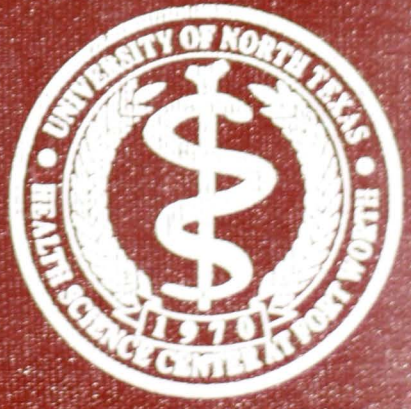
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Bovine retinal pigment epithelial (RPE) cells were cultured under 1, 4.5 and 10 g/l glucose conditions in order to characterize the effects of hyperglycemia upon Na^+/K^+-ATPase. Functional activity of Na^+/K^+-ATPase was measured as ouabain-sensitive Rb^+ uptake. ^3H ouabain was used to assess binding characteristics of Na^+/K^+-ATPase.

The major contributors to rubidium ($^{86}Rb^+$) uptake activity were the ouabain-sensitive Na^+/K^+-ATPase and a bumetanide-sensitive $Na^+/K^+/Cl^-$-cotransporter. Dose response curves for ouabain and bumetanide produced IC_{50} values for $^{86}Rb^+$ uptake of 60-100 nM and 120 nM, respectively. At elevated glucose concentrations, the aldose reductase inhibitor (ARI) AL-1576 stimulated $^{86}Rb^+$ uptake upon chronic treatment.

A sensitive new nonradioactive Rb^+ uptake assay was developed which utilized suppressed conductivity detection and provided several advantages over the radioactive method. The average ouabain IC_{50} value was confirmed to be 100 nM and was not significantly affected by elevated glucose concentrations. The bumetanide sensitive component was responsible for approximately 30% of Rb^+ uptake at all glucose concentrations. Potassium efflux out of the cells was observed that was sensitive to the $Na^+/K^+/Cl^-$-

cotransport inhibitor bumetanide. Elevated glucose appeared to increase Rb^+ transport through potassium channels and also reduced Rb^+ uptake indicating a decrease in Na^+/K^+-ATPase activity. Bovine RPE cells exposed to both high glucose and AL-1576 for one month showed mild stimulation of Rb^+ uptake compared to the activity in high glucose alone.

Ouabain and strophanthidin inhibition of ^3H ouabain binding, in bovine RPE cells, appeared to be unaffected by hyperglycemia. The average IC_{50} values for these compounds were 5.02×10^{-8} M ($K_d = 2.74 \times 10^{-8}$ M) and 8.81×10^{-9} M, respectively.

The results of this study indicate that Na^+/K^+-ATPase activity in bovine RPE is decreased in the hyperglycemic state, and can be stimulated by treatment with an aldose reductase inhibitor administered from the onset of the hyperglycemic insult.

THE EFFECTS OF ELEVATED GLUCOSE

UPON NA^+/K^+-ATPASE IN BOVINE

RETINAL PIGMENT EPITHELIAL CELLS

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THE EFFECTS OF ELEVATED GLUCOSE UPON NA^+/K^+-ATPASE IN BOVINE RETINAL PIGMENT EPITHELIAL CELLS

DISSERTATION

Presented to the Graduate Council of the

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University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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CHAPTER I

INTRODUCTION TO THE STUDY

The retinal pigment epithelial (RPE) layer serves a variety of important functions including: the maintenance of the blood retinal barrier (BRB), modulation of choriocapillaris structure, regulation of retinal ionic flow, phagocytosis of photoreceptor outer segments, recycling of visual pigments, light absorption, retinal adhesion, and protection against free radical damage (Bok, 1990, 1993; Boulton, 1990; Marmor et al., 1980; Negi and Marmor, 1986; Korte et al., 1984; Pfeffer, 1991). The impairment of any of these functions may give rise to a pathological state leading to loss of visual acuity and eventual blindness (Eagle, 1984). The BRB is known to be compromised at the level of the RPE under conditions of diabetes (Blair et al., 1984; Kirber et al., 1980; Krupin et al., 1978; Tso et al., 1977, 1980; Vinores et al., 1990). Therefore, the RPE cell appears to be a useful system for modeling certain aspects of diabetic retinopathy (Khatami, 1991; MacGregor et al., 1986; MacGregor and Matschinsky, 1986).

Na^+/K^+-ATPase, also known as the sodium pump, is an important enzyme that is responsible for the active transport of Na^+ and K^+ in mammalian cells. In addition to maintaining intracellular Na^+ and K^+ concentrations within the normal range, Na^+/K^+-ATPase in RPE may play a role in the regulation of cell volume (Miller et al., 1978) and retinal adhesion (Marmor et al., 1980). The

location of this important cationic pump has been shown, in RPE cells, to be predominantly on the apical membrane (Jaffe et al., 1989; Ostwald and Steinberg, 1980; Rizzolo, 1990; Steinberg and Miller, 1979). The number of ouabain binding sites (reflecting pump number) in cultured RPE cells correlates with increased proliferation and decreased culture density (Jaffe et al., 1989). Experiments conducted *in vitro* may further characterize the modulation of pump activity under pathologic conditions.

Some studies have shown that elevated concentrations of glucose and galactose affect the functioning of Na^+/K^+-ATPase. This enzyme is hypothesized to be a key link between altered tissue metabolism and nerve function in diabetes (Greene et al., 1985). Experiments performed *in vivo* show an increase in Na^+/K^+-ATPase activity in sciatic nerve from rats with galactosemia but an inhibition in the diabetic state (Lambourne et al., 1987). Many of the studies of pump activity in the eye have involved measurements in the lens. Substrate inhibition kinetics are characteristic of the enzyme in lenses from patients with cataracts and in lenses cultured under elevated glucose conditions (250 mM, Garner and Spector, 1986). The authors hypothesized that this effect leads to inhibition of Na^+/K^+-ATPase-dependent K^+ entry into the cell. Pump activity in the lens may be irreversibly impaired soon after the onset of diabetes even in the presence of insulin therapy (Tehrani et al., 1990). Studies with diabetic rabbits have documented a decrease in pump activity in

the RPE (MacGregor and Matschinsky, 1986). As expected, total sodium levels in this layer were elevated under conditions of diabetes.

In animal models of diabetes, several weeks may be required to allow for the progression of the disease. Significant increases in synthesis of $\alpha 2$ and $\alpha 3$ isoforms of ATPase (Specht et al., 1991) were present in the kidney and retina of diabetic rats after four months (not statistically significant at one month), for example. Studies modeling diabetic conditions can be performed acutely in this *in vitro* model because the RPE cells are removed from the compensatory mechanisms provided by the body. Due to the importance of the RPE in maintaining normal retinal function, it is of considerable interest to examine the response of this cell type to hyperglycemia and other metabolic insults.

This dissertation addresses the hypothesis that deficits in Na^+/K^+-ATPase activity (as measured by rubidium uptake) and altered expression of the enzyme (as measured by ouabain binding) are responses of cultured RPE cells to hyperglycemia.

In the present study, the experimental approach for studying the bovine RPE cation transport involved tissue culture techniques. This method offers several advantages over *in vivo* studies (Pfeffer, 1991):

1. Other cell types which could confound or complicate certain procedures and interpretation of the data are excluded.

2. One can obtain larger numbers of cells for study by propagation with resultant proliferation and subculture.

3. The experimenter has a greater degree of control as to substrate and chemical milieu with which the cells come in contact.

CHAPTER II

LITERATURE REVIEW

Retinal Pigment Epithelium

Retinal pigment epithelial cells (RPE) are highly specialized to perform a number of vital functions necessary in maintaining the normal physiology of the retina. Distinctive structural features include a regular hexagonal shape, tight junctions, extensive apical microvilli, and basal membrane infoldings. Melanin granules within the cell function in the absorbance of scattered light allowing for increased visual acuity. Phagosomes are responsible for the degradation of photoreceptor outer segments that are continually shed. RPE cells are involved in the selective transport of ions, water, and nutrients between the choroidal blood supply and the retina. Hyperpolarization of the apical membrane of the RPE is the source of the c-wave of the electroretinogram, (Noell, 1954; Steinberg et al., 1970) that is a result of a light-evoked decrease in retinal $[K^+]o$ (Oakley and Green, 1976; Oakley et al., 1977; Oakley, 1979).

RPE cells play a vital role in the visual cycle (rev. by Bok, 1993). The function of this cycle is to regenerate 11-cis retinaldehyde, the chromophore of the rod and cone photoreceptors used in phototransduction. All-trans retinol is the product of photoisomerization and reduction in the outer segments of the photoreceptors. This molecule passes from the photoreceptors, through the subretinal space to the RPE cells. Reisomerization to 11-cis retinal and

oxidation take place prior to the release of this molecule across the apical membrane of the RPE.

The fact that the RPE layer exhibits tight junctions is important in the maintenance of the blood retinal barrier. Even small molecules are required to traverse the RPE cells prior to gaining access to the photoreceptors (Hewitt and Adler, 1989). Retinal adhesion is maintained by subretinal fluid resorption primarily across the RPE. Frog studies have measured fluid transport from the retinal to the choroidal side of the RPE at a rate of 4-6 $\mu l/cm^2/h$ (Hughes et al., 1984). It has been estimated that approximately 70 percent of this absorption is dependent on ion transport (Negi and Marmor, 1986). The pathogenesis of diabetic macular edema may be linked to impaired RPE fluid transport (Bresnick, 1983).

Human retinal pigment epithelial cells originate during development from the primitive forebrain (Mund and Rodrigues, 1979). The ectoderm is the precursor for this tissue. At the third week of development, small protuberances form on the neural tube called the optic vesicles (rev. by Nordlund, 1989). These vesicles invaginate to form the optic cup around the seventh week of gestation. The outer layer of the cup forms the retinal pigment epithelium, while the inner layer forms the neural retina.

Melanogenesis in human RPE begins around the 7th week of gestation. Melanin forms within the melanosomes through the action of the enzyme tyrosinase. Activity of this enzyme has been detected through the fifth month of

gestation but is apparently absent at birth (Endo and Hu, 1973; Hu and Montagna, 1971). Melanin is thought to have several functions: sunscreen, oxygen scavenger, by-product for detoxification of tyrosine and cysteine radicals, and embryonic inducer (Nordlund, 1989; Chedekel, 1982).

Several ionic transport pathways have been demonstrated in bovine RPE and are shown in Figure 1. Ion transport mechanisms in bovine RPE include:

1. A ouabain-sensitive Na^+/K^+-ATPase (also referred to as the sodium pump)(Miller and Edelman, 1990)
2. A bumetanide-sensitive $Na^+/K^+/Cl^-$-cotransporter (Joseph and Miller, 1991, 1992)
3. A barium sensitive K^+ channel that recycles most of the K^+ that is taken up by the cotransporter or the ATPase (Joseph and Miller, 1991, 1992)
4. An amiloride-sensitive Na^+/H^+ antiporter (Keller et al., 1986, 1987, 1988)
5. A 4,4'-diisothiocyano-2-2'-disulfonic acid stilbene (DIDS)-sensitive Cl^-/HCO_3^- antiporter (Bialek and Miller, 1994)
6. A bepridil sensitive Na^+/Ca^+ exchanger (Fujisawa et al., 1992)
7. A Cl^- transporter that is sensitive to stimulation by Ca^{2+} possibly by α_1 receptor activation (Joseph and Miller, 1991, 1992)(The α_{1c} receptor subtype is hypothesized)(Horie et al., 1993)

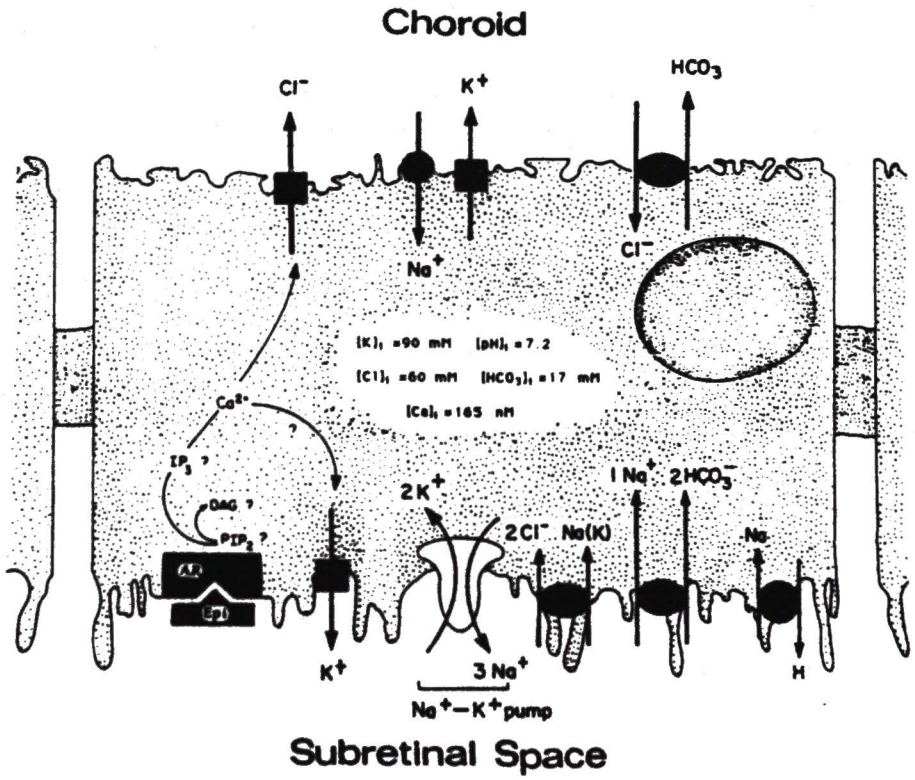


Figure 1. A model of ion transport pathways in bovine RPE. (Modified from Edelman and Miller, 1991).

$Na^+/K^+/Cl^-$-cotransporter

The bumetanide-sensitive $Na^+/K^+/Cl^-$-cotransporter has been observed in a number of cell types including epithelial cells (rev. by Haas, 1989; Kort and Koch, 1990). This molecule has been noted in frog (Adorante and Miller, 1990; Wiederholt and Zadunaisky, 1984), chick embryonic (Frambach and Misfeldt, 1983), monkey (Kennedy, 1992), bovine (Miller and Edelman, 1990) and human retinal pigment epithelial cells (Kennedy, 1990). The cotransporter also was discovered recently in cultured bovine lens epithelial cells (Alvarez and Candia, 1994). This membrane protein is responsible for the electrically silent movement of K^+, Na^+, and Cl^- into the cells with a stoichiometry of 1:1:2, respectively (Geck et al., 1980). This molecule (rev. by O'Grady et al, 1987) is reportedly involved in volume regulation (Adorante and Miller, 1990), fluid transport (Tsuboi and Pederson, 1986), retinal hydration (Li et al., 1994), Cl^- movement (Frambach and Misfeldt, 1983; Miller and Edelman, 1990; Tsuboi et al., 1986), and internal Cl^- control (Joseph and Miller, 1991; Wiederholt and Zadunaisky, 1984). This molecule may be induced in cells undergoing proliferation (Alvarez and Candia, 1994; Vandewalle et al., 1993). Studies with cultured human fibroblasts showed that cotransport was not affected by 10% serum, but was moderately stimulated by epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Owen and Prastein, 1985). In monkey RPE, the $Na^+/K^+/Cl^-$-cotransporter is stimulated by increased osmolarity and inhibited by elevated cAMP (Kennedy, 1992). In contrast, fetal human nonpigmented

ciliary epithelial cell cotransport is stimulated by cAMP (Crook and Polansky, 1994). Ouabain (1 mM) inhibition of Na^+/K^+-ATPase causes a 75% increase in $Na^+/K^+/Cl^-$-cotransporter mediated $^{86}Rb^+$ uptake in rabbit non-pigmented ciliary epithelial cells (Dong et al., 1994). Similarly, rat cardiac myocyte $Na^+/K^+/Cl^-$-cotransporter mediated $^{86}Rb^+$ uptake is stimulated by 10^{-7} M ouabain (Panet et al., 1990). Intracellular loss of potassium may contribute to this effect by increasing the inward driving force for the cotransporter. This transport molecule also may play an important role in the pathogenesis of diabetic complications.

Complications of Diabetes

The disruption of glucose regulating mechanisms in the body produces a number of adverse physiological manifestations (rev. by Guyton, 1986). In diabetes mellitus there is a reduction in glucose utilization by the cells of the body resulting in blood glucose concentrations as high as 300 to 1200 mg/dl. High extracellular glucose exerts a significant osmotic pressure which results in cellular dehydration. Large quantities of glucose are lost in the urine which produces an osmotic diuresis. This also can lead to disturbances in fluid and electrolyte balance. Protein depletion and abnormal fat metabolism also are seen in diabetes.

The regulation of blood glucose is particularly important for the brain and retina since it is the only nutrient that these tissues can utilize in sufficient

quantities to meet their metabolic requirements. As a result, cellular glucose uptake is independent of insulin in these tissues as well as in lens and peripheral nerve (Kornblueth et al., 1953). Under hyperglycemic conditions these tissues are particularly sensitive to diabetic complications.

Chronic elevations in glucose concentrations have been shown to affect the functioning of a number of proteins (rev. by Brownlee et al., 1984). Glucose reacts with the NH_2-terminal residue of the β chain of hemoglobin to form hemoglobin A_{1c} (Holmquist and Schroeder, 1966). This posttranslational modification is dependent on time and degree of severity of diabetes. Hemoglobin A_{1c} may form at three times the normal rate under hyperglycemic conditions (Koenig and Cerami, 1975). Erythrocyte membrane proteins show increased amounts of glycation under diabetic conditions (Miller et al., 1980). Nonenzymatic glycation of peripheral nerve proteins can reportedly increase by two to three fold in diabetic dogs and rats (Vlassara and Brownlee, 1981). Human serum albumin is also subject to nonenzymatic glycation with a resultant decrease in binding of ligands such as bilirubin (50%) and long chain fatty acids (i.e. *cis*-parinaric acid, 20 fold; Shakai et al., 1984). The degree of glucose incorporation into normal human lens crystallin increases with age in a linear fashion. Garlick et al. (1984) also showed a two-fold increase in the level of glycated crystallin in diabetes *versus* control. A value of >10% glycated hemoglobin was associated with an increased risk for progression of retinopathy in a recent clinical study (Brinchmann-Hansen et al., 1992).

Diabetic retinopathy is the major cause of blindness in people from the age of 25 to 74 years (rev. by D'Amico, 1994). Nonproliferative retinopathy involves clinical signs such as hard exudates, intraretinal microaneurysms, hemorrhages, nerve-fiber infarcts and various microvascular abnormalities. The hallmark of proliferative retinopathy is neovascularization originating from the optic disk or retinal vessels. An estimated 12,000 to 24,000 new cases of blindness each year are produced by this diabetic retinopathy (National Society to Prevent Blindness, 1980; Will et al., 1990).

Chronic hyperglycemia appears to be a major cause for the development of retinopathy (Brinchmann-Hansen et al., 1992). Interestingly, it has been shown through ERG studies that the c-wave signal shows a reduced sensitivity to hypoxia under diabetic conditions (Rimmer and Linsenmeier, 1993). This may result from a decrease in ATP production and a reduction in oxidative phosphorylation caused by high concentrations of glucose (the Crabtree effect; rev. by Koobs, 1972). Following a proposed decrease in oxygen demand, an increase in retinal PO_2 may result in a reduction in retinal blood flow. This may lead to inadequate perfusion of other nutrients that are critical to retinal function.

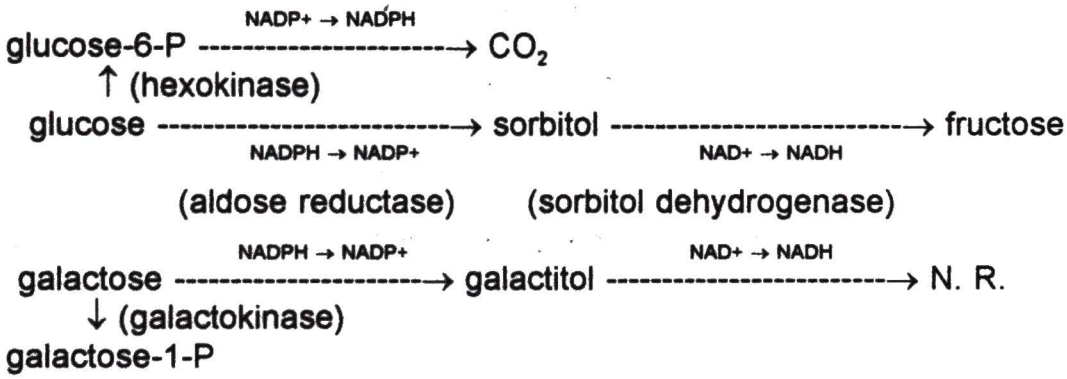
Pathological conditions in the RPE have been studied primarily in animal models. In Royal College of Surgeon (RCS) rats, studies have shown an increase in BRB permeability (Caldwell and McLaughlin, 1983, 1984a) and a redistribution of membrane components such as Na^+/K^+-ATPase (Caldwell,

1987). Choroidal tumors can be associated with a pathological condition in which proliferation, detachment, and atrophy of the RPE are produced. Hyperfluorescence observed under these circumstances is thought to originate from the degenerative RPE layer rather than from vascular leakage (Damato and Foulds, 1990).

The Polyol Pathway

Another biochemical alteration caused by elevated glucose that is of particular importance in ocular tissues involves the polyol pathway (shown in Figure 2). Prolonged hyperglycemia produces an increase in the conversion of glucose to sorbitol *via* a low affinity enzyme known as aldose reductase (rev. by Greene et al., 1987; Hers, 1956; Kador, 1988; Kador et al., 1989; Kinoshita, 1976). Aldose reductase competes with hexokinase for glucose. Under elevated glucose conditions such as chronic diabetes, hexokinase becomes saturated so that sorbitol is produced. In certain tissues this polyol has been shown to accumulate. Rabbit lenses incubated in 35 mM glucose showed increases in sorbitol and sodium that peak at 4 and 5 days, respectively (Kinoshita, 1974). In the lens, this sorbitol buildup has been associated with the development of cataracts. Cultured canine lens epithelial cells produced 550 nmol/mg protein of polyol within 6 h with a concomitant decrease in myo-inositol from 100 to 50 mol/mg protein (Nagata et al., 1989). Rabbit models of diabetes have shown sorbitol to accumulate in all cell layers of the retina

Figure 2. The polyol pathway



(MacGregor et al., 1986). Nerve conduction velocity slows, apparently due to an increase in polyol pathway activity (Greene and Lattimer, 1984b). In addition, glucose interferes with myo-inositol transport in theintestine, lens and other tissues (Cammarata et al., 1991a,b; Caspary et al., 1970). This may contribute to the decrease in myo-inositol which accompanies the diabetic condition (Greene et al., 1987). In this depleted state, myo-inositol appears to modulate Na^+/K^+-ATPase activity (Greene and Lattimer, 1984a; Greene et al., 1987; MacGregor and Matschinsky, 1986). Studies with diabetic rats (four weeks after streptozotocin injection) showed a decrease in sciatic nerve Na^+/K^+-ATPase activity of 40% that was normalized by a 1% myo-inositol diet supplementation (Greene and Lattimer, 1983). A diabetic rat study produced a similar preservation of pump activity with sorbinil, six weeks after induction of diabetes (Simpson and Hawthorne, 1988). Na^+/K^+-ATPase is hypothesized to be a key link between altered tissue metabolism and nerve function in diabetes (Greene et al., 1985).

Table 1 summarizes pathologic changes that have been observed in the retina of diabetic animals (MacGregor and Matschinsky, 1986).

Table 1. Summary of Pathological Changes in the Retina During Diabetes

↑ Glucose concentration

↑ Sorbitol concentration

↑ Retinal Na^+

↓ Myo-inositol concentration

↓ Electroretinogram c-wave

↓ Na^+/K^+-ATPase activity

Aldose Reductase Inhibitors

Inhibitors of aldose reductase (ARIs) have been shown to normalize the effects of diabetes and galactosemia upon the polyol pathway. Treatment with ARIs prevents sorbitol accumulation in the lens and blocks the depletion of tissue myo-inositol in diabetic animals (Finegold et al., 1983; Greene et al., 1985; Greene and Mackway, 1986). Several ARIs have been used as experimental tools *in vitro* as well as *in vivo* (rev. by Kador, 1988).

Example of ARIs include: AL-1576, AL-3152 (Alcon); Sorbinil (Pfizer); Tolrestat (Ayerst); Statil (ICI); and Epalrestat (Ono). Sorbinil treatment preserves normal peripheral nerve myo-inositol content during experimental streptozotocin induced diabetes (Greene and Lattimer, 1984b). This ARI normalized renal sorbitol, myo-inositol and Na^+/K^+-ATPase in streptozotocin diabetic rats (Beyer-Mears et al., 1984; Cohen et al., 1985; Kikkawa et al., 1987). Galactitol increased by more than 30-fold in retinas of galactosemic

dogs (Kern and Engerman, 1991). This polyol accumulation was inhibited 90-96% by sorbinil. Primary human lens epithelial cells exposed to 30 mM galactose for 72 hours produced detectable amounts of galactitol (Lin et al., 1991). In the same study, vacuoles were observe in cells. Polyol and vacuole formations were inhibited by the ARIs AL-1576 and sorbinil with IC_{50} values of 3.6×10^{-8} M and 7.2×10^{-7} M, respectively. Studies performed *in vitro* on human lenses from diabetic patients showed polyol accumulation that was sensitive to inhibition by aldose reductase inhibitors (Chylack et al, 1979). In cultured human RPE cells, exposure to 20-40 mM glucose produced time- and dose-dependent increases in sorbitol and decreases in myo-inositol and rod outer segment phagocytosis (Del Monte et al., 1991). These effects were partially blocked by the ARI sorbinil.

In addition to the inhibitory effect of aldose reductase inhibitors upon polyol accumulation, other benefits have been observed. Treatment with ARIs prevents a delay in reepithelialization observed in experimentally denuded corneas from diabetic rats (Datiles et al., 1983). Retinal capillary basement membrane thickening, a complication of diabetes, has been controlled experimentally (diabetic rat studies) with ARI treatment (Frank et al., 1983; Robison et al., 1983). Changes in ERG patterns observed in diabetic rats have been decreased by the administration of AL-1576 (Chandler et al., 1987). Reductions in nerve conduction velocities (modelled in diabetic rats) have been decreased by treatment with ARIs such as statil (Stribling et al., 1985).

Pathological States of the RPE

Changes in RPE cell function may occur during the progression of diseases such as proliferative vitreoretinopathy (PVR). These cells are thought to lack epidermal growth factor (EGF) receptors *in vivo* under normal conditions (Fassio et al., 1989). RPE cells derived from human eyes with proliferative vitreoretinopathy display these receptors (Nicolai and Eckardt, 1991). The blood-retinal barrier is known to breakdown in this disease, enhancing RPE exposure to serum components such as fibronectin, epidermal growth factor and platelet derived growth factor (Campochiaro et al., 1985). In cultured human RPE cells, epidermal growth factor, platelet derived growth factor, acidic and basic fibroblast growth factor, insulin-like growth factor and serum have been shown to stimulate [^{3}H]-thymidine incorporation and/or $^{86}Rb^{+}$ uptake (Arrindell et al., 1992; Leschey et al., 1990).

The mechanism and location of the breakdown in the blood-retinal barrier (BRB) under diabetic or galactosemic conditions remain controversial. Defects in the BRB appear to be some of the earliest detectable signs of hyperglycemia induced damage (Krupin et al., 1978). Tso and Shih (1977) observed that the RPE was more susceptible to disruption than the retinal vasculature. In studies with rats, Kirber et al. (1980) noted an RPE permeability defect in one half of the subjects, four weeks after streptozotocin induction of diabetes. Leakage of horseradish peroxidase also occurs in spontaneously diabetic rats (Blair et al., 1984). Various types of RPE lesions have been observed after experimental

diabetes induction (Tso et al., 1980), including: dilated smooth endoplasmic reticulum, shrunken nuclear membrane and cellular necrosis (to the extent that discs of outer segments came in direct contact with Bruch's membrane). In some areas, proliferation of RPE took place in the breach created in the blood-retinal barrier. Increased focal permeability of the RPE surface membrane rather than defective tight junctions or vesicular transport is proposed as the mechanism for diabetes-induced BRB breakdown (Vinores et al.,1990).

Animal studies of diabetes which show significant metabolic alterations are typically lengthy (weeks to months in duration). Alternatively, tissue culture experiments are relatively short in duration and allow for the manipulation of a myriad of experimental conditions. Additional *in vitro* studies on the effects of metabolic insults, such as elevated glucose, would provide further insights into the role of the RPE in retinal pathologies.

The Na^+/K^+-ATPase Molecule

Several early experiments were critical to the understanding of Na^+/K^+-ATPase function (rev. by Skou, 1989). Dean (1941) postulated that a sodium pump existed in the cell membrane to pump sodium out and potassium into the cell. Much of the important work of the next decade was performed by Ussing and Zerahn on perfused frog skin (rev. by Ussing, 1988). The identification of the sodium pump as the membrane-bound Na^+/K^+-ATPase was a result of several serendipitous experiments by Skou using crab nerve (1957, rev. 1989).

The inhibition of Na^+/K^+-ATPase by cardiac glycosides was reported by Schatzmann (1953). Post et al. (1960) showed the relationship between cation effect and Na^+/K^+-ATPase in membrane fragments.

Na^+/K^+-ATPase is an E_1E_2-type enzyme situated in the plasma membrane (Boldyrev, 1993; rev. by Gennis, 1989; Glynn and Karlish, 1990; Sachs and Munson, 1991). This molecule exists in two distinguishable forms that have different binding properties. Both high and low affinity ATP binding sites are present with $K_{1/2}$ values of 0.1 and 2 mM, respectively (Askari et al., 1988). In the E_1 conformation the cytoplasmic binding sites (high Na^+ affinity, low K^+ affinity, high ATP affinity) are exposed. The phosphorylated form of the E_2 has ionic binding sites facing the extracellular space (high K^+ affinity, low Na^+ affinity; Jorgensen, 1982). An important tool to the understanding of the pump has been fluorescein isothiocyanate (FITC). This molecule reacts with Lys-501 (Farley et al., 1984) of the enzyme. This area of the pump appears to be important for both high and low affinity binding of ATP (Ellis-Davies and Kaplan, 1993).

