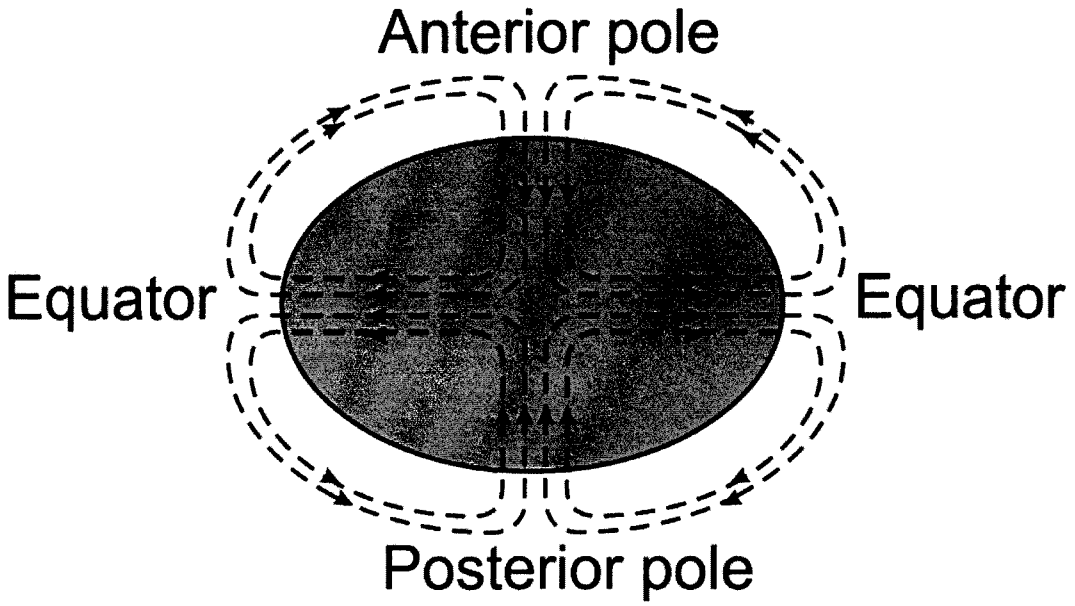



**Figure 34. Lines of ionic current in the lens.** Current flows inward at both poles of the lens and outward at the equator, thereby circulating around and through the lens. The circulation of ions through the lens is believed to drive fluid movement because water flow follows solute flux. The inward movement of fluids via electroosmosis primarily between the fiber cell extracellular space shuttles glucose to nuclear fiber cells where it can be utilized for anaerobic metabolism. Metabolic waste products are then shuttled out of the lens at the equator. Thus, the circular movement of fluid created by sodium transport at the surface of the lens can significantly affect the fiber cell cytoplasmic content throughout the lens. (Figure from Donaldson et al., (2001)). News Physiol Sci. 16:118-123.

maintains high Na,K-ATPase activity. Therefore, the lens epithelium may provide the necessary Na,K-ATPase activity for the whole lens and could be responsible for the circulating currents that move substrates to the innermost fiber cells. In this manner, these circulating currents could shuttle glucose and remove waste products faster than can be explained by simple diffusion.

Modulation of Na,K-ATPase activity in lens cells could also be an important protective mechanism. Damage to the lens caused by oxidative stress or UV light could cause membrane damage and result in the influx of $Na^+$. Lens fibers could potentially counteract this event by activating PTPs, and dephosphorylating the Na,K-ATPase α1 subunit, thereby, increasing Na,K-ATPase activity and restoring the resting membrane potential (**Figure 35**). Since protein synthesis is low in lens fibers, the synthesis of new Na,K-ATPase proteins would be unlikely. The Lens epithelium could also aid in counteracting the $Na^+$ influx by either activating PTKs to phosphorylate the Na,K-ATPase and increase activity or via the synthesis of new Na,K-ATPase proteins for long-term regulation (**Figure 36**).

In cataract, the precise molecular mechanism for development of lens opacity is still not known. However, indirect evidence suggests there may be a link between

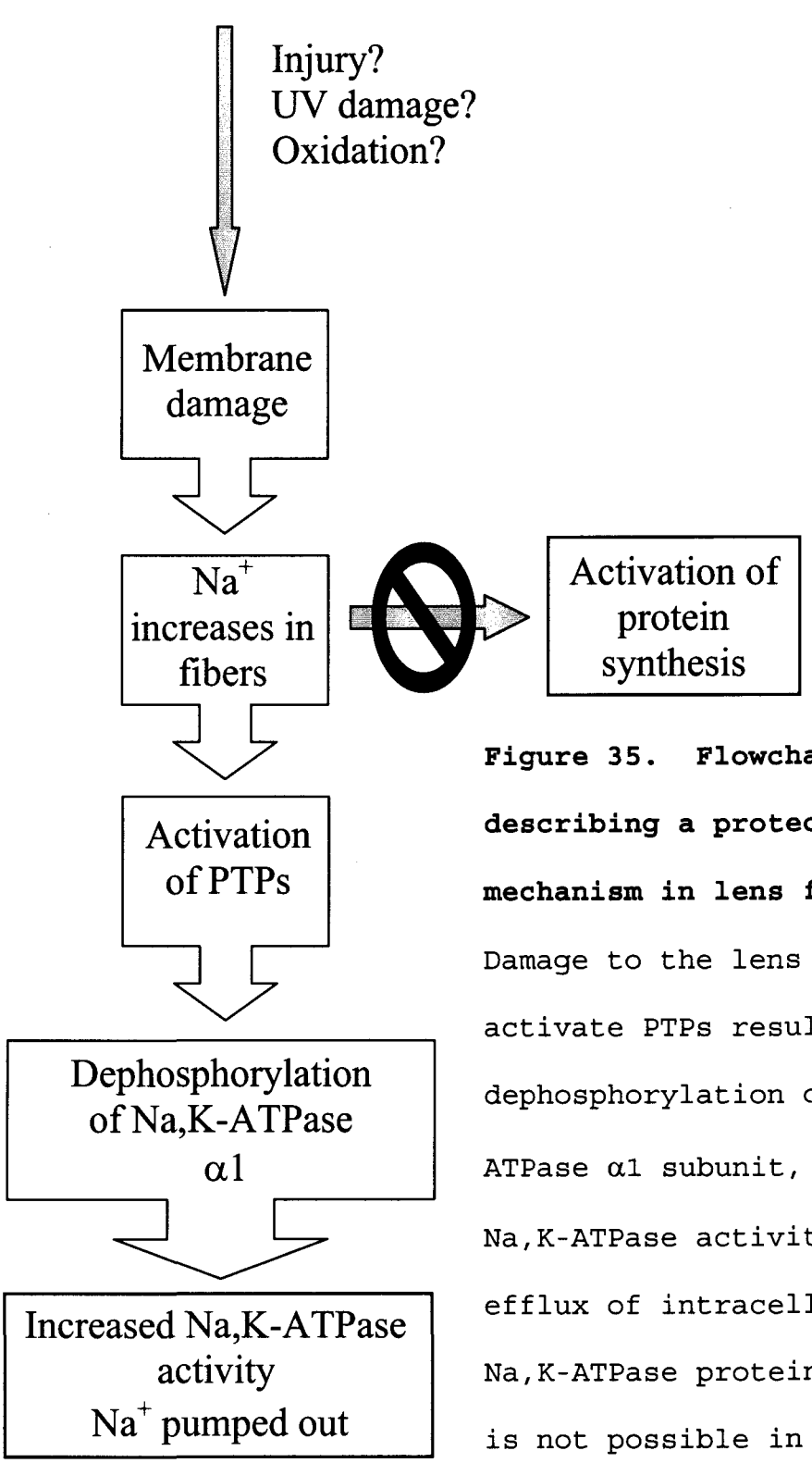



**Figure 35. Flowchart describing a protective mechanism in lens fibers.** Damage to the lens might activate PTPs resulting in the dephosphorylation of Na,K-ATPase α1 subunit, increased Na,K-ATPase activity, and efflux of intracellular $Na^+$. Na,K-ATPase protein synthesis is not possible in lens fiber cells.

