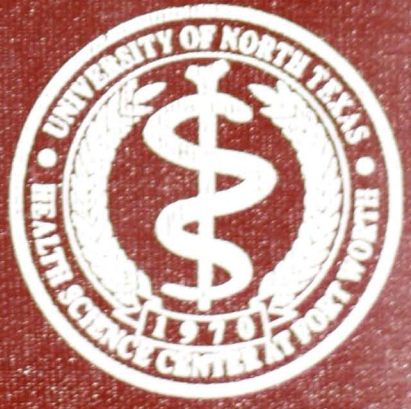
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The basic cellular requirement of volume regulation utilizes many different channel and transport pathways working in concord to maintain a constant cell volume. Among these are distinct pathways spontaneously activated by changes in cell volume that modulate the gain or loss of certain organic osmolytes, such as myo-inositol (MI). The major goal of this dissertation is to characterize and identify the mechanism involved in the MI effux pathway and explore its relationship with intracellular polyol accumulation in cultured bovine lens epithelial cells (BLECs).

Hypertonic exposure of BLECs causes an increase in MI uptake and aldose reductase enzymatic activity, two events which ultimately influence osmolyte efflux. A biphasic efflux pathway induced by rapid cell swelling (hypotonic-induction) was demonstrated in BLECS switched from hypertonic to physiologic medium. Also, intracellular polyol accumulation from galactose exposure resulted in enhanced activation of the MI efflux pathway (polyol-induction). Chloride channel inhibitors effectively blocked MI efflux suggesting a relationship between anion (chloride) movement and intracellular MI loss from cell to medium.

Expression of a chloride channel regulatory protein, pI_{Cln}, was demonstrated by Northern blot analysis in cultured BLECs. Hypertonic exposure upregulates the

expression of pI_{Cln} mRNA while hypotonicity downregulates expression. The volume-sensitivity for transcription of pI_{Cln} mRNA in BLECs lends stong support for its role in both anion and osmolyte loss associated with the MI efflux pathway.

The MI efflux pathway functions as a "relief valve" in cell volume regulation by providing a conduit to alleviate intracellular osmotic stress. The mechanism which evolved to function under normal cellular circumstances in relief of excessive accumulation of intracellular osmolytes (i.e. polyols), may, by design, inadvertently promote the loss of essential intracellular volume and nonvolume regulatory organic solutes. Ironically, under certain pathological conditions, this mechanism, designed to protect the cell from intracellular osmotic stress, may instead be detramental to the cell by promoting the excessive loss of osmolytes essential for normal cell function.

CHARACTERIZATION OF THE MYO-INOSITOL EFFLUX PATHWAY

IN CULTURED BOVINE LENS EPITHELIAL CELLS

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CHAPTER I

INTRODUCTION

The lens is a transparent, biconvex structure held in position behind the pupil by fine fibers called suspensory ligaments (zonules) which pass between the ciliary body and the lens. The lens is a unique organ devoid of vasculature and innervation and is totally enclosed within a capsule (basement membrane). The mathematician Francisco Maurolycus of Messina proposed in 1554 that the role of the lens was to form an image in a similar fashion as a convex lens focuses rays of light into an image. This role was eloquently demonstrated in the book *Oculus* (1619) by a Jesuit from Bavaria named Christopher Scheiner, who cut away the sclera from the posterior pole and observed image formation on the retina in both animal and human eyes (Brown and Bron, 1996). Because of this fundamental role of focusing light on the retina, the transparency of the lens is essential for the normal vision process.

The lens has a unique feature in that it continues to grow throughout life as the germinative zone of the epithelium divides and new lens fibers are laid down (Paterson and Delamere, 1992). Both epithelial and fiber cells are internalized and contained within the lens capsule from an early period in embryonic development, so any change in cell morphology or damage to cellular architecture can lead to altered transparency and opacification of the lens. Alterations in this transparency, as in the case of cataract formation, lead to partial and even total blindness.

There are many different types of cataracts, derived as a result of a wide range of etiologies, that are either present at birth or develop during an individual's lifetime. The lens has a limited range of responses to physical or metabolic assault, and thus cataract formation may arise from a variety of influences including metabolic disorders (Rose, 1983). Cataract formation has been linked to radiation and chemical exposure, congenital anomolies, trauma, the normal aging process (senile cataracts), and metabolic disorders such as diabetes and galactosemia (sugar cataracts). For nearly 40 years, the etiology of sugar cataractogenesis has been a major focus of lens research. Investigators have identified multiple metabolic defects associated with the onset and development of sugar cataracts; however, the mechanisms initiating this particular form of cataracts remain unresolved.

Cataracts are the leading cause of blindness worldwide, with an annual economic impact estimated to be over $3.4 billion in the United States alone (Cataract Management Guideline Panel, 1993). With life expectancies of Americans increasing with each passing decade, the number of cataract cases would be expected to increase along with the cost to treat these individuals. Should a factor be found to delay the onset of cataracts by ten years, the number of cataract operations is estimated to decrease annually by 45%, as well as significant economic savings through reduced medical expenses (Kupfer, 1984).

LENS DEVELOPMENT

Preceding the earliest stages of lens formation, the embryonic ectoderm over the

notochord thickens to form the neural plate. This plate subseqently folds up to form the neural tube around the end of the third week of embryonic development (Moore and Persaud, 1993; Donaldson, 1976). At the beginning of the fourth week, the cranial end of the neural tube grows rapidly and forms lateral evaginations which develop into hollow diverticula called optic vesicles, while the surface ectoderm adjacent to the vesicles thickens to form lens placodes. These placodes are the primordial structure to the lenses and are induced by a signal produced by the optic vesicles (Moore and Persaud, 1993). The central portion of the lens placode now begins to invaginate to form the lens pit, with the edges of this structure eventually fusing to form a spherical lens vesicle around 32 days of development. The hollow lens vesicle shortly pinches off from the surface ectoderm from which it was derived to develop into the lens of the eye (Donaldson, 1976).

The cells of the posterior vesicle wall begin a remarkable elongation, gradually obliterating the cavity of the lens vesicle by approximately the sixth week of development. These cells form the highly transparent primary lens fibers of the embryonic lens. In contrast, the cells of the anterior wall of the lens vesicle do not change appreciably as they become the anterior lens epithelium, the major cells of focus in this project. These cuboidal cells show large indented nuclei with two nucleoli and other organelles including small mitochondria, Golgi apparatus, smooth and rough endoplasmic reticulum, ribosomes, polysomes, and lysosomes.

The region of the lens between the anterior and posterior poles forms a distinct rim called the equatorial zone (Brown and Bron, 1996). It is in the pre-equatorial region

that the taller, peripherial epithelial cells exhibit a proliferative capacity to become the germinative zone of the lens. In this mitotic zone, lens epithelial cells begin to differentiate and give rise to secondary lens fibers through a process of growth and elongation (Donaldson, 1976). These fiber cells undergo a process of terminal differentiation, followed by pyknosis of the nucleus which ultimately disappears along with other cell organelles. Recent studies by Chamberlain et al. (1987) and Beebe et al. (1980) indicate that certain growth factors such as fibroblast growth factor (FGF) and lentropin (later identified as insulinlike growth factor) may play important roles in lens fiber differentiation.

Mitosis of epithelial cells is associated with DNA synthesis; however, elongation of fiber cells involves an increase in transcriptional activity and RNA synthesis as specific structural, membrane and other proteins are formed (Brown and Bron, 1996).

Proteins, such as the crystallins, are synthesized in large quantities as the genetic machinery of lens fiber cells shuts down when the cells begin to mature. As secondary lens fiber cells continue to be laid down after maturation, the earlier fibers are displaced progressively deeper into the lens. The earliest ones formed comprise the fetal nucleus, the later the infantile nucleus, with the latest added forming the adult nucleus. Formation of the cortex, the outermost portion of lens fibers, begins to occur between 20 and 25 years of age and continues throughout life but at a slower rate than nucleus formation (Donaldson, 1976).

Around the sixth week of embryonic development, the cells at the equatorial zone as well as those of the posterior wall begin the secretion of the lens capsule. The lens is

fully encapsulated around midterm, once the more slowly secreted anterior lens capsule is completed (Brown and Bron, 1996). As lens epithelial cells continue to divide and differentiate into secondary lens fiber cells throughout life, the lens capsule is obliged to remodel itself to accomodate the continuous increase in volume. The human lens continues to grow througout life, as compared to that of other species, such as dog, rat or rabbit where growth plateaus in the latter part of the life span (Brown and Bron, 1996; van Heyningen, 1977).

LENS ANATOMY, BIOLOGY AND METABOLISM

Compared to other tissues, the lens has a relatively low water content and a particularly high protein content. About 90% of these proteins consists of the lens crystallins, which reside in the lens fiber cells and provides the high refractive index necessary for the lens's optical function (Brown and Bron, 1996). Half-lives of these proteins are measured in decades. Normal lens transparency is due to the highly ordered arrangement of lens fiber cell proteins, small differences in refractive index between light-scattering components, and the specialized arrangement of the individual lens fibers within the lens (Paterson and Delamere, 1992). Lens transparency is highly dependent on protein structure and integrity; therefore, the development of opacification leading to cataract formation may be the result of relatively small changes in these parameters which might include aggregration, changes in tissue hydration, breakdown of the cellular membrane, and changes in the structure of the cytoskeleton. Under the normal aging

process and certainly with cataract formation most of these changes can and do occur (Paterson and Delamere, 1992).