In the Post-Albers model (Albers et al., 1968; Post et al., 1965) of the Na^+, K^+-pump cycle, the E_1 conformation has three Na^+ ions bound to the cytoplasmic surface. The enzyme is then phosphorylated by ATP at a specific aspartate residue. As ADP dissociates from the enzyme the inorganic ions become occluded (i.e. they cannot be released from the membrane). FITC apparently reports a conformational change in the dephosphorylated enzyme

that is involved in the cation transport (Smirnova and Faller, 1993). Phosphorylation causes a stabilization of the enzyme into a low affinity for Na^+ and the binding sites facing the cell exterior (resulting in transport of the ions, E_1-P to E_2-P).

The E_2-P conformation has extracellular binding sites with a high K^+ affinity. When K^+ binds, the dephosphorylation of the protein is catalyzed and the ion becomes occluded. The pump inhibitor vanadate binds to a low affinity ATP binding site (Cantley et al., 1978) and stabilizes the E_2(K) complex (Glynn and Richards, 1982). The rate-limiting step in transport appears to be the "de-occlusion" or release of K^+ from the enzyme. ATP acts as both a substrate and an allosteric effector in the process (Gennis, 1989). In addition, there is an inverse correlation between membrane microviscosity and Na^+/K^+-ATPase activity (Boldyrev, 1993).

Experiments have been performed to determine the interaction of Rb^+, a K^+ analog, with the Na^+/K^+-ATPase molecule (Forbush, 1987b; Hasenauer et al., 1993). The rate constant for release of $^{42}K^+$ or $^{86}Rb^+$ from a phosphorylated pump is 5-15/s. In the presence of K^+, Cs^+, or Rb^+, $^{86}Rb^+$ is released from two distinct sites (the "s" or slow site and the "f" or fast site). Rubidium occlusion sites appear to be confined within the protein and connected by heterogeneous access channels. When ATP binds to its low affinity site, the binding and release of Rb^+ are accelerated. ATP, in this instance, does not affect Rb^+ binding to the pump molecule. Na^+ is a competitive inhibitor of Rb^+ at the

occluded sites, while being an effector at the allosteric site (possibly activating the access channels). Ouabain is a channel inhibitor that reduces the rates of binding and release of Rb^+. Another pump inhibitor, vanadate, caused Rb^+ to be released in a P_i stimulated manner but 25-fold slower.

Na^+/K^+-ATPase consists of two subunits, namely α and β. The α-subunit (MW ~ 90,000) is the catalytic site that is responsible for ATP as well as cardiac glycoside binding (Kawakami et al., 1985; Shull et al., 1985; Sweadner, 1989). The β-subunit is approximately 300 amino acids in length (MW ~ 55,000) and passes through the plasma membrane once near the amino terminus, the apparent cytoplasmic portion of the molecule (Craig and Kyte, 1980; Farley et al., 1986; Kawakami, 1988). This subunit is predicted to be in an α helical structure from the amino terminus to Asn157 (the first site of glycosylation). The carboxy-terminal portion of the molecule appears to be arranged in a β-pleated sheet (Brown et al., 1987). It is proposed that the β-subunit regulates the number of heterodimers transported to the plasma membrane

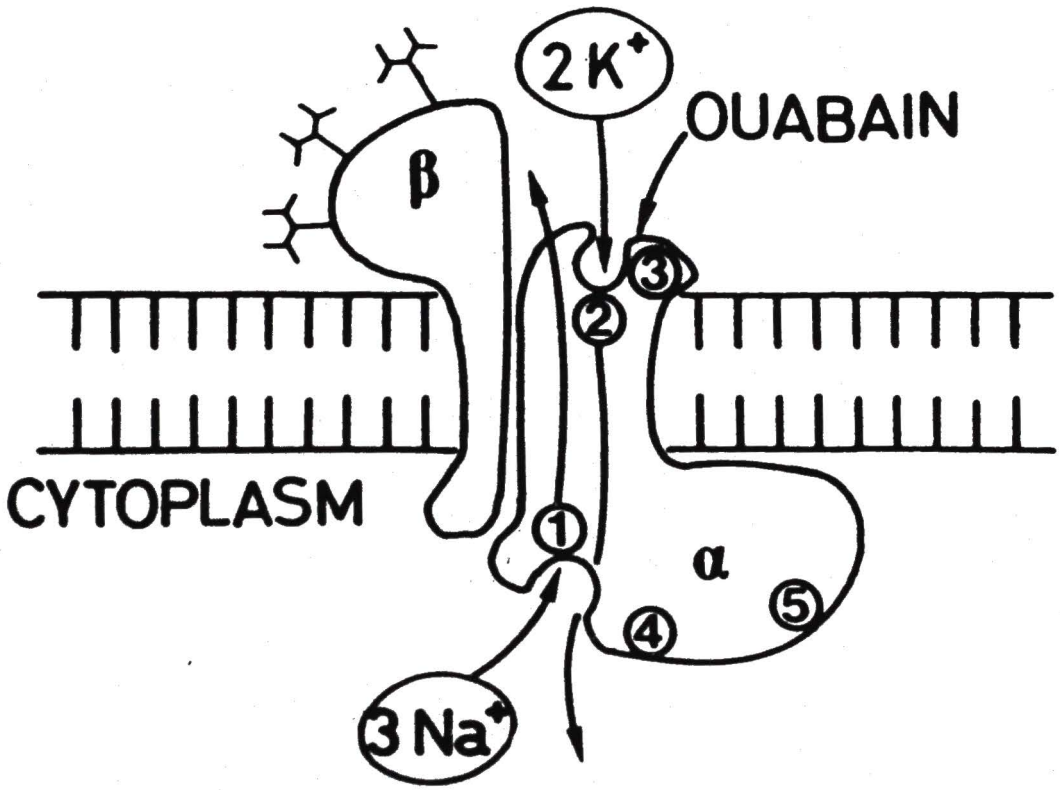


Figure 3. Model of the Na^+/K^+-ATPase αβ heterodimer (Modified from Horisberger et al. 1991).

Binding sites:

1. Na^+
2. K^+
3. Ouabain and other cardiac glycosides
4. Phosphorylation
5. ATP

(McDonoughet al., 1990). The membrane insertion signal appears to consist of a transmembrane segment of approximately 28 amino acids near the amino terminus (Kawakami and Nagano, 1988). In studies on *Xenopus* oocytes injected with α- and β-subunit-specific mRNAs, it is apparent that both are required for functional Na^+/K^+-ATPase expression (Noguchi et al., 1987). A third, γ-subunit, recently has been proposed and may form part of the cardiac glycoside binding site (Collins and Leszyk, 1987).

Several different isoforms of the Na^+/K^+-ATPase subunits have been characterized (α1, α2, α3, β1, β2, β3)(Good et al., 1990; Herrera et al., 1987; Martin-Vasallo et al., 1989; Mercer et al., 1986; Pagliusi et al., 1989; Shull et al., 1986a,b; Sweadner, 1989; Young et al., 1987). It is thought that the molar ratio of the α/β-subunits in the holoenzyme is 1:1. The amount of the different isoforms vary with the type of tissue and the stage of development. For instance, 5-day-old rats have substantial amounts of α1 and α3-subunits in the pineal gland, while the α3 and β2 forms predominate in the adult animals (Shyjan et al., 1990a). α1-subunits are expressed in a wide variety of tissues (such as the kidney), while α2-subunits are found predominantly in the heart, brain (myelinated axons), and lung (Horisberger et al., 1991; Sweadner, 1989). The α3-subunit is found in neural cells of the brain (Schneider et al., 1988; Schneider and Kraig, 1990). Rat studies have shown the α2 and α3 isoforms to be highly sensitive to ouabain ($K_i = 10^{-6}$ M to 10^{-7} M) while the α1 form is

much less sensitive ($K_i = 10^{-5}$ M to 10^{-3} M)(Brodsky, 1990; Shyjan et al., 1990a,b; Skou, 1962, 1988; Sweadner, 1979).

In studies using a cDNA probe similar to the rat brain α1 isoform, labeling was noted in photoreceptor inner segments, inner nuclear layers, ganglion cell bodies, plexiform layers and outer nuclear layers (Hieber et al., 1989). In another experiment, rat photoreceptors were shown to contain predominantly α3 and β2 isoforms of Na^+/K^+-ATPase (Schneider and Kraig, 1990). In experiments with transfected HeLa cells, the α3-subunit shows about a four-fold lower K_m for Na^+ than does the α1 or α2 (Munzer et al., 1994). It has been proposed that the α3 isoform may be adapted to function in cells with hyperpolarizing potentials (Shyjan et al., 1990a).

Several studies have been performed to determine the portion of the α-subunit that confers ouabain sensitivity. Experiments using chimeric α1-subunit molecules show that alterations in hydrophobic domains H_1-H_4 produce changes in sensitivity to cardiac glycosides (Lingrel et al., 1991; Schultheis, et al, 1993). Charged amino acids on the extracellular loop between H_1 and H_2 confer ouabain resistance (Price and Lingrel, 1988; Price et al., 1989, 1990). Another group of investigators has found that substitution of a tyrosine in the H_3-H_4 region produces increased ouabain resistance (Canessa et al, 1993).

The function of the glycosylated β-subunit is still under investigation. A great degree of amino acid sequence homology exists between β-subunit from *Torpedo electroplaque* (Kawakami et al., 1986), sheep kidney (Shull et al., ,

1986a), HeLa cells (Noguchi et al., 1986), rat brain and kidney (Young et al., 1987), and chick brain (Takeyasu et al., 1987). This subunit may be involved in the insertion of the Na^+/K^+-ATPase molecule into the membrane (Geering, 1991). *Xenopus* oocyte studies show the synthesis of the β-subunit to increase the proportion of trypsin-resistant α-subunits (Geering et al., 1989). The extracellular domain of the β-subunit is sufficient and required for proper assembly with the α-subunit (Hamrick et al., 1993). A portion of the α-subunit near the carboxyl-terminal is critical for assembly with the β-subunit (Lemas et al., 1992). The carbohydrates on the exterior of the protein molecule make them available for mediating cell to cell interactions (adhesion)(Treuheit et al., 1993). An adhesion molecule on glial cells is thought to be involved in intercellular associations (Gloor et al.,1990). β1 and β2 isoforms show homology to adhesion molecules such as those found on glia (Gloor et al., 1990; Treuheit et al., 1993). Mediation of cell to cell interaction between neurons and glia also have been proposed for the β3-subunit (Pagliusi et al., 1989). β2-subunits have been identified in the kidney, brain, thymus, pineal gland and heart (Shyjan et al., 1990 a and b). These varying tissue distributions suggest that different isoforms of the enzyme may modify its function in different tissues (Shyjan and Levenson, 1989).

Some experiments have probed the subunit composition of RPE Na^+/K^+-ATPase. The presence of the α1-subunit has been observed in rat retinal pigment epithelium (Gundersen et al., 1991; McGrail and Sweadner, 1986).

One study dealing with human RPE has shown a correlation between Na^+/K^+-ATPase catalytic activity and α-subunit immunoreactivity (Mircheff et al., 1990). The β1 isoform has been detected in RPE (Rizzolo and Heiges, 1991) and may contribute to polarity of the pump in this cell type (Bok, 1993).

It is possible that, within a given tissue, Na^+/K^+-ATPase isoform distribution is not uniform. For example, gradient of isoforms have been observed in the nonpigmented ciliary epithelium (NPE, Ghosh et al., 1990, 1991; Martin-Vassallo et al., 1989) that correlated with diminishing Na^+/K^+-ATPase activity from anterior (pars plicata) to posterior (ora serrata). These authors also hypothesized that this unique isoform distribution could impact the functioning of this ocular cell layer (NPE).

One important means of regulating Na^+/K^+-ATPase activity is through protein phosphorylation by protein kinases (Ling and Cantley, 1984, Racker, 1985). In studies with ^{32}P incorporation, Na^+/K^+-ATPase (from duck salt glands) is phosphorylated by protein kinases A and C (Chibalin et al., 1991). This phosphorylation appears to take place at serine and threonine residues on the α-subunit (Chibalin et al., 1992). It has been shown that cAMP injection into *Xenopus laevis* oocytes causes pump activation while phorbol ester and diacylglycerol produce inhibition (Vasilets et al., 1990). Ouabain binding measurements in this study indicate that protein kinase regulation of Na^+/K^+-ATPase is related to the number of pump molecules in the active state. The

apparent ouabain affinity appears unchanged. Protein kinase C is present in rat RPE (Heth and Schmidt, 1988).

Experiments with bovine lenses have shown Na^+/K^+-ATPase to be important in the maintenance of normal light transmission through the eye (Miller et al., 1979). Ouabain, at $1x10^{-4}$ M, produces cortical opacification that is similar to that seen with $1x10^{-4}$ M dexamethasone.

Some Effects of Pathological States Upon Na^+/K^+-ATPase

Many animal studies have shown the effects of abnormal physiology on Na^+/K^+-ATPase. In a cataract model using the Nakao mouse, a 50% decrease in lens pump activity leads to cataract formation (Iwata and Kinoshita, 1971). A decrease in Na^+/K^+-ATPase immunostaining in the ciliary epithelium was found in a study with RCS rats compared to their controls (Yamaguchi et al., 1991). In this same rat strain, an increase in lens pump number was noted at 4 and 8 weeks (Garner and Garner, 1994). Renal Na^+/K^+-ATPase activity is lower in Dahl salt-sensitive rats (DS;Rodriguez-Sargent, 1981). A mutation in the form of a leucine substitution of glutamine at position 276 of the α1-subunit has been observed in DS rats (Herrera and Ruiz-Opazo, 1990). A reduction in Na^+/K^+-ATPase activity also may lead to cataract formation in this rat strain (Rodriguez-Sargent et al., 1987,1989). Hypoxia may cause an inhibition of photoreceptor Na^+/K^+-ATPase (Linsenmeier and Steinberg, 1984).

Effects of Hyperglycemia Upon Na^+/K^+-ATPase

Diabetic conditions may cause the expression of Na^+/K^+-ATPase isoforms to be altered. For instance, $\alpha 1$ and $\beta 1$-subunits are upregulated in rat intestinal mucosa two weeks after diabetes induction (Barada et al., 1994). Poulsom and coworkers (1990) observed an increase in $\alpha 2$ mRNA levels in the retinas of galactosemic rats. Studies involving spontaneously diabetic BB/Wor rats have shown a significant increase in $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform synthesis in the retina after 4 months (Specht et al., 1991). In addition, blood glucose concentrations were approximately 323 mg/dl in diabetic *vs* controls while the glycated hemoglobin concentrations were about two fold higher in the diabetic animals. This change in isoform composition may alter the susceptibility of the enzyme to effects of endogenous compounds, such as digitalis-like substance, (Okamoto et al., 1991) and thus the progression of diabetic complications.

A variety of effects on Na^+/K^+-ATPase have been observed under diabetic or galactosemic conditions in animal experiments. A decrease in pump activity in streptozotocin diabetic rat erythrocytes was accompanied by an increase in cell volume and a decrease in cytosolic K^+/Na^+ ratio (Kowluru et al., 1989). ATPase activity in ZDF/Gmi-fa rat (a model of NIDDM) retina was reduced after four, eight, and 16 weeks of diabetes by seven, 11 and 14% respectively (Eichberg et al., 1993). Rat kidney Na^+/K^+-ATPase activity can become irreversibly impaired in as early as 15 days after diabetes onset even in the presence of daily insulin injections (blood glucose held to approximately

6.7 mM; Tehrani et al., 1990). In studies performed *in vitro* in rat kidney, Na^+/K^+-ATPase glycation produced a shift in the steady state kinetics from substrate activation to Michaelis-Menten kinetics. Total inhibition of K^+ occlusion occurred and approximately 0.8 moles of glucose were incorporated per mole of enzyme into the catalytic subunit some distance from the amino terminus (Garner et al., 1990). Yokoyama et al. (1994) showed that 0.04 g/kg ascorbate combined with 10% galactose in the drinking water intensified the loss of pump activity in rat lens epithelium. In rats, diabetes has produced a reduction in activity of sciatic nerve and dorsal root ganglia while the galatosemic animals showed an increase in sciatic nerve and no change in dorsal root ganglia ouabain-sensitive Na^+/K^+-ATPase activity (Lambourne et al., 1988). Bovine kidney studies with AL-1576 (in the presence of elevated glucose-6-phosphate) produced a 20-30% increase in the rate of ATP hydrolysis. 10^{-6} M AL-1576 caused a shift in kinetics of hydrolysis from that of substrate inhibition to substrate activation indicating that the drug may be able to normalize a defective (glycated) pump (Garner and Spector,1987). In the same study, neither sorbinil nor AL-1576 prevented the *in vitro* glycation of Na^+/K^+-ATPase but both drugs inhibited modification of the α-subunit by fluorescein isothiocyanate (FITC). In a study with alloxan-induced diabetes in rats, a decrease in vagus nerve Na^+/K^+-ATPase activity of 39% was overcome by the administration of ganglioside inner ester derivatives (Bianchi et al., 1991).

Conflicting evidence exists concerning the effects of galactosemia or diabetes on ATPase activity. In studies with bovine lens epithelial cells, 40 mM galactose was shown to have no effect upon Na^+/K^+-ATPase activity as measured by $^{86}Rb^+$ uptake (Cammarata et al., 1991a,b). Exposure to 30 mM galactose produced an increase in $^{86}Rb^+$ efflux that may have been related to membrane permeability (Kawaba et al., 1986). Unaker and Tsui (1980) saw a gradual decrease in lens Na^+/K^+-ATPase activity after feeding rats 50% galactose chow. A decrease in retinal Na^+/K^+-ATPase activity under both diabetic and galactosemic conditions was observed in a two-month study using both rats and dogs (Kern et al., 1994). Genetically hyperglycemic mice (C57BL-KS-J strain, db/db), a model for non-insulin-dependent diabetes show no polyol accumulation or decrease in erythrocyte sodium pump activity (Kowluru and Kowluru, 1992). Other authors (Bianchi et al., 1990) showed no significant difference in optic or sciatic nerve ATPase activity in the db/db mouse strain.

Another experiment assessed the effect of 50% galactose feeding in the absence and presence of ARIs upon Na^+/K^+-ATPase activity in rat lenses (Unakar et al., 1989). At about 12 days, the enzyme activity was approximately 54 percent of the control values. The galactosemic animals treated with the ARI Eisai E-0722 (structurally related to sorbinil) displayed a retention of 83%, 85%, and 94% Na^+/K^+-ATPase activity at 10 to 12 days at 0.15, 0.5, and 1 (mg/kg body weight per day), respectively. The same research group has

shown that a 50% galactose diet supplemented with 50 mg/kg of sorbinil was almost totally effective in preserving lens Na^+/K^+-ATPase activity over galactose alone (Unakar and Tsui, 1983). Lambourne et al. (1988) reported a 54% decrease in rat sciatic nerve ouabain resistant ATPase activity with stretozotocin-induced diabetes, while 20% galactose feeding produced an increase of 225%. Treatment with the aldose-reductase inhibitors tolrestat, ponalrestat, and sorbinil prevented the increase in activity due to high galactose. These drugs were ineffective, however, in the diabetic model. In a study by Greene and Lattimer (1984a), treatment with sorbinil completely prevented the decline in ATPase activity caused by streptozotocin-induced diabetes. Substrate inhibition kinetics are prevalent in human lenses in either mature onset diabetes or incubations with high glucose (250 mm; Garner and Spector, 1986).

Several experiments have been performed on tissues and cells derived from human subjects. Human erythrocytes showed a reduction in pump activity within 30 minutes of high glucose exposure while malonyldialdehyde (MDA, a lipid peroxidation product) appeared only after four hours (Rajeswari et al., 1991). In patients with diabetes complicated by hypertension, an elevation in an endogenous digitalis-like substance has been shown (Okamoto et al., 1991). Corneal endothelial cells grown at 4.5 g/l glucose for 15-25 days show a 76% decrease in Na^+/K^+-ATPase activity compared with the controls (1g/l glucose; Whikehart et al., 1993). In the same study, assays performed directly in high

glucose after culturing the cells in low glucose produced no significant loss in Na^+/K^+-ATPase activity. A significant decrease in ouabain binding capacity and/or pump activity has been shown in erythrocytes and platelets from diabetic patients (Mazzanti et al., 1990; Noda et al., 1990; Umeda et al., 1989). In one of these studies (Umeda et al., 1989) the ARI, ponalrestat, produced a stimulation of Na^+/K^+-ATPase activity as well as an increase in ouabain binding.

Studies with Na^+/K^+-ATPase in RPE Cells

Na^+/K^+-ATPase is present in virtually all eukaryotic cells but is particularly active in cells involved in transport such as the RPE. There is ample evidence to support the presence of high concentrations of the enzyme on the apical surface of this retinal cell (Bok and Filerman, 1979; Frambach et al., 1988; Ostwald and Steinberg, 1980). The apical localization of Na^+/K^+-ATPase in RPE may be linked to its association with ankyrin and fodrin, cytoskeletal proteins found on the basolateral membrane of the cells (Gundersen et al., 1991; Nelson and Hammerton, 1989). Another possible cause of this polarity could be minor sequence differences in the protein structure (Bok, 1993). Single amino acid substitutions can cause a shift from apical to basolateral (influenza HA, Brewer and Roth, 1991), for instance. Primary cultures of chick embryonic RPE have shown Na^+/K^+-ATPase to be present both in the apical and basolateral membrane (Rizzolo, 1990). Cultured human RPE cells also exhibited Na^+/K^+-ATPase molecules predominantly at the apical surface, but on

the basolateral as well (Hu et al., 1994). Maximal Na^+/K^+-ATPase pump numbers per cell are observed in sparse, proliferating cultures of human RPE (Jaffe et al., 1989).

Several authors have examined the functioning of the pump *in vitro* through the aid of electrophysiologic techniques. Primary *Xenopus laevis* RPE grown on microporous membrane filters develop transepithelial resistance of approximately 400 Ohm/cm^2 (Defoe and Easterling, 1994). Experiments with perfused bovine RPE/choroid preparations show that the resistance established by Na^+/K^+-ATPase can be negated by the addition of 10^{-5} M ouabain on the apical side of the RPE (Miller and Edelman, 1990). Similar studies with bullfrogs, *Rana catesbeiana*, showed cAMP stimulation of pump activity within one minute that persists for several hours (Hughes et al., 1988).

In studies with human RPE cells in culture, hypertonic stress was shown to affect Na^+/K^+-ATPase activity (Yokoyama et al., 1993). An exposure to medium containing an additional 150 mM NaCl for 66 hours produced a two-fold increase in Na^+/K^+-ATPase activity (as measured by inorganic phosphate liberation). A time course experiment showed a significant increase in the activity of the enzyme that reached a plateau after 24 hours. Yokoyama and colleagues (1993) were unable to determine whether the increase in Na^+/K^+-ATPase activity was due to activation or induction of the enzyme. Taurine and myo-inositol concentrations also were elevated in cells exposed to 150 mM NaCl supplemented medium.

Experiments have been performed in sodium iodate injected rabbits to determine the distribution of Na^+/K^+-ATPase in regenerating RPE (Korte and Wanderman, 1993). A gradient of intensity and localization of pump activity was noted from least mature cells at the distal portion of the regenerating sheet to the more mature proximal RPE. In early stages of regrowth, minimal Na^+/K^+-ATPase activity was observed over the entire plasma membrane with the exception of the portion facing the basement membrane. As the cells matured, the activity became localized to the apical membrane, exhibiting normal polarity with the cells. These studies show that under certain circumstances, the RPE is able to recover its normal polarity.

Elevated Glucose and RPE

Limited information is available concerning the effect of elevated glucose or galactose specifically upon RPE cellular function or morphology. Diabetes produces morphological alterations in the RPE layer which appear early in the progression of the disease. RPE plasma membrane basal folding increased by an average of 33% in diabetic rats (Grimes and Laties; 1980, 1984; Kirber et al., 1980). This effectively doubled the membrane area overlying Bruch's membrane. Insulin-mediated stimulation of glucose uptake and lactate production was decreased significantly in RPE cells from diabetic donors compared with normal controls (Miceli and Newsome, 1991). Oxygen consumption was similar in both groups, however. The ARI, sorbinil, was

shown to ameliorate the deterioration of the c-wave of pigmented diabetic rat electroretinograms (MacGregor and Matschinsky, 1985).

Some work has focused on myo-inositol and the polyol pathway in RPE cells. Galactitol accumulation was shown in human RPE cells exposed to 30 mM galactose for 72 hours (Reddy et al, 1992a). In the same study, a decrease in myo-inositol, taurine and a number of free amino acids was observed under high galactose conditions. These effects were inhibited by the ARI AL-3152. Growth of primary bovine RPE in 40 mM glucose has been shown to down-regulate ^{3}H-myo-inositol transport and DNA synthesis (Khatami, 1991, 1992). Exposure of cultured human RPE cells to 20-40 mM glucose produces time- and dose-dependent increases in sorbitol content and decreases in myo-inositol content (Del Monte et al., 1991). Human RPE cells exposed to 20 mM glucose showed a sorbitol increase from 5.0 to 149.4 nmoles/mg protein within seven days (Nakamura et al., 1992). These effects were partially inhibited by the ARI, sorbinil. In addition, myo-inositol decreased from 40.8 nmol/mg protein at 5 mM glucose to 13.4 nmol/mg protein in the 20 mM glucose group. A decrease in phagocytosis of rod outer segments upon exposure of the cells to 20 mM glucose was observed. Decreases in myo-inositol content also have been observed in rabbit and cat RPE cells (Marano and Matschinsky, 1989).

One large study performed by Reddy and coworkers (1991, 1992b) examined the efficacy of several different aldose reductase inhibitors for

preventing polyol formation in cultured human lens (HLE) and retinal pigment epithelial cells (HRPE). The cells were exposed to 30 mM D-galactose in the presence or absence of an ARI for 72 hours. In the absence of aldose reductase inhibitors, galactitol concentrations were found to be 1362±167 nmol/mg protein and 1523±309 nmol/mg protein in HLE and HRPE, respectively. The order of potency for inhibition of galactitol accumulation in both cell types was AL-4114 > AL-3152 > AL-1576 > tolrestat > statil > sorbinil. Higher doses (typically ten fold) of the ARIs were required to inhibit polyol formation in the HRPE compared to the HLE. An experiment was also conducted to examine the effect of serum on the ARI mediated inhibition of polyol synthesis. Dog lens epithelial cells were used in this study since it was determined that HLE and HRPE cells could not tolerate serum free conditions for the required length of time. Comparing serum free to 20% serum conditions, no effect was observed for the compounds tested (AL-4114, AL-3152, sorbinil, and tolrestat).

Few studies have examined the effects of elevated glucose upon pump activity specifically in RPE cells. Plasma membrane preparations from RCS rat RPE showed higher Na^+/K^+-ATPase activity than their controls (Braunagel et al., 1988). Experiments using RCS rats showed Na^+/K^+-ATPase in RPE to redistribute laterally after tight junctions begin to break down (Caldwell and McLaughlin, 1984b). The same authors noticed that, upon detachment from Bruch's membrane, the enzyme could be found on the basal membrane of that

cell. Samples from alloxan-diabetic rabbit RPE showed increased intracellular glucose, sorbitol and sodium (MacGregor and Matschinsky, 1986b). The same experiment produced a decrease in Na^+/K^+-ATPase activity and myo-inositol content. Experimental diabetes produces a large increase in rabbit RPE sodium content while apparently having no effect on potassium concentrations (MacGregor and Matschinsky, 1986a). A decrease was also noted in Na^+/K^+-ATPase activity in the diabetic animals. These studies did not address the efficacy of therapeutic intervention upon pump function, however. The authors suggested that, due to the barrier and transport functions of the RPE that this cell type could prove useful in study of the pathological consequences of diabetes.

This dissertation addressed the hypothesis that deficits in Na^+/K^+-ATPase activity (as measured by rubidium uptake) and altered expression of the enzyme (as measured by ^3H ouabain binding) are responses of cultured RPE cells to hyperglycemia.

CHAPTER III

METHODS

Bovine RPE Isolation and Culture (Basu et al., 1983; Boulton, 1990; Edwards, 1982, Li, 1991)

Fresh bovine eyes typically were received by noon (packed in saline on wet ice). Intact eyes were soaked in Betadyne solution for approximately five minutes, rinsed in ethanol and finally rinsed in DPBS$^-$ (Dulbecco's Phosphate Buffered Saline without calcium and magnesium) containing 10x (100 µg/ml) gentamicin. Fat and muscle were removed from the eyes using large scissors and forceps. Eyes then were rinsed again in Betadyne, ethanol, and DPBS$^-$. A scalpel was used to make an incision 5-10 mm behind the limbus. Large scissors were used to continue the incision around the entire globe. The anterior segment was removed along with the vitreous. Typically, the retina detached with the vitreous and was clipped at the optic nerve area with small scissors (a small portion of retinal tissue was left behind so that the action of the trypsin did not perforate through to the choroid resulting in fibroblastic contamination). The eye cup (with exposed RPE layer) was placed in small cap for support and 0.25% trypsin/2.65 mM Na/EDTA (Gibco) was added (the eye cup was only filled to within approximately five mm from the top to prevent the enzyme solution from spilling over behind the choroid). Incubation of the cells in EDTA has been shown to effectively loosen their attachment to Bruch's

membrane (Vielkind and Crawford, 1988). A sterile specimen cup was used to house the eye cup and its support. The preparation was placed in a 37°C CO_2 incubator for about 30 min. The enzyme solution was removed *via* pasteur pipette and fresh solution was added and the incubation carried out for another 15 min. At this time the second enzyme solution was removed (the remnants of the neural retina in the area of the optic disc then could be removed). A final treatment of trypsin/EDTA was administered and the RPE cells were dislodged by repeated trituration.