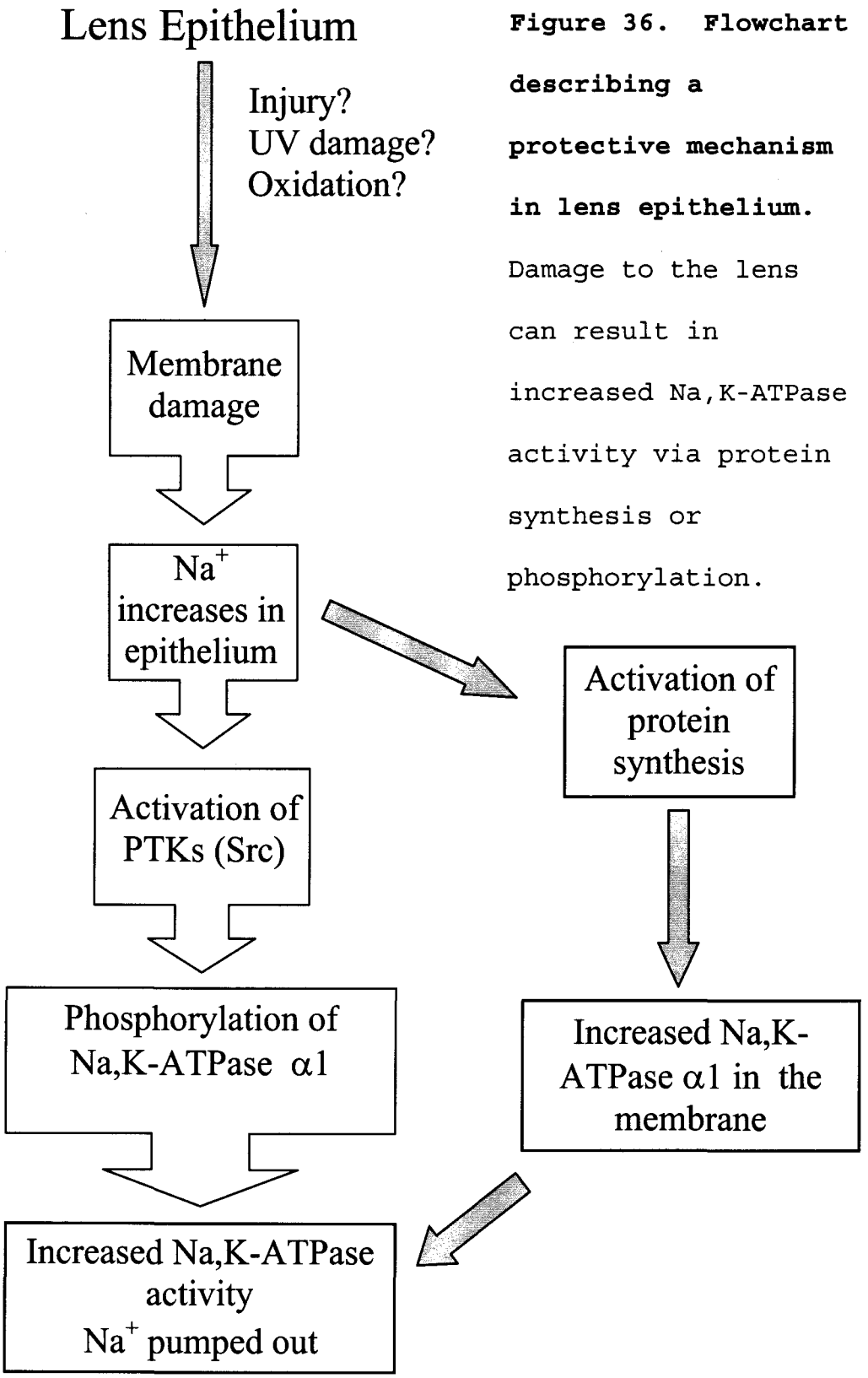



**Figure 36. Flowchart describing a protective mechanism in lens epithelium.** Damage to the lens can result in increased Na,K-ATPase activity via protein synthesis or phosphorylation.

cortical cataract and Na,K-ATPase activity. The majority of human cataractous lenses display abnormally high sodium contents and the degree of lens opacification is more severe in lenses with higher sodium values (Duncan and Bushell, 1975). Endogenous digitalis-like compounds (DLC) known to inhibit Na,K-ATPase activity have been discovered in normal human lenses. The concentration of DLCs was determined to be higher in the cataractous human lenses (Lichtstein et al., 1993). Ouabain, a specific inhibitor of Na,K-ATPase activity has been shown to induce lens opacification using an *in vitro* model (Marcantonio et al., 1986). Though it is not known whether tyrosine phosphorylation of the Na,K-ATPase causes cataract, studies have shown that inhibition of PKA activity results in the formation of cortical cataracts and this is associated with higher sodium levels (Calvin et al., 2003). It remains to be determined whether increased tyrosine phosphorylation of the Na,K-ATPase α1 protein is associated with cataract formation. Future studies are needed to test whether inhibition of Na,K-ATPase activity is one of the many mechanisms involved in the disease.

## REFERENCES


Achen, M. G., M. Clauss, H. Schnurch and W. Risau (1995). The non-receptor tyrosine kinase Lyn is localised in the developing murine blood-brain barrier. *Differentiation*. **59**: 15-24.

Al-Khalili, L., M. Yu, et al. (2003). Na+,K+-ATPase trafficking in skeletal muscle: insulin stimulate translocation of both alpha 1- and alpha 2-subunit isoforms. *FEBS Lett*. **536**: 198-202.

Aperia, A., Fryckstedt, S., Svensson, L., Hemmings, H.C.J., Nairon, A.C., and Greengard, P. (1991). Phosphorylated Mr 32,000 dopamine- and cAMP-regulated phosphoprotein inhibits Na,K-ATPase activity in renal tubule cells. *Proc Natl Acad Sci*. **88**:2798-2801.

Askari, A. (1987). ($Na^+K$)-ATPase: on the number of ATP sites of the functional unit. *J of Bioenerg and Biomembr*. **19**:359-374.

Beebe, D. C., J. T. Parmelee and K. S. Belcher (1990). Volume regulation in lens epithelial cells and differentiating lens fiber cells. *J Cell Physiol*. **143**: 455-459.

Beguin, P., Beggah, A.T., Chibalin, A.V., Burgener-Kairuz, P., Jaisser, F., Mathews, P.M., Rossier, B.C., Cotecchia, S., and Geering, K. (1994). Phosphorylation of the $Na^+,K^+$-ATPase α-subunit by protein kinase A and C *in vitro* and intact cells. Identification of a novel motif for PKC-mediated phosphorylation. *J of Biol Chem*. **269**:24437-24445.

Bertorello, A.M., Aperia, A., Walaas, I., Nairn, A.C., and Greengard, P. (1991). Phosphorylation of the catalytic subunit of $Na^+,K^+$-ATPase inhibits the activity of the enzyme. *Proc Natl Acad Sci*. **88**:11359-11362.

Blanquet, P. R. and F. Croquet (1995). Activation of phosphotyrosine phosphatase activity is associated with decreased differentiation in adult bovine lens. *J Cell Physiol*. **165**: 358-366.

Blanquet, P. R. and F. Croquet (1995). A high phosphotyrosine phosphatase activity is present in differentiating bovine lens cells. *Cell Biol Int*. **19**: 121-128.

Blaustein, M. P. and W. J. Lederer (1999). Sodium/calcium exchange: its physiological implications. *Physiol Rev*. **79**: 763-854.

Blanco, G., and Mercer, R.W. (1998). Isozymes of the Na,K-ATPase: heterogeneity in structure, diversity in function. *Amer Phys Soc*. F633-F650.

Blostein, R. (1999). Structure-function studies of the sodium pump. *Biochem Cell Biol*. **77**:1-10.

Boldyrev, A., and Kurella, E. (1996). Mechanism of oxidative damage of dog kidney Na/K-ATPase. *Biochem Biophys Res Commun*. **222**:483-487.

Borchman, D., Pateraon, C.A., and Delamere, N.A. (1989). Oxidative inhibition of $Ca^2$ -ATPase in the rabbit lens. *Invest Ophthalmol & Vis Sci*. **30**:1633-1637.

Bozulic, L.D., Dean, W.L., Delamere, N.A. (2003). The influence of Lyn kinase on Na,K-ATPase in porcine lens epithelium. *Am J Physiol (Cell). In press*.

Bozulic, L.D., Dean, W.L., Delamere, N.A. (2003). The influence of PTP-1B on Na,K-ATPase in lens. *Cellular Physiology. In press*.

Calvin, H., Wu, K., Li, W., Guo, L., Banerjee, U., and Fu, J. (2003). Induction of cortical cataracts in cultured mouse lenses with H-89, an inhibitor of protein kinase A. *Curr Eye Res*. **27**:269-278.

Candia, O. A. and A. C. Zamudio (2002). Regional distribution of the Na(+) and K(+) currents around the crystalline lens of rabbit. Am J Physiol Cell Physiol. **282**: C252-62.

Cappiello, M., Del-Corso, A., and Mura, U. (1994). Glutathione dependent modification of bovine lens aldose reductase. *Exp Eye Res.* **58**:491-501.

Chen, Y.H., Pouyssegur, J., Courtneidge, S.A., and Van Obberghen-Schilling, E. (1994). Activation of Src family kinase activity by the G protein-coupled thrombin receptor in growth-responsive fibroblasts. *J of Biol Chem.* **269**:27372-27377.

Chibalin, A. V., M. V. Kovalenko, et al. (2001). Insulin- and glucose-induced phosphorylation of the Na(+),K(+)adenosine triphosphatase alpha-subunits in rat skeletal muscle. Endocrinology. **142**: 3474-3482.

Chibalin, A. V., L. A. Vasilets, H. Hennekes, D. Pralong and K. Geering (1992). Phosphorylation of Na,K-ATPase alpha-subunits in microsomes and in homogenates of Xenopus oocytes resulting from the stimulation of protein kinase A and protein kinase C. *J Biol Chem.* **267**: 22378-22384.

Cho, M. J., T. I. Pestina, S. A. Steward, C. A. Lowell, C. W. Jackson and T. K. Gartner (2002). Role of the Src family kinase Lyn in TxA2 production, adenosine diphosphate secretion, Akt phosphorylation, and irreversible aggregation in platelets stimulated with gamma-thrombin. *Blood.* **99**: 2442-2447.

Cicmil, M., J. M. Thomas, T. Sage, F. A. Barry, M. Leduc, C. Bon and J. M. Gibbins (2000). Collagen, convulxin, and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelets. Evidence for the involvement of Src family kinases. *J Biol Chem.* **275**: 27339-27347.

Cleverley, K.E., Betts, J.C., Blackstock, W.P, Gallo, Jean-Marc, and Anderson, B.H. (1998). Identification of novel *in vitro* PKA phosporylation sites on the low and middle molecular mass neurofilament subunits by mass spectrometry. *Biochem.* **37**:3917-3930.