The lens is surrounded by a capsule which is the thickest basal lamina (basement membrane) in the body. This structure is rich in type IV collagen and also contains type I and III collagens in addition to a number of other extracellular matrix components such as, laminin, fibronectin, heparan sulphate proteoglycan and entactin (Marshal et al, 1991). The capsule is thicker anteriorly because posterior lens fibers have a limited capacity to secrete new lamina (Parmigiani and McAvoy, 1991). The lens capsule is freely permeable to water, ions and other small molecules, and to small proteins, but restricts the movement of larger colloidal material such as albumin and hemoglobin (Friedenwald, 1930; Paterson and Delamere, 1992).

The lens epithelium forms an anterior subcapsular monolayer that can be easily removed with the anterior capsule and subcultured in growth medium. In its intact state, the epithelium can be functionally divided into two zones. The central zone, whose cells normally do not undergo division, plays an important role in transporting a variety of solutes between the lens and the aqueous humor. The cells of the equatorial zone are those which divide and undergo a dramatic elongation and differentiation process to give birth to all fiber cells which constitute the bulk of the lens (McAvoy, 1981).

In the cataractogenic process, damage to the metabolic and synthetic activity of the epithelial cells can have a significant effect on the overall integrity and structural stability of the lens, leading to opacifications of the cortical and nuclear regions. From studies with tracer molecules (Gorthy et al, 1971; Rae and Stacey, 1979), it has been

determined that the epithelial monolayer is not a tight barrier as once believed. Recent studies in whole bovine lens by Cammarata et al (1997) have shown that there is a large passive flux of the osmolyte, myo-inositol, between the extralenticular environment and the anterior aspect of the lens under physiological conditions. Lo and Harding (1983) suggested that the passage of high molecular weight solutes between epithelial cells in both frog and human lens is restricted by zonular occludens; however, the existence of these structures has been disputed (Kuszak and Brown, 1991). Kistler and Bullivant (1989) have shown the existence of low-resistance gap junctions allowing cell-to-cell communications between epithelial cells. It has also been suggested that gap junctions exist between the lens epithelial cells and underlying lens fiber cells which may be different from junctions found between individual fiber cells (Brown et al, 1990). A proposal by Goodenough et al. (1980) suggests that ions and metabolites gain access to the fiber cell cytoplasm via gap junctions connecting the closely apposed apical membranes of the epithelial and fiber cells. It is widely accepted that the epithelium plays an important role in regulating the passage of ions and metabolites between the aqueous humor and the cytoplasm of the lens fiber cells (Kinsey and Reddy, 1965).

The interior of the lens is filled with lens fiber cells of differing age, with the younger cells forming the outer cortical region and older fiber cells comprising the lens nuclear region. A specialization of lens cortex and nuclear fibers cells, apparently directed toward maintainence of transparency, is their lack of normal cellular organelles such as nuclei and mitochondria. Low-resistant gap junctions are also a unique feature of the densely packed fiber cells allowing this tissue to function like a syncytium rather than

a group of individual cells (Paterson and Delamere, 1992). Consequently, the hypothesis that normal fiber cell functions are maintained through direct connections to the epithelium has prompted numerous studies of the role of epithelial cell dysfunction in cataract formation (Bassnett et al, 1994).

The chief energy source of the lens (van Hayningen, 1969) is glucose which is supplied primarily from the newly formed aqueous humor flowing immediately across the lens anterior surface. Glucose, as well as other sugars, enters the lens by facilitated diffusion, using energy generated by the sodium gradient across the cell membrane through activity of the Na-K ATPase pump. Transport of glucose appears to occur across both surfaces of the lens, and recent evidence suggests that the lens contains a specific glucose transporter (Lucus and Zigler, 1987; Lucas and Zigler, 1988).

Transparency of the lens cannot be maintained when the whole organ is cultured in a glucose free medium; however, if adequate glucose is present transparency can be maintained in the absence of oxygen (Reddy and Kinoshita, 1962), indicating that ATP production in the lens is predominantly an anaerobic event. Glucose metabolism in the lens may follow one of several different pathways (Figure 1). Since the lens is devoid of a blood supply and derives its oxygen from the aqueous humor, anaerobic glycolysis is the primary source of glucose metabolism due to the scarcity of oxygen. The glycolytic pathway is regulated by the enzyme hexokinase, which catalyzes the conversion of glucose into glucose 6-phosphate. This enzyme is found in limited supply within the lens and is the rate limiting step in this pathway (van Heyningen, 1965; Green et al., 1955).

More than 70% of the energy produced in the lens is through anaerobic

glycolysis; however, 20-30% of generated energy comes from the much more efficient aerobic metabolism which utilizes only 3% of the total lens glucose (Brown and Bron, 1996). Aerobic metabolism of glucose by the Kreb's cycle is limited primarily to the lens epithelium and the superficial lens fibers. ATP generated in these layers provides energy for ion pumps, mitosis in the epithelium, and the major synthetic requirements of differentiating cells (Brown and Bron, 1996).

In addition to the glycolysis and aerobic metabolism of glucose, 10-20% of lens glucose is metabolized via the pentose phosphate pathway, also known as the hexosemonophosphate shunt (Kinoshita, 1965). Although the pentose phosphate pathway does not generate a large quantity of ATP in the lens, it is an important source of NADPH, which is utilized in the lens for nucleotide, fatty acid, and sterol synthesis. This source of NADPH is critical to a number of other pathways including the maintenance of glutathione in its reduced state (GSH) by the enzyme glutathione reductase and for the reduction of hexose sugars to sugar alcohols, in the polyol (sorbitol) pathway, a pathway extensively hypothesized to play a pivitol role in the development of sugar catarcts (Brown and Bron, 1996; Paterson and Delamere, 1992; Kinoshita, 1974).

THE POLYOL PATHWAY AND SUGAR CATARACTOGENESIS

Excess glucose in the lens is shunted into other pathways, such as the polyol pathway, which accounts for approximately 5% of normal lens glucose metabolism. This pathway (Figure 2) is responsible for the conversion of glucose into the sugar alcohol, sorbitol, via an enzyme called aldose reductase. Diabetic conditions involving

hyperglycemia causes more glucose to enter this pathway resulting in the accumulation of metabolic products within the lens cells (Basher and Roberts, 1995). In the normal human lens, the activity of aldose reductase is several times lower than that seen in either the dog or rat (Halder and Crabbe, 1984); however, recent evidence strongly suggests that aldose reductase activity significantly increases in the cortex of human lenses after the onset of diabetes (Akagi et al., 1987). In a study by Ederer et al (1981), it was shown that the prevalence of cataracts is significantly higher in diabetic patients compared to the general population. Both cortical and epithelial lens tissue damage has been documented in human diabetics (Creighton et al., 1978). The polyol pathway, although responsible for metabolizing a relatively small amount of the total lens glucose, has been the source of great interest for nearly 40 years as a candidate for the genesis of diabetic-induced and age-related cataracts.

Beginning in 1959, van Heyningen eloquantly demonstrated with a series of studies that polyols generated by the polyol pathway could be detramental to the lens (van Heyningen, 1959; van Heyningen, 1990). Later studies varified these initial findings and established the prominent role of the polyol pathway in the development of sugar cataracts (Kinoshita, 1974; Kinoshita, 1990). The polyol-osmotic hypothesis (osmotic stress hypothesis, Figure 3) proposes that the polyol pathway initiates the cataractogenic process through prolonged exposure and accumulation of sugar alcohols (polyols) in lenticular tissue, resulting in increased membrane permeability, sodium influx and hydration followed eventually by cell disruption (Kinoshita, 1974; Kador and Kinoshita, 1984; Kawaba et al, 1985). The polar character of polyols renders them relatively

impervious to the cell membrane so elevated intracellular accumulation of sugar alcohol creates a hyperosmotic effect which, if prolonged, may induce lens hydration to counteract the osmotic gradient produced. Water imbibition by the lens causes architectural changes that lead to rapid lens opacification (Kinoshita, 1974; Basher and Roberts, 1995). In this hypothesis, Kinoshita (1974) proposed that the key factor in initiating the cataractous process in the lenses of galactosemic and diabetic animals was the polyol pathway enzyme aldose reductase.

Galactose is also a substrate of aldose reductase and is similarly reduced in the polyol pathway to galactitol (or dulcitol). With a higher affinity for aldose reductase than glucose, increased levels of galactose are more rapidly reduced to galactitol. Sorbitol undergoes further metabolism in the polyol pathway in its conversion to fructose by the enzyme sorbitol dehydrogenase. However, galactitol is able to accumulate intracellularly at much higher levels because of the inability of sorbitol dehydrogenase to further metabolize this sugar alcohol. In 1971, van Heyningen showed that rats fed high galactose diets accumulated very high concentrations of galactitol (dulcitol) in the lens paralleled by a large influx of water. The overall hyperosmotic effect from galactose is much greater with a more rapid onset and swelling of lens cells occuring (Kinoshita, 1962; Kador and Kinoshita, 1984). Recent studies by Lin et al. (1990) have shown that human lens epithelial cells cultured in high galactose medium accumulate significant amounts of intracellular galactitol via the polyol pathway, with the appearance of vacuoles similar to those seen in experimental animal models which develop cataracts. Pathologically similar observations from studies with intact lens and epithelial cell

cultures have led investigators to conclude that the etiology of human diabetic cataracts may also involve the polyol pathway (Reddy et al., 1992).