The cell suspension was placed in a 50 ml centrifuge tube containing Dulbecco's Minimal Essential Media (DMEM), with 1, 4.5, or 10 g/l glucose (corresponding to 5.55, 25.0 and 55.5 mM glucose), 110 mg/l sodium pyruvate, 584 mg/l L-glutamine) containing amphotericin B (2.5 µg/ml), gentamicin (10 µg/ml), 0.2 mM glutamine and 10% Fetal Bovine Serum (Defined FBS, Hyclone) to stop the trypsinization. The cells were centrifuged in a table top centrifuge at low speed for five minutes. The medium was aspirated, decanted, and replaced with approximately five ml of fresh medium to remove traces of the enzyme. The cells were resuspended and a small aliquot was used to determine the number of cells with a hemocytometer. Trypan blue (0.4%) was added to the slide in order to assess viability (usually > 95%) of the harvest. An average yield of cells was $0.5\text{-}2.0 \times 10^6$ per eye. The cells were seeded into six well plates (Costar) at approximately 5×10^4 cells per cm^2. In three to four days the medium was replaced with fresh medium lacking amphotericin.

Feeding was performed semi-weekly. The cells typically reached confluence within 7-14 days. The RPE then were passaged and grown in 75 cm^2 or larger flasks. The cells were passaged (1:3 or 1:5 split ratio) by rinsing three times in $DPBS^-$ followed by a room temperature incubation in 0.05% trypsin/0.5 mM Na/EDTA. The action of the trypsin solution was terminated by the addition of excess medium containing 10% FBS. Cells of passages two through five were diluted for further culture or seeded into 24 well plates (Costar, surface area of each well = 2 cm^2 ; volume of media in each well = 1 ml) for the examination of ^3H thymidine incorporation, rubidium uptake, ouabain binding etc. Media, antibiotics, trypsin, trypan blue and glutamine were products of Gibco (Grand Island, NY).

^3H Thymidine Incorporation (Leschey et al., 1990)

Confluent cells were washed with serum free DMEM three times and incubated for 16 h at (37°C) with or without the compound of interest (i.e. insulin). The RPE were then incubated for two hours in DMEM containing 2 µCi/ml ^3H thymidine. Cells were washed three times with $DPBS^-$ and five times with ice-cold 5% TCA. One ml of 0.1 N sodium hydroxide (NaOH) containing 0.1% sodium dodecyl sulphate (SDS) was added to each well. A 100 µl aliquot was removed and added to 15 ml Optifluor (Packard) prior to counting on a LKB Rackbeta System.

$^{86}Rb^{+}$ Uptake in Suspended Cells (Blachley et al., 1986)

Quantification of Na^{+}/K^{+}-ATPase activity *via* $^{86}Rb^{+}$ uptake provided some advantages over other assays involving the liberation of inorganic phosphate:

1. It was unnecessary to make membrane preparations (i.e. cell homogenates) which would have been necessary to access the cell interior.
2. The data were not as subject to interferences from other types of ATPases that may have been present.
3. The activity of the enzyme was measured in living cells.

In addition, $^{86}Rb^{+}$ and $^{42}K^{+}$ are approximately equivalent when used to study potassium movement across the RPE (Miller and Steinberg, 1982). $^{86}Rb^{+}$ has been used as a K^{+} analog when studying the $Na^{+}/K^{+}/Cl^{-}$ cotransporter because it has nearly the same affinity as K^{+} for the carrier (Geck et al., 1980; O'Grady et al., 1986; Owen and Prastein, 1985).

Small disposable cation exchange columns (0.4x10 cm, 1 ml Dowex 50W-X8, tris form, pH 7.0) were washed with a buffer solution containing BSA (5 ml 10 mM Hepes pH 7.0, 1 mg/ml BSA) to block potential protein binding sites. The columns were washed with five ml 250 mM sucrose, 10 mM Hepes pH 7.0, and allowed to drain.

Cells (primary through passage five) were suspended in experimental buffer (5 mM KCl, 140 mM NaCl, 3 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose,

pH 7.0, room temperature). The suspension was split into two samples, one of which contained ouabain. $^{86}Rb^+$ tracer was added to both samples. Aliquots (200 µl) were removed at timed intervals, passed through cation exchange columns, immediately washed with two ml sucrose buffer and allowed to drain into scintillation vials. Samples then were mixed with 15 ml of Optifluor and counted *via* scintillation spectroscopy (LKB Rackbeta 1219) for three to five min.

$^{86}Rb^+$ Uptake in Attached Cultured RPE Cells (Jaffe et al., 1989)

The RPE cells of passage two through five were seeded into 24 well plates for uptake studies. Preliminary experiments were performed by adding $^{86}Rb^+$ (typically 200,000 CPM, New England Nuclear) to the assay medium. The method of Jaffe et al. (1989) was adopted since it had been used with RPE cells. Cells were rinsed three times in uptake buffer (warmed to 37°C in a H_2O bath, uptake buffer (g/l) NaCl, 6.78;$CaCl_2 2H_2O$, 0.14; $MgSO_4 7H_2O$, 0.2; glucose, 1.0, 4.5 or 10.0; 1 mM sodium phosphate, pH 7.0-7.2). Chemicals were supplied by Sigma Chemical Co. and J. T. Baker. Uptake buffer containing experimental compounds then was added. Incubations were performed in a CO_2 incubator at 37°C for various lengths of time. Uptake buffer without RbCl was removed and buffer (1 ml) containing RbCl 0.6 g/l was added. Jaffe et al. (1989) reported a greater amount of Rb^+ uptake if the cells were pre-incubated in the absence of RbCl. Ouabain (1-2.5 mM) was added to some

wells as a control to determine specific uptake through the Na^+/K^+-pump. $^{86}Rb^+$ was added immediately and the plates were gently shaken for one min prior to their return to the incubator. At the end of a specified time (typically 30 min) the uptake buffer was removed. The plates were washed three times with one ml cold DPBS. One ml of 0.1 N NaOH was added to solubilize the cells. The entire sample was mixed with Optifluor (15 ml, Packard) and counted in a beta counter (LKB Rackbeta 1219) for three to five min. **(Note: Standard assay conditions = 20 min pre-incubation in the absence of RbCl and 30 min incubation in the presence of RbCl, 37°C. Compounds of interest such as ouabain and bumetanide were present during both the pre-incubation and incubation periods).**

Nonradioactive Rubidium (Rb^+) Uptake in Attached Cultured RPE Cells (; Dionex, 1989; Longo et al., 1991)

A method for simultaneous quantitation via HPLC of sodium, potassium and rubidium in cultured cells was modified for use with RPE cells. This method provided several key advantages over the use of $^{86}Rb^+$:

1. The use of radioactivity (for the overall study) was decreased.
    a. Exposure of the experimenter to isotopes was minimized.
    b. Record keeping associated with radioisotopes was reduced.
    c. Storage of the hazardous material ($^{86}Rb^+$) was no longer needed.
    d. Liquid scintillation cocktail and vials were not required.

e. Disposal of radioactive waste was minimized.

f. The use of $^{86}Rb^{+}$, which has a short half-life, was no longer required.

2. Quantitation of intracellular sodium, potassium and rubidium took place simultaneously providing more information regarding inorganic cation transport than the analysis of rubidium uptake alone.

3. Injection of the samples onto the HPLC eventually was accomplished by an autosampling device at a considerable time savings.

RPE cells of passage two through five were grown in 24 well plates for various lengths of time as described for the radioactive assay. In the early studies, each treatment was run in quadruplicate allowing for six possible drug concentrations. In later experiments, as variability was more effectively controlled, the treatments were performed in triplicate allowing eight possible experimental drug combinations. The cells were rinsed two to three times in uptake buffer (warmed to 37°C in a water bath, uptake buffer (g/l) NaCl, 6.78;$CaCl_2 \cdot 2H_2O$, 0.14; $MgSO_4 \cdot 7H_2O$, 0.2; glucose, 1, 4.5, or 10; 1 mM sodium phosphate, pH at 7.2). Experimental agents were then added and incubations were carried out for various lengths of time at 37°C in a water bath (early studies) or CO_2 incubator. **(Note: Standard assay conditions = 20 min ouabain pre-incubation in the absence of RbCl and 30 min incubation in the presence of RbCl, pH = 7.2, 37°C. Compounds of interest such as ouabain and bumetanide were present during both the pre-incubation and incubation**

periods). Variations from this protocol were stated in the appropriate graphs. To terminate the progression of rubidium uptake, the buffer was removed and the cells were rinsed immediately two to three times with ice-cold 100 mM $MgCl_2$. After removal of the final rinse by aspiration, the dry plates were frozen for later analysis.

Upon thawing, ethanol (0.1 ml) was added to the samples and then evaporated by placing the experimental plates on an orbital shaker. Ethanol was used to denature cellular proteins while avoiding their resuspension in the following extraction (on the orbital shaker) with 500 µl of deionized water. The samples were loaded onto the Dionex BioLC equipped with a gradient pump module (GPM-1), a pulsed electrochemical detector (PED-1), an autosampler (ASM-2), an advanced computer interface (ACI), an Autoion™ 450 chromatography software package, and a Dell™ 486D/33 computer. Sample volume in the injection loop was 25 µl. Detection of inorganic cations was accomplished via suppressed conductivity. The Dionex system was equipped with a CMMS-II cation micromembrane suppressor. The CMMS-II consisted of two anion exchange membranes situated between three high capacity anion exchange screens. Chemical suppression occurred as an acid-base reaction across these semipermeable membranes. Hydroxide ions from the regenerant (0.1 M tetrabutylammonium hydroxide, TBAOH, flow 2 ml/min) traverse the membrane to react with hydronium ions in the eluant, forming water.

Simultaneously, eluant anions cross into the regenerant and replace the hydroxide ions. The signal to noise ratio is therefore improved in two ways:

1. The conductivity of the acidic eluant is reduced by its conversion to water.
2. Sample cation conductances are increased by pairing with hydroxide counter ions which have a higher conductance.

Separation of the cations of interest was achieved through the use of a cation exchange column (Fast Cation 1, Dionex Corp., Sunnyvale, Calif.). The same column was used for all studies. Elution of the compounds of interest took place within five min of injection. This was accomplished with an isocratic protocol of 0.3 mM DL-2,3 diaminopropionic acid (DAP) and 20 mM HCl, at a flow rate of 2 ml/min. These eluants were recommended in the technical bulletin that accompanied the Fast Cation 1 column. Several tests were performed on different DAP and HCl eluant protocols. The above conditions were experimentally confirmed as producing the best compromise of elution speed and peak separation. A 12 min rinse protocol was performed between experimental groups (i.e. between triplicate or quadruplicate runs). This consisted of a 0.5 min pulse with 15 mM DAP and 75 mM HCl and 11.5 min of the normal 0.3 mM DAP/20 mM HCl protocol. This rinse protocol eluted $MgCl_2$ (used in terminating the Rb^+ uptake in the cells) from the column. Samples from uptake assays were compared to a standard curve consisting of increasing concentrations of a mixture of NaCl, KCl, and RbCl. Standards ranged from

0.25 to 25 nmoles/25 µl injection. Two 24 well plates (plus standards) could be analyzed per day. A typical experiment would yield 150 chromatograms (including standards). Although integration of the peaks was performed automatically, *via* the Dionex AI-450 chromatography software package, each chromatogram was examined visually to insure that the peaks were properly identified. The chromatograms that form the data presented in this dissertation number in the thousands.

Ouabain Binding Studies (Blachley et al., 1986; Jaffe et al., 1989; Nørgaard et al., 1983; Takeyasu et al, 1987)

Bovine RPE cells of passage two through four were grown in 24 well plates under varying glucose conditions for different lengths of time. The cells were rinsed with K^+-free bicarbonated Ringer solution (g/l: NaCl, 6.801; $CaCl_2 \cdot 2H_2O$, 0.153; $MgCl_2 \cdot 6H_2O$, 0.158; NaH_2PO_4, 0.103; $NaHCO_3$, 2.453; glucose, 1, 4.5, or 10; Na_3VO_4, 0.183; pH 7.2). Vanadate binds to Na^+/K^+-ATPase like P_i to form a very stable intermediate (Cantley et al., 1978). Cultures were incubated at room temperature (under gentle shaking conditions) in K^+-free Ringer's solution, with concentrations typically of 0.5 nM ^3H ouabain (32 Ci/mmole). The cells were rinsed two to three times with ice cold K^+-free bicarbonated Ringer solution to terminate the experiment. This step was accomplished in less than five seconds. The samples were solubilized with 0.4 N NaOH and aliquots were added to Optifluor liquid scintillation cocktail and

analyzed by scintillation spectroscopy. Nonspecific binding was defined as binding of radiolabelled ouabain in the presence of 10^{-4} M unlabelled ouabain. **(Note: Unless otherwise noted standard assay conditions for binding were 2 h at room temperature (25°C)).**

Protein Determinations

Samples were solubilized in 0.1 N NaOH for analysis in the Pierce BCA Protein Assay. Aliquots of the samples were processed according to the assay guidelines and compared to standards of purified Bovine Serum Albumin (BSA). At the end of the incubation period, OD measurements were obtained by either a Beckman DU70 Spectrophotometer or a Beckman Biomek 1000 equipped with an OD reader set at 540 or 562 nm, respectively.

Statistical Analysis and Data Calculations

Data were plotted using the Origin (MicroCal Software, Inc.; Northampton, MA) software package. Sigmoidal fitting of the data was obtained with this package by the use of the Boltzman equation.

$$\frac{A1 - A2}{1 + e^{(x-x0)/dx}} + A2$$

Where: x0 = center

dx = width

A1 = Y initial

A2 = Y final

IC_{50} values were obtained from points on the sigmoidal curves that produced 50% inhibition. For analysis of linearity of a given response, correlation coefficients (*r*) were obtained by linear regression analysis according to the equation:

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \Sigma y^2}}$$

Means, standard deviations (SD) and standard error of the means (SEM) were obtained using the Excel (Microsoft, Inc.) software package. Unpaired student's t tests were used to analyze for differences between mean values (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Equilibrium binding experiments with ^3H ouabain used the Law of Mass Action Model for a competing ligand. Nonspecific binding was defined as binding in the presence of 10^{-4} M unlabelled ouabain. A Scatchard analysis was performed according to the linear equation:

$$[B] = -1/K_d([B]) + B_{max}/K_d$$

where:

y axis = Bound/Free

x axis = Bound

[B] = Bound ligand concentration

[L] = Free ligand concentration

K_d = Dissociation constant

B_{max} = Number of receptors

Slope = $-1/K_d$

Y Intercept = B_{max}/K_d

X Intercept = B_{max}

Hill coefficients (n_H) were calculated using the Comp computer software package according to the equation:

$$\log(B/B_{max}-B) = n_H \log[L] - \log K_d$$

$K_i = IC_{50} / [1 + n / K_d]$, where n is the concentration and K_d the dissociation constant of radiolabelled ligand used in the competitive binding assay.

CHAPTER 4

RESULTS

^{3}H Thymidine Uptake in Bovine RPE

A control study was performed to examine the insulin responsiveness of RPE cell proliferation. A 16 hour exposure to insulin produced a dose dependent increase in ^{3}H thymidine incorporation in bovine RPE (Figure 4). Control samples did not contain insulin. Leschey et al. (1990) showed a similar increase in thymidine incorporation in two of the three human RPE cell lines tested.

Protein Determinations

For the purpose of normalization, protein values were measured in selected experiments. These typically ranged from 50 to 100 µg/well, reflecting differences in the numbers of cells/well for individual experiments. Within each study, the cells were seeded at the same density (typically 3-5x10^4 cells/cm^2) prior to their differential glucose exposure. The glucose concentrations of 1, 4.5, and 10 g/l correspond to 5.55, 25.0 and 55.5 mM, respectively. These concentrations were chosen to provide varying degrees of hyperglycemia. 5.55 mM glucose would simulate the normal condition. Mild and severe hyperglycemia would be modelled by the 25.0 and 55.5 mM concentrations, respectively. Experimentally, 20-50 mM glucose and 30 mM galactose

exposure have been used to model hyperglycemic conditions by other investigators. Table 2 is a summary of protein values for the present studies.

Table 2. Summary of Protein Values

(Expressed as µg/well)

Gluc. (g/l)	1-6 Days	SEM	1-6 Days +ARI	SEM	≥7 Days	SEM	≥7 Days +ARI	SEM
1	47.3	2.7	53.1	4.7	102.5	-	122.4	11.7
4.5	46.8	3.9	52.1	8.9	104.4	4.7	116.2	9.2
10	40.3	2.5	45.3	1.5	101.8	8.9	129.4	-

Days = days of growth under varying glucose concentrations,
ARI = 10^{-6} M AL-1576

There were no significant differences between the 1, 4.5 or 10 g/l glucose samples at a given duration of glucose exposure. In addition, no significant differences were observed with AL-1576 exposure. Therefore, it was unnecessary to express each experiment on a mg protein basis. More protein was present in cells grown for longer time periods. RPE cells typically are not contact inhibited and will continue to grow when cultured on plastic even in the absence of serum (Bryan and Campochiaro, 1986).

Preliminary Studies of $^{86}Rb^{+}$ Uptake in Suspended Cells Using the Dowex Column Technique

To assess the activity of Na^{+}/K^{+}-ATPase *in vitro* under different assay conditions, $^{86}Rb^{+}$ (a functional analog of K^{+}) uptake studies were performed. Preliminary experiments involved human red blood cells (RBC's) using Dowex columns. This method had been reported in the literature to be a simple way of measuring $^{86}Rb^{+}$ uptake. Human RBC's were used because they were plentiful compared to the slow growing RPE cells. An initial study (data not shown) provided time course and ouabain inhibition data for this simple system. $^{86}Rb^{+}$ uptake was both time dependent and susceptible to ouabain inhibition (81.4% inhibition at the 50 min time point). Correlation coefficients for regression (control CPM as a function of time) approached one indicating a linear response between 10 and 50 minutes.

In an experiment involving suspended bovine RPE (ouabain pre-incubation was 10 min), $^{86}Rb^{+}$ uptake was linear for between 20 and 80 min (Figure 5). Maximal ouabain inhibition occurred within 20 minutes of initiation of uptake and was 59.6% at 80 min. Each sample consisted of 6×10^{5} cells in this experiment. The cells were harvested via gentle scraping from the culture plate.

A control study was performed to assess whether the uptake correlated with cell number (Table 3). Ouabain (2.5 mM) pre-incubations were conducted for 10 min. Uptake was dependent upon cell number. The percent of ouabain

inhibition at 30 min was 47, 39, and 25 for the 1.875 x 10^6, 3.75 x 10^6 and 7.5 x 10^6 (cells seeded per well) groups, respectively.

Table 3. $^{86}Rb^+$ Uptake in Suspended Bovine RPE Cells

(Dependence on Cell Number)

(Cell Number x 10^6)

Cell #	1.9	1.9	3.8	3.8	7.5	7.5
Minutes	Control	Ouabain	Control	Ouabain	Control	Ouabain
0	116	114	197	138	244	140
10	356	202	649	340	1388	770
20	454	330	1209	568	2770	1557
30	389	206	1356	829	2721	2032

($^{86}Rb^+$ uptake expressed as counts per minute (cpm). Cells were grown at 4.5 g/l glucose for 2 weeks prior to the uptake experiment.)

Additional time course experiments were performed upon bovine RPE cells grown at 4.5 g/l glucose using the Dowex columns (Table 4). A considerable amount of $^{86}Rb^+$ uptake remained in the presence of 2.5 mM ouabain. This phenomenon was addressed in later experiments.

Table 4. A Time Course Study of $^{86}Rb^{+}$ Uptake in Suspended Bovine RPE Cells (Controls vs 2.5 mM Ouabain)

Minutes	Control	SEM	Ouabain	SEM	% Inhib.
0	134	7.5	124	11.6	7.5
10	353	24.7	255	21.2	27.6
20	548	66.0	358	15.0	34.7
30	672	40.9	458	16.5	31.8
40	800	13.8	459	53.3	42.6
50	858	43.8	550	20.6	35.9
60	905	43.8	531	81.6	41.3

($^{86}Rb^{+}$ uptake expressed as counts per minute (cpm). Values are means of triplicate determinations.)

After performing several studies, it was determined that the Dowex column method was too labor intensive to be used extensively. This technique also required a large number of cells per sample (>5 x 10^5). The objective was to grow cells in 24 well plates so that studies could be conducted on confluent monolayers in an attempt to more closely mimic the cell conditions *in vivo* (Jaffe

et al., 1989). Unlike the red blood cells, the RPE cells are adherent and cell to cell interactions play a role in their normal function.

$^{86}Rb^{+}$ Uptake in Adherent Bovine RPE Cells

Rubidium uptake experiments were performed using a confluent monolayer of RPE cells grown in 24 well plates. The rubidium uptake method of Jaffe et al. (1989) was adopted because it was relatively simple and had been employed in other studies with RPE cells.

Preliminary time course experiments were performed to determine optimal ouabain pre-incubation times in a 30 min uptake protocol (data not shown). These studies showed inhibition to plateau between 17 and 32 min. The standard pre-incubation time for the rubidium uptake assay in adherent cells was set at 20 min.

A large percentage of $^{86}Rb^{+}$ uptake was insensitive to ouabain inhibition. Since bovine RPE cells were known to possess a $Na^{+}/K^{+}/Cl^{-}$-cotransporter (Miller and Edelman, 1990), bumetanide was used to inhibit rubidium uptake through this mechanism. Experiments were performed to obtain dose response data for ouabain in the presence of high dose bumetanide and vice versa (Table 5, Figures 6 and 7). The objective was to eliminate the effect of either Na^{+}/K^{+}-ATPase or the $Na^{+}/K^{+}/Cl^{-}$-cotransporter to produce a more accurate estimate of the IC_{50} for each compound. The ouabain IC50 values (in the presence of 10^{-5} M bumetanide) were 5.97×10^{-8} M and 9.55×10^{-8} M for 1 and

4.5 g/l glucose, respectively. These data agreed with previous dose response curves in the absence of bumetanide. The IC_{50} values for bumetanide (in the presence of 10^{-5} M ouabain) measured in the presence of 1 and 4.5 g/l glucose were nearly identical; 1.22×10^{-7} M and 1.14×10^{-7} M respectively. A similar IC_{50} for bumetanide has been noted in turkey erythrocyte (2.5×10^{-7} M, Palfrey et al, 1980), hagfish erythrocyte (6.0×10^{-7} M, Ellory and Wolowyk, 1990), human erythrocyte (1.6×10^{-7} M, Ellory and Stewart, 1982), human fibroblasts (1.0×10^{-7} M, Owen and Prastein, 1985), and cultured bovine lens epithelial cells (1.2×10^{-7} M; Alvarez and Candia, 1994).

Table 5. The % Inhibition of $^{86}Rb^{+}$ Uptake Produced by Ouabain (in the Presence of 10^{-5} M Bumetanide) and Bumetanide (in the Presence of 10^{-5} M Ouabain) in Bovine RPE Cells Cultured at 1 and 4.5 g/l Glucose for 48 Hours (Means of Quadruplicate Samples)

Drug (varied)	Glucose (g/l)	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
Ouabain	1	26.1	33.0	67.0	84.9	85.4
Ouabain	4.5	13.7	19.0	49.8	81.1	78.1
Bumet.	1	35.6	36.5	53.0	72.9	75.7
Bumet.	4.5	50.6	47.3	62.1	80.1	80.9

High dose ouabain (present in the bumetanide dose response experiment) produced greater inhibition of uptake at 4.5 g/l glucose compared to the 1 g/l control ($P<0.05$ at all bumetanide concentrations). This supported the idea of an increased sensitivity of Na^+/K^+-ATPase to ouabain at higher glucose concentrations. Conversely, in the presence of high dose bumetanide (present in the ouabain dose response experiment), cells grown at 4.5 g/l glucose showed less inhibition of rubidium uptake when compared to 1 g/l controls ($P<0.05$ for all ouabain concentrations).

Experiments were performed to determine the effect of number of cells seeded per well upon ouabain dose response of $^{86}Rb^+$ uptake after two days of growth (Figure 8). Increased glucose concentrations did not appear to alter the IC_{50} for ouabain. However, at 10 g/l of glucose, ouabain produced the greatest amount of inhibition (57%). RPE grown at higher glucose concentrations tended to be more sensitive to ouabain inhibition and exhibited lower IC_{50} values. The least ouabain sensitive group appeared to be sparsely seeded cells grown at 1 g/l of glucose. In other words, sparse proliferating cultures appear to be less susceptible to inhibition by this drug. Similar results were seen when the experiment was performed after four days of growth in varying glucose concentrations.

An experiment was performed which tested the acute (50 min) and chronic (48 hour) effect of various concentrations of the aldose reductase inhibitor (ARI) AL-1576 upon $^{86}Rb^+$ uptake. During the acute experiments, in

which AL-1576 was only present during the assay, the ARI had no significant effect on rubidium uptake (Figure 9). However, an effect was observed after a 48 hour exposure to the ARI (Figure 10). Stimulation of rubidium uptake was statistically significant in two different groups exposed to elevated glucose. Interestingly, chronic ARI exposure had no effect on rubidium uptake under the 1 g/l (normal) glucose condition.

Nonradioactive Rubidium (Rb^+) Uptake in Attached Cultured RPE Cells: Development and Use of a High Pressure Liquid Chromatographic Method for Measuring Intracellular Concentrations of Rb^+ and Other Cations

A pilot experiment was performed to examine the possible utility of a nonradioactive rubidium (Rb^+) uptake assay that employed HPLC and conductivity detection of Rb^+ and other cations. Figures 11 and 12 show representative chromatograms from control cells and those exposed to a combination of 10^{-4} M ouabain and 10^{-4} M bumetanide. The y axis, peak height, is measured in μS. S denotes a siemen which is the conductance of an element that has the resistance of 1 ohm. As expected, ouabain inhibition caused the sodium peak to increase. The rubidium peak was large in the control sample but undectectable in the sample containing both ouabain and bumetanide. Representative standard curves for sodium, potassium and rubidium are shown in Figure 13. These were established simultaneously by utilizing a mixture containing standards of Na^+, K^+ and Rb^+. Correlation

coefficient values (peak area vs. amount of the respective analyte injected) approached 1.00 for all three cations in this and subsequent experiments. Elution of the cations of interest took place within 5 minutes. Over the course of these studies, from 11/92 to 9/94, the same Dionex Fast Cation I column was used; the elution time decreased only slightly from 1.30, 2.00, and 2.33 to 1.18, 1.91, and 2.20 minutes for Na^+, K^+, and Rb^+, respectively.

Dose response curves for ouabain inhibition were obtained using both rubidium uptake methods (Figures 14-15). The added benefit of the nonradioactive assay was that sodium, potassium, and rubidium could be measured simultaneously. The sodium values on this trial experiment were too variable to obtain dose response curves. In later studies, it became apparent that sodium would be the most difficult of the three ions to measure. This was due to the large concentration of sodium chloride that was present in the assay buffer. After the first experiment with the nonradioactive assay, care was taken to insure that rinsing with $MgCl_2$ was thorough. The IC_{50} values for ouabain inhibition of rubidium uptake produced from the nonradioactive assay were slightly smaller than those obtained from the radioactive assay making it appear to be more sensitive. The ouabain IC_{50} values for 1 g/l glucose were 1.99×10^{-7} M for the $^{86}Rb^+$ and 5.74×10^{-8} M for the nonradioactive Rb^+ assay. At 4.5 g/l glucose the IC_{50} values were 9.99×10^{-8} M and 3.32×10^{-8} M for the radioactive and nonradioactive assays, respectively. The new Rb^+ uptake assay was sensitive (nmoles/mg protein) and give comparable results to the radioactive

method. It also provided more information about two important cations involved in transport, sodium and potassium.

Another added value of the new assay related to "nonspecific" rubidium uptake which was significant in the $^{86}Rb^+$ assay. Maximum inhibition of rubidium uptake by high dose ouabain plus bumetanide was typically no greater than 80% in the $^{86}Rb^+$ uptake assay. Thus, a measurable portion of the rubidium transport activity was unaccounted for (i.e. rubidium binding to plastic culture dish, etc.). In the nonradioactive assay, however, the combination of high dose bumetanide and ouabain typically produced 90-100% inhibition of Rb^+ uptake.

Experiments were performed to ensure that the 30 min Rb^+ incubation was in the linear portion of the uptake curve (Figures 17-18). For both 1 and 4.5 g/l glucose-exposed samples, correlation coefficients were near unity. Therefore, the standard condition, 30 minutes of Rb^+ incubation, was maintained in the nonradioactive assay. This allowed direct comparisons between the $^{86}Rb^+$ and the Rb^+ uptake methods. Time course data from these studies showed that rubidium increased over time, while potassium decreased, and sodium remained constant (Figures 18-20).

A study was performed to assess the dependence of Rb^+ uptake on the concentration of Rb^+ added (Figure 21). This experiment was performed upon cells exposed to medium containing 1, 4.5, and 10 g/l glucose for 72 hours. The concentrations of Rb^+ used routinely in these studies, 0.6 g/l, was shown to

saturate the membrane transporters for this ion. Unpaired Student's t-test analyses showed that at concentrations of Rb^+ producing detectable uptake all samples were significantly different from the 1 g/l glucose ($P<0.05$; with the exception of the 10 g/l glucose, 3mg/ml Rb^+ data point). Rb^+ uptake was saturable, however, with a decreased maximal value under conditions of elevated glucose. This experiment was repeated and the maximal Rb^+ uptake again was depressed under the 10 g/l condition.

A study was executed to determine if supersaturating concentrations of RbCl (3.0 g/l) would reveal a difference in uptake at various glucose concentrations (48 h growth, Figure 22). Rb^+ uptake expressed as nmoles/mg protein was decreased with increasing glucose concentrations. Potassium concentrations decreased while sodium increased relative to intracellular rubidium under simulated diabetic conditions.

An experiment was performed to assess the effect of barium chloride ($BaCl_2$), a potassium channel blocker, on K^+ concentrations measured in the Rb^+ uptake assay. At the 1 and 4.5 g/l glucose doses (Figure 23), higher doses of $BaCl_2$ appeared to inhibit the leakage of potassium. This inhibition was not observed in the 10 g/l glucose group. No effect of $BaCl_2$ on rubidium uptake was noted at 1, 4.5 or 10 g/l glucose (72 h) (data not shown). In addition, the Na^+/H^+ exchange blocker amiloride (up to 10^{-5} M) produced no consistent effect on either rubidium uptake or intracellular sodium concentrations (data not shown).