Corey, S., A. Eguinoa, K. Puyana-Theall, J. B. Bolen, L. Cantley, F. Mollinedo, T. R. Jackson, P. T. Hawkins and L. R. Stephens (1993). Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells. *Embo J.* **12**: 2681-2690.

Corey, S.J., and Anderson, S.M. (1999). Src-related protein tyrosine kinases in hematopoiesis. *Blood.* **93**: 1-14.

Couchman, G. M., R. Bentley, M. S. Tsao, K. Raszmann, J. A. McLachlan and D. K. Walmer (1997). p53lyn and p56lyn: a new signaling pathway in human endometrium and endometrial adenocarcinomas. *J Soc Gynecol Investig.* **4**: 103-109.

Cui, G., W. L. Dean and N. A. Delamere (2002). The influence of cycloheximide on Na,K-ATPase activity in cultured human lens epithelial cells. *Invest Ophthalmol Vis Sci.* **43**: 2714-2720.

Davson, H. (1990). The lens. Physiology of the Eye. H. Davson. New York, Pergamon Press: 139-201.

Dean, W.L., Chen, D., Brandt, P.C., and Vanaman, T.C. (1997). Regulation of platelet plasma membrane $Ca^{2+}$-ATPase by cAMP-dependent and tyrosine phosphorylation. *J Biol Chem.* **272**:15113-15119.

Dean, W.L., Delamere, N.A., Borchman, D., Moseley, A.E., and Ahuja, R.P. (1996). Studies on lipids and the activity of $Na^+,K^+$-ATPase in lens fiber cells. *Biochem J.* **314**:961-967.

De Franceschi, L., E. Villa-Moruzzi, L. Fumagalli, C. Brugnara, F. Turrini, R. Motta, E. Veghini, C. Corato, S. L. Alper and G. Berton (2001). K-Cl cotransport modulation by intracellular Mg in erythrocytes from mice bred for low and high Mg levels. *Am J Physiol Cell Physiol.* **281**: C1385-1395.

DeFranco, A. L., V. W. Chan and C. A. Lowell (1998). Positive and negative roles of the tyrosine kinase Lyn in B cell function. *Semin Immunol.* **10**: 299-307.

Delamere, N.A., and Dean, W.L. (1993). Distribution of lens sodium-potassium adenosine triphosphatase. *Investigative Ophthalmol Vis Sci.* **34**:2159-2163.

Delamere, N.A., Manning, R.E., Liu, L., Moseley, A.E. and Dean, W.L. (1998). Na,K-ATPase polyepetide upregulation responses in lens epithelium. *Investigative Ophthalmol Vis Sci.* **39**:763-768.

Delamere, N.A., Paterson, C.A., and Cotton, T.R. (1983). Lens cation transport and permeability changes following exposure to hydrogen peroxide. *Exp Eye Res.* **37**:45-53.

Delamere, N. A., W. L. Dean, J. M. Stidam and A. E. Moseley (1996). Influence of amphotericin B on the sodium pump of porcine lens epithelium. *Am J Physiol.* **270**(2 Pt 1): C465-473.

den Hertog, J., C. E. Pals, M. P. Peppelenbosch, L. G. Tertoolen, S. W. de Laat and W. Kruijer (1993). Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. *Embo J.* **12**: 3789-3798.

Donaldson, P., Kistler, J., and Mathias, R.T. (2001). Molecular solutions to mammalian lens transparency. *News Physiol Sci.* **16**:118-123.

Duncan, G. and A. R. Bushell (1975). Ion analyses of human cataractous lenses. *Exp Eye Res.* **20**: 223-230.

Ellis, D.K., Carr, A., and dePòmerai, D.I. pp60c-src expression in transdifferentiating cultures of embryonic chick neural retina cells. *Development.* **101**:847-856.

Feraille, E., Carranza, M.L., Gonin, S., Begun, P., Pedemonte, C., Rousselot, M., Caverzasio, J., Geering, K., Martin, P., and Favre, H. (1999). Insulin-induced stimulation of Na, K-ATPase activity in kidney proximal tubule cells depends on phosphorylation of the α-subunit at Tyr-10. *Mol Biol Cell.* **10**:2847-2859.

Feraille, E., Carranza, M.L., Rousselot, M., and Favre H. (1997). Modulation of the $Na^+,K^+$-ATPase activity by a tyrosine phosphorylation process in rat proximal convoluted tubule. *J of Physiol.* **498**:99-108.

Forrester, J.V., Dick,A.D., McMenamin, P., and Lee, W.R. (1996). The Eye. W.B. Saunders Company. Oval Road, London, England.

Fox, J. E. (1996). Platelet activation: new aspects. Haemostasis 26 Suppl **4**: 102-31.

Frangioni, J. V., P. H. Beahm, V. Shifrin, C. A. Jost and B. G. Neel (1992). The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell.* **68**: 545-560.

Frank, D. A. and A. C. Sartorelli (1986). Regulation of protein phosphotyrosine content by changes in tyrosine kinase and protein phosphotyrosine phosphatase activities during induced granulocytic and monocytic differentiation of HL-60 leukemia cells. *Biochem Biophys Res Commun.* **140**: 440-447.

Gao, J., X. Sun, et al. (2000). Isoform-specific function and distribution of Na/K pumps in the frog lens epithelium. J Membr Biol. **178**: 89-101.

Garner, M.H., Bahador, A., Nguyen, B., Wang, R., and Spector, A. (1992). $Na^+,K^+$-ATPase of cultured bovine lens epithelial cells $H_2O_2$ effects. *Exp Eye Res.* **54**:321-328.

Garner, M.H., and Spector, A. (1980). Selective oxidation of cysteine and methionine in normal and senile cataract lenses. *Proc Natl Acad Sci.* **27**:1274-1277.

Geering, K. (1997). Na, K-ATPase. *Curr Opin Nephrol Hypertens.* **6**:434-439.

Grandaliano, G., Monno, R., Ranieri, E., Gesualdo, L., Schena, F.P., Martino, C., and Ursi, M. (2000). Regenerative and proinflammatory effects of throombin on human proximal tubular cells. *J Ameri Soc Nephrol.* **11**:1016-1025.

Guptasarma, P., Balasubramanian, D., Matsugo, S., and Saito, I. (1992). Hydroxyl radical mediated damage to proteins, with special referance to the crystallins. *Biochem.* **31**:4296-4303.

Harding, j. (1991). Cataract: biochemistry, epidemiology, and pharmacology. Chapmand & Hall. New York, NY.

Hibbs, M. L., D. M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S. A. Stacker and A. R. Dunn (1995). Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell*. **83**: 301-311.

Hightower, K. R. and R. Farnum (1985). Calcium induces opacities in cultured human lenses. *Exp Eye Res*. **41**: 565-568.

Hirao, A., I. Hamaguchi, T. Suda and N. Yamaguchi (1997). Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets. *Embo J*. **16**: 2342-2351.

Horisberger, J.D. (1994). The $Na^+,K^+$-ATPase: structure function relationship. R.G. Landes Company. Georgetown, TX.

Isakov, N., and Biesinger, B. (2000). Lck protein tyrosine kinase is a key regulator of T-cell activation and a target for signal intervention by Herpesvirus saimiri and other viral gene products. *Euro J Biochem*. **267**:3413-3421.

Jaffe, N.S., and Horwitz, J. (1992). Lens and cataract. Gower medical publishing. New York, NY.

Kanagawa, M., S. Kaya, H. Umezu, S. Watanabe, K. Togawa, A. Shimada, T. Imagawa, S. Mardh and K. Taniguchi (1999). Direct evidence for in vivo reversible tyrosine phosphorylation of the N-terminal domain of the H/K-ATPase alpha-subunit in mammalian stomach cells. *J Biochem. (Tokyo)* **126**: 266-270.

Kanagawa, M., S. Watanabe, S. Kaya, K. Togawa, T. Imagawa, A. Shimada, K. Kikuchi and K. Taniguchi (2000). Membrane enzyme systems responsible for the Ca(2+)-dependent phosphorylation of Ser(27), the independent phosphorylation of Tyr(10) and Tyr(7), and the dephosphorylation of these phosphorylated residues in the alpha-chain of H/K-ATPase. *J Biochem. (Tokyo)* **127**: 821-828.

Khundmiri, S. J. and E. Lederer (2002). PTH and DA regulate Na-K ATPase through divergent pathways. Am J Physiol Renal Physiol. **282**: F512-22.

Kobayashi, S., Debdutta, R., and Spector, A. (1983). Sodium/potassium ATPase in normal and cataractous human lenses. *Curr Eye Res.* **2**:327-334.

Kovacic, B., Ilic, D., Damsky, C.H., and Gardner, D.G. c-Src activation plays a role in endothelin-dependent hypertrophy of the cardiac myocyte. *J Biol Chem.* **273**:35185-35193.

Kutz, C., M. Paintz and E. Glusa (1998). Inhibition of thrombin-induced contractile responses by protein kinase inhibitors in porcine pulmonary arteries. *Exp Toxicol Pathol*. **50**: 497-500.

Kwak, Y. G., S. K. Park, K. P. Cho and S. W. Chae (1996). Reciprocal modulation of ATP-sensitive K+ channel activity in rat ventricular myocytes by phosphorylation of tyrosine and serine/threonine residues. *Life Sci*. **58**: 897-904.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Levine, R.L. (1983). Oxidative modification of glutamine synthetase.I. Inactivation is due to loss of one histidine residue. *J Biol Chem.* **258**:11823-11827.