With the development of aldose reductase inhibitors (ARI), the polyol pathway gained further support in the involvement of the cataractous process . The first aldose reductase inhibitor, tetramethyleneglutaic acid (TMG) blocked polyol accumulation and prevented cataract formation in lenses maintained in either high galactose or glucose medium (Kinoshita et al., 1968; Chylack and Kinoshita, 1969). Sorbinil, another ARI, was first shown to prevent cataract formation in diabetic rats (Fukishi et al., 1980) and was more recently shown to inhibit galactose-induced cataract formation in galactose-fed dogs (Sato et al., 1991). These aldose reductase inhibitors interact with a nucleophilic residue at a common stereospecific site on the enzyme to block polyol formation (Kador et al., 1981).

Physiological surrogates such as the bovine lens epithelial cell (BLECs) culture model and whole lens models, as well as comparative animal models, have been used to make the association between the metabolism of excess sugar via the polyol pathway and cataract formation. Isolated lenses cultured in either 35 mM glucose or galactose can produce conditions which simulate hyperglycemia or hypergalactosemia. These conditions result in an increase in hydration concomitant with polyol accumulation followed by lens opacification (Chylack and Kinoshita, 1969). As swelling occurs, initial membrane permeability changes result in a decrease in the K/Na ratio as sodium enters by diffusion (Kador, 1994). Besides this major electrolyte imbalance, the lenticular levels of reduced glutathione, adenosine triphosphate (ATP), myo-inositol, and free amino acids

begin to decrease, and in due course, lenticular protein synthesis shuts down (Kador and Kinoshita, 1984).

Although it is well established that the aldose reductase pathway leads to sugar cataract formation in animal lenses (Kinoshita, 1974), the mechanism in which high sugar levels or intracellular polyol accumulation culminates in the initiation of sugar cataracts has been debated extensively. Since lens levels of myo-inositol, an essential factor for cell growth (Eagle et al, 1957), are depleted in parallel with polyol accumulation, an alternative theory was proposed for the genesis of sugar cataracts during diabetes and experimental hyperglycemia. The initial observations for this theory were made by Winegrad and Greene (1976) who associated hyperglycemia with altered myo-inositol metabolism and impairment of nerve conductance seen in experimental diabetes. Their proposal, an extrapolation of the polyol-osmotic hypothesis, was called the sorbitol/myo-inositol hypothesis (Figure 4), also known as the myo-inositol depletion hypothesis (Greene et al, 1987; Winegrad, 1987). They suggested that the initiation of pathological processes in tissues prone to show complications under hyperglycemic (diabetic) or hypergalactosemic conditions was fundamentally linked to the depletion of intracellular myo-inositol. Deficiencies of this essential osmolyte cause severe disturbances in cell growth, differentiation and homeostasis including a reduction of Na-K ATPase activity (Charalampous, 1971), amino acid transport (Lembach and Charalampous, 1967), and nucleic acid synthesis (Tsukagoshi et al, 1966). Also, myo-inositol plays an important role as a substrate for phosphoinositide synthesis which are involved in mediating the actions of hormones (Berridge, 1987; Diecke et al., 1995)

It was Greene et al. (1987) who first doubted the polyol-osmotic hypothesis, because the concentration of sorbitol in certain diabetically damaged tissues is many times lower than that seen in the lens; therefore, sorbitol in these tissues is unlikely to have an osmotic effect significant enough to cause damage. Narayanan (1993) suggested that inititation of the cataractous process in the lens under hyperglycemic (diabetic) or hypergalactosemic conditions results from the direct loss of intracellular myo-inositol upon alteration of lens cell permeability. This hypothesis is supported by the findings that dietary myo-inositol supplementation delay or reduce diabetic complications including cataract (Greene et al., 1975; Beyer-Mears et al., 1989; Ruf et al., 1992). Reduced myo-inositol levels have been shown in lenses from diabetic patients (Pirie and van Heyningen, 1964; Belpoliti and Maraini, 1993; Varma et al., 1979) and lenses from hyperglycemic (McCaleb and Sredy, 1992) and galactosemic (Broekhuyse, 1968; Kawaba et al., 1986) rats.

Polyol accumulation in tissues via the polyol pathway promotes intracellular myo-inositol (MI) depletion by several means. Those resulting from the inhibition of myo-inositol uptake mechanisms have been extensively studied and described; however, alternative depletion routes, such as efflux mechanisms, have not stimulated a congruent interest for investigation. Broekhuyse showed in 1968 that lenses incubated in the presence of high galactose medium showed a significant decrease in the uptake of myo-inositol. This result was confirmed in a later set of experiments by Kawaba et al., (1986) which indicated polyol accumulation inhibited myo-inositol uptake in rat lens. They showed that the aldose reductase inhibitor sorbinil normalized intracellular myo-inositol

levels by blocking polyol synthesis in galactose-exposed lens. Lens epithelial cells cultured in high galactose medium showed significant reduction in myo-inositol uptake (Cammarata et al., 1990). In glucose-stressed lens and lens epithelial cell cultures, myo-inositol transport is inhibited by both glucose and its sugar alcohol, sorbitol (Beyer-Mears et al., 1991; Cammarata et al., 1991; Cammarata et al., 1992a). In these studies, the restoration of intracellular myo-inositol content with aldose reductase inhibitors was also shown. The primary focus of these studies mentioned above revolved around the depletion of intracellular myo-inositol from inhibited uptake mechanisms.

In an alternative set of experiments, Broekhuyse (1968) demonstrated an increase in myo-inositol efflux in lenses incubated in the presence high galactose medium. This phenomenon, later referred to as myo-inositol run-out, was also demonstrated in rat lens (Kawaba et all., 1986). Although both groups of investigators acknowledge the occurence of myo-inositol efflux from lenticular tissue, they speculated efflux resulted from polyol accumulation; however, no convincing data was given as to how this observed efflux was mediated by the lens. Reddy et al. (1992) similarly demonstrated reduction of intracellular myo-inositol in galactose maintained human lens epithelial cells. They too showed a distinct recovery of intracellular myo-inositol levels when polyol accumulation was blocked with an aldose reductase inhibitor. Their explanation for these events was in concord with earlier theories in that elevated intracellular polyol accumulation somehow changes cell permeability. Due to localization of polyols in the epithelial layer, Belpoliti and Maraini (1993) proposed that initiation of age-related and diabetic cataract formation may occur at the lens epithelial cell level. This conclusion was drawn from data showing

significantly lower myo-inositol levels in the lens epithelia of cataractous lenses from diabetic patients. This evidence coupled with earlier observations regarding the unidentified efflux mechanisms of myo-inositol depletion seen in galactose maintained lenses laid the framework for this research project.

OSMOREGULATION AND MYO-INOSITOL EFFLUX

Hypertonic medium conditions cause cultured bovine lens epithelial cells to shrink, initially accumulating electrolytes through uptake mechanisms such as the Na^+, K^+, Cl^- cotransporters (O'Donnell, 1993). Further osmotic balance is achieved through the uptake of osmotically active, but chemically inert, nonperturbing organic solutes termed "osmolytes" (Garcia-Perez and Burg, 1991) to compensate for osmotic stress. Cells use both electrolytes and organic solutes (organic osmolytes) for volume regulation. In animal cells, organic osmolytes are typically grouped into three distinct classes (Strange *et al*, 1996): 1) polyols (e.g., sorbitol, myo-inositol), 2) amino acids and their derivatives (e.g., taurine, alanine, glycine and proline), and 3) methyl-amines (e.g., glycerophosphorylcholine, betaine). Under hypertonic stress, cells accumulate these particular organic compounds at elevated concentrations to adjust their intracellular osmolality. Perturbing solutes, such as Na^+, K^+, and Cl^-, inhibit enzymes and other cellular processes at elevated concentrations, whereas organic osmolytes have relatively little effect on normal cell function, even at high concentrations (Nakanishi and Burg, 1989). Lenticular tissue can be subjected to various degrees of osmotic stress in metabolic disorders such as hyperglycemia (diabetes), hypergalactosemia and renal disfunction

which would initiate response from these osmoregulatory mechanisms.

Three putative organic osmolytes have been identified in lens epithelial cells: sorbitol (Carper et al., 1990), myo-inositol (Cammarata et al., 1992a; Cammarata et al, 1992b), and taurine (Yokoyama et al., 1993). In cultured bovine lens epithelial cells, hypertonicity induces an increase in the Na^+-myo-inositol cotransporter uptake activity and an accumulation of intracellular myo-inositol (Cammarata and Chen, 1994), with very similar results seen in taurine uptake experiments. It has also been reported in these cells that hypertonic stress rapidly upregulates Na^+-myo-inositol cotransporter mRNA with maximum induction occuring within 8 hours. Furthermore, aldose reductase mRNA expression is upregulated in hypertonic exposure, with maximum induction by 24 hours (Zhou et al., 1994). The characteristic hypertonic-induced upregulation of mRNA expression for the myo-inositol transporter and aldose reductase played a pivitol role in the development of assays used to monitor myo-inositol efflux in bovine lens epithelial cell cultures.