Bumetanide was tested, in the absence of ouabain, for its effect in the Rb^+ uptake assay. Figure 24 shows that for cells grown at 1 and 4.5 g/l glucose for 72 hours, high doses of bumetanide increased potassium concentrations in the cells. In routine experiments this agent was included as a control condition, at a concentration of 10^{-4} M (in one set of triplicate wells), to measure the portion of Rb^+ uptake that occurred through the $Na^+/K^+/Cl^-$-cotransporter.

Table 6. Summary of IC_{50} for Ouabain Inhibition of Rubidium Uptake (30 Minute Incubation) in Bovine RPE (Expressed as nM)

g/l Gluc.	$^{86}Rb^+$	SEM	*n*	Rb^+	SEM	*n*
1	129	23.5	8	56.6	9.30	9
4.5	211	133	8	109	55.0	10
10	64.1	32.3	4	68.2	20.0	4

(Each value of *n* represents one 24 well plate with triplicate or quadruplicate ouabain dose response curves ranging from 10^{-10} M to 10^{-4} M)

Several studies were conducted in an attempt to examine the effect of elevated glucose upon the ouabain IC_{50} for rubidium uptake. Over the course of these studies, the IC_{50} values were consistent. A summary table (Table 6) shows the data from 30 minute uptake experiments in both the radioactive and nonradioactive assays. No difference in mean ouabain IC_{50} values was noted

between the two assays when comparing within a single glucose concentration. In addition, varying the concentration of medium glucose up to 10 g/l for periods up to 28 days did not alter the ouabain IC_{50} values.

Table 7. Summary of % Inhibition of Rubidium Uptake Produced by 10^{-4} M Ouabain

Glucose (g/l)	$^{86}Rb^+$	SEM	*n*	Rb^+	SEM	*n*
1	40.7	5.7	8	66.4***	3.1	22
4.5	43.4	4.1	10	62.4**	3.6	21
10	47.2	5.0	7	76.3***	2.9	9

(Each value of *n* represents a different experiment performed in triplicate or quadruplicate. **$P<0.01$, ***$P<0.001$ Rb^+ compared to respective $^{86}Rb^+$ value)

Table 7 shows a summary of the maximal inhibition of rubidium uptake produced by 10^{-4} M ouabain for cells grown up to 28 days in varying glucose conditions. There was a significant difference between the two assays at every glucose concentration. In the nonradioactive assay, a significant difference was observed between the 4.5 and 10 g/l glucose concentrations ($P<0.05$). The difference in the same assay between the 1 and 10 g/l doses was significant at the 10% level ($P<0.10$).

Table 8. Summary of % Inhibition of Rubidium Uptake Produced by 10^{-4} M Bumetanide

Glucose (g/l)	% Inhibition Rb^+	SEM	*n*
1	29.8	5.5	5
4.5	30.0	2.8	6
10	30.1	5.6	4

(each value of *n* denotes one experiment performed in triplicate)

Maximal inhibition (as a percentage of the total) produced by bumetanide alone is summarized in Table 8. No effect in this value was noted from increased glucose concentrations (for up to 28 day exposures). Approximately 30% of rubidium uptake occurred by way of the bumetanide sensitive cotransporter, while about 60-70% took place through the ouabain-sensitive Na^+/K^+-ATPase (see Table 7 on the previous page).

Table 9. Summary of % Inhibition of Rubidium Uptake by a Combination of 10^{-4} M Ouabain and 10^{-4} M Bumetanide

Glucose (g/l)	% Inhibition Rb^+	SEM	*n*
1	97.3	0.6	24
4.5	94.5*	1.2	25
10	92.1**	1.3	3

(*$P<0.05$, **$P<0.01$ compared to 1 g/l group, each value of *n* denotes one experiment performed in triplicate)

Table 9 shows a summary table of percent inhibition of rubidium uptake by the addition of both high dose ouabain and bumetanide. Elevated glucose caused a slight decrease in inhibition by both agents when compared to the 1 g/l glucose group.

Table 10. Summary of Rubidium Uptake in Bovine RPE Cells Exposed to 1, 4.5 and 10 g/l Glucose Concentrations for Up to One Month (In the Presence and Absence of 10^{-6} M AL-1576) (Expressed as nmoles/Sample)

Gluc. (g/l)	Days	Cont.	O	B	O+B	Cont. ARI	ARI+ O	ARI+ B	ARI+ O+B
1	7	42.2	10.1	32.4	4.2	40.4	10.8	31.7	3.0
4.5	7	39.5	8.2	25.4	2.6	40.0	10.1*	31.6	2.
10	7	33.5	6.7	30.1	2.6	30.9	5.8	31.0	2.1
1	14	45.6	10.3	39.9	2.4	50.3	10.2	40.0	2.3
4.5	14	45.4	7.3	34.4	2.3	48.3	8.3	34.7	2.0
10	14	43.1	12.0	42.5	3.9	49.1	8.9*	32.9*	4.9
1	28	54.6	8.0	35.8	0	51.1	8.3	37.4	0
4.5	28	48.6	7.7	35.6	4.5	45.1	6.8*	33.4	3.8
10	28	37.2	5.5	33.5	2.6	51.1*	6.9	35.4	0

(Each sample consists of one well in the 24-well culture plate. Means of triplicate samples. O represents 10^{-4} M ouabain, B represents 10^{-4} M bumetanide, ARI represents 10^{-6} M AL-1576, Days = days cultured in varying glucose with or without the ARI, Cont. represents control samples without the ARI at various [glucose]. *$P<0.05$, **$P<0.01$ when compared to their respective controls.)

A one month study was performed to examine the effects of elevated glucose upon rubidium uptake at various time points (Table 10). Some cells were grown in the presence of the ARI AL-1576 (10^{-6} M) to measure any effects of the compound on the intracellular concentration of the cations of interest. Although the ARI appeared to stimulate rubidium uptake, few statistically significant differences were observed between the controls versus their respective ARI counterparts. Figure 25 shows a summary of control data at various time points. As the cells grew over the course of the study, increased amounts of all the ions of interest were noted. In general, with increased glucose concentrations, rubidium uptake was decreased. In many cases hyperglycemia also caused decreases in intracellular potassium. Sodium concentrations were consistent between the three glucose concentrations at each time point. The net result was that the sodium was increased relative to the fall in rubidium and potassium under high glucose conditions. For example, sodium comprised 25.4%, 26.7%, and 32.0% of the total intracellular cations at the 1, 4.5, and 10 g/l glucose concentrations, respectively (control samples at the one month time point).

Ouabain Binding Studies

To determine if the number of Na^+/K^+-ATPase molecules changed with exposure to varying glucose concentrations, ^3H ouabain binding was measured. Two separate time course binding experiments were performed to establish

subsequent experimental protocols. These were accomplished by titrating nonradioactive ouabain against a constant amount of ^3H ouabain. The first study, utilizing cells grown in 1 g/l glucose medium for 2 days, produced IC_{50} values of 2.63×10^{-7} M, 7.60×10^{-8} M, and 8.98×10^{-8} M for time points of 40, 88 and 94 minutes, respectively. In addition, Hill slopes or coefficients for the same experiment were 0.85, 1.15, and 0.82, respectively. Nonspecific binding was defined as the binding that occurred in the presence of 10^{-4} M nonradioactive ouabain. Specific binding ranged from 76.1% for the 40 min time point to 92.1% for the 94 min time point.

The second study, shown in Figure 26, involved RPE cells grown in medium containing 4.5 g/l glucose. IC_{50} values for ouabain inhibition of ^3H ouabain binding were similar between 60 and 120 minutes. Maximal binding was not significantly different (at the 5% level) between the 90 and 120 min time points, therefore the 120 min time point was chosen as the standard assay condition. Specific binding at the 120 min time point was 84.4%. Hill slopes were all approximately 1.0 which indicated single site binding curves. These results were consistent since RPE cells were shown in the literature to contain only the $\alpha 1$ isoform of the enzyme. Other isoforms of Na^+/K^+-ATPase show differing affinities for ouabain (Brodsky, 1990; Shyjan et al., 1990a,b; Skou, 1962, 1988; Sweadner, 1979).

Cells exposed to 4.5 g/l glucose for 72 hours were tested for saturation of ouabain binding sites (Figure 27). Specific binding was saturated at

approximately $1.5x10^{-7}$ M ^{3}H ouabain per well, corresponding to 39.8 µg of protein ($3.33x10^{-9}$ M/mg protein). This value agreed favorably with published results with human RPE cells (5×10^{-8} M; Jaffe et al., 1989; this value may have represented fewer cells per sample).

A Scatchard plot for ^{3}H ouabain binding in bovine RPE is shown in Figure 28. The $B_{max} = 6.02 \times 10^{-10}$ M/well or 1.51×10^{-8} moles/mg protein. The K_d value found in the bovine cells 2.74×10^{-8} M for ^{3}H ouabain binding was comparable to that found by Jaffe et al., 1989 (2.07×10^{-8} M) for cultured human RPE cells.

RPE cells were grown for 48 hours in 1, 4.5 and 10 g/l glucose medium and assayed for ^{3}H ouabain binding (Figure 29). The amount of specific binding was 94.1%, 94.8%, 94.6% for the 1, 4.5, and 10 g/l glucose samples, respectively. IC_{50} values for ouabain inhibition were virtually identical under the different conditions.

RPE cells were grown in 1 and 10 g/l glucose (due to availability of the compound, 40 and 11.4 pmoles/well, corresponding to 80 and 22.8 nM ^{3}H ouabain were used, respectively) for 72 hours to determine the effect on ouabain binding (data not shown). Specific binding for the two highest doses of radioactive ouabain was between 89-93% for all glucose concentrations. Although the amount of radioactive ouabain added was higher at 1 g/l glucose, the IC_{50} values for inhibition by nonradioactive ouabain were similar (83.0 and 56.9 nM for 1 and 10 g/l glucose, respectively).

Table 11 shows a summary of IC_{50} data for inhibition by nonradioactive ouabain of ^3H ouabain binding. IC_{50} values were similar with the average under all conditions being 5.02×10^{-8} M. Hill coefficients were all approaching 1.00 indicating single site binding. In addition, these values were comparable to IC_{50} values obtained for ouabain inhibition of rubidium uptake (56 nM to 211 nM).

Table 11. Summary of Bovine RPE ^3H Ouabain Binding Data

Glucose (g/l)	Days of Exposure to Glucose	Ouabain IC_{50}(nM)	Hill Coefficient
1.0	2	53.3	1.12
4.5	2	41.7	1.02
10.0	2	52.0	0.96
1.0	3	83.0	0.89
10.0	3	56.9	0.85
1.0	10	27.0	1.20
4.5	10	49.2	1.16
4.5	12	38.1	0.94

IC_{50} values represent means of triplicate determinations

A binding experiment was conducted with strophanthidin, a more potent Na^+/K^+-ATPase antagonist than ouabain, in an attempt to discern any possible

differences in antagonist binding under varying glucose conditions for 1 week (Figure 30). IC_{50} values were similar under the three glucose conditions and averaged 8.81 x 10^{-9} M. As expected, these IC_{50} values were lower than those found using nonradioactive ouabain as the antagonist. Hill numbers were 1.02, 1.14, and 1.30 for 1, 4.5, and 10 g/l glucose samples respectively.

The effect of acute (2 h) exposure to AL-1576 on ^{3}H ouabain binding was examined (Table 12). No significant effect was observed by the aldose reductase inhibitor in this study. Similarly, an earlier study (data not shown) had produced no effect with AL-1576 during a 1 hour incubation with varying glucose concentrations.

Table 12. The Effect of a 120 Minute Incubation with the ARI AL-1576 on ^{3}H Ouabain Binding in Bovine RPE (Expressed as DPM Bound)

Glucose	Control	10^{-8} M ARI	10^{-7} M ARI	10^{-6} M ARI	10^{-5} M ARI
1 g/l	1712±168	1495±224	1440±287	1471±208	1777±102
4.5 g/l	1502±112	1791±188	1614±104	1625±86	1615±56

(Means±SD of triplicate samples. Cells were cultured with two glucose concentrations for 10 days in the absence of the ARI)

Another experiment was performed to examine the effects of elevated glucose and an aldose reductase inhibitor upon ouabain inhibition of ^{3}H ouabain

binding. In the first experiment, (Table 13) the RPE cells were exposed to 10 g/l glucose and 10^{-6} M AL-1576 for 48 hours. The ARI also was present during the binding assay. There was a slight decrease in ^{3}H binding in one sample that was exposed to the ARI. However, the overall effect of the ARI was negligible.

Table 13. The Effect of 48 Hour Exposure to 10 g/l Glucose and AL-1576 on ^{3}H Ouabain Binding (Expressed as DPM Bound)

[Ouabain]M	Control (10 g/l Glucose)	$1x10^{-6}$ M AL-1576
$1x10^{-7}$	1118±28	1105±62
$3x10^{-7}$	501±37	551±24
$1x10^{-6}$	430±7	325±24**
$1x10^{-4}$	351±25	233±59

Means±SD of triplicate samples (**P<0.01, relative to the respective control in the absence of the ARI, AL-1576).

A similar experiment was performed in which the cells were exposed to varying glucose concentrations and 10^{-6} M AL-1576 for 1 week to determine the effect on strophanthidin inhibition of ^{3}H ouabain binding Table 14. When the samples exposed to elevated glucose and the ARI were compared to their respective controls, there was a significant difference in only three experiments,

two of which corresponded to the smallest strophanthidin concentration used (10^{-11} M). The effects at elevated glucose were not consistent over all concentrations of strophanthidin tested, however, and were considered negligible. Interestingly, the samples exposed to 1 g/l glucose were unaffected by the ARI.

Table 14. The Effect of a 1 Week Exposure to 10 g/l Glucose and 10^{-6} M AL-1576 on Strophanthidin Inhibition of ^{3}H Ouabain Binding (Expressed as a % of Total DPM Added)

Strophanthidin	$1x10^{-11}$ M	$1x10^{-9}$ M	$1x10^{-7}$ M	$1x10^{-5}$ M
Control (1g/l)	3.29±0.04	2.93±0.07	0.27±0.03	0.02±0.02
ARI (1g/l)	3.28±0.07	3.03±0.12	0.28±0.03	0.02±0.01
Control (4.5g/l)	3.41±0.10	3.27±0.12	0.31±0.02	0.01±0.03
ARI (4.5g/l)	3.69±0.04*	3.23±0.09	0.30±0.02	0.02±0.04
Control (10g/l)	3.77±0.25	3.52±0.21	0.36±0.01	0.04±0.03
ARI (10g/l)	4.19±0.05*	3.89±0.12	0.32±0.01*	0.02±0.00

Mean±SD of triplicate samples (*$P<0.05$, relative to the respective control in the absence of ARI).

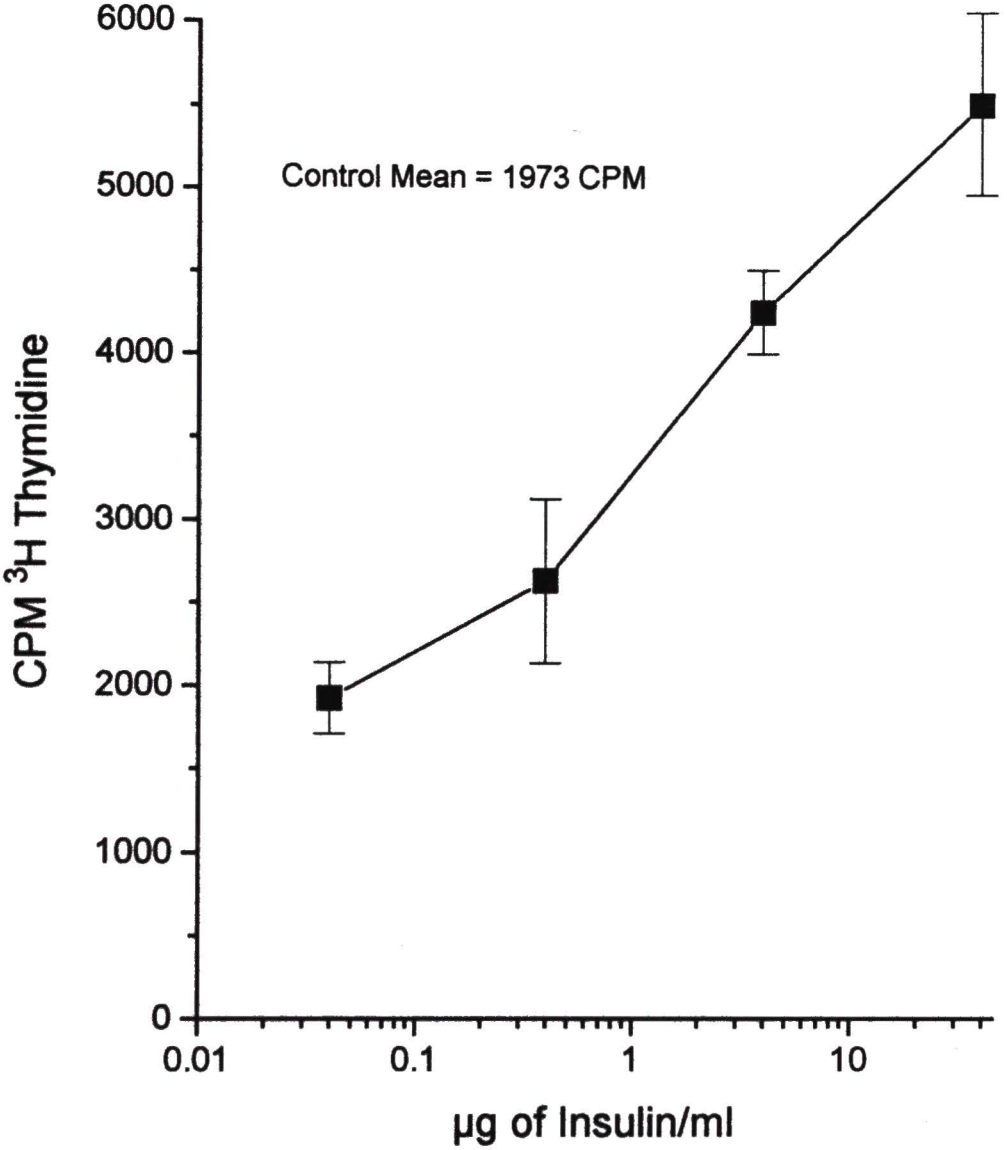


Figure 4. The effect of varying insulin concentrations on ^{3}H thymidine incorporation in bovine RPE cells cultured at 4.5 g/l glucose for 72 h. Insulin exposure 16 h. Control incubation 16 h in the absence of insulin. Means±SEM of quadruplicate determinations.

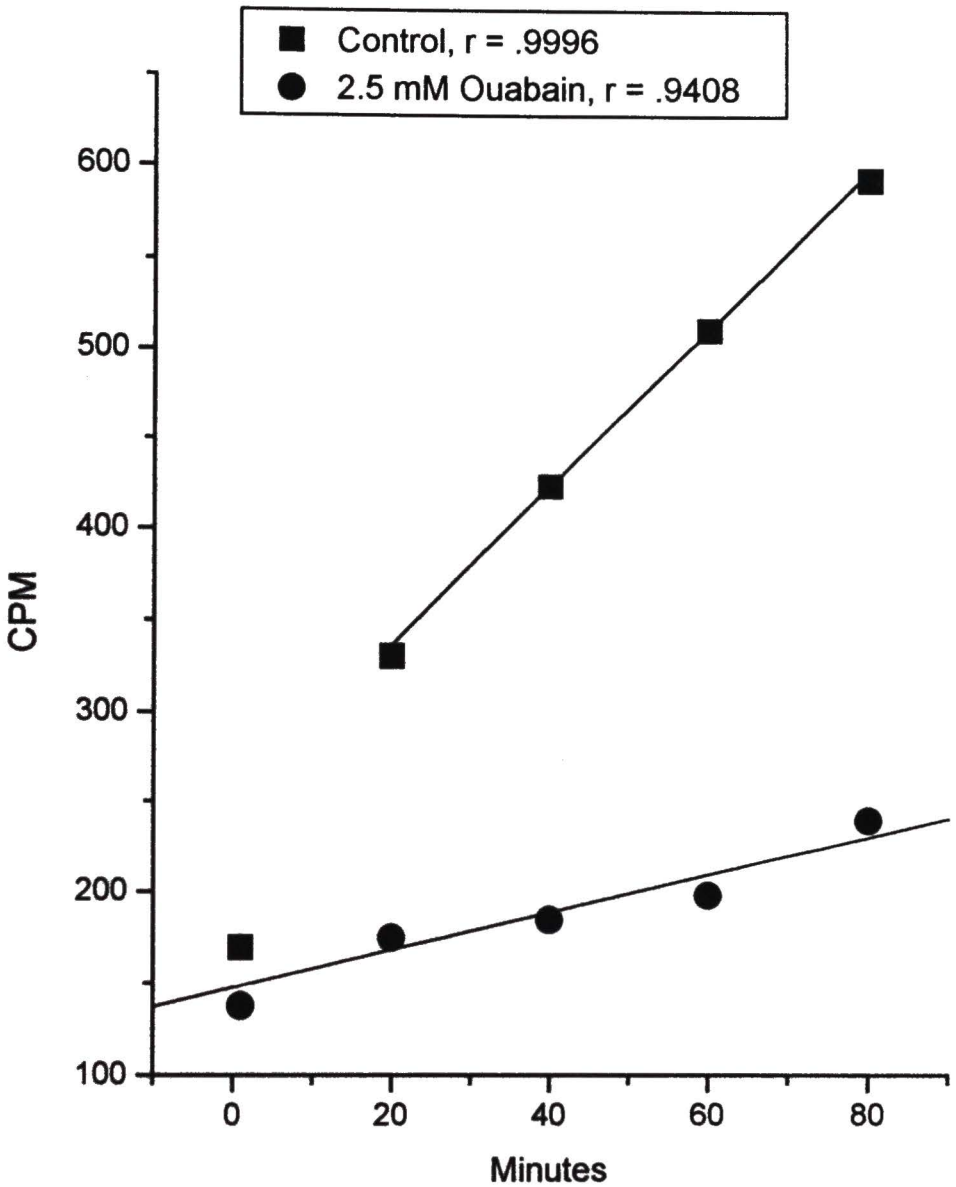


Figure 5. Time dependence of $^{86}Rb^{+}$ uptake in suspended bovine RPE cells in the presence and absence of 2.5 mM ouabain. Ouabain pre-incubation time = 5 min. $6x10^{5}$ cells per sample. Room temperature. Separation of intracellular and extracellular $^{86}Rb^{+}$ was performed with Dowex 50W-X8 cation exchange columns.

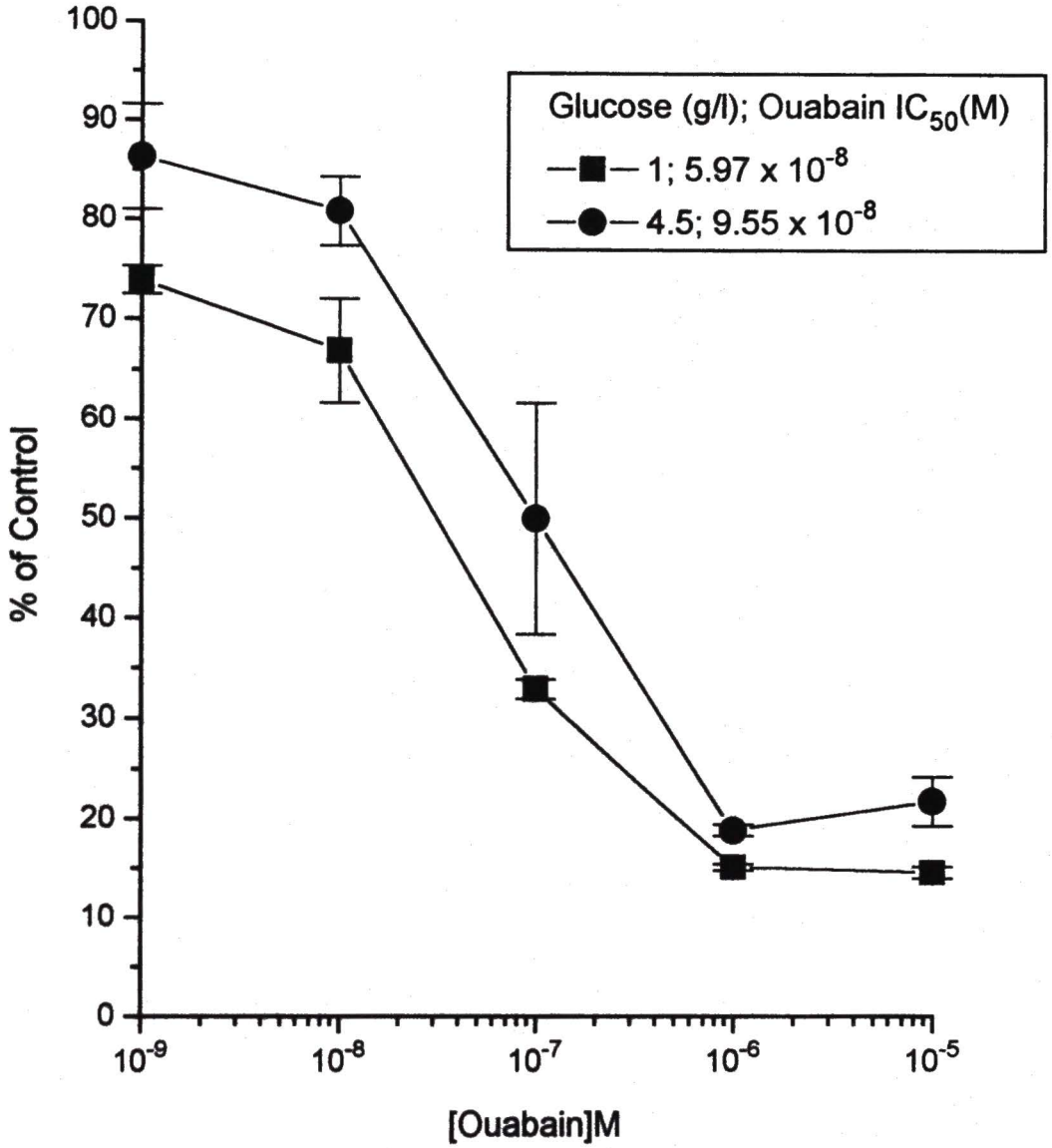


Figure 6. Concentration-dependent inhibition by ouabain (in the presence of 10^{-5} M bumetanide) of $^{86}Rb^{+}$ uptake in bovine RPE cells exposed to varying glucose concentrations for 48 h. Control samples for each glucose concentration were not exposed to ouabain or bumetanide. Standard assay conditions. Data points represent means ±SEM of quadruplicate samples.

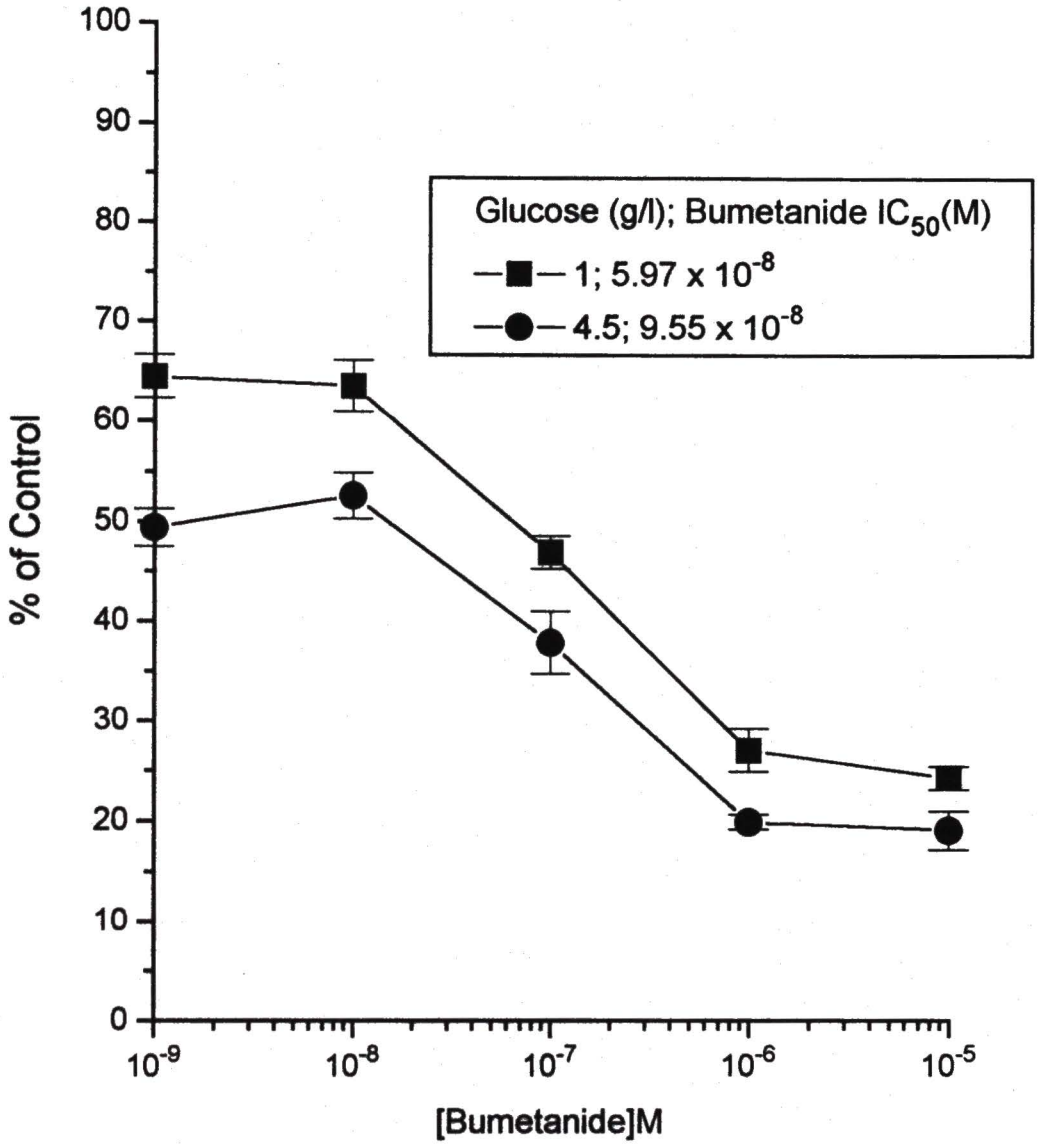


Figure 7. Concentration-dependent inhibition by bumetanide (in the presence of 10^{-5} M ouabain) of $^{86}Rb^{+}$ uptake in bovine RPE cells exposed to varying glucose concentrations for 48 h. Control samples for each glucose concentration were not exposed to ouabain or bumetanide. Standard assay conditions. Data points represent means±SEM of quadruplicate samples.