Lichtstein, D., Gati, I., Samuelov, S., Berson, D., Rozenman, Y., Landau, L., and Deutsch, J. (1993). Identification of digitalis-like compounds in human cataractous lenses. *Eur J Biochem.* **216**:216-268.

Li, R. Y., A. Ragab, F. Gaits, J. M. Ragab-Thomas and H. Chap (1994). Thrombin-induced redistribution of protein-tyrosine-phosphatases to the cytoskeletal complexes in human platelets. *Cell Mol Biol (Noisy-le-grand)*. **40**: 665-675.

Liu F, Chernoff J. (1997). Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. *Biochem J.* **1**:139-145.

Mahabeleshwar, G.H., and Kunda, G.C. (2003). Tyrosine kinase, p56lck regulates cell motility and nuclear factor kappa B-mediated secretion of urokinase type plasminogen activator through tyrosine phosphorylation of I kappa B alpha following hypoxia/reoxygenation. *J Biol Chem*. In Press.

Maraini, G., and Pasino, M. (1983). Active and passivve rubidium influx in normal human lenses and senile cataracts. *Exp Eye Res*. **36**:543-550.

Marcantonio, J.M.,Duncan, G., Davies, P.D., and Bushell, A.R. (1980). Classification of human senile cataracts by nuclear colour and sodium content. *Exp Eye Res*. **31**:227-237.

Marcantonio, J.M.,Duncan, and Rink, H. (1986). Calcium-induced opacification and loss of protein in the organ-cultured bovine lens. *Exp Eye Res*. **42**:617-630.

Mariotti, A., Kedeshian, P.A., Dans, M., Curatola, A.M., Gagnoux-Palacios, L., and Giancotti, F.G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol*. **155**:447-458.

Mathias, R. T., J. L. Rae and G. J. Baldo (1997). Physiological properties of the normal lens. *Physiol Rev*. **77**: 21-50.

Matsuda, T., Y. Murata, N. Kawamura, M. Hayashi, K. Tamada, K. Takuma, S. Maeda and A. Baba (1993). Selective induction of alpha 1 isoform of (Na+ + K+)-ATPase by insulin/insulin-like growth factor-I in cultured rat astrocytes. *Arch Biochem Biophys* **307**: 175-182.

Moeslein FM, Myers MP, Landreth GE. (1999). The CLK family kinases, CLK1 and CLK2, phosphorylate and activate the tyrosine phosphatase, PTP-1B. *J Biol Chem.* **274**:26697-26704.

Nakai, Y., W. L. Dean, Y. Hou and N. A. Delamere (1999). Genistein inhibits the regulation of active sodium-potassium transport by dopaminergic agonists in nonpigmented ciliary epithelium. *Invest Ophthalmol Vis Sci.* **40**: 1460-1466.

Narkar, V. A., T. Hussain, et al. (2002). Activation of D2-like receptors causes recruitment of tyrosine-phosphorylated NKA alpha 1-subunits in kidney. *Am J Physiol Renal Physiol.* **283**: F1290-5.

Okafor, M.C., Dean, W.L., and Delamere, N.A. (1999). Thrombin inhibits active sodium-potassium transport in porcine lens. *Investigative Ophthalmol Vis Sci.* **40**:2033-2038.

Okafor, M. C. and N. A. Delamere (2001). The inhibitory influence of endothelin on active sodium-potassium transport in porcine lens. *Invest Ophthalmol Vis Sci.* **42**: 1018-1023.

Omri, B., Blancher, C., Neron, B., Marty, M.C., Rutin, J., Molina, T.J., Pessac, B., and Crisanti, P. (1998). Retinal dysplasi in mice lacking p56lck. *Oncogene.* **16**:2351-2356.

Omri, B., Crisanti, P., Marty, M.C., Alliot, F., Fagard, R., Molina, T., and Pessac, B. (1996). The Lck tyrosine kinase is expressed in brain neurons. J *Neurochem.* **67**:1360-1364.

Parmelee, J. T. and D. C. Beebe (1988). Decreased membrane permeability to potassium is responsible for the cell volume increase that drives lens fiber cell elongation. *J Cell Physiol.* **134**: 491-496.

Pathan, N.I., Geahlen, R.L., Harrison, M.L. (1996). The protein tyrosine kinase Lck associates with and is phosphorylated by Cdc2. *J Biol Chem.* **217**:27517-27523.

Reddan JR. Giblin FJ. Dziedzic DC. McCready JP. Schrimscher L. Reddy VN. (1988). Influence of the activity of glutathione reductase on the response of cultured lens epithelial cells from young and old rabbits to hydrogen peroxide. *Exp Eye Res.* **46**:209-221.

Resing, K.A., and Ahn, N.G. (1997). Protein phosphorylation analysis by electrospray ionization-mass spectrometry. *Method Enzymol.* **283**:29-44.

Roche S.I., Fumagalli S., Courtneidge S.A. (1995). Requirement for src family protein tyrosine kinases in G2 for fibroblast cell division. *Science.* **269**:1567-1569.

Shi, H.G., Mikhaylova, L., Zichittella, A.E., and Arguello, J.M. (2000). Functional role of cysteine residues in the (Na,K)-ATPase α subunit. *Biochimica et Biophysica Acta.* **1464:**177-187.

Siyanova, E. Y., M. S. Serfas, I. A. Mazo and A. L. Tyner (1994). Tyrosine kinase gene expression in the mouse small intestine. *Oncogene.* **9**: 2053-2057.

Stanley, E., S. Ralph, S. McEwen, I. Boulet, D. A. Holtzman, P. Lock and A. R. Dunn (1991). Alternatively spliced murine lyn mRNAs encode distinct proteins. *Mol Cell Biol.* **11**: 3399-3406.

Suryawan A, Davis TA. (2003). Protein-tyrosine-phosphatase 1B activation is regulated developmentally in muscle of neonatal pigs. *Am J Physiol Endocrinol Metab.* **284**:E47-E54.

Sussman, M.R. (1988). Purification of integral plasma membrane proteins by reverse-phase high performance liquid chromatography. *Anal Bioch.* **169**:395-399.

Takahashi, T., Shirasawa, T., Miyake, K., Yahagi, Y., Maruyama, N., Kasahan, N., Kawamura, T., Matsumura, O., Mitarai, T., and Sakai, O. (1995). Protein tyrosine kinases expressed in glomeruli and cultured glomerular cells: Flt-1 and VEGF expression in renal mesangial cells. *Biochem Biophys Res Commun.* **209**:218-226.

Tamiya, S., W. L. Dean, et al. (2003). Regional distribution of Na,K-ATPase activity in porcine lens epithelium. Invest *Ophthalmol Vis Sci*. **44**:4395-4399.

Therien A.G., Blostein, R. (2000). Mechanisms of sodium pump regulation. *Am J of Physiol Cell Physiol*. **279**: C541-C566.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. *Annu Rev Cell Div Biol*. **13**:513-609.

Togawa, K., T. Ishiguro, S. Kaya, A. Shimada, T. Imagawa and K. Taniguchi (1995). Reversible phosphorylation of both Tyr7 and Tyr10 in the alpha-chain of pig stomach H+,K(+)-ATPase by a membrane-bound kinase and a phosphatase. *J Biol Chem*. **270**: 15475-15478.

Tonks, N. K., M. F. Cicirelli, C. D. Diltz, E. G. Krebs and E. H. Fischer (1990). Effect of microinjection of a low-Mr human placenta protein tyrosine phosphatase on induction of meiotic cell division in Xenopus oocytes. *Mol Cell Biol*. **10**: 458-463.

Tonks, N. K., C. D. Diltz and E. H. Fischer (1988). Characterization of the major protein-tyrosine-phosphatases of human placenta. *J Biol Chem*. **263**: 6731-6737.

Torigoe, T., R. O'Connor, D. Santoli and J. C. Reed (1992). Interleukin-3 regulates the activity of the LYN protein-tyrosine kinase in myeloid-committed leukemic cell lines. *Blood*. **80**: 617-624.

Van Iwarden P.R., Driessen, A.J., and Konings, W.N. (1992). What we can learn from the effects of thiol reagents on transport proteins. *Biochem Biophys Acta*. **1113**:161-170.

Walker, J. L., L. Zhang and A. S. Menko (2002). Transition between proliferation and differentiation for lens epithelial cells is regulated by Src family kinases. *Dev Dyn*. **224**: 361-372.

Wallick, E. T. and A. Schwartz (1988). Interaction of cardiac glycosides with Na+,K+-ATPase. *Methods Enzymol*. **156**: 201-213.

Whikehart, D.R., Edelhauser, H.F., and Woods, W.D. (1997). How might 12(R) HETTE cause the inhibition of Na,K-ATPase? *Mol Vis.* **3**:1-7.

Yi, T. L., J. B. Bolen and J. N. Ihle (1991). Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. *Mol Cell Biol.* **11**: 2391-2398.

Yingst, D. R., J. Davis and R. Schiebinger (2000). Inhibitors of tyrosine phosphatases block angiotensin II inhibition of Na(+) pump. *Eur J Pharmacol.* **406**: 49-52.

Yoshizumi, M., Abe, J.H., and Huang, Q.B. (2000). Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J Biol Chem.* **275**:11706-11712.