The depletion of intracellular myo-inositol in bovine lens epithelial cells involves several identified mechanisms: 1) the suppression of the myo-inositol transporter with intracellular polyol accumulation (Cammarata et al., 1992, Cammarata and Chen., 1994), 2) the downregulation of mRNA expression for the myo-inositol transporter with polyol accumulation (Zhou et al., 1994), and 3) the promotion of myo-inositol efflux from the cell to medium with intracellular polyol accumulation (Broekhuyse, 1968; Kawaba et al., 1986; Reddy et al., 1992). As previously mentioned, the latter mechanism is the least reported and understood of the three and consequently provided the basis for this

research.

Characteristics of the efflux pathway have been carried out mainly in mammalian cells (Jackson and Strange, 1993; Roy and Malo, 1992; Seibens and Spring, 1989; Strange et al., 1993; Reeves and Cammarata, 1996) and cells of lower vertebrates (Ballatori and Boyer, 1992; Haynes and Goldstein, 1993; Kirk et al., 1992). These experiments show volume regulatory loss of organic osmolytes from cells occurs by passive concentration gradient-driven efflux from the cell to medium. Appearantly, osmolyte efflux is mediated by passive Na^+-independent transport pathways which do not exhibit saturation behavior (Strange et al., 1996). These findings led Roy and Malo (1992) to initially hypothesize that organic osmolyte loss is by diffusion (efflux) through anionic membrane channels, rather than saturable carrier-mediated transport. This hypothesis is supported by the shared characteristic of swelling-activated organic osmolyte efflux mechanisms being inhibited by anion transport inhibitors (Solis et al., 1988; Kimelberg et al., 1990; Kirk et al., 1990; Garcia-Romeu, 1991; Sanchez-Olea et al., 1993; Strange et al., 1993; Reeves and Cammarata, 1996) which specifically block chloride channels.

The release (efflux) of myo-inositol from cell to medium has been demonstrated in MDCK cell cultures (Nakanishi and Burg, 1989), MDCK cells grown on filters (Yamauchi *et al*, 1991) and more recently with C6 glioma cells (Strange *et al,* 1993). These early studies dealt solely with the characterization of myo-inositol efflux attributed to cell swelling (swelling-activated). Recent studies have indicated that volume-sensitive anion channels are involved in myo-inositol efflux in C6 glioma cells (Strange *et al*,

1993, Jackson and Strange, 1993, and Jackson *et al*, 1994), Madin-Darby canine kidney cells (Bagnasco *et al*, 1993), and bovine lens epithelial cell (Reeves and Cammarata, 1996). Furthermore, parallels have been shown which demonstrate links between myo-inositol efflux attributed to cell swelling and that which had been previously attributed to rapid intracellular polyol accumulation (as seen in sugar cataractogenesis). There is strong evidence which suggests both polyol-activated myo-inositol efflux, originally described in lens tissue, and swelling-activated myo-inositol efflux are modulated through a common transport pathway associated with Cl^- movement (Reeves and Cammarata, 1996).

Interest in identification and the molecular characterization of the protein(s) involved in the efflux process have increased significantly over the past several years. Paulmichl et al. (1992) described the cloning of a cDNA that codes for a protein termed, I_{Cln} (or pI_{Cln}) which has subsequently been cloned from ocular ciliary epithelium (Anquita et al., 1995). When overexpressed in *Xenopus* oocytes, pI_{Cln} gives rise to an outwardly rectifying anion conductance (Paulmichl et al., 1992), is inhibited by conventional anion (chloride) channel inhibitors, external ATP and other nucleotides, is inactivated by strong depolarization, and has an anion selectivity of $SCN^->I^->Br^->Cl^-$ (Paulmichl et al., 1993). The characteristics of pI_{Cln} are almost indistinguishable from those of another volume-sensitive organic osmolyte-anion channel (VSOAC) which was first described by Jackson et al. (1994). The similarities in these characteristics strongly suggests that pI_{Cln} is either the VSOAC channel, a functional subunit of the channel, or a channel regulator (Paulmichl et al., 1992; Krapivinsky et al., 1994; Strange et al., 1996).

It was initially proposed by Paulmichl et al. (1992) that pI_{Cln} is a channel forming protein based on evidence that mutations of a putative nucleotide binding site on the protein prevented inhibition by extracellular nucleotides and altered channel voltage dependence. They also proposed that the putative channel consists of a dimer of the protein, with the pore being formed by an eight-stranded antiparallel β-barrel. Krapivinsky et al. (1994) has more recently reported that pI_{Cln} encodes for an abundant, soluble, highly acidic protein predominantly located in the cytosol of MDCK and heart muscle cells. In contrast, they determined that the cytoplasmic location and biochemical characteristics of the protein were unexpected for an anion channel and subsequently proposed that the pI_{Cln} protein is not an anion channel, but a regulatory protein for the channel. The contrasting views between the pI_{Cln} channel protein and pI_{Cln} regulatory protein hypotheses will undoubtedly require much additional work, including that at the molecular level, to understand fully the structure and cellular function of this protein. Regardless of the different interpretations, both proposals strongly suggests that pI_{Cln} is involved in the cellular process of anion/osmolyte efflux and cell volume regulation.

Strange et al. (1996) recently proposed that pI_{Cln}, due to its β-barrel structure, may be "porinlike in nature", that is it may be similar to a primitive class of channels that transport organic solutes in a nonselective fashion across bacterial outermembranes. Porins insert spontaneously into lipid bilayers and the functional and electrophysiological characteristics of porins are very similar to pI_{Cln} and VSOAC. It was proposed that cell swelling releases pI_{Cln} from its binding site in the cytosol, allowing the protein to insert

spontaneously into the cell membrane (Strange et al., 1996). The "anchor-insertion" model, as suggested, would be characterized by the off state of the channel representing the cytosolically located form of pI_{cln} with an abrupt switching of the channel into the on state reflecting its insertion into the cell membrane.

In an alternative model, studies have suggested that the channel regulatory protein, pI_{cln}, is tethered to the cell membrane in the vicinity of the channel protein through actin binding sites (Paulmichl et al., 1992; Krapivinsky et al., 1994; Coca-Prados et al., 1995). The volume-sensitive pI_{cln} is proposed to regulate anion and osmolyte movement, possibly through the Cl^- channel ClC-3 (Coca-Prados et al., 1995). In the future, the incorporation of extensive molecular, immunohistochemical, and electrophysiological studies will be required in order to answer questions regarding similarities and differences between pI_{cln} and VSOAC, the anchor-insertion model for pI_{cln} (Strange *et al*, 1996), the functional relationship between pI_{cln} and ClC-3, and chloride channel/channel regulator controversy.

Using Northern analysis, recent findings in our lab (Reeves et al., 1997, Chapter 3) have indicated that expressional levels of pI_{cln} mRNA in bovine lens epithelial cells under hypertonic and hypotonic medium conditions are predictably characteristic of an anion/osmolyte channel responding to fluctuations in cell volume and/or intracellular osmolality. The chloride channel, ClC-3, was also identified by northern analysis in bovine lens epithelial cell cultures maintained under physiologic conditions. Great interest in the identification of the protein(s) involved in osmolyte efflux has emerge in

recent years, and it is my intention in this research project to explore and demonstrate possible relationships between myo-inositol efflux, polyol accumulation, cell volume fluctuations and the cell volume regulatory protein, pI_{cln}.

RESEARCH GOAL AND SPECIFIC AIMS

An extensive amount of research has centered around the organic osmolyte, myo-inositol, regarding its role in the pathogenic mechanisms associated with long-term diabetic (hyperglycemic) complications, such as reduced nerve conductance and cataractogenesis. Nearly 30 years ago, the efflux of myo-inositol, triggered by diabetic conditions favoring cataract formation, was observed in lenticular tissue (Broekhuyse, 1968). This efflux pathway has been described as one of several methods of intracellular myo-inositol depletion in the lens; however, little is known of the factors involved in the initiation and culmination of these events typically associated with intracellular polyol accumulation. Investigations concerning the myo-inositol efflux pathway and the intracellular depletion of myo-inositol, related to polyol formation, could generate information that might explain the origin of pathological complications associated with diabetes.

To define the underlying mechanisms that activate the myo-inositol efflux pathway in bovine lens epithelial cells, experimental protocols were first designed to stimulate or inhibit myo-inositol release from hypertonically adapted cells once the extracellular osmolality was reduced. An alternative set of experiments was designed

specifically to monitor the effect of polyols on myo-inositol efflux under conditions in which medium osmolality was not reduced but were favorable for rapid polyol accumulation. Once these initial parameters were attained, it was possible to begin to answer questions related to the overall goal of this project.

The major goal of this research is to characterize and identify the mechanism(s) involved in the myo-inositol effux pathway and explore its relationship with intracellular polyol accumulation in cultured bovine lens epithelial cells. The specific aims to achieve this goal were as follows:

1) Develop a reproducible assay which can easily and accurately monitor myo-inositol efflux from cell to medium in cultured bovine lens epithelial cells.

2) Determine the effect of rapid and prolonged intracellular polyol accumulation on myo-inositol efflux and compare/contrast that to the swelling-activated (tonicity-activated) efflux pathway in bovine lens epithelial cells.

3) Utilizing anion channel inhibitors and ion substitution studies, characterize the relationship between anion movement and myo-inositol efflux in cultured bovine lens epithelial cells .

4) Determine if the myo-inositol efflux pathway is concentration-gradient driven and investigate if myo-inositol movement occurs bidirectionally through the pathway.

5) Utilizing Northern analysis, characterize the mRNA expression of pI_{cln}, a regulatory protein for a swelling-activated chloride channel, under conditions favoring activation of cell volume regulatory mechanisms and myo-inositol efflux in cultured bovine lens epithelial cells.