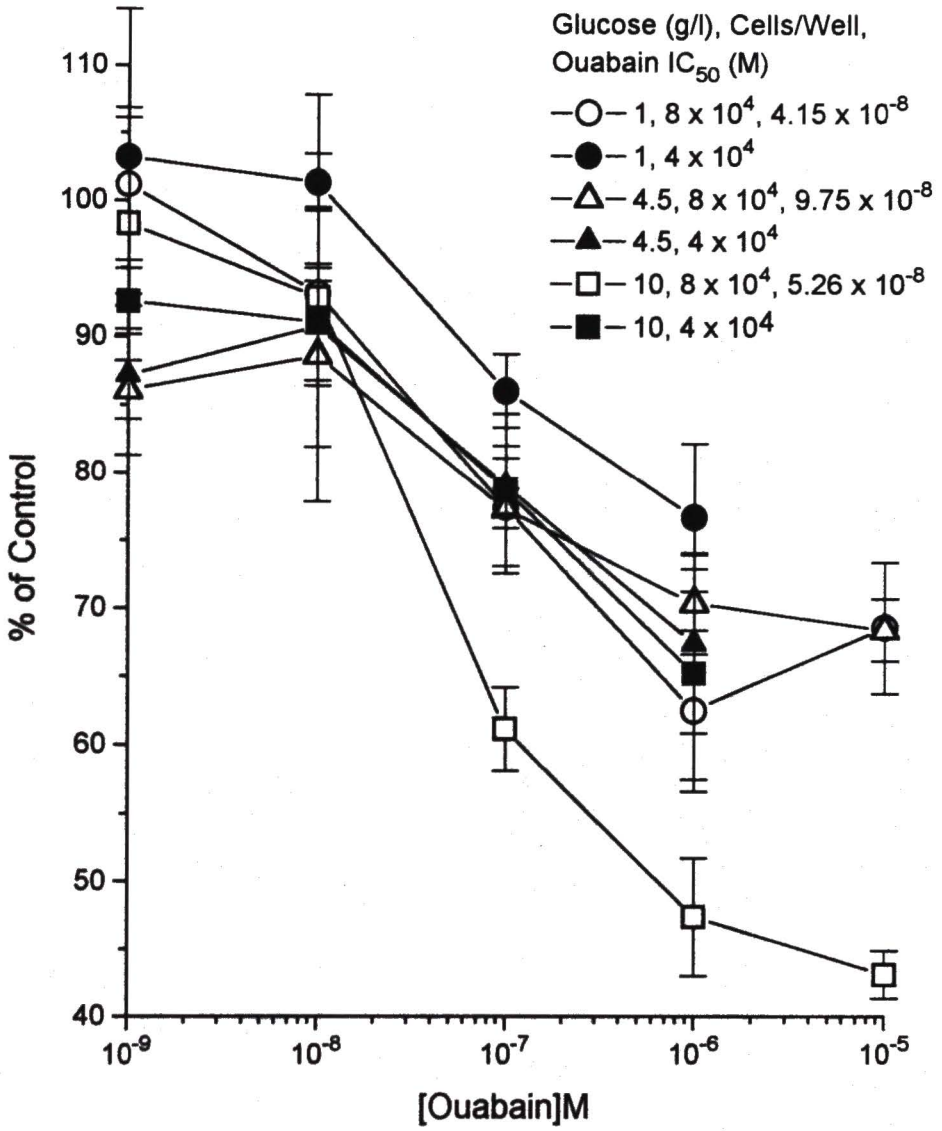


Figure 8. Effect of seeding density upon ouabain inhibition of bovine RPE $^{86}Rb^{+}$ uptake at three glucose concentrations. Control samples were not exposed to ouabain. Experiments were performed two days post seeding. The values represent means ±SD of quadruplicate determinations. Ouabain pre-incubation = 20 min. $^{86}Rb^{+}$ incubation = 30 min.

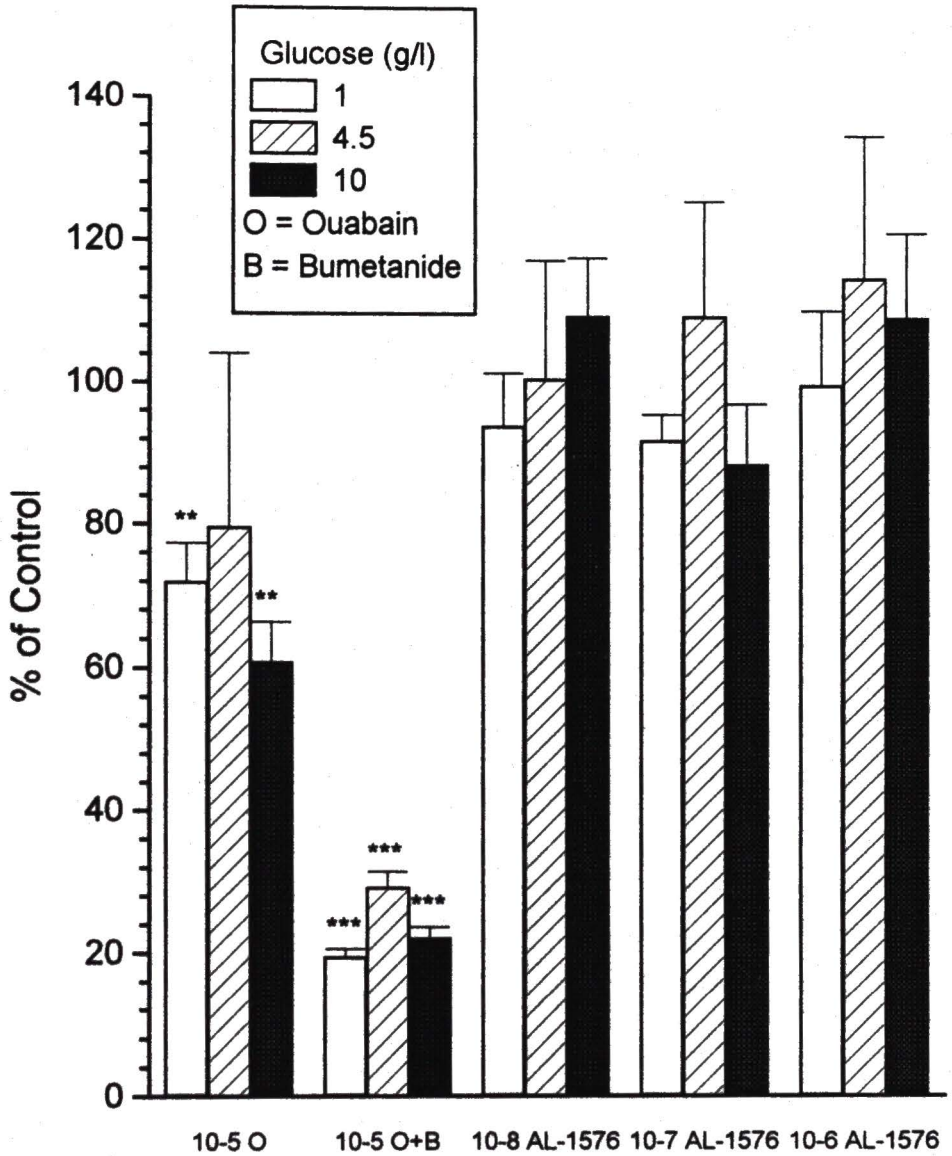


Figure 9. The effect of an acute (50 min) exposure to AL-1576 on $^{86}Rb^{+}$ uptake in bovine RPE cells cultured under varying glucose concentrations for 48 h. Bars represent means±SD of quadruplicate samples. Control samples were not exposed to ouabain or AL-1576. Difference from respective controls **P*<0.05, ***P*<0.01, ****P*<0.001.

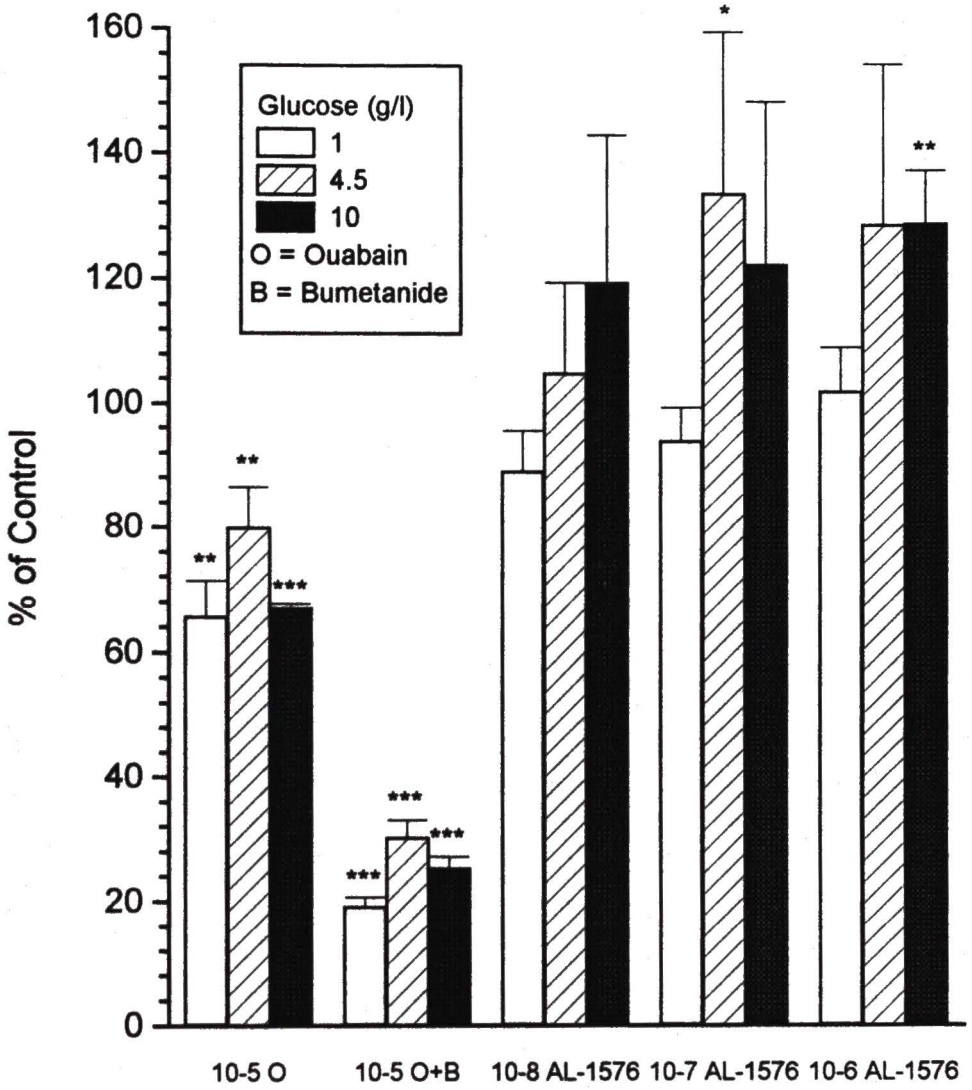


Figure 10. The effect of a 48 h exposure to AL-1576 and varying glucose concentrations on $^{86}Rb^{+}$ uptake in bovine RPE cells. Bars represent means±SD of quadruplicate samples. Control samples were not exposed to ouabain or AL-1576. Comparisons with respective controls *P<0.05, **P<0.01, ***P<0.001.

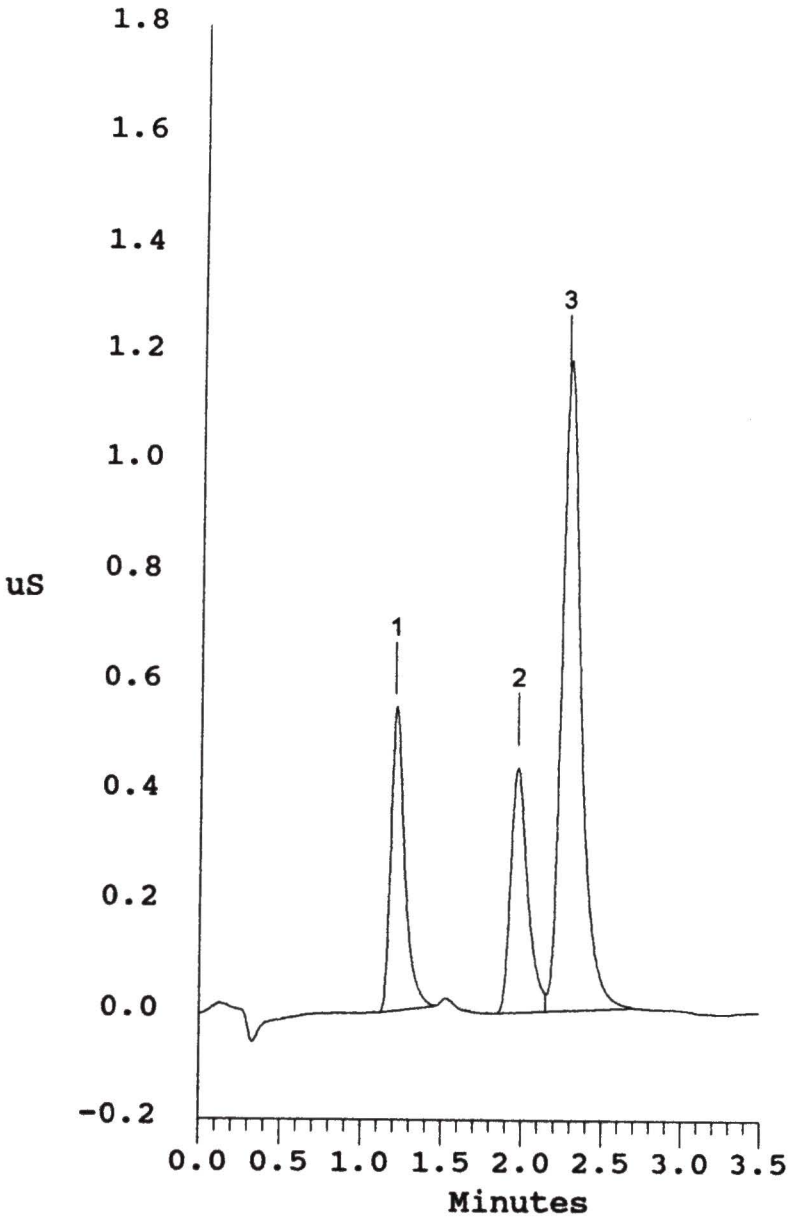


Figure 11. Representative chromatogram of bovine RPE cell extract after performing the standard Rb^+ uptake assay (1 g/l glucose in the absence of ouabain). Elution conditions are those described in the Methods Section. The three prominent peaks correspond to Na^+ (peak 1), K^+ (peak 2), and Rb^+ (peak 3). The unit of conductivity is 10^{-6} sieman (μS). A siemen is defined as the conductance of an element with resistance of 1 ohm.

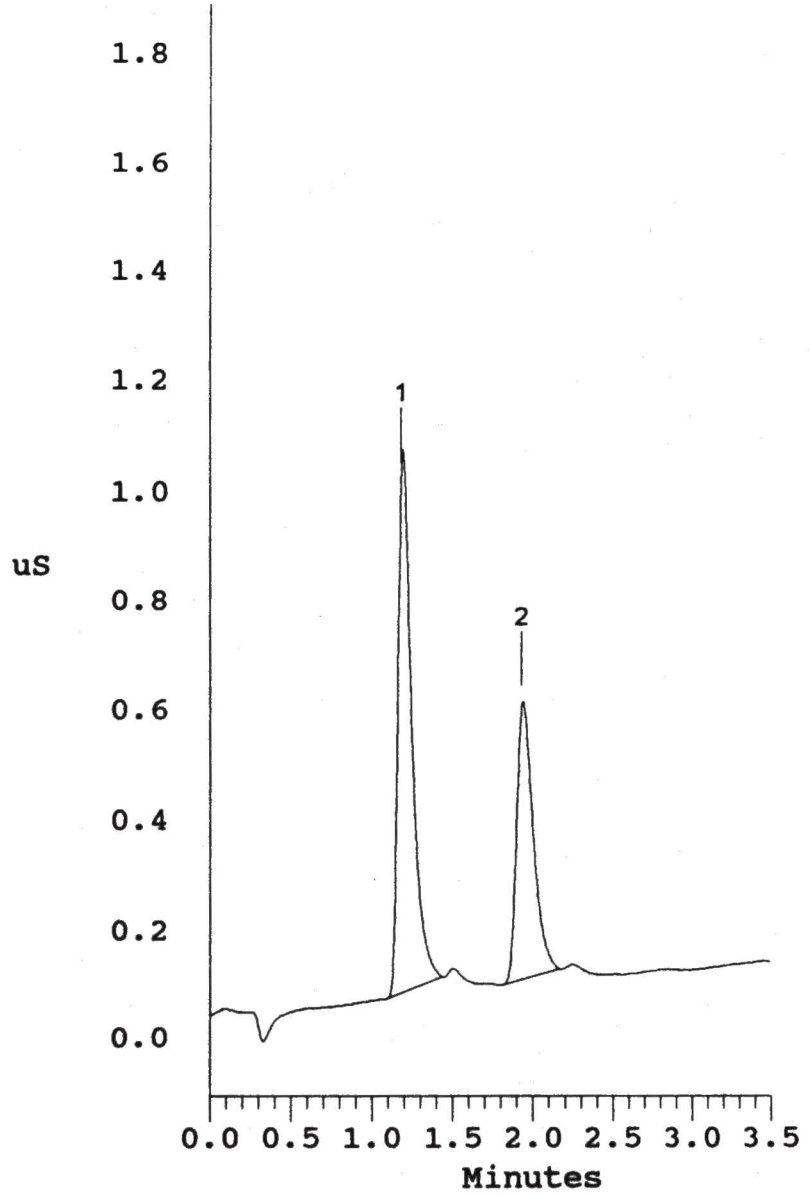


Figure 12. Representative chromatogram of bovine RPE cell extract after performing the standard Rb^+ uptake assay (1 g/l glucose, in the presence of 10^{-4} M ouabain and 10^{-4} M bumetanide). Sample volume injected was 25 µl (of a total extract volume of 500 µl). Elution conditions are those described in the Methods Section. The two prominent peaks correspond to Na^+ (peak 1) and K^+ (peak 2). Rb^+, with a retention time of 2.27 min, was below the limit of detection in this sample. The unit of conductivity is 10^{-6} sieman (µS). A sieman is defined as the conductance of an element with resistance of 1 ohm.

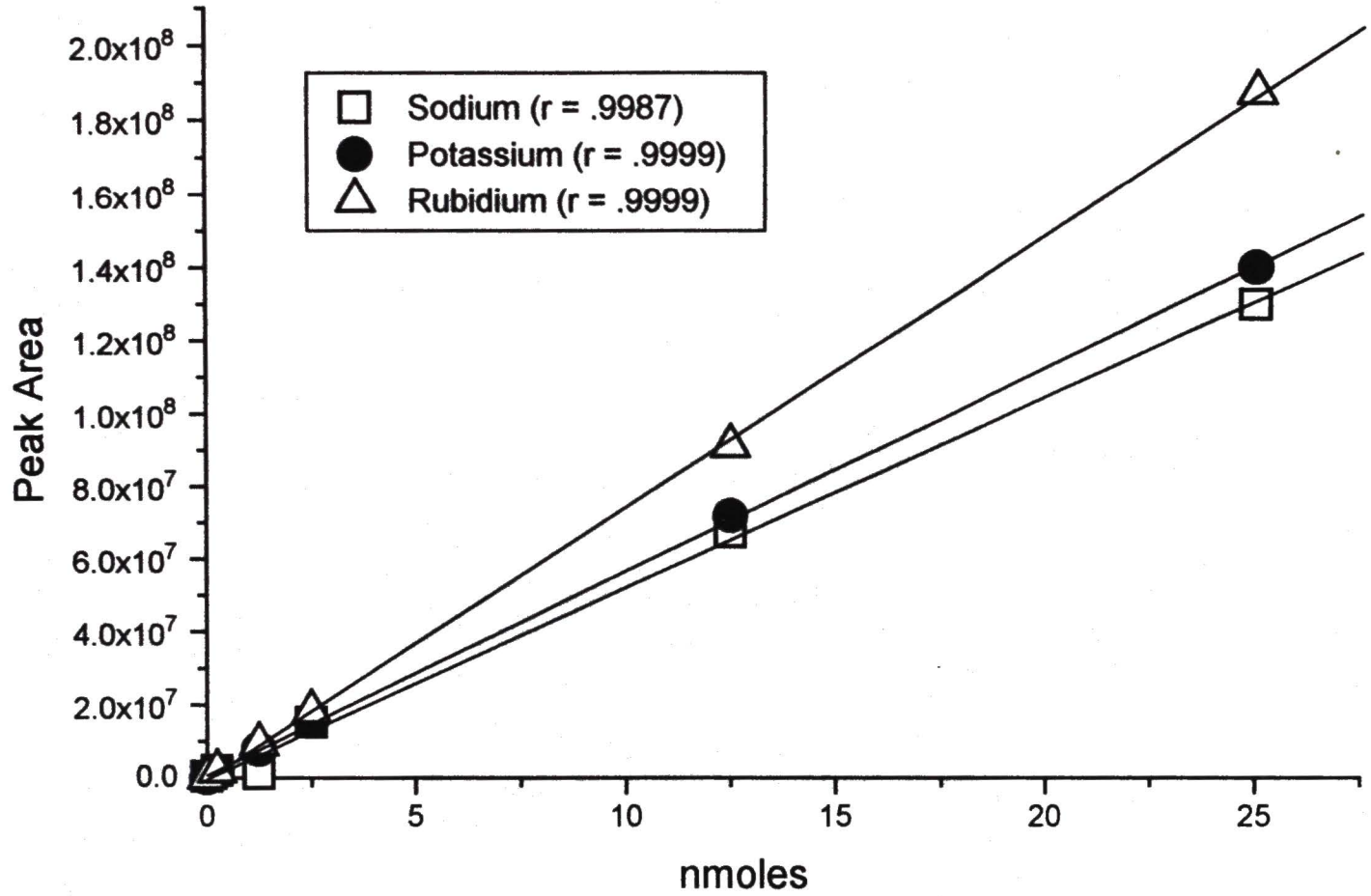


Figure 13. Sample standard curves for sodium, potassium, and rubidium in the nonradioactive rubidium uptake assay.

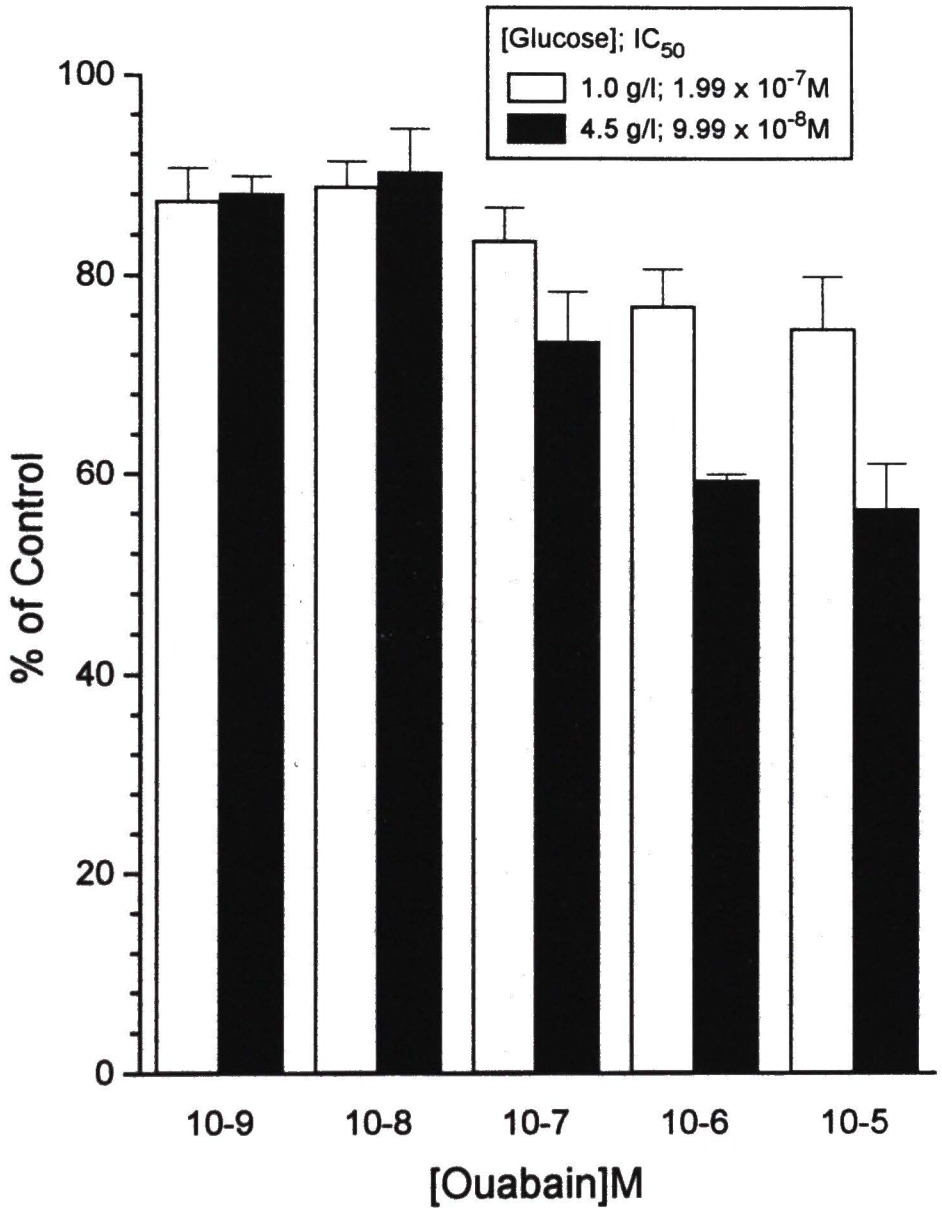


Figure 14. Concentration-dependent inhibition by ouabain of $^{86}Rb^{+}$ uptake in bovine RPE exposed to two glucose concentrations for 48 h. Standard assay conditions. Control samples for each glucose concentration were not treated with ouabain. Bars represent means ±SEM of quadruplicate samples.