Yudowski, G.A., Efendiev, R., Pedemonte, C.H., Katz, A.I., Berggren, P.O., and Bertorello, A.M. (2000). Phosphoinositide-3 kinase binds to a proline-rich motif in the Na,K-ATPase alpha subunit and regulates trafficking. *Proc Natl Acad Sci.* **97**:6556-6561.

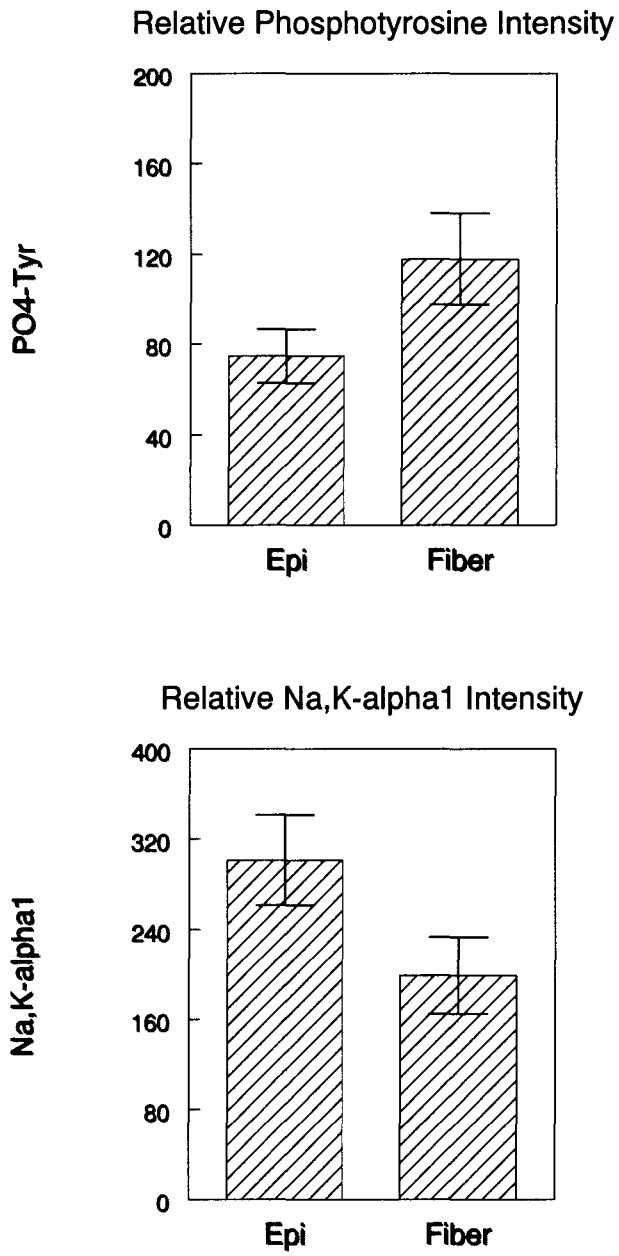
Zhou, J. and A. S. Menko (2002). The role of Src family kinases in cortical cataract formation. *Invest Ophthalmol Vis Sci* **43**: 2293-2300.

Zhou, S., K. L. Carraway, 3rd, et al. (1995). Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nature.* **373**: 536-9.

# APPENDIX I

Some experimental results acquired over the course of this study supporting the regulation of Na,K-ATPase by tyrosine phosphorylation were not added to chapters 2 and 3 of this study because these chapters contain the text of published papers. Some of the figures in the appendix were obtained during the studies described in the papers, but were deleted by editors of the papers or were decided to be unnecessary for the manuscripts. However, these experiments nevertheless contributed to the conclusions of the published work and therefore are presented in the appendix.

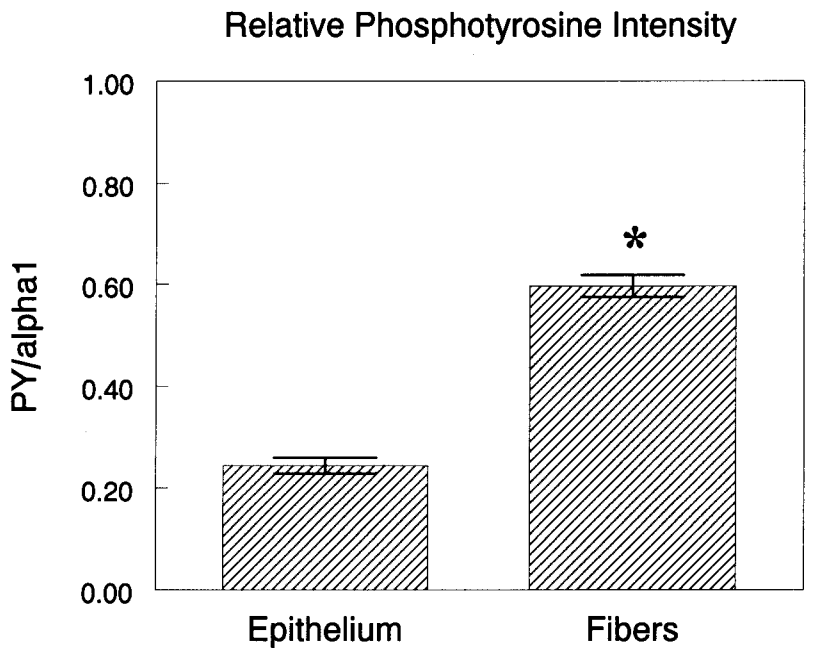
**Figure 1. 100 kDa phosphotyrosine (PY) and Na,K-ATPase α1 band densities.** Experiments were carried out in order to quantify the 100kDa phosphotyrosine and 100 kDa Na,K-ATPase α1 band densities from endogenous epithelium and fiber membrane preparation.



Lens epithelium and fiber membrane preparation (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against tyrosine phosphoproteins. Nitrocellulose membranes were exposed to x-ray film and the bands quantified using Kodak 1D image-analysis software. The data are presented as arbitrary units and are the mean ± SE (*vertical bar*) of normalized results from 7 independent determinations.

The intensity of the 100 kDa PY band appeared to be greater in lens fibers while the 100 kDa α1 band was higher in the lens epithelium.

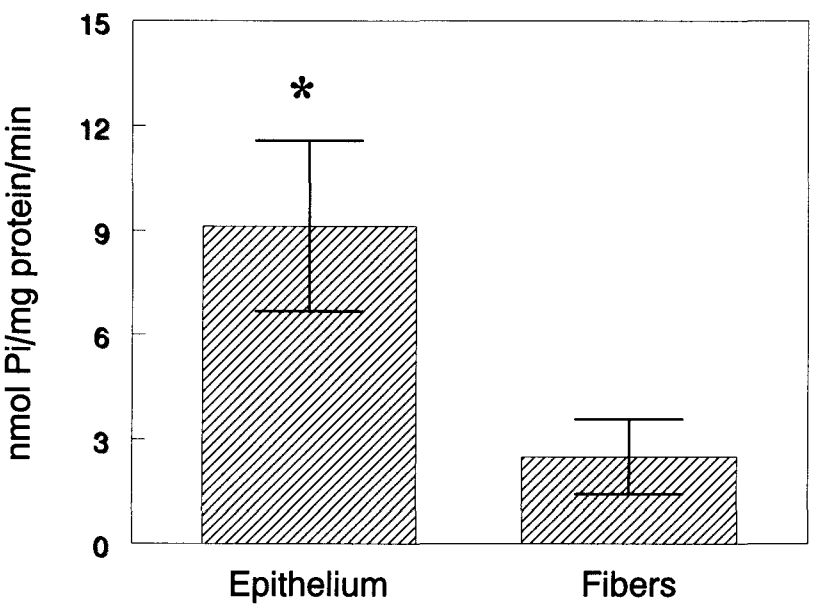
**Figure 2. Ratio of 100 kDa phosphotyrosine (PY) band intensity to Na,K-ATPase α1 band intensity.** Data from the previous figure was used in order to compare the ratio of 100kDa phosphotyrosine and 100 kDa Na,K-ATPase α1 band densities from endogenous epithelium and fiber membrane preparation.



The ratio of 100 kDa phosphotyrosine (PY) band intensity to Na,K-ATPase α1 band intensity was determined in lens epithelium and fiber membrane preparation. The data are presented as arbitrary units and are the mean ± SE (*vertical bar*) of normalized results from 7 independent determinations. *Indicates a significant difference from epithelium ($P<0.01$).

Lens fibers display a significantly higher degree of tyrosine phosphorylation of the 100 kDa band immunopositive for Na,K-ATPase α1 than lens epithelium.