The cellular importance of myo-inositol as a precurser to important membrane phospholipids and second messengers necessitates the investigation into the mechanism of depletion of intracellular myo-inositol levels well below physiologic concentrations. The essential cellular role of myo-inositol, combined with its intracellular osmoregulatory function, give significance and credibility to the results obtained in this study regarding the efflux pathway of this osmolyte.

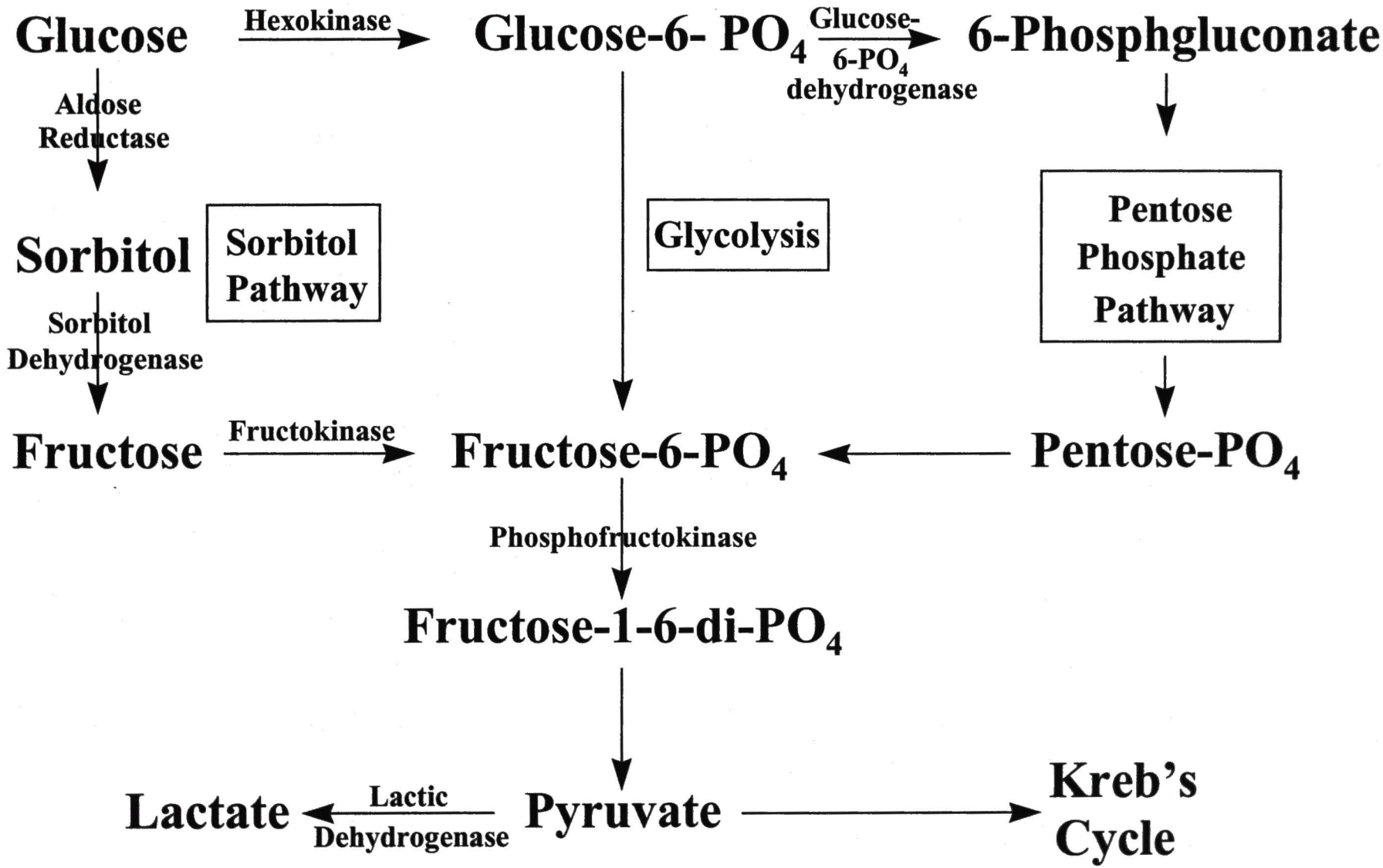

Figure 1. Glucose metabolism in the lens
(Modified from Paterson and Delamere, 1992)

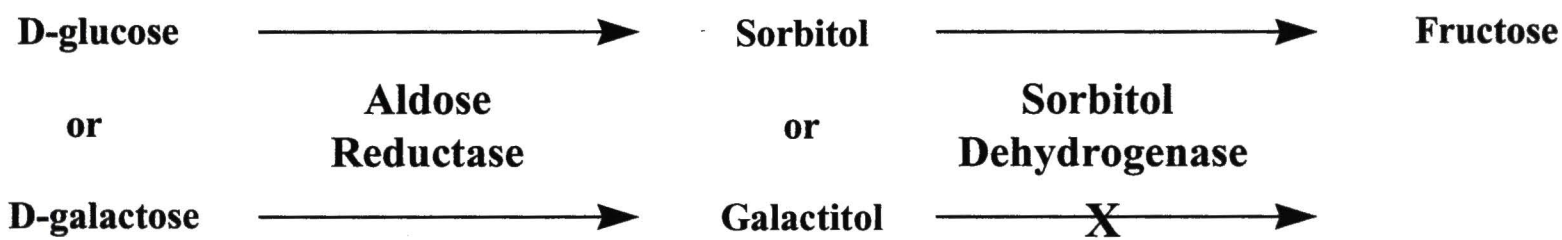

Figure 2. Polyol (Aldose Reductase) Pathway

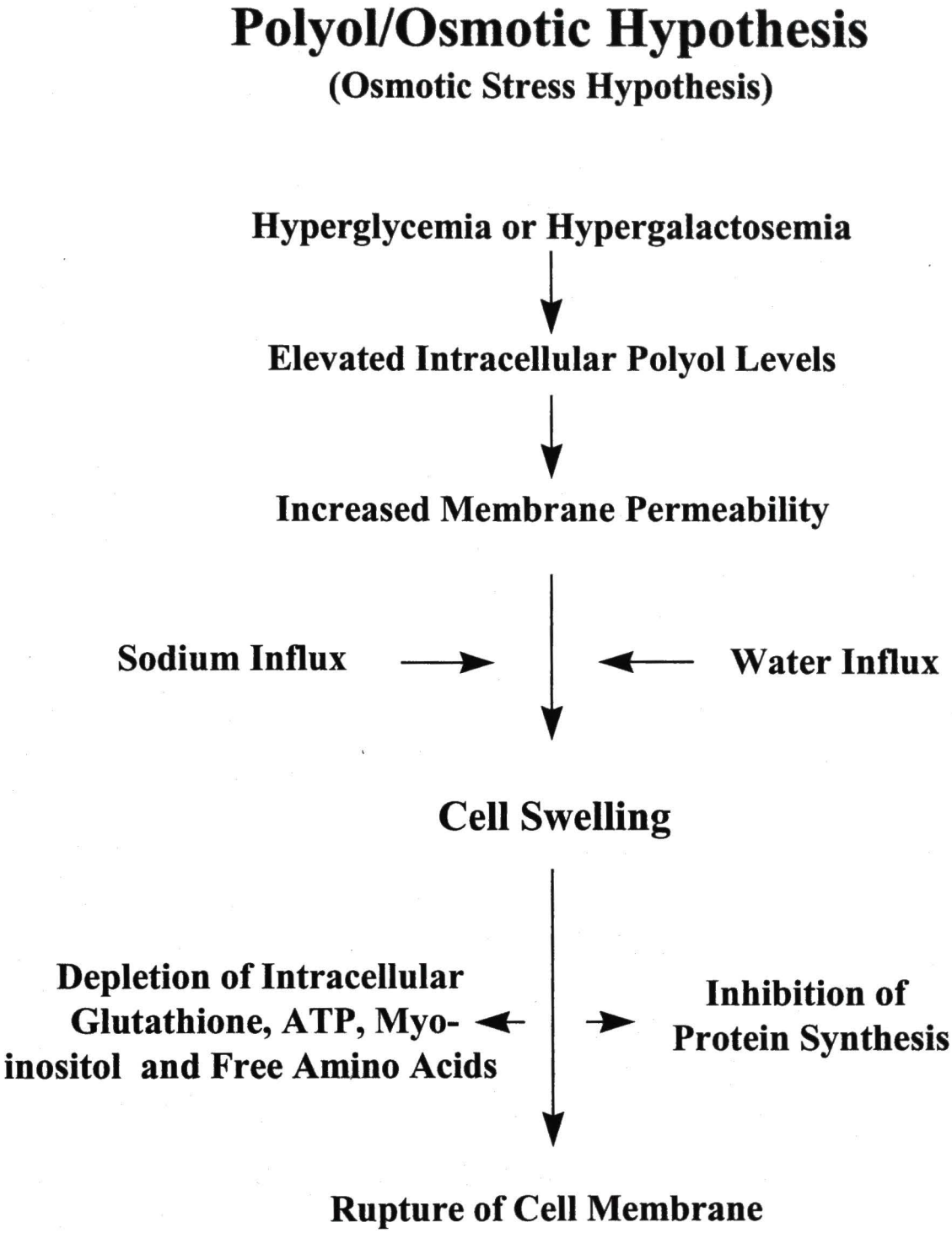

Figure 3. Polyol/Osmotic Hypothesis (Modified from Kinoshita, 1974)