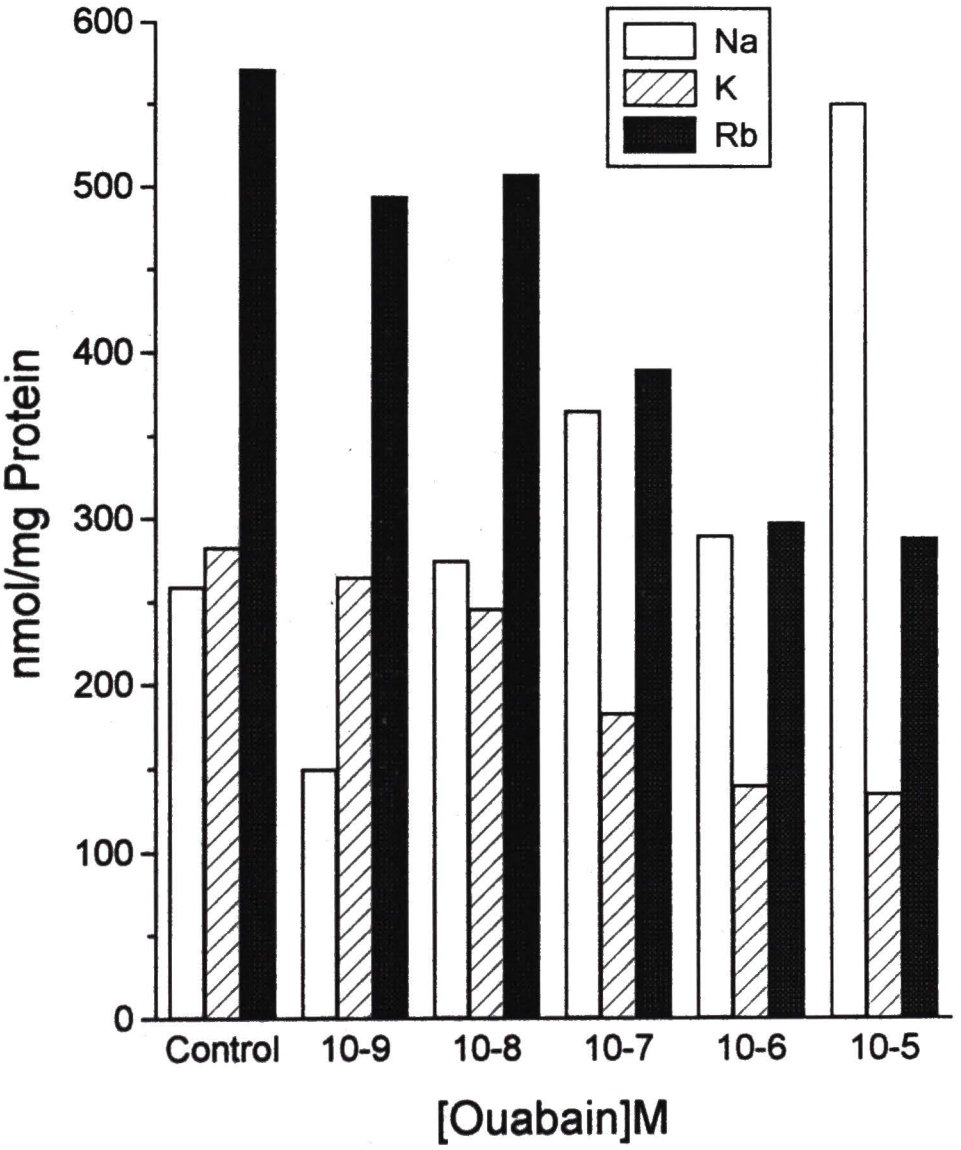


Figure 15. Concentration-dependent effects of ouabain on intracellular Rb^+, Na^+, and K^+ concentrations of bovine RPE cells exposed to 1 g/l glucose for 48 h, in the standard Rb^+ assay. Values are means of duplicate samples. (The IC_{50} value for ouabain inhibition of Rb^+ uptake was 5.74×10^{-8} M)

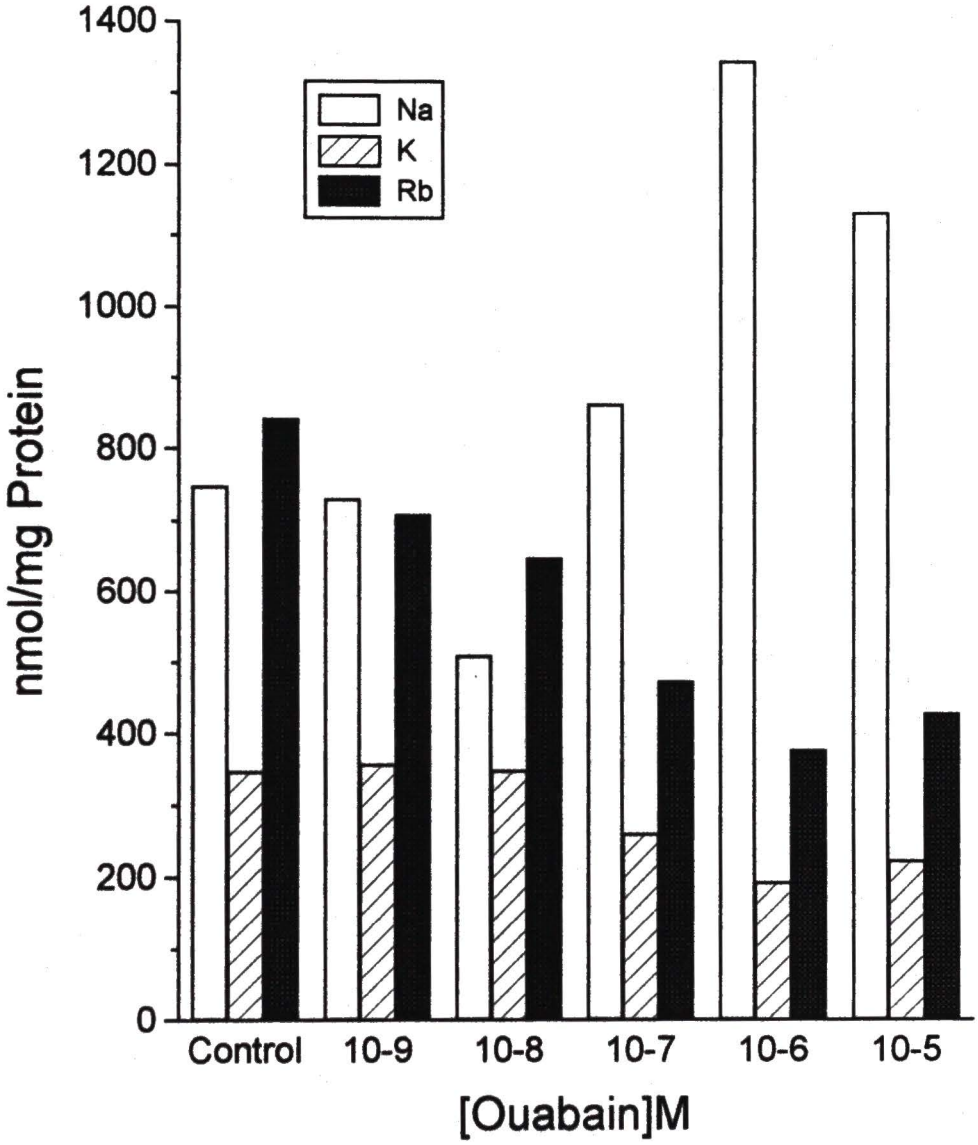


Figure 16. Concentration-dependent effects of ouabain on intracellular Rb^+, Na^+, and K^+ concentrations of bovine RPE cells exposed to 4.5 g/l glucose for 48 h, in the standard Rb^+ assay. Values are means of duplicate samples. (The IC_{50} value for ouabain inhibition of Rb^+ uptake was 3.32×10^{-8} M.)

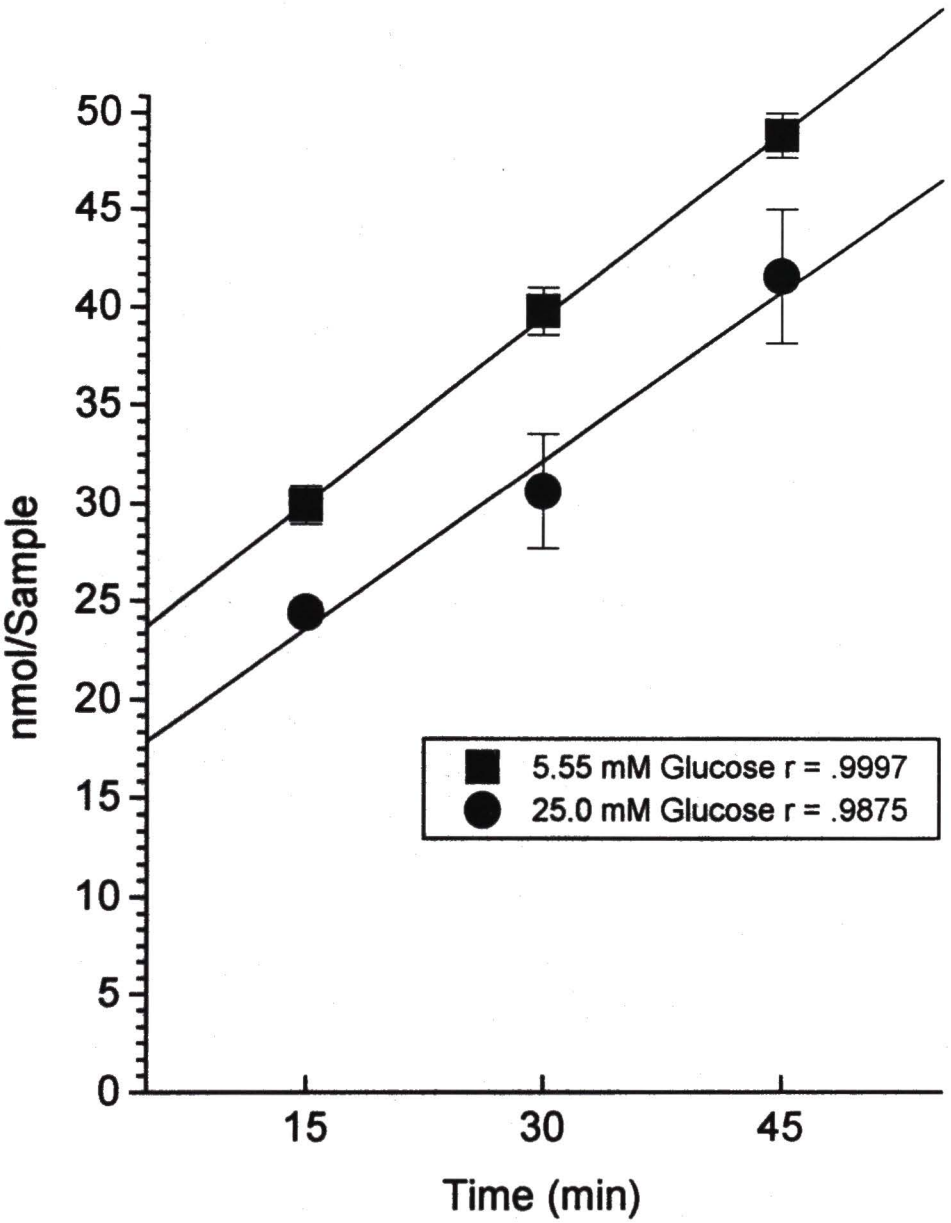


Figure 17. Time course of Rb^+ uptake in bovine RPE cells grown at varying glucose concentrations for 10 days. Ouabain pre-incubation 20 min. Data points represent means±SEM for quadruplicate samples.

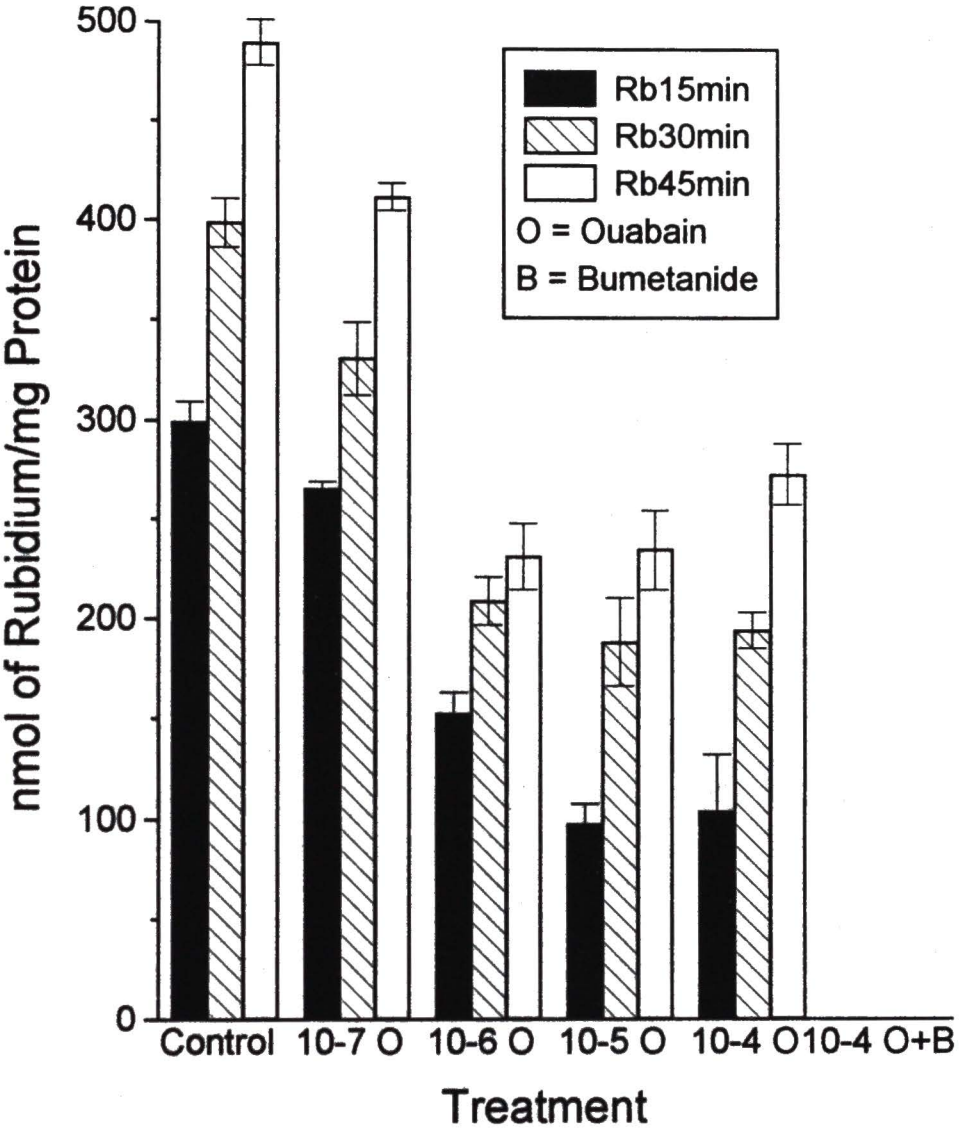


Figure 18. Concentration-dependent inhibition by ouabain of Rb^+ uptake in bovine RPE cells exposed to 1 g/l glucose for 10 days, as a function of incubation time with Rb^+. 20 min pre-incubation with or without ouabain. Rb^+ in samples containing both ouabain and bumetanide was not detectable. Means ±SEM of quadruplicate samples.

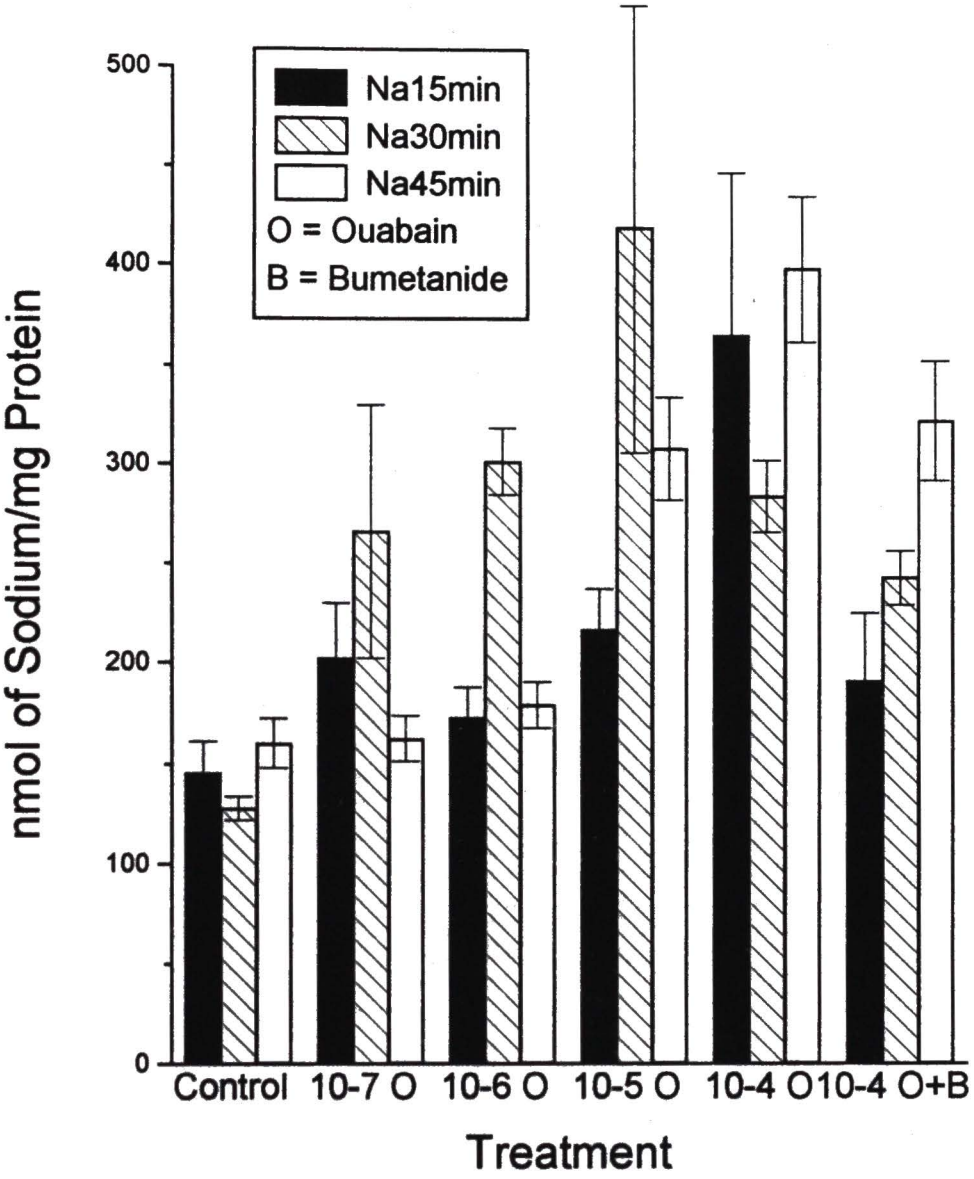


Figure 19. Concentration-dependent effect of ouabain on intracellular Na^+ in bovine RPE cells exposed to 1 g/l glucose for 10 days, as a function of incubation time with Rb^+. 20 min pre-incubation with or without ouabain. Means±SEM of quadruplicate samples.

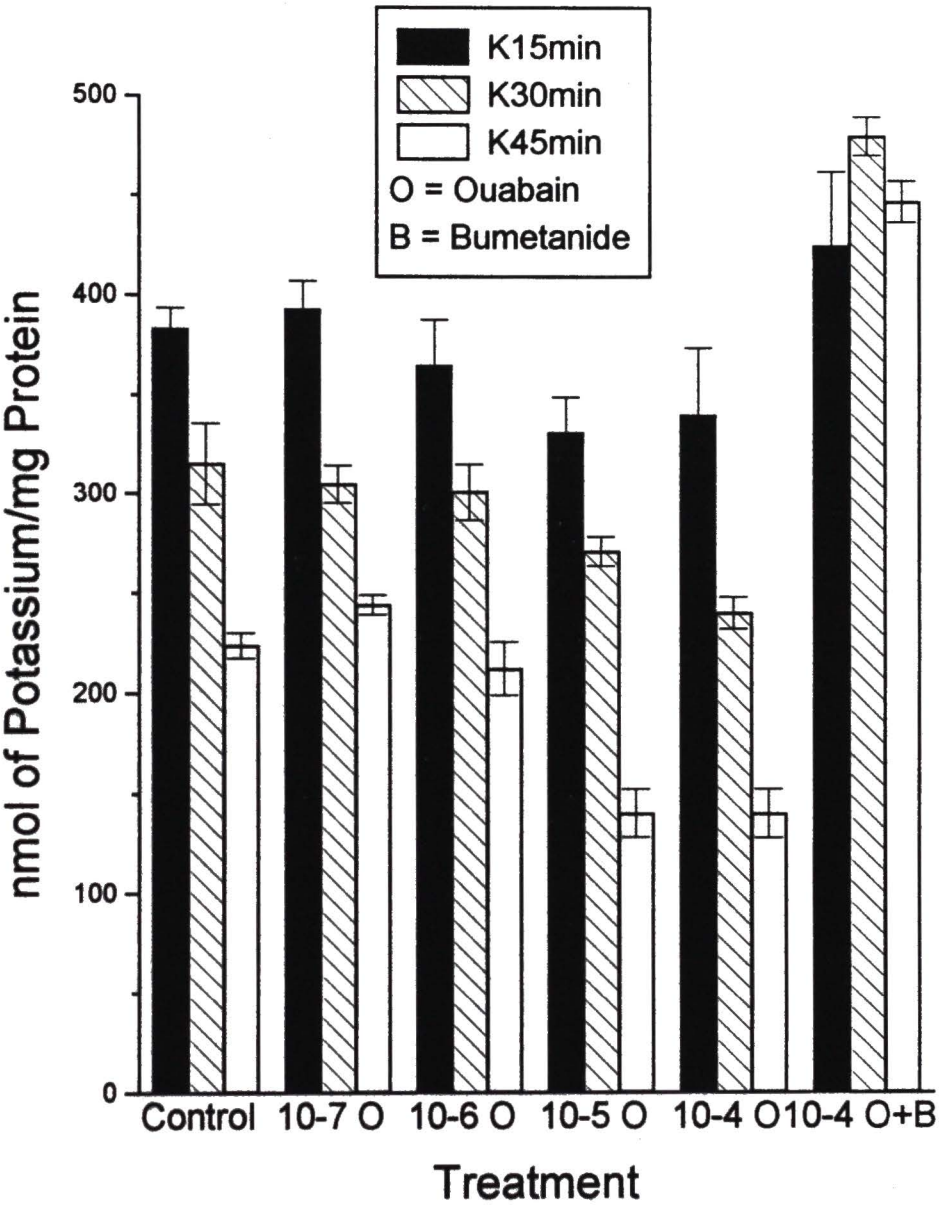


Figure 20. Concentration-dependent effects of ouabain on intracellular K^+ concentrations in bovine RPE cells exposed to 1 g/l glucose for 10 days, as a function of incubation time with Rb^+. 20 min pre-incubation with or without ouabain. Means±SEM of quadruplicate samples.

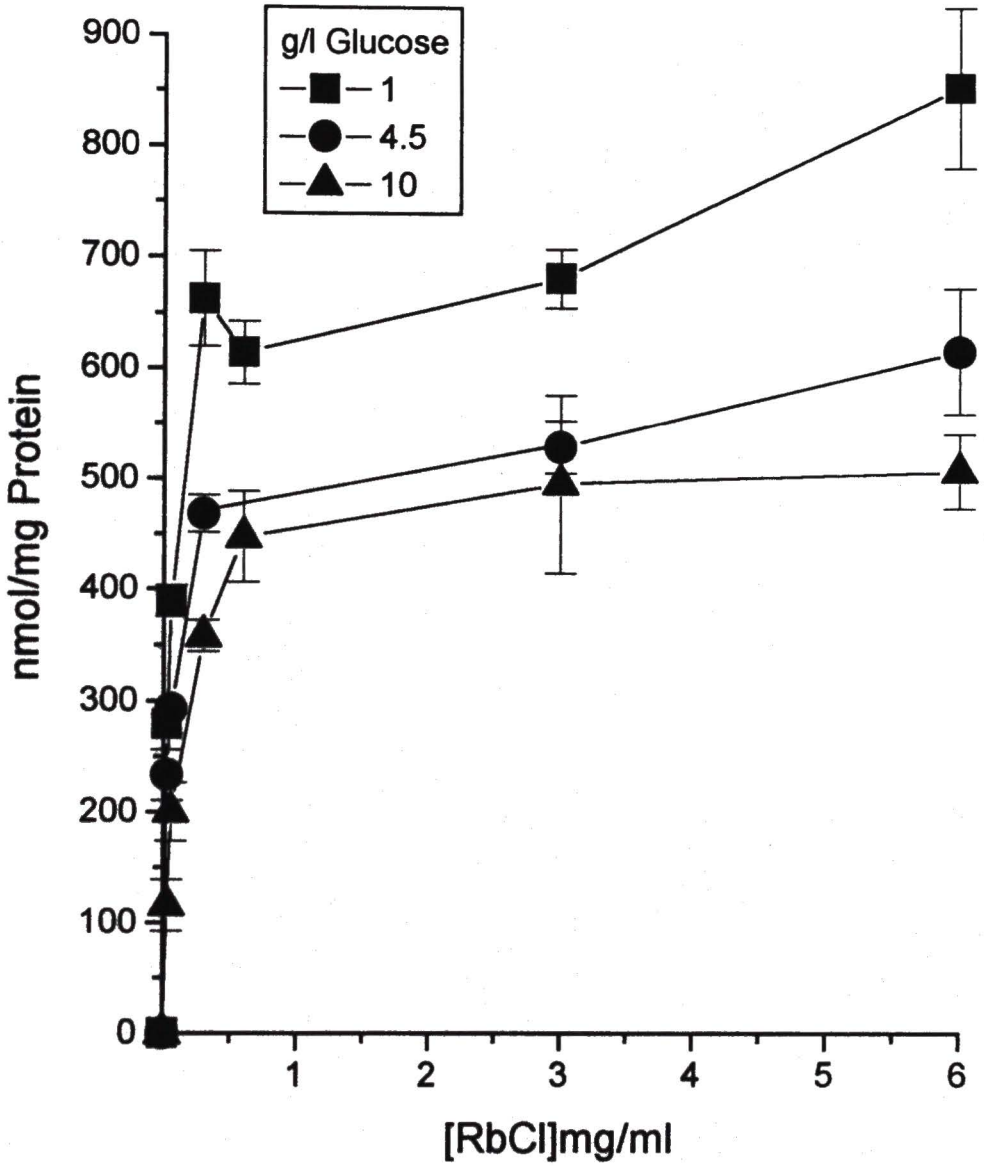


Figure 21. Dependence of Rb^+ uptake in bovine RPE cells on media Rb^+ concentrations, at different media glucose concentrations (72 h exposure prior to Rb^+ uptake assay). Data points represent means±SD of triplicate samples.

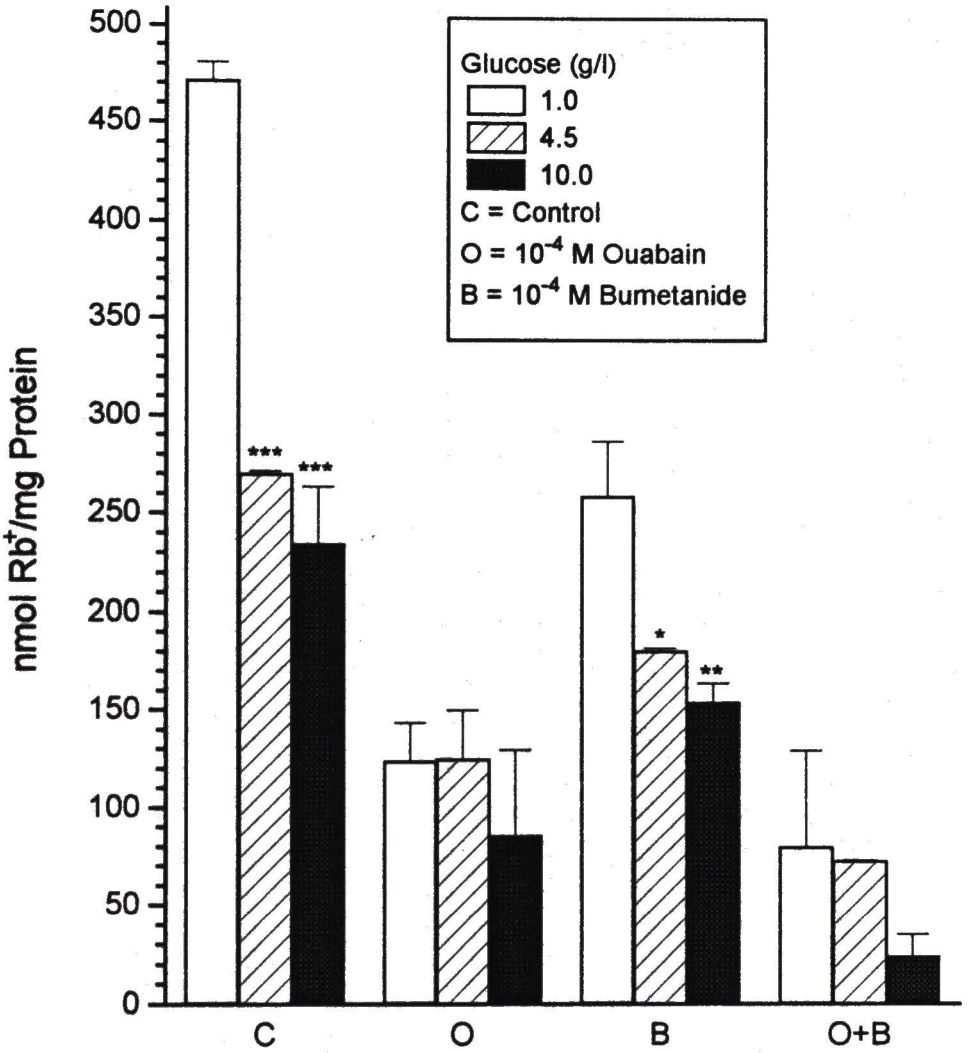


Figure 22. Rb^+ uptake by bovine RPE cells in assay buffer containing 3.0 g/l RbCl, at different media glucose concentrations (48 hour exposure prior to Rb^+ uptake assay). The conditions varied from the standard protocol only in the use of 3.0 g/l instead of 0.6 g/l RbCl. Data points are means±SD of quadruplicate determinations. *P<0.05, **P<0.01, ***P <0.001 when compared to the respective 1 g/l glucose sample.

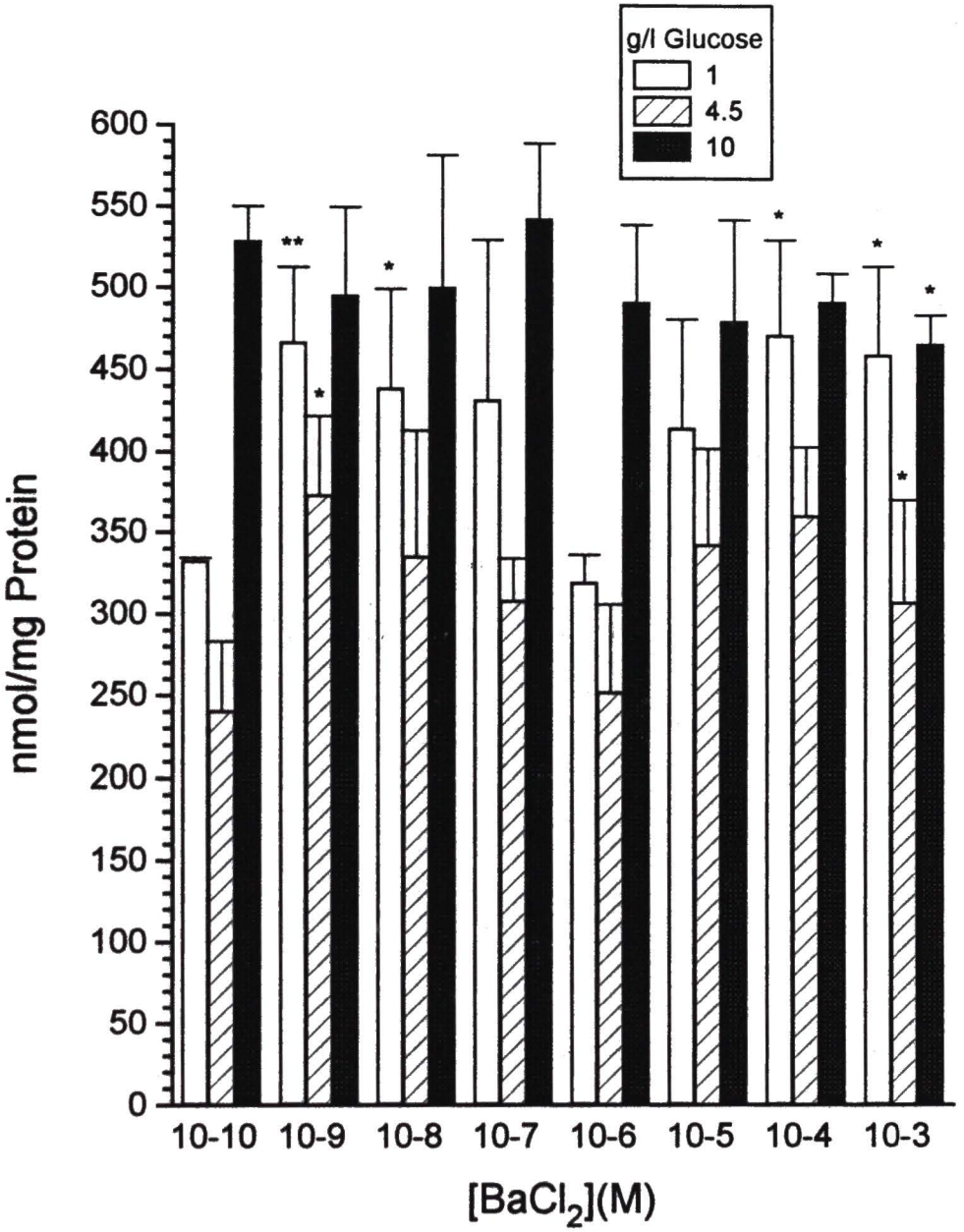


Figure 23. The effect of $BaCl_2$ on intracellular K^+ concentrations in bovine RPE cells during the Rb^+ uptake assay. The cells were exposed to various glucose concentrations for 72 h and assayed under standard conditions. Bars represent means±SD of triplicate determinations. *P<0.05, **P<0.01 compared with lowest concentration of $BaCl_2$.