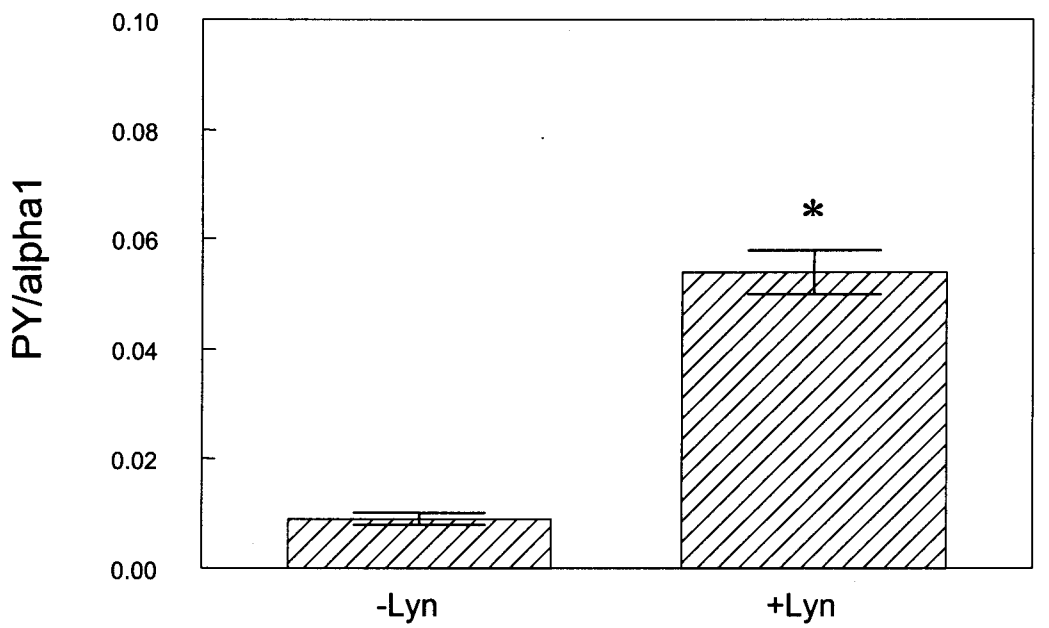
**Figure 3. Na,K-ATPase activity (ouabain-sensitive ATP-hydrolysis) measured in epithelium and fiber membrane preparation.** Experiments were carried out to compare the endogenous Na,K-ATPase activity between the two cell types. Earlier studies suggested that Na,K_ATPase activity was higher in the lens epithelium.



Lens epithelium and fiber membrane preparation was incubated in ATP-containing buffer for 45 minutes. Na,K-ATPase activity was defined as the difference between ATP hydrolysis in the presence and absence of ouabain. The data are shown as mean ± SE (*vertical bar*) of results from 5 independent determinations from several pools of membrane preparation. *Indicates a significant difference from fibers ($P<0.05$).

Na,K-ATpase activity was found to be significantly higher in lens epithelium than lens fibers. The higher degree of tyrosine phosphorylation in lens fibers might account for lower activity in these cells.

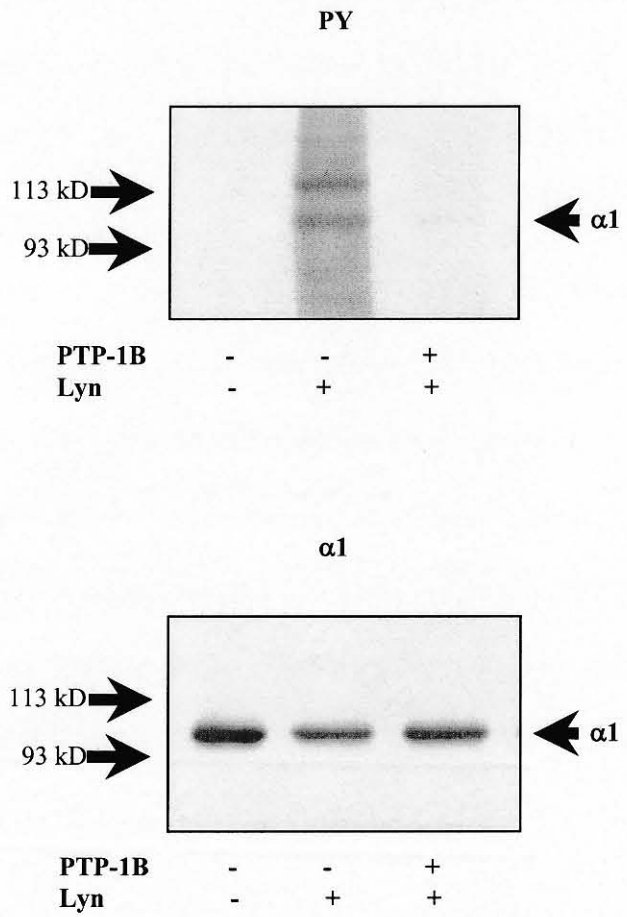
**Figure 4. The influence of Lyn kinase on Na,K-ATPase α1 subunit isolated by immunoprecipitation from lens epithelium membrane preparation.** Studies were carried out to determine if there is a difference between the PY/alpha1 100 kDa band intensities from immunoprecipitated samples treated with and without Lyn kinase.



The ratio of the 100 kDa phosphotyrosine band intensity to Na,K-ATPase α1 band intensity determined in immunoprecipitated samples treated with and without Lyn kinase. Raw data was normalized against common band densities between blots, setting the resultant ratios at arbitrary units. Each bar represents the mean +/- S.E. of three independent determinations. * indicates a significant difference from control ($P < 0.001$).

Tyrosine phosphorylation of Na,K-ATPase α1 was significantly higher in Lyn-treated immunoprecipitates.

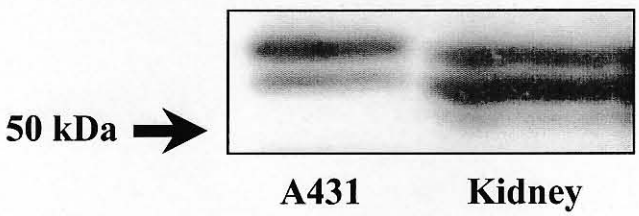
**Figure 5. The influence of PTP-1B on Lyn kinase-treated lens epithelium membrane preparation.** Experiements were carried out to determine if Lyn-kinase induced tyrosine phosphorylation of lens epithelium membrane preparation could be reversed with PTP-1B treatment.



Lens epithelium membrane preparation was incubated with partially purified Lyn kinase in ATP containing buffer for 20 minutes. Membrane preparation was then pelleted and incubated in the presence of PTP-1B (40 Units) for 20 minutes. Treated membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against phosphotyrosine residues (upper). The phosphotyrosine blot was stripped and reprobed with an antibody directed against Na,K-ATPase α1 (lower).

Lyn kinase tyrosine phosphorylated several membrane proteins, including a 100 kDa phosphotyrosine band that co-migrated with the Na,K-ATPase α1 protein. The 100 kDa phosphotyrosine band was observed in the sample obtained from Lyn kinase-treated lens epithelium membrane preparation but not from membrane preparations that had subsequently been treated with PTP-1B. PTP-1B reversed the Lyn effect.

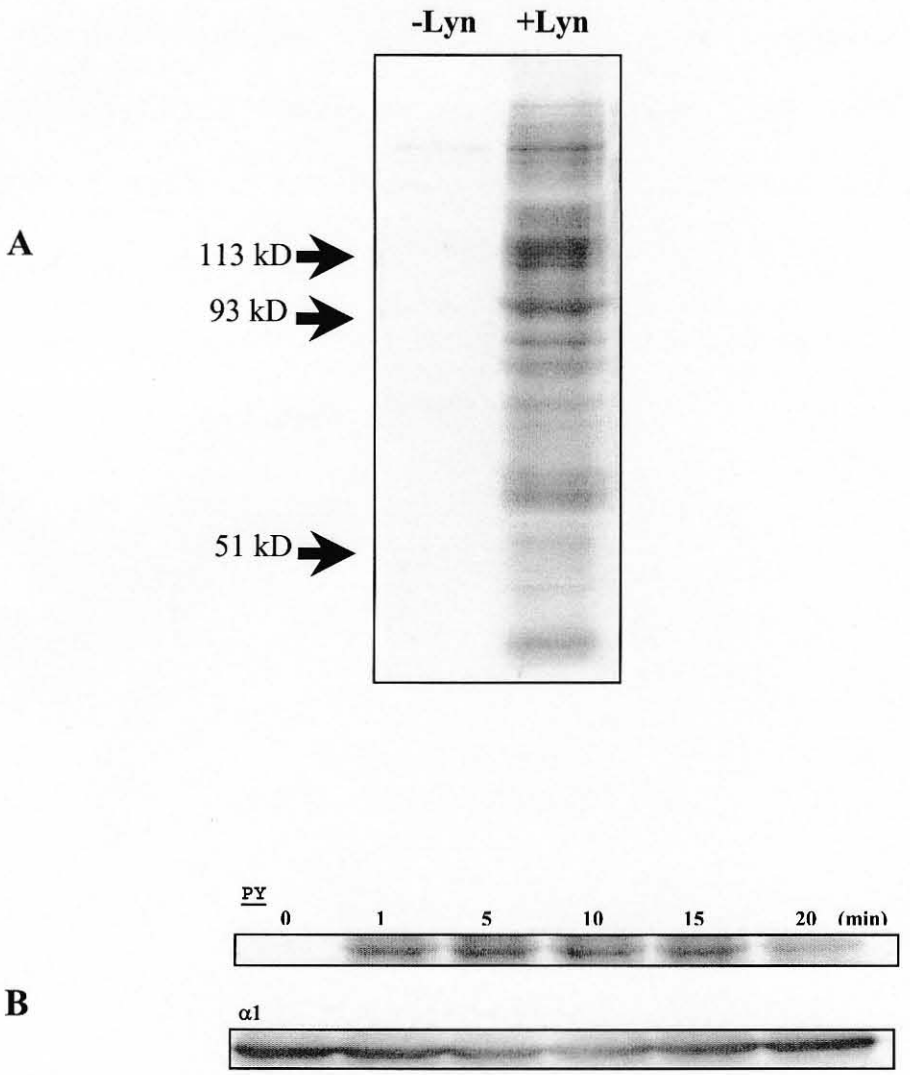
**Figure 6. Detection of Lyn kinase in kidney outer medulla membrane preparation.** Studies were carried out in membrane preparations isolated from porcine kidney in order to determine if Lyn kinase could be detected in a non-lens tissue where the major Na,K-ATPase isoform is α1.