Sorbitol/Myo-inositol Hypothesis

(Myo-inositol Depletion Hypothesis)

Hyperglycemia or Hypergalactosemia

↓

Elevated Intracellular Polyol Levels

↓

Inhibition of Myo-inositol Uptake

↓

Decreased Phosphoinositide Synthesis

↓

Diacylglycerol Levels Lowered

↓

Decreased PKC and
Na/K ATPase Activity

↓

Elevated Intracellular Sodium Levels
Followed by Water Influx and Cell Swelling
(Activation of Myo-inositol Efflux Pathway)

↓

Disruption of Normal Cellular Functions

Figure 4. Sorbitol/Myo-inositol Hypothesis (Modified from Greene et al, 1987; Winegrad, 1987; Narayanan, 1993)

Myo-inositol efflux was first demonstrated in lens by Broekhuyse (1968); however, the mechanism(s) eliciting this response is not well understood. In the following chapter, myo-inositol efflux was characterized as an event associated with cell swelling and intracellular polyol accumulation, with the possibility of both methods of efflux being mediated through a common pathway. Anion channel inhibitors and Cl^- substitution studies demonstrated a relationship between the pathways of chloride and osmolyte (myo-inositol) loss from the cell to the medium.

CHAPTER II

The following manuscript was published in ***Investigative Ophthalmology and Visual Science,*** 1996;**37**:619-629.

Osmoregulatory Alterations in Myo-inositol Uptake by Bovine Lens Epithelial Cells. Part 5: Mechanism of the Myo-inositol Efflux Pathway.

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Short Title: Osmoregulation of the Myo-inositol Efflux Pathway

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Key Words: hypertonicity, osmoregulation, myo-inositol efflux, chloride channel, lens epithelial cells, cell culture

Purpose. **Cultured bovine lens epithelial cells (BLECs) exposed to sodium hypertonicity respond with an accumulation of intracellular myo-inositol. Utilizing BLECs initially maintained at hypertonicity and reacting to a decrease in medium osmolality, a mechanism for the *tonicity-activated* release of myo-inositol was recognized. Alternatively, BLECs acclimated to sodium hypertonicity and subsequently transferred to high sodium osmolality plus hypergalactosemia rapidly accumulate intracellular galactitol, an experimental manipulation which permitted characterization of the role of sugar alcohols in *polyol-activated* myo-inositol efflux. The authors identify a *communal transport route* for *tonicity-activated* and *polyol-activated* myo-inositol release from cell to medium and demonstrate an association for myo-inositol efflux with chloride movement.**

Methods. **Two distinct experimental approaches were designed in order to delineate the physiological circumstances which initiate myo-inositol efflux. For tonicity-induced inositol efflux, BLECs were maintained at confluence in sodium hypertonic medium (473±6 mosm) for 48 hours and afterwards, the medium replaced with isotonic medium (285±4 mosm) containing 40 mM galactose ± Sorbinil. For polyol-induced inositol release, hypertonically adapted BLECs were transferred to fresh sodium hypertonic medium containing 40 mM galactose (513±10 mosm).**

Results. **Upon reduction in medium osmolality, intracellular myo-inositol was lost due to a rapid, transient efflux during the first 30 minutes, followed by a slow, sustained decrease in efflux over the next 12 hours. Inhibition of aldose reductase activity substantially diminished myo-inositol efflux from cell to galactose-**

containing, isotonic medium. Administration of phloretin significantly inhibited both *tonicity-activated* and *polyol-activated* myo-inositol release, as did the chloride channel blocker, niflumic acid.

Conclusions. **In cultured bovine lens epithelial cells, tonicity-activated movement of myo-inositol from cell to medium and myo-inositol efflux as induced by intracellular polyol accumulation appear to be interactively associated with chloride movement and moderated via a common anionic (chloride) channel and/or carrier-mediated transport protein.**

When extracellular osmotonicity increases, cells shrink because of water loss. Cells subjected to a hypertonic salt environment initially regulate their volume by electrolyte uptake, a transitory reaction. Thereafter, the accumulated electrolyte is replaced with the concentration of compatible "organic osmolytes," in order to counterbalance elevated extracellular osmotonicity and prevent water loss.[1] Three putative organic osmolytes have been identified in bovine lens epithelial cells (BLECs); sorbitol[2], myo-inositol (MI)[3,4] and taurine.[5] Return of hypertonically-adapted BLECs to isotonicity causes cell swelling followed by a gradual return toward original volume via a forfeiture from cell to medium, of (among other osmolytes) myo-inositol, a process termed regulatory volume decrease. Thus, the mechanism of *tonicity-activated* myo-inositol release represents one plausible manipulation which accounts for the depletion of myo-inositol in cultured lens cells, associated with hypertonic insult. The mechanism of tonicity-sensitive organic osmolyte release, however, is poorly characterized.

The etiology of sugar cataractogenesis has been a major focus of lens research. In the past thirty years, investigators have identified multiple metabolic defects associated with the onset and development of sugar cataract. However, the mechanism(s) responsible for the onset of this disorder remain largely speculative. Hyperglycemia is a major contributory factor to the pathophysiological complications of diabetes in humans. In animal models and physiologic surrogates like the bovine lens epithelial cell culture model, the metabolism of excess glucose has been associated with sugar cataract formation. One explanation for sugar cataractogenesis is the activation of glucose to sorbitol by aldose reductase. Yet the precise metabolic dysfunction, initiated by polyol

accumulation, and culminating in the genesis of sugar cataract, remains elusive. Polyols accumulate in diabetic complications-prone tissues and promote intracellular myo-inositol deficit via an alteration of lens cell permeability.[6] Thus, the mechanism of *polyol-activated* myo-inositol release represents a second plausible experimental manipulation which accounts for the depletion of myo-inositol in cultured lens cells, associated with hyperglycemic insult. The mechanism of polyol-sensitive organic osmolyte release is, likewise, poorly characterized.

Cultured BLECs respond to the initial stages (2-6 hours) of sodium hypertonic stress with the upregulation of Na^+- MI cotransporter gene expression[7,8] followed by increased *de novo* synthesis of the Na^+- MI cotransporter protein(s),[9] promoting an intracellular accumulation of myo-inositol. Long-term exposure to sodium hypertonicity (>24 hours) results in enhanced sorbitol formation and accumulation due to upregulation of aldose reductase gene expression.[2,8] The replacement of myo-inositol by polyol as the major intracellular accumulating osmolyte impacts not only on the myo-inositol uptake system,[8,10] but ultimately signals myo-inositol release from cell to medium.

This report is concerned with documenting an association between myo-inositol release from cell to medium by hypertonically adapted cultured lens epithelial cells shifted to isotonic medium compared with myo-inositol efflux due to intracellular formation and accumulation of polyol. Evidence presented indicates that the *tonicity-activated* loss of intracellular myo-inositol and the *polyol-activated* depletion of intracellular myo-inositol are mediated via the same transport pathway. Further, anion channel blockers, as well as chloride substitution from the medium were employed to

clarify whether myo-inositol efflux is associated with chloride movement.

MATERIALS AND METHODS

Cell culture

Anterior lens capsules with attached epithelial cells were aseptically detached from lenses of Bovine (*Bos taurus*) eyes brought to the lab on ice from a local slaughterhouse. Bovine lens epithelial cells (BLECs) were isolated and cultured as previously reported by Cammarata *et al.*[11] in 10 % adult bovine serum-supplemented Eagle's minimal essential medium (MEM, 257 ± 2 mOsm). All culture medium contained 15 μM myo-inositol. Myo-inositol efflux studies were performed with confluent monolayers of cells in 25-cm^2 or 150-cm^2 culture flasks. All studies were performed with cell cultures representing 2nd-passage cells.

Determination of myo-[^{3}H]inositol efflux

Bovine lens epithelial cells grown to near confluence in serum-supplemented isotonic medium (MEM) were switched to sodium hypertonic medium (MEM + 116 mM additional NaCl, 473 ± 6 mOsm), hereafter referred to as SHM, for 48 hours at 37°C in a humidified 5% CO_2-95% air atmosphere. During the latter half of the 48 hours, the cells were preloaded with 0.5 μCi/ml myo-[^{3}H]inositol (Amersham, Arlington Heights, IL). After 24 hours, the cells were rinsed three times (5 ml/rinse) with isotope-free SHM to remove free myo-[^{3}H]inositol.

In the experiment to determine the effect of decreasing medium osmolality and its relationship to myo-inositol release from cell to medium, the cells were switched to 5 ml of fresh Eagle's minimal essential medium supplemented with incremental adjustments of

NaCl to the following osmolarities: 480 ± 4 mOsm (+116 mM), 457 ± 5 mOsm (+92.8 mM), 404 ± 4 mOsm (+69.6 mM), 367 ± 9 mOsm (+46.4 mM), 324 ± 8 mOsm (+23.2 mM) and 279 ± 3.7 mOsm (no additional NaCl supplementation).