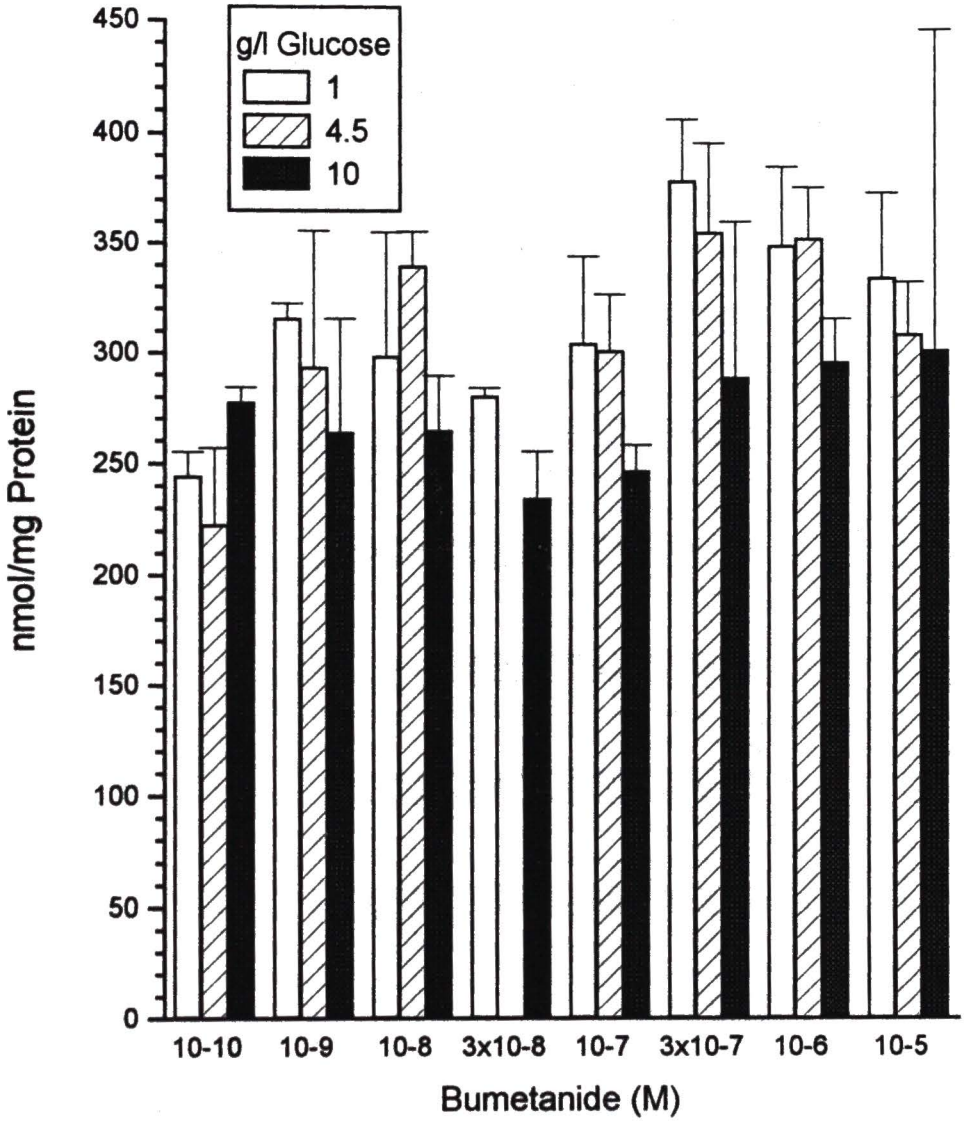


Figure 24. The effect of bumetanide upon loss of intracellular K^+ from bovine RPE cells during the Rb^+ uptake assay (after a 72 h exposure to varying glucose concentrations). Standard assay conditions. Bars represent means±SD of triplicate determinations.

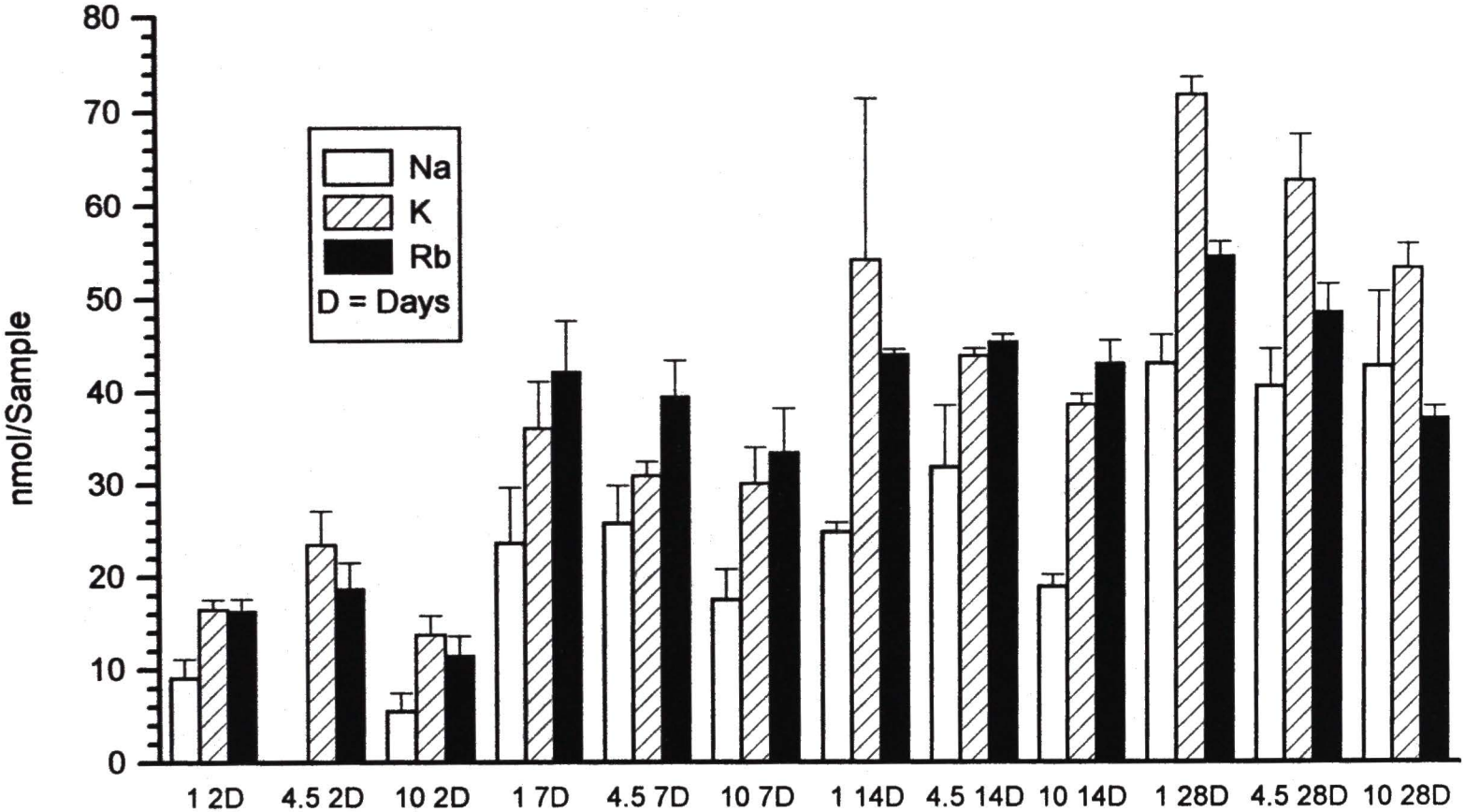


Figure 25. Effects of exposure time of bovine RPE cells to varying glucose concentrations on intracellular Rb^+, K^+, and Na^+ concentrations. Cells were exposed to 1, 4.5, or 10.0 g/l glucose for 2, 7, 14 or 28 days. Standard Rb^+ uptake assay. Data points represent means ±SD of triplicate determinations.

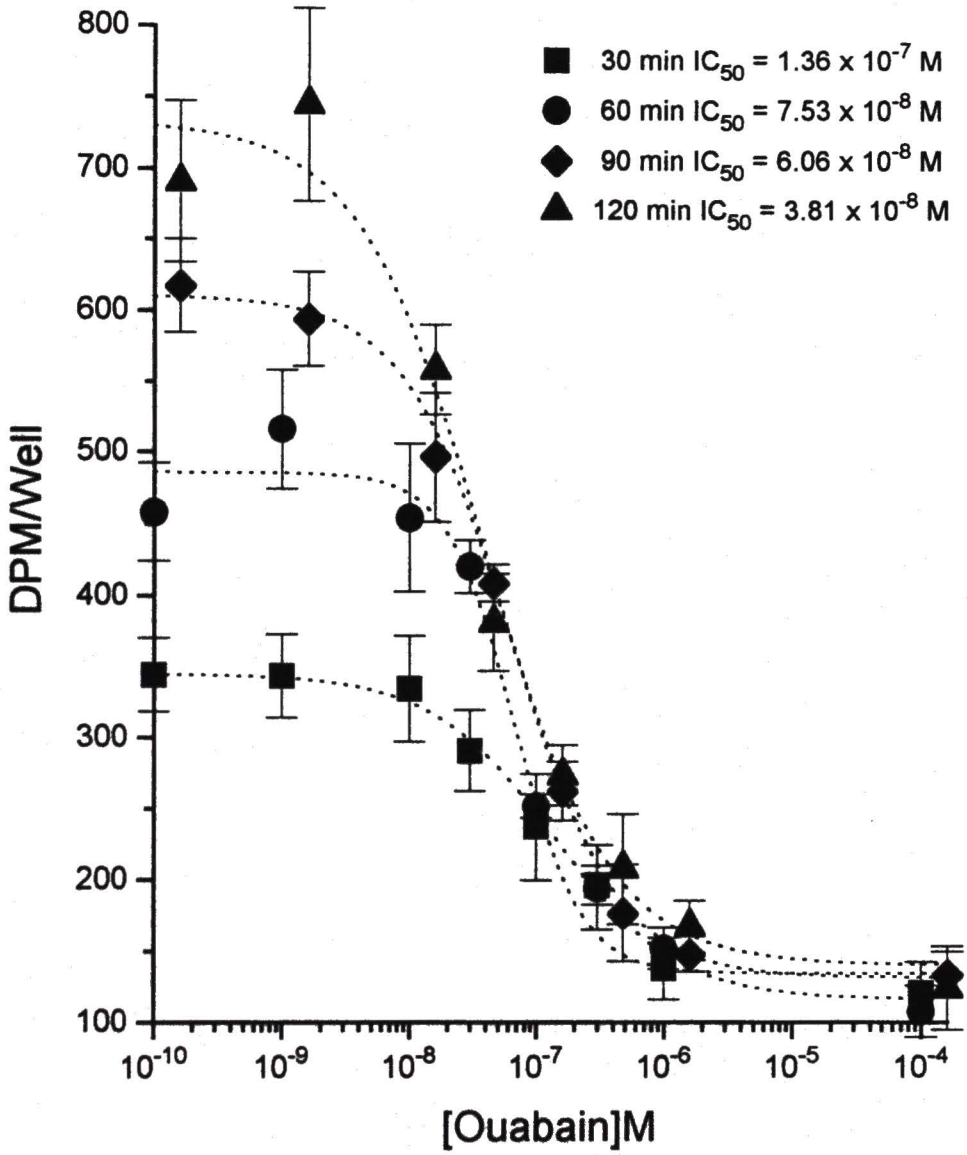


Figure 26. Time course for ouabain inhibition of ^{3}H ouabain binding in bovine RPE cultured at 4.5 g/l glucose for 12 days. Room temperature incubations. Values are means±SD of triplicate determinations.

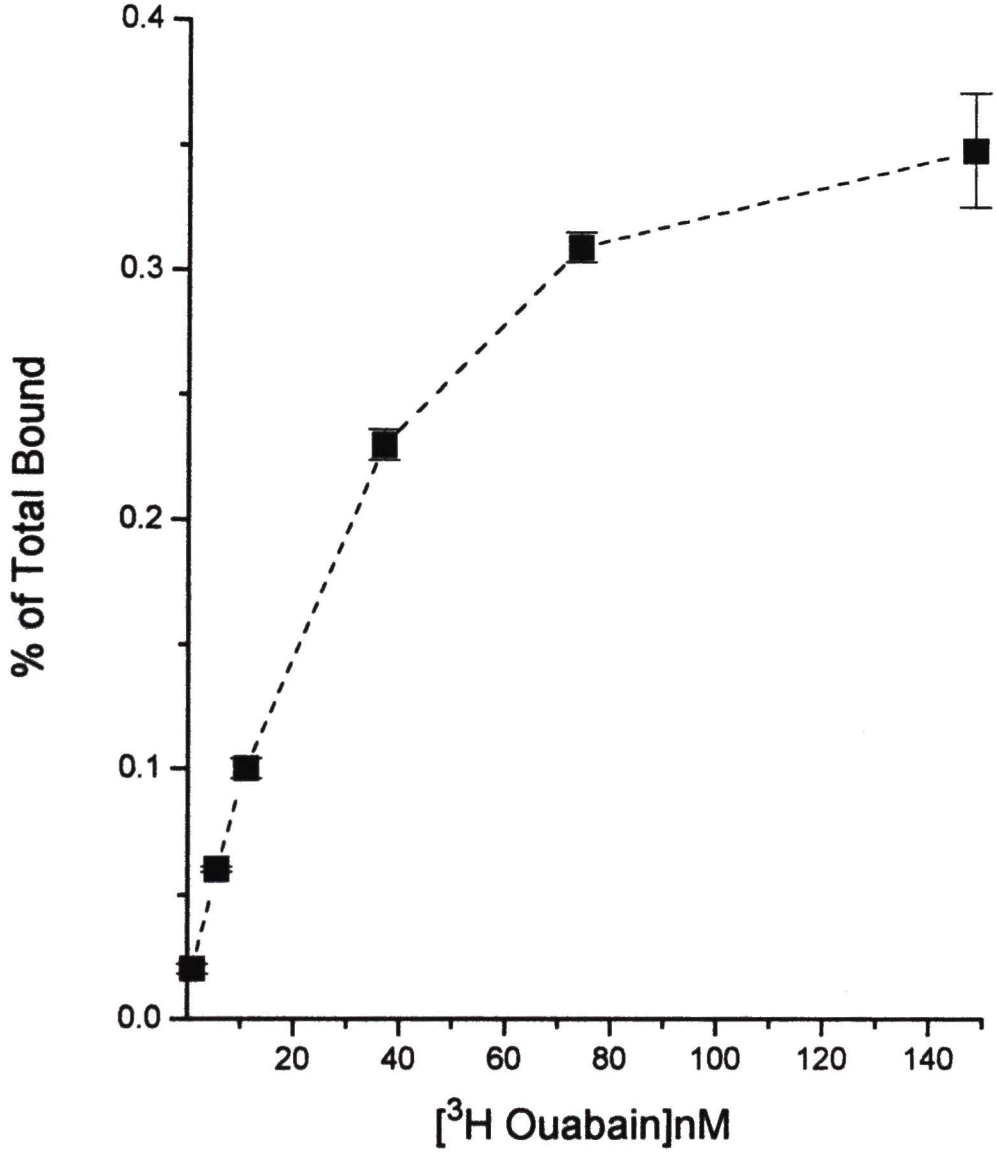


Figure 27. Saturation of ^{3}H ouabain binding in bovine RPE cells. 2 h incubation at room temperature. Cells were exposed to 4.5 g/l glucose for 72 h prior to the binding assay. Data points represent means±SD of duplicate samples.

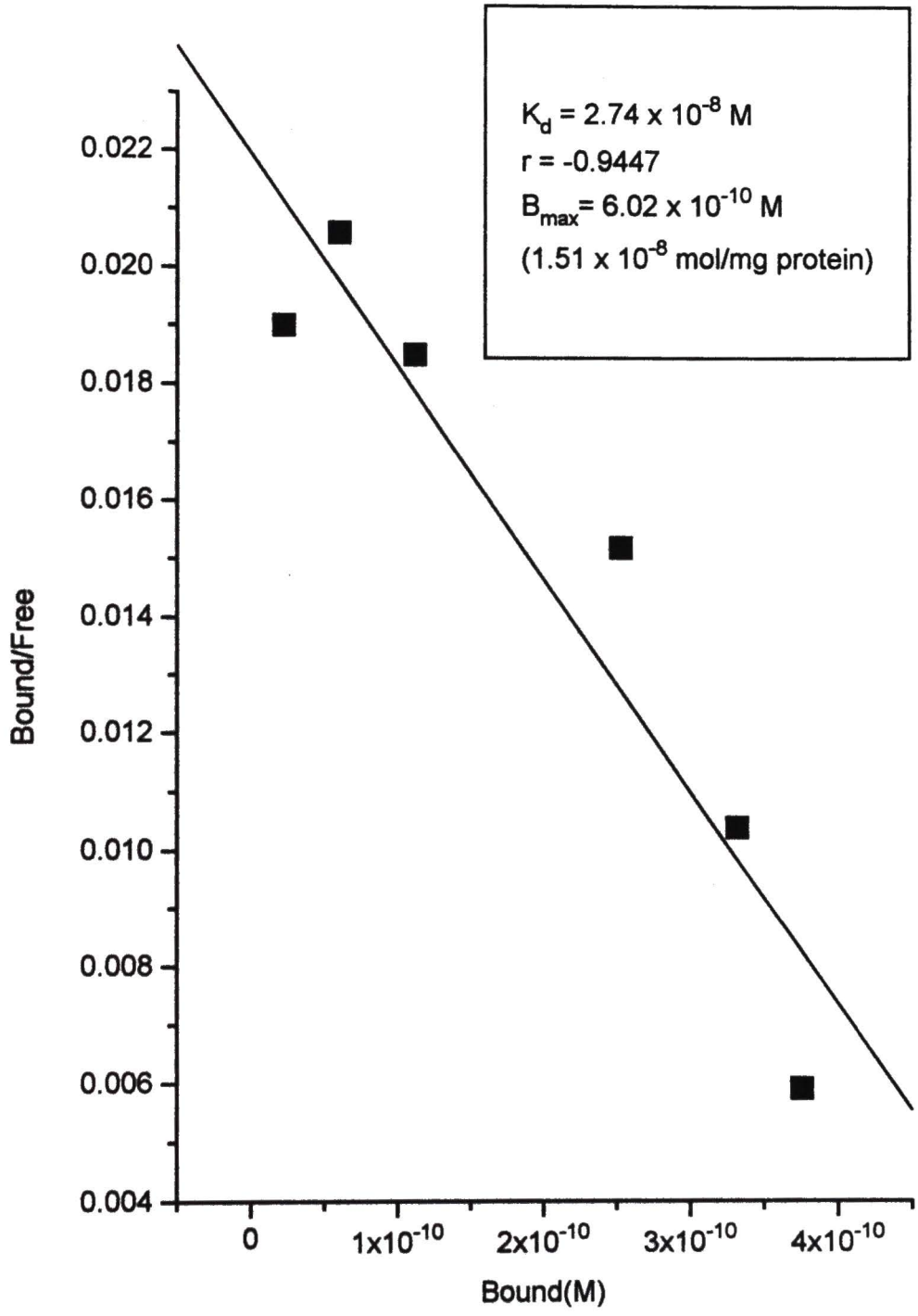


Figure 28. Scatchard plot of ^{3}H ouabain binding in bovine RPE cells grown at 4.5 g/l glucose for 72 h. Standard binding conditions.

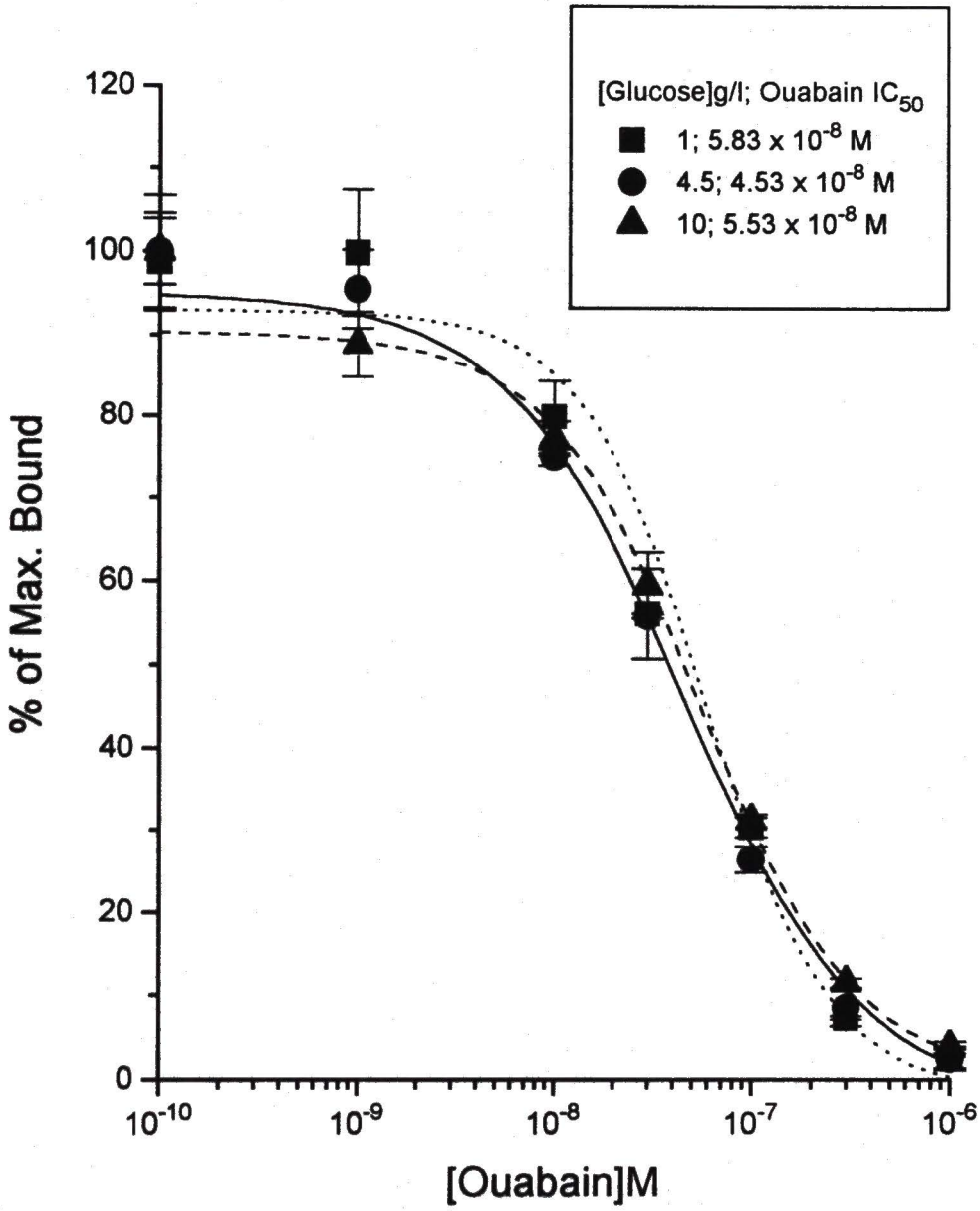


Figure 29. Ouabain inhibition of ^{3}H ouabain binding by bovine RPE cells (exposed to varying glucose concentrations for 48 h prior to the binding assay). Standard binding conditions. Data points represent means±SD of triplicate determinations.

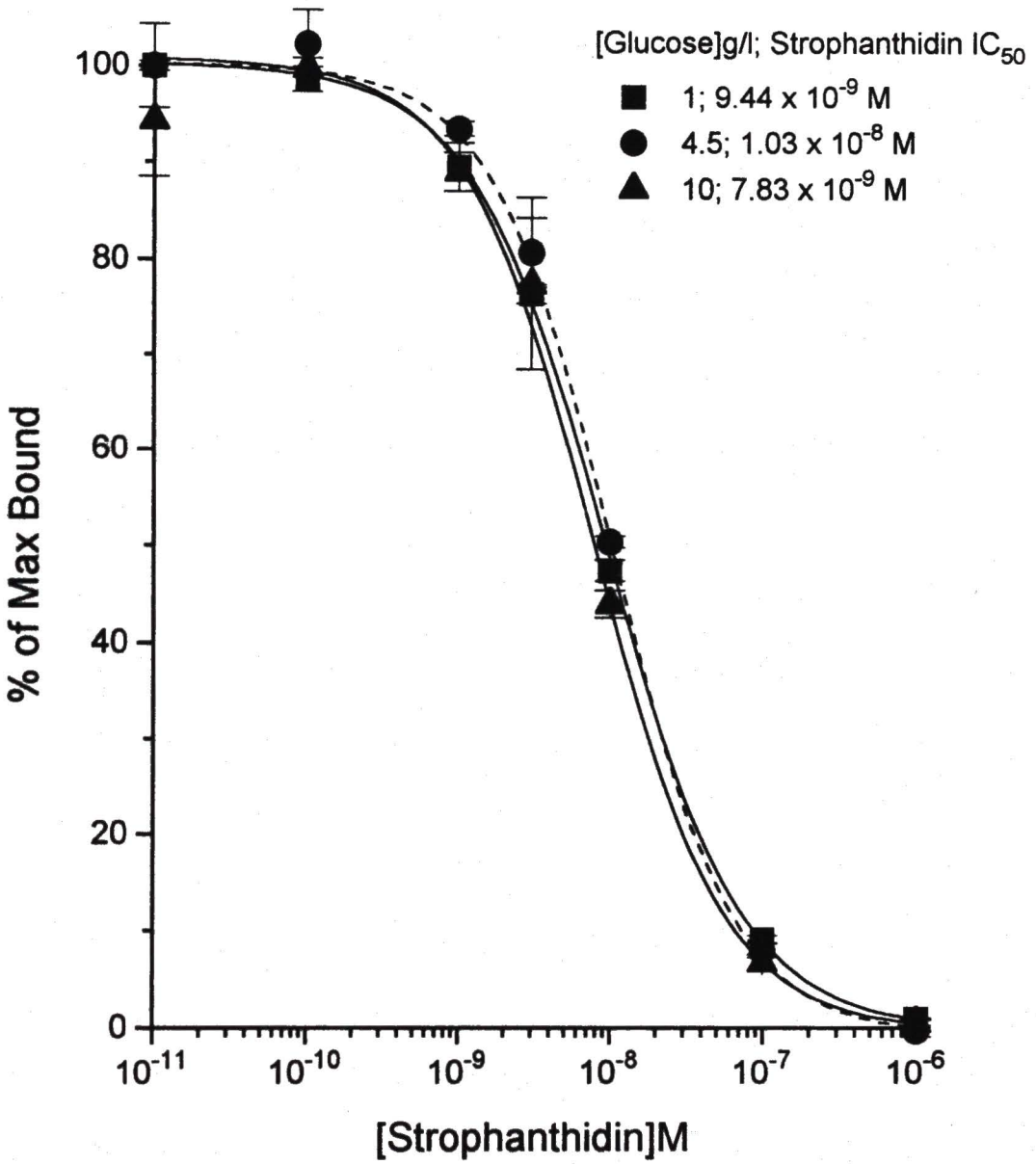


Figure 30. Strophanthidin inhibition of ^{3}H ouabain binding by bovine RPE cells in varying glucose concentrations (exposed to varying glucose concentrations for 1 week prior to binding assay. Standard binding conditions. Data points represent means±SD of triplicate determinations.

CHAPTER V

DISCUSSION

^{3}H Thymidine Incorporation

Stimulation of RPE proliferation with insulin indicated that even though this cell type may not take up glucose in an insulin dependent manner, insulin may act as a growth factor. Leschey et al. (1990) showed a similar increase in thymidine incorporation in cultured human RPE cells after insulin exposure. In diabetes, the blood retinal barrier is largely dependent on the viability of the RPE (Blair et al., 1984; Kirber et al., 1980; Krupin et al., 1978; Tso et al., 1977, 1980; Vinores et al., 1990) and proliferation of RPE takes place near the breach created in the blood-retinal barrier. Therefore, insulin responsiveness in this cell type may contribute to the progression of diabetic retinal complications for patients utilizing insulin therapy.

Rubidium Uptake

Rubidium uptake was chosen as the means of measuring activity Na^{+}/K^{+}-ATPase because of its relative simplicity and wide use in the literature. $^{86}Rb^{+}$ and $^{42}K^{+}$ were approximately equivalent when used to study potassium movement across the RPE (Miller and Steinberg, 1982). Studies utilizing liberation of phosphate to measure pump activity in RPE showed that only a small portion of the response is due to the Na^{+}/K^{+}-ATPase (MacGregor and

Matschinsky, 1986)(It was realized from these experiments that the signal to noise ratio for the phosphate assay would not be acceptable.)

Several preliminary studies utilizing $^{86}Rb^+$ were performed as a foundation for this work. In protocols using suspended cells, dose and time dependent inhibition with ouabain were demonstrated. These early results suggested a change to a more feasible approach whereby the RPE cells were grown in 24 well plates and tested for the effects of hyperglycemia. These experiments led to the subsequent development of a nonradioactive rubidium uptake assay. Several agents were tested for their effects in this system.