Kidney membrane preparation (50 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody directed against Lyn kinase. A431 non-stimulated cell lysate (20 μg) was used as a positive control for Lyn kinase expression.

Lyn isoforms A and B were both detected in kidney medulla membrane preparation.

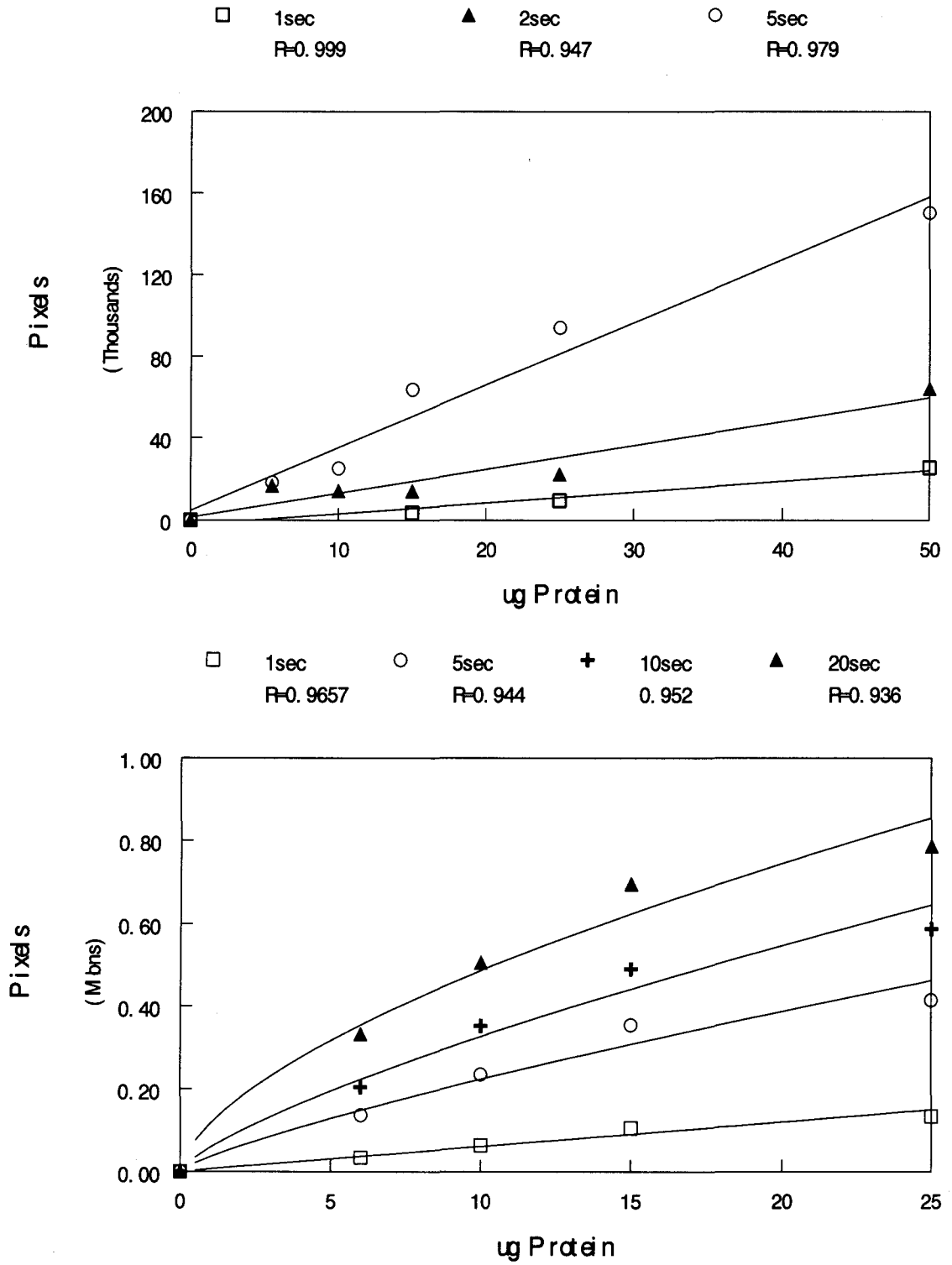
**Figure 7. The influence of Lyn kinase on kidney outer medulla membrane preparation.** Studies were carried out in membrane preparation isolated from porcine kidney in order to compare the response to Lyn kinase in a non-lens tissue where the major Na,K-ATPase isoform is α1.



Panel A: phosphotyrosine western blot. Kidney membrane preparation (25 μg) was incubated with partially purified Lyn kinase (2 Units) in ATP-containing buffer for 1 minute. Panel B: time course for tyrosine phosphorylation of proteins at ~100 kDa. Kidney membrane preparation (25 μg) was incubated with Lyn kinase (2 Units) in ATP-containing buffer for 0-20 minutes. The phosphotyrosine western blot (upper) was stripped and reprobed with a monoclonal antibody directed against Na,K-ATPase α1 (lower).

Lyn phosphorylated many bands including a 100kDa band that co-migrated with Na,K-ATPase α1.

**Figure 8. Quantitation of Western blot data.** Studies were carried out to determine if increased pixel/band strength correlated with increased protein concentration.

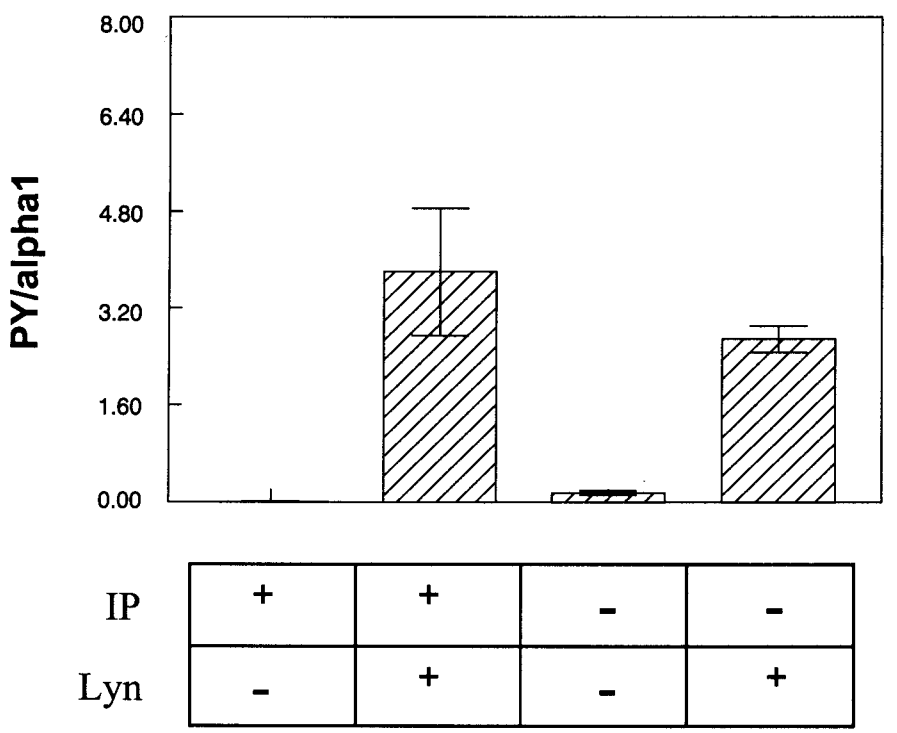


Different amounts of lens epithelium membrane preparations were subjected to SDS-PAGE and western blot analysis. Blots were exposed to film for 1-5 seconds for PY (upper) analysis or 1-20 seconds for alpha 1 (lower) analysis. Kodak imaging software was used to determine the total pixels present in the 100 kDa band at different concentrations and exposure times. The experiments described in chapters 2-4 were all in the range where pixel/band vs. protein plot shows an increase with increasing membrane protein added. Lines were drawn using linear regression.

The ratio of pixel/band strength is the relevant measurement, not the amount of protein added since some of the experiments were immunoprecipitations with a different relationship between μg protein and signal. The amount of phosphorylated tyrosine residues and Na,K-ATPase signals vary from preparation to preparation. This experiment is really a measure of film sensitivity. Though the pixel/band intensities increases with the amount of protein, the slope of each line depends on the exposure time of each blot. Since the exposure times of the experimental blots are not known and internal standards were not run on all blots, PY to Na,K-ATPase α1 pixel

intensity does not reflect the actual stoichiometry of tyrosine phosphorylation.