For the majority of experiments, when cultured cells were treated with pharmacological agents, an equilibration of 2 hours in SHM, in the presence of each treatment condition was performed. Control cells were transferred to fresh SHM for the same 2 hour period. (Myo-[^{3}H]inositol efflux was monitored at the end of this initial 2 hour equilibration period and was negligible). Thereafter the cells were switched to 5 ml of fresh isotonic MEM *containing 40 mM galactose* (285 ± 4 mOsm, hereafter defined as isotonic galactose medium or IGM) in the continued presence of the treatment condition. The rapid reduction in medium osmolality coupled to the simultaneous exposure of BLECs to 40 mM galactose, permitted an evaluation of both the tonicity-activated and intracellular polyol-exacerbated myo-[^{3}H]inositol efflux mechanisms.

For those experiments designed to determine polyol-activated myo-[^{3}H]inositol efflux in the absence of tonicity-activation, BLECs previously adapted for 48 hours to hypertonicity were transferred to fresh sodium hypertonic medium or sodium hypertonic medium containing 40 mM galactose (513 ± 10 mOsm), hereafter defined as hypertonic galactose medium or HGM. Thus, for these experiments, medium osmolality was not reduced, so that tonicity-activation of the myo-inositol efflux pathway was not initiated.

For efflux measurements in Na^+- or Cl^--free medium , Na^+ was replaced with N-methyl-D-glucamine (NMDG) and Cl^- was replaced with gluconate. The protocol for efflux measurements using the Na^+- and Cl^--free conditions was the same as that

described above with the following medium modifications. For the Na^+-free experiments, control cells previously adapted to hypertonic conditions as described above were transferred to *serum-free* medium consisting of NaCl (116 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), glucose (5.5 mM) and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, 10 mM), pH 7.4 at 322 ± 3 mOsm. For the Na^+-free cells, NaCl was replaced with 136 mM NMDG, pH 7.4 at a final medium osmolarity of 321 ± 6 mOsm. For the Cl^--free experiments, control cells previously adapted to hypertonic conditions were transferred to *serum-free* Eagle's minimal essential medium (MEM, 264 ± 5 mOsm), pH 7.4. For the Cl^--free cells, all chloride salts were replaced by corresponding gluconic acid salts, pH 7.4 at a final medium osmolarity of 273 ± 6 mOsm. Myo-[^{3}H]inositol efflux was monitored for 2 and 4 hours in each respective medium.

The medium was collected for liquid scintillation counting at specified time intervals up to 12 hours. Triplicate sets of flasks were collected for each time point. The efflux medium was removed from each culture flask at the predetermined intervals and transferred to a 15 ml polystyrene centrifuge tube and spun at 2,500g at room temperature for 10 minutes. Aliquots of 1 ml were taken from each centrifuge tube for liquid scintillation counting (Packard Tri-Carb TR1600, Meriden, CT). The culture flasks were drained overnight at 4°C followed by the addition of 5 ml of 2% sodium carbonate in 0.1 N sodium hydroxide for 24 hours to ensure cell lysis. Triplicate 50 µl aliquots were removed from each flask for protein determination by the method of Bradford.[12] Released radioactivity is expressed as pmol myo-inositol per total mg protein in the sample flask.

Determination of intracellular myo-inositol, galactitol and galactose content

Intracellular myo-inositol, galactitol and galactose content were quantified by anion exchange chromatography and pulsed electrochemical detection using a Dionex BioLC chromatography system (Dionex, Sunnyvale, CA) as previously described.[3,10] Bovine lens epithelial cells grown in MEM to partial confluence in 150-cm^2 culture flasks were acclimated for 48 hours in SHM and subsequently incubated with fresh SHM in the presence and absence of 0.2 mM phloretin for an additional 2 hours. The cells were consequently switched to IGM, in the continued presence and absence of 0.2 mM phloretin (Sigma, St. Louis, MO) for 4, 8, and 12 hours before dispersion with trypsin in serum-free isotonic galactose medium and centrifuged at 2500*g* at 4°C for 8 min. The cells were resuspended in 0.9 ml of 0.3 N zinc sulphate (Sigma Chemical, St. Louis, MO) and cell disruption was accomplished by rapid freezing in liquid nitrogen and thawing at 37°C. This process was repeated three times. Thereafter, the samples were transferred to a 5-ml Dounce homogenizer and subjected to five strokes while being maintained in an ice bath. The homogenate was centrifuged at 18,000*g* at 4°C for 20 min, and the cell pellet was saved for protein determination. The supernatant was adjusted to 1 ml with 0.3 N zinc sulphate and 1.0 ml of 0.3 N barium hydroxide (Sigma Chemical, St. Louis, MO) was added. The supernatant was centrifuged at 2,500*g* at 4°C for 8 min and then stored without further modification at -20°C for subsequent myo-inositol, galactose and galactitol analysis.

All inhibitors, if not soluble in water, were dissolved in either ethanol or dimethyl sulfoxide (DMSO) at concentrations no greater than 0.2% of solvent. Control

experiments were performed to monitor the effects of ethanol or DMSO on myo-[^{3}H]inositol efflux from BLECs, resulting in no significant difference ($P > 0.35$) in efflux ± solvents. Medium osmolalities for all experiments were measured using a Wescor, model 5500, vapor pressure osmometer (Wescor, Inc., Logan, UT).

Chemicals

Inhibitors used in this study include: the PKA and PKC inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine•2HCl (H-7), 1-(5-isoquinolinesulfonyl)-homopiperazine•2HCl (HA-1077) and hypericin (Biomol, Plymouth Meeting, PA), the aldose reductase inhibitor, Sorbinil (Pfizer Inc., Groton, CT), the sugar transport/PKC inhibitor, phloretin (Sigma, St. Louis, MO), the anion channel blockers, R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid (IAA-94, Biomol, Plymouth Meeting, PA), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS, Sigma, St. Louis, MO), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, Biomol, Plymouth Meeting, PA), niflumic acid (Sigma, St. Louis, MO) and 1,9-dideoxyforskolin (Calbiochem, San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Statistical analysis

Data are presented as means ± SE. The statistical significance, defined as $P < 0.05$, was evaluated by either one-tailed or two-tailed Student's t test where applicable.

RESULTS

Tonicity-activated myo-[^{3}H]inositol efflux

As shown in Fig. 1, myo-[^{3}H]inositol efflux increased by incremental progression as

hypertonically adapted cells were shifted to media of regressing osmolality. The resultant cell swelling after medium transfer triggered a myo-[^{3}H]inositol release that was transient in character. When medium osmolality was reduced from 480 mOsm to 400 mOsm by removing NaCl, myo-[^{3}H]inositol efflux increased approximately two-fold. Below a medium osmolality of 400 mOsm, the cells responded to the swelling pressure with a dramatically increased myo-[^{3}H]inositol efflux, approaching eight-fold as physiological conditions were realized.

When the extracellular osmolality was reduced from 473 ± 6 mOsm (sodium hypertonic medium, SHM) to 285 ± 4 mOsm (isotonic galactose medium, IGM), approximately 32% myo-[^{3}H]inositol was released into the medium in the first 30 min, and then continued to increase at a slower, but sustained efflux over the next 12 hours (Fig. 2A). Inhibition of galactitol formation by the inclusion of the aldose reductase inhibitor, Sorbinil (0.1 mM) to IGM, significantly decreased myo-[^{3}H]inositol efflux ($P < 0.015$) over the course of 12 hours.

In a separate experiment, hypertonically adapted BLECs transferred to galactose-free, MEM (257 ± 2 mOsm, a condition which would not favor extensive polyol formation) displayed an efflux pattern similar to that of BLECs switched into IGM + Sorbinil (Fig. 2B).

Effect of intracellular galactitol

BLECs maintained in SHM were transferred to fresh sodium hypertonic medium supplemented with 40 mM galactose (referred to as hypertonic galactose medium, HGM). As illustrated in Fig. 3, the high ambient salt environment complemented with galactose

(HGM) stimulated myo-[^{3}H]inositol efflux. By comparison, cells maintained in SHM *without the addition of galactose,* displayed a much reduced myo-[^{3}H]inositol efflux over the course of 8 hours.

Myo-[^{3}H]inositol efflux can be blocked by a putative membrane transport inhibitor

Phloretin is a known inhibitor of sugar transport pathways.[13] To determine whether phloretin could interfere with the myo-inositol efflux pathway, BLECs maintained in SHM (with and without phloretin) were subsequently transferred to IGM in the continued presence or absence of 0.2 mM phloretin, and the level of myo-[^{3}H]inositol in the medium determined. Phloretin significantly blocked myo-[^{3}H]inositol efflux ($P < 0.035$) as compared to cells maintained in phloretin-free, isotonic galactose medium over the 12 hr efflux assay (Fig. 4). Thus, despite conditions which otherwise favored tonicity-activated efflux, an effective treatment to impede myo-[^{3}H]inositol efflux was uncovered by phloretin. A similar experiment was repeated with phloretin utilizing cells maintained in sodium hypertonic medium supplemented with galactose. As illustrated in Fig. 5B, little difference in efflux was observed between hypertonically acclimated BLECs switched to fresh sodium hypertonic medium (SHM) compared with those replaced with SHM and 40 mM galactose ± phloretin, after the initial 2 hour efflux period. However, as intracellular galactitol increased with longer exposure of the cells to the galactose medium, enhancement of myo-[^{3}H]inositol release from cell to medium became increasingly evident after 4 and 8 hours, respectively. The addition of 0.2 mM phloretin to the hypertonic, galactose-supplemented medium *entirely* impeded this polyol-activated mode of myo-[^{3}H]inositol efflux.