It became apparent after several rubidium uptake experiments that a large component of rubidium uptake was not sensitive to ouabain. Rb^+ could be used as a K^+ analog when studying the $Na^+/K^+/Cl^-$-cotransporter because it has nearly the same affinity as K^+ for the carrier (Geck et al., 1980; O'Grady et al., 1986; Owen and Prastein, 1985). Jaffe et al. (1989) also had observed that only 40-50% of bovine RPE rubidium uptake was ouabain sensitive. For these reasons, bumetanide, a $Na^+/K^+/Cl^-$-cotransport blocking agent, was tested for its efficacy in inhibition of rubidium uptake. A significant fraction of uptake was blocked by high dose bumetanide. These data indicated the presence of a $Na^+/K^+/Cl^-$-cotransporter in the bovine RPE and confirmed recent reports of others (Miller and Edelman, 1990; Joseph and Miller, 1991,1992) regarding the existence of the significant amounts of the cotransporter in bovine RPE. The IC_{50} values for bumetanide (in the presence of 10^{-5} M ouabain) were similar at

two concentrations of glucose: 1.22×10^{-7} M and 1.14×10^{-7} M for 1 and 4.5 g/l glucose, respectively. These values agreed with data obtained from other species (Palfrey et al, 1980; Ellory and Wolowyk, 1990; Ellory and Stewart, 1982; Owen and Prastein, 1985) and from cultured bovine lens epithelial cells (1.2×10^{-7} M; Alvarez and Candia, 1994). High doses of both ouabain and bumetanide inhibited rubidium uptake by greater than 90%. The remaining rubidium uptake was probably due to its movement through K^+ channels (Alvarez and Candia, 1994).

The data presented for bovine RPE cells regarding the effect of cell seeding density upon $^{86}Rb^+$ uptake agree with published results for human RPE cells. In that study, pump activity, measured by $^{86}Rb^+$ uptake was greater in sparse cultures than in dense ones (Jaffe et al., 1989). The mechanism for this phenomenon is uncertain but the same authors have noted in unpublished studies with cultures of similar density, but varying proliferation rates, that cell to cell interaction may be a more important modulator of pump activity than the state of proliferation. Qualitatively, during early, sub-confluent stages, increased numbers of cells were observed in areas around the edges of the culture wells in the studies described herein. In fact, if the cells were seeded at a low density, they failed to proliferate. Bryan and Campochiaro (1986) found that RPE cells produced heat stabile factors that promoted their own growth. After 48 hours of culturing in RPE-conditioned media cell number was increased by 110% compared to cells cultred under identical conditions in the

absence of conditioned media. These results and observations suggest that cell to cell interactions play an important role in the proliferation of bovine RPE cells.

Results with the nonradioactive rubidium uptake assay documented numerous advantages of this assay over the radioactive assay. The need for a radioactive probe, with associated costs, hazards, record keeping, disposal, etc. was eliminated. Simultaneous quantitation of intracellular sodium, potassium and rubidium was possible which provided more information regarding inorganic cation transport than the analysis of rubidium uptake alone. For instance, the leakage of potassium and the sensitivity of that leakage to bumetanide could be monitored. After the initial method development was complete, an autosampling device was instituted that resulted in a considerable time savings. A greater degree of ouabain inhibition was observed in the nonradioactive assay, providing a more sensitive measurement to use in these studies.

One unexpected result in these studies was the high potency of ouabain as an inhibitor of Rb^+ uptake. Traditionally, the $\alpha 1$ isoform, thought to be present in the RPE cell (McGrail and Sweadner, 1986; Gundersen et al., 1991), has produced ouabain IC_{50} values from 10^{-3} M to 10^{-5} M in other tissues (Shyjan et al., 1990a,b; Skou, 1962, 1988; Sweadner, 1979). In the present study, ouabain IC_{50} values averaged from 5.66×10^{-8} M to 2.11×10^{-7} M. In a comprehensive study of cation transport in the bovine lens epithelium, Bonting (1963, 1965) reported ouabain IC_{50} values for Na^+/K^+-ATPase activity from

between 3.98 x 10^{-8} M and 9.77 x 10^{-8} M. These results suggested a difference in the susceptibility of the bovine enzyme to inhibition. It was apparent that in the traditional $^{86}Rb^+$ uptake assay (containing K^+ in the assay buffer) that $^{86}Rb^+$ and K^+ were competing for a site on the pump molecule. Therefore, the ouabain IC_{50} values could be different from those reported here where the standard condition consisted of $^{86}Rb^+$ and/or Rb^+ alone in the assay medium. The omission of K^+ from the experimental medium increased the signal to noise ratio, therefore, increasing the sensitivity of the assay. Since the K^+-binding site is near the ouabain-binding site on the extracellular face of the α subunit (Fig. 3), it is possible that the ouabain binding affinity is influenced by the presence or absence of bound K^+.

An interesting finding of this work was that high doses of bumetanide decreased potassium efflux over the experimental time course (20 min pre-incubation without Rb^+, and 30 min incubation in the presence of Rb^+). Movement of ions through the $Na^+/K^+/Cl^-$-cotransporter was traditionally thought to be inwardly directed (Geck, 1986) with the exception of the squid axon, where influx and efflux were shown (Altamirano, et al., 1987). Typical potassium concentrations (in the assay buffer) for rubidium uptake assays are approximately 5 mM. Recently, Alvarez and Candia (1994) reported outwardly directed cotransport in cultured bovine lens epithelial cells. In these studies, the cells were incubated in a solution containing low potassium and high sodium (2 mM and 110 mM, respectively). Therefore, cotransport proceeded

down the potassium gradient and against the sodium gradient. In the present studies a similar condition existed. Since potassium was replaced by rubidium in the assay buffer, potassium efflux occurred, via the cotransporter, down a steep concentration gradient. One advantage of this system was the increase in rubidium signal. Since Rb^+ did not compete with external K^+ to any great extent, Rb^+ uptake was substantial and this cation represented a large portion (50-60%) of the intracellular cations. One disadvantage of this approach was that the typical accumulation of sodium that occurs in RPE under conditions of diabetes (MacGregor and Matschinsky, 1986b) may have been masked by the fact that sodium was being transported out of the cell by the $Na^+/K^+/Cl^-$-cotransporter. Dose dependent increases in sodium were observed, however, under the influence of ouabain inhibition. For cells, *in vivo*, the cotransporter is generally considered to move potassium against a concentration gradient (i.e. from 5mM outside the cell to 90 mM internally) and sodium down a gradient. It was apparent that flow of cations in bovine RPE cells in culture could be manipulated by altering conditions of the assay.

Since the RPE cells are in close association with rods and cones, they maintain the concentration gradient of sodium and potassium amidst large fluctuations in these ions. In the subretinal space, the area between the photoreceptors and the apical membrane of the RPE, potassium has been shown to rise from 2 mM under conditions of light to 5 mM in the dark (Edelman et al., 1988). These authors have shown that this elevation in K^+

reverses the direction of transport of Cl^-. This phenomenon is a physiological example of the importance of the function of the $Na^+/K^+/Cl^-$-cotransporter in the movement of cations. The value of an assay which can monitor cellular ion fluctuations in response to varying physiologic conditions is therefore underscored.

Amiloride and $BaCl_2$ did not affect rubidium uptake in the cultured bovine RPE cells. The highest dose of amiloride used, 10^{-5} M, may not have been adequate to observe an effect. RPE cells are reported to contain K^+ channels (Joseph and Miller, 1991; Miller and Edelman, 1990) as well as Na^+/H^+ exchange mechanisms (Joseph and Miller, 1991; Keller et al., 1986, 1987, 1988; Miller and Edelman, 1990). Those studies were performed as real time measurements of membrane potentials using either fresh tissues or cultured cells. Any effects of amiloride or $BaCl_2$ on rubidium uptake may have gone undetected due to the large amount of this cation moving through the $Na^+/K^+/Cl^-$-cotransporter and Na^+/K^+-ATPase. In studies with cultured bovine lens epithelial cells, Alvarez and Candia (1994) also were unable to show an effect of $BaCl_2$ on $^{86}Rb^+$ uptake. Due to the low residual rubidium uptake in the presence of bumetanide and ouabain, movement through the potassium channels appeared to be a relatively minor contributor to Rb^+ uptake in bovine RPE cells.

$BaCl_2$ did produce an effect on potassium efflux from the RPE cells, however. Several concentrations of $BaCl_2$ appeared to inhibit the leakage of

potassium that typically was observed during the course of a rubidium uptake assay. These data would indicate the presence of a barium-sensitive potassium channel. Interestingly, the 1 and 4.5 g/l glucose concentrations showed an apparent inhibition of potassium leakage while in the 10 g/l only one group appeared to be affected by $BaCl_2$. These data emphasize the versatility of the Rb^+ assay. Since they were unable to monitor potassium movement, Alvarez and Candia (1994)(using the traditional $^{86}Rb^+$ uptake assay) were unable to discern any effect of barium on the potassium channel.

One important contribution of this research was the finding that experimental hyperglycemia produced observable decreases in Na^+/K^+-ATPase activity as measured by rubidium uptake (Figures 21, 22, and 25). Traditionally, studies of the effects experimental diabetes on RPE function have been conducted *in vivo* (MacGregor and Matschinsky, 1985, 1986a,b) or under galactosemic conditions in culture (Reddy et al., 1992 a,b). Utilizing high glucose in place of high galactose would appear to be a more physiologically relevant experimental design. The use of tissue culture techniques allowed for greater manipulation of the exposure conditions than an *in vivo* approach would provide. Del Monte et al. (1991) performed studies on cultured human RPE cells, originating from a single donor to characterize the effect of varying glucose (20-40 mM) on sorbitol, myo-inositol and rod outer segment (ROS) phagocytosis. Uptake of ROS was inhibited by elevated glucose. Time- and

dose-dependent increases in sorbitol and decreases in myo-inositol were observed in the cultured RPE cells.

Dose response curves for ouabain inhibition of Rb^+ uptake were unaffected by hyperglycemia. These data were quite consistent in both the radioactive and nonradioactive assay systems. The ouabain IC_{50} values ranged from 5.66×10^{-8} M to 2.11×10^{-7} M. In a published study with rats 4 months after the onset of diabetes, the strophanthidin-inhibition constant for Na^+/K^+-ATPase also was unaltered (Specht et al., 1991). These findings were quite relevant since bovine (used in this research) and rat RPE cells are thought to contain the $\alpha 1$ isoform of the enzyme.

Examination of the effect of inhibition of rubidium uptake by either 10^{-4} M ouabain, bumetanide or a combination of the two drugs revealed some interesting trends. The fact that ouabain inhibition appeared to be greater at elevated glucose concentrations provides evidence that either there is a subtle difference in the Na^+/K^+-ATPase molecule or that the altered internal environment of the cell is causing an alteration in pump function. As sugar alcohols increase within the RPE, the cells will attempt to osmoregulate by decreasing intracellular concentrations of compounds such as taurine and other amino acids (Reddy, 1992a). The data indicated that the contribution of the $Na^+/K^+/Cl^-$-cotransporter to the total rubidium uptake remained a constant proportion (30%) of the total rubidium uptake under the assay conditions and simulated conditions of hyperglycemia employed. If small changes in this value

did occur, they might not have been detected as a statistical significance in the studies performed. Additional experiments would be required to make definitive conclusions about the effects of simulated hyperglycemia on the $Na^+/K^+/Cl^-$-cotransporter. Simultaneous administration of ouabain and bumetanide produced between 92% and 97% inhibition of rubidium uptake overall (in the nonradioactive assay). These numbers agreed well with published studies on osteoblasts (Whisenant et al, 1991), adenocarcinoma cells (Kim et al., 1988), bovine lens epithelial cells (Alvarez and Candia, 1994), nonpigmented ciliary epithelial cells (Crook et al., 1992b), and monkey RPE (Kennedy, 1992). A combination of the two drugs appeared to cause a slight decrease in the amount of total inhibition of uptake under high glucose conditions. In other words, the residual (generally regarded as movement through K^+ channels) grew larger. Data discussed previously regarding the lack of inhibition by $BaCl_2$ on K^+ efflux at the 10 g/l glucose dose support this idea. These data indicate that potassium channels (with altered sensitivity to $BaCl_2$) may play a greater role in cation homeostasis under hyperglycemic conditions, or that one of the other channels is permissive to K^+ transport out of the cell.

Acute exposure (50 min) to the aldose reductase inhibitor AL-1576 consistently failed to produce any significant stimulation of rubidium uptake. In contrast, Garner and Spector (1987) were able to produce a 20-30% increase in the rate of ATP hydrolysis by glycated bovine renal Na^+/K^+-ATPase *in vitro* by a 90 minute exposure of this altered enzyme to AL-1576. This difference may be

explained in several ways. Even though both RPE and renal ATPase molecules appear to react with antibodies against the α1 isoform (Gundersen et al., 1991; Herrera et al., 1987; McGrail and Sweadner, 1986; Sweadner, 1989), there may be small differences in their protein structure that render these sodium pumps more or less susceptible to stimulation by an ARI. Similarly, a single amino acid substitution in the α1-subunit of Na^+/K^+-ATPase of Dahl salt-sensitive rats caused a change in the hydropathy profile and a decrease in $^{86}Rb^+$ uptake compared to the salt-resistant strain. The α3 isoform, for instance, contains an amino acid substitution in the sodium binding domain (Shull et al. 1986b). The ion transport properties of this subunit are therefore altered probably due to subtle conformational changes in the molecule. The uptake of Rb^+ may not show differences with acute ARI exposure since it is a composite of cation movement through Na^+/K^+-ATPase and the $Na^+/K^+/Cl^-$- cotransporter.

Another reason for the lack of ARI stimulation upon acute (50 min) exposure may be due to need for a more aggressive experimental approach. This could involve a longer drug treatment or a prevention protocol in which the compound is added simultaneously with the introduction of elevated glucose. In cells that contain measurable aldose reductase activity, this protocol would prevent the large increases in intracellular sorbitol. Kowrulu et al. (1989) found that decreased rat erythrocyte Na^+/K^+-ATPase activity was prevented by administration of the ARI, sorbinil simultaneously with experimental diabetes induction with streptozotocin. In studies with human RPE cells, Del Monte and

coworkers (1991) found that there was a sharp increase in intracellular sorbitol and fructose between the time of seeding and two days in culture (20 and 40 mM glucose). Likewise, a large decline in myo-inositol was observed during the same time. All of these changes appeared to plateau at approximately one week (the duration of their studies).

Additional evidence for the presence of aldose reductase in human RPE cells was provided by Reddy et al. (1992 a,b) in which galactitol formation was observed with exposure of the cells to high galactose medium. This galactitol accumulation was inhibited by various ARIs. Interestingly, many of the published studies have utilized elevations in galactose rather than glucose. As shown by the polyol pathway on page 15, galactitol does not appear to be metabolized by sorbitol dehydrogenase (Van Heyningen, 1959; Kinoshita, 1974). Therefore, galactitol accumulation is readily observed in experimental situations. On the other hand, sorbitol can be converted to fructose by sorbitol dehydrogenase. This fact may make perturbation of the cells by elevated glucose more difficult to observe.

The fact that changes in rubidium uptake in the 4.5 and 10 g/l *versus* 1.0 g/l samples were not immediate may relate to the course of other events initiated by the onset of hyperglycemia. Glucose interferes with myo-inositol transport in the intestine, lens, RPE and other tissues (Cammarata et al., 1991a,b; Caspary et al., 1970; MacGregor and Matschinsky, 1986). This may contribute to the decrease in myo-inositol which accompanies the diabetic

condition (Greene et al., 1987). Studies with cultured human RPE cells (Del Monte et al., 1991) show this decrease to be dramatic at 40 mM glucose, even at one day of exposure, but less so at the 10 and 20 mM concentrations. In the myo-inositol depleted state, Na^+/K^+-ATPase activity is decreased (Greene and Lattimer, 1984a; Greene et al., 1987; MacGregor and Matschinsky, 1986). This depletion of myo-inositol appears to require several days of elevated glucose exposure.

The magnitude of sorbitol accumulation observed in culture under hyperglycemia and hypergalactosemia appears to be smaller than the effect of experimental diabetes *in vivo*. For example, Griffin et al., (1987) observed a 30 and 24 fold reduction in lens and sciatic nerve sorbitol, respectively compared to the diabetic controls upon dosing with 0.50 mg/kg/d of AL-1576. Studies with cultured human RPE cells produced only a three to four fold decrease in galactitol accumulation with the ARIs AL-3152 or AL-4114 (Reddy et al., 1992a,b). The maximal concentration of galactitol accumulation was approximately 375 nmoles/10^6 cells in the absence of ARIs. This may relate to the expression of aldose reductase in cultured cells. Also, some leakage of intracellular polyol occurs, especially from cells exposed chronically to a high aldose insult. However, with cells maintained in culture, the chronic effects of accumulation of polyol in both the intracellular and extracellular media are likely decreased by changing/renewing the culture media at frequent intervals. Assuming a maximal accumulation of sorbitol at three to four fold, resultant

changes in the behavior of the sodium pump may be difficult to observe. Other investigators have remarked that RPE cells are resistant to injury by factors such as hypoxia and prolonged exposure to room temperature conditions (MacDonald, 1994; Nash et al., 1994).

In summary, elevations in glucose produce a number of physiological effects which may impact on the functioning of Na^+/K^+-ATPase. Sorbitol tends to accumulate, while myo-inositol becomes depleted within the cells. Under these conditions, for RPE cells cultured at high glucose concentrations, sodium pump activity was depressed. Once these changes had taken place, acute exposure of the ARI AL-1576 during the assay, had no effect on pump activity as measured by rubidium uptake. On the other hand, if the ARI were dosed simultaneously to the onset of hyperglycemia, modest stimulation of Na^+/K^+-ATPase was observed.

Ouabain Binding

A simple approach to ouabain binding was possible due to the large number of binding sites per cell. Human RPE cells were shown to contain from 1.1×10^6 to 6.6×10^6 sites per cell for confluent and sparse cultures, respectively (Jaffe et al., 1989). K_d values for bovine RPE ouabain binding in this dissertation (2.74×10^{-8} M) were similar to K_d values for human RPE (2.07×10^{-8} M) published by the same authors. Ouabain inhibition of ^3H ouabain binding resulted in K_i values of 5.14×10^{-8} M, 4.02×10^{-8} M, and 5.02×10^{-8} M

for RPE cells exposed to 1, 4.5, and 10 g/l glucose, respectively, for 48 hours (assuming equivalent K_d values under the three glucose conditions). These results support the idea that elevations in glucose concentrations did not affect ouabain binding in this cell type.

The use of strophanthidin, a more potent antagonist of ^3H ouabain binding, confirmed studies with nonradioactive ouabain. Strophanthidin IC_{50} values for binding (~ 1×10^{-8} M) were approximately five-fold more potent under 1, 4.5, and 10 g/l glucose conditions as compared to previous experiments with ouabain (average of IC_{50} values ~ 5×10^{-8} M). The magnitude of this increase in potency was similar to published studies with rat brain Na^+/K^+-ATPase (Sweadner, 1979). One week of exposure of the RPE cells to varying concentrations of glucose did not change the strophanthidin IC_{50} value for ^3H ouabain binding. Therefore, the numbers for K_i at 1, 4.5, and 10 g/l glucose were similar, 8.01×10^{-9} M, 9.94×10^{-9} M, and 7.55×10^{-9} M, respectively (assuming equivalent K_d values under the three glucose conditions). Had there been a shift in isoform composition from $\alpha 1$ to $\alpha 2$, or $\alpha 3$, a discernable effect on the strophanthidin IC_{50} would have been expected. $\alpha 2$ and $\alpha 3$ isoforms are 10 to 1000 fold more sensitive to inhibition by agents such as ouabain and strophanthidin. In addition, minimal (nonspecific) and maximal binding values were not significantly different ($P>0.50$) for the two inhibitors at the three glucose concentrations. These data indicated no change in pump number under 1, 4.5 or 10 g/l glucose.

The aldose reductase inhibitor AL-1576 appeared to have no effect on ^{3}H ouabain binding in bovine RPE cells. This phenomenon was noted in three different experimental protocols. These data agreed with published results from *in vitro* kidney studies (Garner, 1987). In these studies, sorbinil and AL-1576 had no direct effect on ouabain binding. However, both drugs competed with fluorescein isothiocyanate for a low-affinity ATP binding site on the Na^{+}/K^{+}-ATPase molecule indicating a direct interaction of these compounds with the sodium pump.

In conclusion, cultured bovine RPE cells contain Na^{+}/K^{+}-ATPase molecules that are inhibited by low concentrations of ouabain under the experimental conditions used. The sensitivity to ouabain did not appear to change upon exposure to elevated concentrations of glucose for up to one month. Similarly, ^{3}H ouabain binding was not altered by exposure of the cells to experimental hyperglycemia. Pump activity, as measured by Rb^{+} uptake, decreased after a one month exposure to high glucose. Under this "chronic hyperglycemic" insult, the ARI AL-1576 produced a modest increase in rubidium uptake, when administered in a prevention protocol for the duration of the hyperglycemia. These results indicate that the altered environment of the Na^{+}/K^{+}-ATPase molecule under conditions of simulated diabetes may affect its functioning in the RPE cell and that prevention of such alterations in the Na^{+}/K^{+}-ATPase and its environment by an aldose reductase inhibitor can improve functional activity of this important ion transporter in the plasma membrane.

Future Studies

Future studies could exploit the utility of the Rb^+ uptake method to examine different components of cation transport in bovine RPE under conditions of hypertonic stress such as high sodium. Studies with activators of protein kinase C such as diacylglycerol or a phorbol ester could be performed in an attempt to activate the sodium pump after exposure of the RPE cells to hyperglycemia. Additional studies with the $Na^+/K^+/Cl^-$-cotransporter, in which the potassium in the assay buffer was varied, could prove interesting. Manipulation of the experimental conditions could show subtle differences in the functioning of this molecule under diabetic conditions. The data presented in this dissertation indicated the possibility of a small change in the proportion of ion flux through the potassium channel. This could be explored further in studies of the residual rubidium uptake in the presence of high dose ouabain and high dose bumetanide. Also, dose response curves for the inhibition of potassium efflux by $BaCl_2$ under differing culture conditions would be valuable.

Second messenger studies also would complement this work. Examining the amount of agonist stimulated cAMP production under hyperglycemic conditions would be of interest since cAMP was shown to cause pump activation (Vasilets et al., 1990). For example, cAMP production is reduced in the RPE from the Royal College of Surgeon Rat, a model of inherited retinal dystrophy (Gregory et al., 1992). These authors showed PGE_1 and PGE_2 to stimulate cAMP production in rat RPE. The $Na^+/K^+/Cl^-$-

cotransporter also can be stimulated by cAMP (Palfrey et al., 1980). Preliminary studies were conducted to examine the effect of elevated glucose on PGE_2 stimulated cAMP in the bovine RPE. After 5 days of growth in 1 and 10 g/l glucose, the EC50 values for PGE_2 stimulated cAMP production were, 8.69×10^{-8} M and 7.8×10^{-8} M, respectively.

Similarly, inositol phosphate (PI) turnover could be monitored under hyperglycemic conditions. Since myo-inositol concentrations are reduced in the diabetic condition, the effect of ARIs and myo-inositol supplementation on inositol phosphate turnover could be compared. An agonist such as carbachol could be examined since it has been shown to stimulate PI turnover in human RPE cells (Crook et al., 1992a).

CHAPTER VI

SUMMARY

Bovine retinal pigment epithelial (RPE) cells were cultured with 1, 4.5, or 10 g/l glucose, corresponding to 5.55, 25.0 and 55.5 mM, respectively up to 1 month in order to characterize the effect of hyperglycemia on the Na^+/K^+-ATPase. Functional activity on Na^+/K^+-ATPase was measured as ouabain-inhibitable rubidium uptake.

The present work contributed to the understanding of retinal pigment epithelial cell (RPE) function through the development of a new nonradioactive rubidium uptake assay which allowed the simultaneous analysis of intracellular sodium, potassium, and rubidium. This method utilized cation exchanged coupled with conductivity detection *via* the Dionex BioLC. The rubidium uptake assay could be manipulated by a number of agents such as ouabain (a Na^+/K^+-ATPase inhibitor) and bumetanide (a $Na^+/K^+/Cl^-$-cotransport blocking agent) to provide information regarding different transport properties of bovine RPE cells. The bumetanide sensitive $Na^+/K^+/Cl^-$-cotransporter was shown to transport potassium in the outward direction under certain conditions. This result was different from the traditional view but had been reported in the literature in studies with bovine lens epithelial cells (Alvarez and Candia, 1994).

Chronic hyperglycemia provided greater total ouabain inhibition of Rb^+ uptake, but did not change the ouabain IC_{50} value. In addition, elevated

glucose did not appear to effect the proportion of bumetanide-sensitive inhibition of rubidium uptake. A combination of bumetanide and ouabain produced less inhibition under hyperglycemic conditions suggesting a greater importance of potassium channels in total cation flux. Prolonged elevations in glucose decreased Na^+/K^+-ATPase activity as measured by rubidium uptake. Intervention with the aldose reductase inhibitor AL-1576 provided a modest stimulation of pump activity (as measured by rubidium uptake) when dosed chronically and simultaneously to hyperglycemic onset. This result indicated an additional benefit of aldose reductase treatment in the treatment of diabetic complications for which diminished Na^+/K^+-ATPase activity may play a role.

Studies were performed to assess the effect of hyperglycemia upon ^3H ouabain binding. The K_d for ouabain binding was 2.74×10^{-8} M for RPE cells grown at 4.5 g/l glucose. Cells grown at 1, 4.5 or 10 g/l glucose produced similar IC_{50} values (average IC_{50} of 5.02×10^{-8} M corresponding to a K_i of 4.84×10^{-8} M) for nonradioactive ouabain inhibition of ^3H ouabain binding. A more potent antagonist of ^3H ouabain binding, strophanthidin, was tested and produced an average IC_{50} of 8.81×10^{-9} M (corresponding to a K_i of 8.50×10^{-9} M). AL-1576 did not affect ^3H ouabain binding. In conclusion, chronic hyperglycemia did not affect Na^+/K^+-ATPase binding to ouabain.

The results from rubidium uptake and ouabain binding in bovine RPE suggested that the decrease in Na^+/K^+-ATPase observed under hyperglycemic conditions is not a result of a shift in the expression of the enzyme isoform but

the result of secondary physiological responses within the cell such as decreased myo-inositol and increased sorbitol concentrations (see Figure 31). The prevention of these pathological changes, through therapeutic intervention with an aldose reductase inhibitor, resulted in the added benefit of preserving Na^+/K^+-ATPase activity.

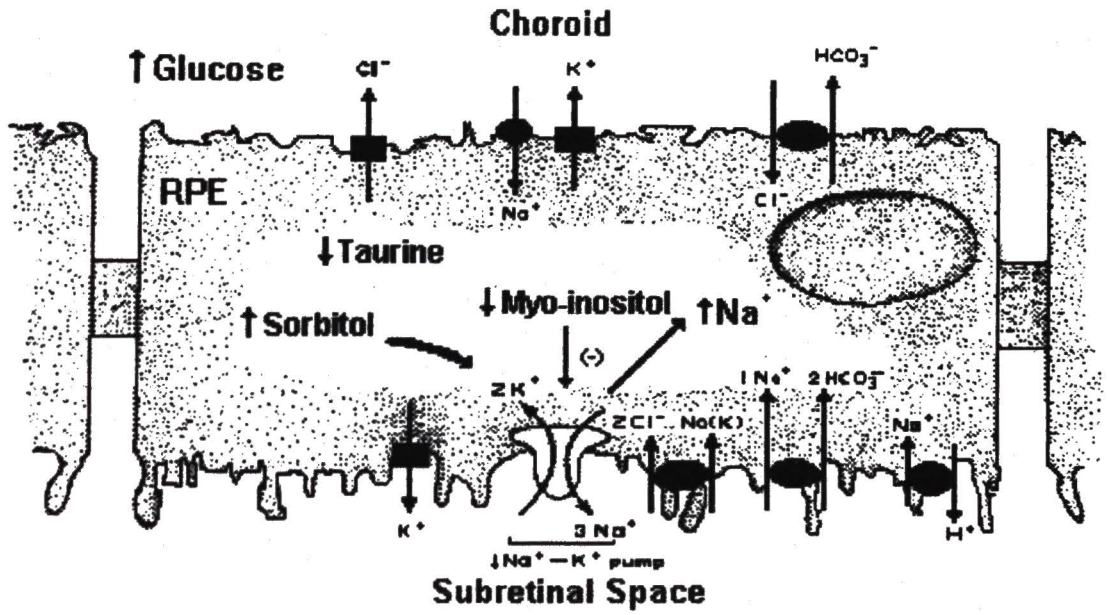


Figure 31. Summary of ion transport phenomena in bovine RPE under hyperglycemic conditions. (Modified from Edelman and Miller, 1991)

APPENDIX

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
$BaCl_2$	barium chloride
BRB	blood retinal barrier
BSA	bovine serum albumin
C	centigrade
cAMP	cyclic adenosine-3',5'-monophosphate
Cl^-	chloride
cm	centimeter(s)
CO_2	carbon dioxide
CPM	counts per minute
d	day(s)
dl	deciliter(s)
DMEM	Dulbecco's Minimal Essential Media
DPBS	Dulbecco's Phosphate Buffered Saline
EC_{50}	concentration of drug that produces 50% of the maximal effect
EDTA	ethylenediaminetetraacetic acid, disodium salt
ERG	electroretinogram
FBS	fetal bovine serum
g	gram(s)
h	hour(s)
^3H	tritium
IC_{50}	concentration of drug that produces 50% of the maximal inhibition
K^+	potassium
K_d	dissociation constant
kg	kilogram(s)
K_i	inhibition constant
l	liter(s)
LYS	lysine
M	molar
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
µl	microliter(s)
mm	millimeter(s)
mM	millimolar
mol	mole(s)

LIST OF ABBREVIATIONS CONTINUED

MW	molecular weight (daltons)
Na^+	sodium
NIDDM	non insulin dependent diabetes mellitus
nM	nanomolar
nm	nanometer(s)
PVR	proliferative vitreoretinopathy
Rb^+	nonradioactive rubidium
$^{86}Rb^+$	radioactive rubidium
RPE	retinal pigment epithelial cell
SD	standard deviation
SEM	standard error of the mean

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Note: The Joint Commission on Biochemical Nomenclature of the International Union of Biochemistry (IUB) and the International Union of Pure and Applied Chemistry (IUPAC) suggest the term "glycation" rather than "glycosylation" or glucosylation" for the nonenzymatic reaction between glucose or other sugars, and free amino groups of proteins.