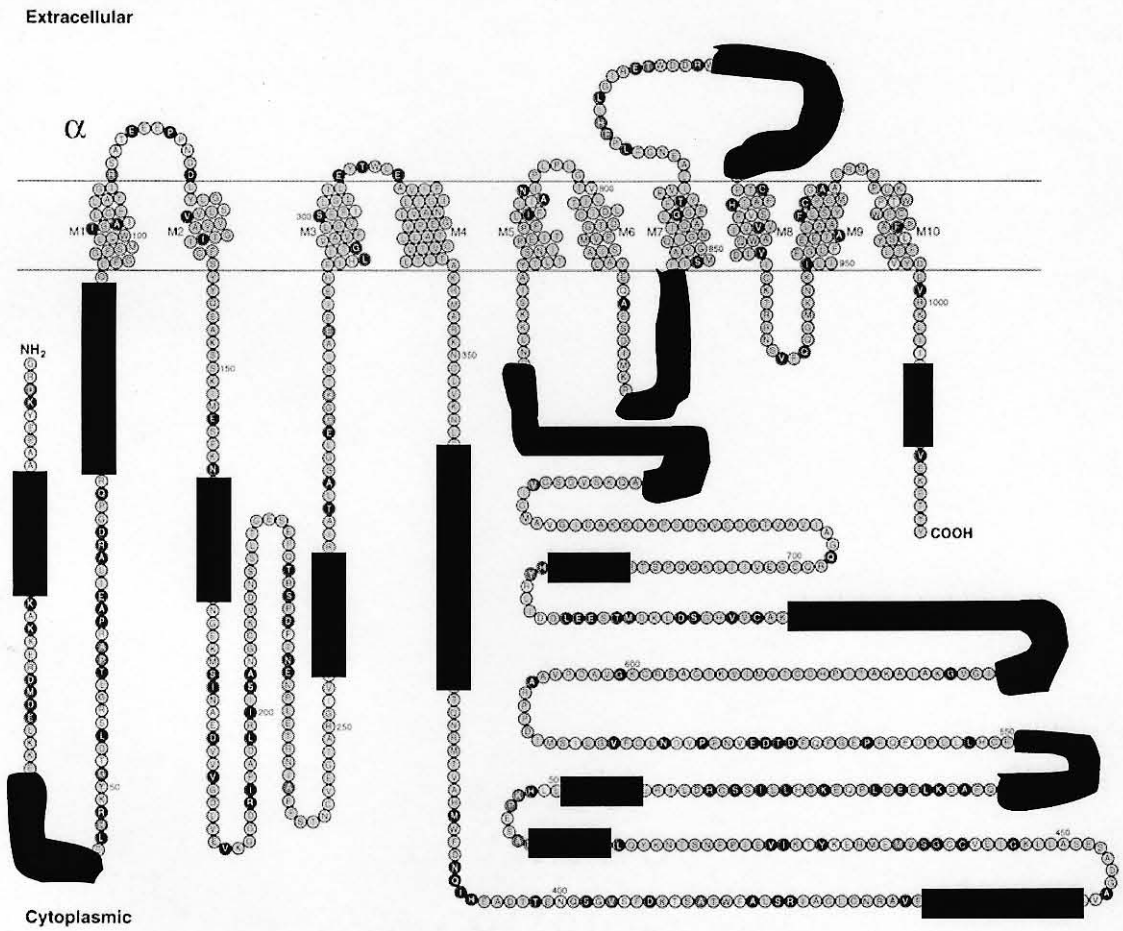
**Figure 9. Quantification of immunoprecipitated Na,K-ATPase α1 subunit from Lyn kinase-treated kidney membrane preparation.** To consider the possibility that tyrosine phosphorylation of 100 kDa membrane proteins other than the Na,K-ATPase α1 subunit takes place during Lyn treatment, the ratio of phosphotyrosine band intensity to Na,K-ATPase α1 band intensity was calculated for Lyn-treated kidney membrane immunoprecipitates and compared with the ratio calculated for non-immunoprecipitated Lyn-treated kidney membrane samples.



The ratio of the 100 kDa phosphotyrosine band intensity to alpha1 band intensity determined in immunoprecipitated and non-immunoprecipitated samples following incubation in the presence or absence of partially purified Lyn kinase in ATP-containing buffer for 20 minutes. Each bar represents the mean +/- S.E. of four independent determinations.

The similarity of the phosphotyrosine band intensity ratios suggests that Na,K-ATPase α1 is the major phosphorylated protein at 100 kDa. PY data for Lyn-treated kidney membrane preparation (-IP in figure) is an underestimate based on the fact that the PY signal was beyond the linear range of pixel response shown in figure 8. Therefore this value is an underestimate of the PY/alph1 ratio.

**Figure 10. Na,K-ATPase α1 peptides identified by MALDI-TOF analysis overlayed in black on the Na,K-ATPase α1 subunit.**




Database search analysis resulted in a 20% coverage of the Na,K-ATPase α1 protein sequence from trypsin-digested lens epithelium membrane proteins.

# APPENDIX II

## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ACN | Acetonitrile |
| ADP | Adenosine Diphosphate |
| ATP | Adenosine Triphosphate |
| BCA | Bicinchoninic Acid |
| BSA | Bovine Serum Albumin |
| cdc2 | Cell Division Control 2 |
| CLK | cdc-2-Like Kinase |
| D-1 | Dopamine-1 |
| Da | Dalton |
| DLC | Digitalis-Like Compounds |
| DTT | Dithithreitol |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGFR | Epidermal Growth Factor Receptor |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| Epi | Epithelium |
| ET-1 | Endothelin-1 |
| ExPasy | expert Protein Analysis System |
| GAP | GTPase Activating Protein |
| G2 | GAP 2 |
| HEK 293 | Human Embryonic Kidney Cell Line |
| HEPES | Hydroxyethyl Piperazineethanesulfonic Acid |

| | |
|---|---|
| H,K-ATPase | Hydrogen Potassium Adenosine Triphosphatase |
| HL-60 | human leukemic Cell Line |
| HLE-B3 | Human Lens Epithelium Cell Line |
| *In vitro* | Latin: Outside the Living Cell |
| IP | Immunoprecipitation |
| Jurkat | Human T Cell Leukemia |
| kDa | Kilodalton |
| IgG | Immunoglobulin G |
| M | Mitosis |
| MALDI-TOF | Mass Assisted Laser Desorption Ionization-Time of Flight |
| Na,K-ATPase | Sodium Potassium Adenosine Triphosphatase |
| OK | Opossum Kidney |
| PAGE | Polyacylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| $P_i$ | Inorganic Phosphate |
| PKA | Protein Kinase A |
| PKC | Protein kinase C |
| PI3-K | Phosphoinositol-3 Kinase |
| PMCA | Plasma Membrane Calcium ATPase |
| PMSF | Phenylmethylsulfonyl Fluoride |
| PP1 | {{4-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine}} |
| PTK | Protein Tyrosine Kinase |

| | |
|---|---|
| PTPases | Protein Tyrosine Phosphatases |
| PTP-1B | Protein Tyrosine Phosphatase-1B |
| PY | Phosphotyrosine |
| PY20 | Anti-Phosphotyrosine Antibody |
| SDS | Sodium Dodecyl Sulphate |
| SE | Standard Error |
| SH2 | Src-homology 2 |
| SH3 | Src-homology 3 |
| Tris | Trizma® -Tris-hydroxymethylaminomethane |
| TTBS | Tween-Tris Buffered Saline |
| Tween 20 | Polyoxyethylenesorbitan Monolaurate |
| Y10 | Tyrosine-10 |
| W/V | Weight/Volume |

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Conformational Modulation of Troponin T by Configuration of the $NH_2$-Terminal Variable Region and COOH-Terminal Domain
March 1999

Allosteric Modulation by the Transducin α-Subunit Helical Domain
January 2000

Modulation of Sodium Channel Expression by Schwann Cells in Spinal Sensory Neurons and Possible Regulatory Pathways
May 2000

Recycling of $Na^+,K^+$-ATPase in the Regulation of Kidney Sodium Reabsorption
November 2000

Posttranslational Modification of $Na^+,K^+$-ATPase in Lens Cells
January 2001

A Novel Role for Ceramide in the Regulation of Protein Synthesis
March 2001

Regulation of Na+,K+-ATPase Activity in Lens Cells by TyrosinePhosphorylation
October 2003

**ABSTRACTS**

**A1** Delamere, NA, Okafor MC, Bozulic LD. Studies on Tyrosine Phosphorylation and Modulation of Na,K-ATPase Activity in Lens Epithelium. US-Japan - Cooperative Cataract Research, Hawaii, Nov 3-Nov 7, 2002.

**A2** Bozulic LD, Dean WL, Delamere NA. Tyrosine Phosphorylation by Src-Family Tyrosine Kinases Alters Lens Na,K-ATPase Activity. Transport 2002 International Scientific Symposium, Miami, FL, May3-May 4, 2002. Annual Vision Research Conference, Signal Transduction I, Fort Lauderdale, FL, May 5-May 10, 2002.

**A3** Delamere, NA, Okafor MC, Dean WL, Bozulic LD. Modulation of Lens Na,K-ATPase Activity by a Tyrosine Phosphorylation Mechanism. International Congress of Eye Research, Geneva, Switzerland, Oct 6-Oct 19, 2002.

**A4** Bozulic LD, Dean WL, Delamere NA. Studies on Na,K-ATPase Activity in Lens Fiber Cells. International Congress of Eye Research, Geneva, Switzerland, Oct 6-Oct 19, 2002.

**A5** Bozulic LD, Dean WL, Delamere NA. The Influence of Protein Tyrosine Phosphatase-1B on Na,K-ATPase Actviity in Lens. Louisville Research Day, Louisville, KY, November 4, 2003.

**PUBLICATIONS**

**P1** Bozulic LD, Dean WL, Delamere NA. (2003). The influence of Lyn kinase on Na,K-ATPase in porcine lens epithelium. American Journal of Physiology (Cell) In press.

**P2** Bozulic LD, Dean WL, Delamere NA. (2003). The influence of PTP-1B on Na,K-ATPase in porcine lens epithelium. Journal of Cellular Physiology. In Press.

**P3** Bozulic LD, Dean WL, Delamere NA. (2003). The influence of Src-family tyrosine kinases on Na,K-ATPase activity in lens epithelium. Submitted.

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| Participant | International Congress of Eye Research October 2002 |
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