Phloretin did not appear to be limiting the uptake of galactose nor interfere with the conversion of galactose to galactitol. Table 1 shows that the intracellular concentrations of galactose and galactitol in BLECs maintained in isotonic galactose medium, in the presence and absence of phloretin, were equivalent as determined by anion exchange chromatography for 4, 8, and 12 hours. The intracellular myo-inositol content of BLECs adapted to hypertonicity and later transferred to IGM in the presence of phloretin was significantly greater than BLECs switched to IGM alone (refer to Table 1), supporting a direct blocking of inositol release.

Phloretin has been classified as a weak protein kinase C inhibitor.[14] In order to rule out the possibility that phloretin was not inhibiting myo-inositol efflux through a secondary effect as a PKC inhibitor, we utilized three recognized protein kinase inhibitors, each at a concentration of 6 μM; HA-1077, H-7 and hypericin.

Hypertonically adapted BLECs were separately exposed to the protein kinase inhibitors for 2 hours prior to transfer to IGM, and thereafter remained in the continued presence of inhibitors, for a 2 hr efflux period. On the assumption that the known PKC inhibitors were effective, the inhibition of PKC had no significant inhibitory effect ($P > 0.05$) on myo-[^{3}H]inositol efflux (Fig. 6). On the other hand, inclusion of 0.2 mM phloretin under the same experimental protocol significantly inhibited efflux ($P < 0.03$).

Blockers of anionic channels inhibit myo-[^{3}H]inositol efflux

Recent studies indicate that volume-sensitive anion channels are involved in myo-[^{3}H]inositol efflux in C6 glioma cells[15-17] and Madin-Darby canine kidney (MDCK) cells.[18] The effect of various anion transport inhibitors on myo-[^{3}H]inositol release was

evaluated with cultured lens epithelial cells. As illustrated in Fig. 7, 0.01 mM IAA-94 or 0.1 mM NPPB or 0.5 mM SITS had no significant suppressive effect ($P > 0.2$) on myo-[^{3}H]inositol release subsequent to medium osmolality reduction. Niflumic acid (0.5 mM), however, did block swelling-induced myo-[^{3}H]inositol release by 23% ($P < 0.05$). Increasing the niflumic acid concentration to 1 mM blocked myo-[^{3}H]inositol release by > 51% (data not shown). 1,9-Dideoxyforskolin (0.1 mM) blocked myo-[^{3}H]inositol efflux by > 33% ($P < 0.02$). In a separate experiment, niflumic acid (0.5 mM) was, likewise, added to hypertonically adapted BLECs for 2 hours prior to, and after transfer to, fresh sodium hypertonic medium containing 40 mM galactose (HGM). As shown in Fig. 5A, a significant inhibition of myo-[^{3}H]inositol efflux (27%, $P < 0.005$) was observed after an 8 hr efflux period.

Effect of ion substitutions on myo-[^{3}H]inositol efflux

Tonicity-activated myo-[^{3}H]inositol release was essentially unchanged by replacement of Na^+ with NMDG (Fig. 8A). Na^+ substitution with NMDG marginally increased myo-[^{3}H]inositol efflux by 7-9% after a 4 hour efflux period. Myo-inositol efflux was markedly suppressed by substitution of Cl^- with gluconate (Fig. 8B). Replacement of Cl^- with gluconate curtailed myo-[^{3}H]inositol efflux by 30%.

DISCUSSION

To define the underlying mechanisms that activate the myo-inositol efflux pathway in lens cells, protocols were designed to; (1) stimulate or inhibit myo-inositol release from hypertonically adapted cells when the extracellular osmolality was reduced (tonicity-activation) and (2) inhibit myo-inositol release from galactose-supplemented

hypertonically adapted cells under conditions where medium osmolality was not altered but polyol accumulation was favored (polyol-activation). Thus, our experimental manipulations assured that we could evaluate whether a common transport pathway modulates the release of myo-inositol from cell to medium by *independently* monitoring tonicity-activated inositol efflux and polyol-activated inositol efflux. While this experimental approach is indirect, it was reinforced by the fact that several treatment conditions analogously inhibited both *tonicity-activated* and *polyol-activated* myo-inositol efflux.

Cellular model

When cultured bovine lens epithelial cells were transferred from sodium hypertonic medium to an isotonic medium, the immediate response to the decreased osmolality was a rapid release of myo-[^{3}H]inositol from the cells followed by a slower, sustained inositol efflux (Fig. 2). Thus, reduction in medium osmolality with resultant cell swelling causes a tonicity-activated *biphasic* movement of myo-[^{3}H]inositol from cell to medium. An analogous result has previously been shown for sorbitol release by Bagnasco *et al.*[19] using a continuous line of epithelial cells from rabbit inner medulla. Biphasic myo-inositol release from cell to medium has previously been demonstrated by Nakanishi and Burg[20] with MDCK cell cultures, Yamauchi *et al.*[21] with MDCK cells grown on filters, and more recently, Strange et al.[15] with C6 glioma cell cultures. The present study extends these earlier efforts in an important and major way. The above mentioned studies dealt solely with the characterization of a swelling-activated (which we operationally refer to as tonicity-activated) myo-inositol transport pathway. In the current study myo-inositol

efflux resulting from polyol-activation (i.e. myo-inositol release as mediated by the intracellular osmotic stress initiated by polyol accumulation) was jointly characterized and contrasted with the tonicity-activated mode of myo-inositol release. As illustrated in Fig. 2, it is noteworthy that the pattern of myo-[^{3}H]inositol release from hypertonically adapted cells transferred to IGM or IGM + Sorbinil was indistinguishable during the initial 30 min efflux period. This was likely due to the fact that an insufficient level of galactitol had been synthesized, so that a considerable intracellular osmotic stress had yet to be achieved. Thereafter, as intracellular galactitol accumulated, a greater release of myo-inositol was evident from those cells maintained in the galactose-supplemented medium in the absence of aldose reductase inhibitor as compared with the cells treated with the aldose reductase inhibitor. These data clearly document that the formation and accumulation of intracellular galactitol further exacerbates the myo-[^{3}H]inositol efflux already stimulated by cell swelling attributable to the medium osmolality reduction. Tonicity-induced myo-inositol release from hypertonically adapted BLECs transferred to *galactose-free,* isotonic medium (minimal essential medium, MEM) was similar to the degree of myo-inositol release into *galactose-containing*, isotonic medium *in the presence of an aldose reductase inhibitor* (Fig. 2B). That is, tonicity-induced myo-inositol release is independent of galactitol-induced myo-inositol release. Furthermore, as illustrated in Fig. 3, an increase in intracellular polyol formation, *in the absence of medium osmolality reduction*, likewise, stimulated polyol-activated myo-[^{3}H]inositol flux from cell to medium. Such data not only establishes that inositol movement from cell to medium is relatively inactive in the "resting" hypertonically adapted cells, but also that

the inclusion of 40 mM galactose to SHM, a situation which initiates rapid galactitol formation, triggers the polyol-activated inositol release mechanism. Therefore, both the cellular swelling which results from a rapid reduction in medium tonicity *and* intracellular osmotic stress due to polyol formation and accumulation effectively stimulate myo-inositol transport from cell to medium. It remained to be determined whether the routes of inositol efflux initiated by the two autonomous sources share a common permeability pathway.

Inhibition of myo-[^{3}H]inositol release by phloretin

The inhibitory effects of 0.2 mM phloretin on tonicity-induced (Fig. 4) and polyol-induced (Fig. 5B) myo-[^{3}H]inositol release is noteworthy because it provided a useful tool for further experimentation. At present, it is not clear if the observed inhibition of inositol release from cultured BLECs by phloretin is due to its direct interaction with a carrier and/or channel protein or an indirect secondary effect. One possible explanation for phloretins' inhibitory effect on inositol efflux could be due to the fact that it is a known inhibitor of sugar transport pathways.[13] That is, phloretin might be acting by limiting the uptake of galactose, which, in turn, might result in a diminished intracellular pool of galactitol due to the reduction of substrate availability. If such a reduction in the formation and accumulation of galactitol were to occur, it would relieve intracellular polyol-induced osmotic stress. This, in turn, would lessen the quantity of myo-inositol necessary to be released in order for the cell to achieve equilibrium with the extracellular medium, in essence giving the *appearance* that myo-inositol efflux was directly blocked. Table 1 shows that the intracellular concentration of galactose in BLECs maintained in

isotonic galactose medium in the presence and absence of phloretin was equivalent as determined by anion exchange chromatography for 4, 8, and 12 hours. Likewise, the conversion of galactose to galactitol appeared unaffected by the phloretin treatment. It was noted, however, that the intracellular galactose content decreased with time (despite the continued presence of galactose in the medium), irrespective of whether phloretin was included or omitted. Galactitol accumulation, on the other hand, readily increased over the course of the 12 hours. Under these experimental conditions, aldose reductase gene expression is upregulated.[8] It is highly probable that the rate of conversion of galactose to galactitol (an upregulated enzymatic reaction) exceeds the rate of galactose uptake (a carrier-mediated reaction). Thus, it is not likely that phloretin is acting indirectly to block myo-inositol release by lowering intracellular osmotic stress through diminished galactose uptake or reduced polyol formation. That is, phloretin does not appear to be functioning as a sugar membrane transport inhibitor (nor an inhibitor of aldose reductase) under these experimental conditions. Indeed, the intracellular myo-inositol content of BLECs adapted to hypertonicity and later transferred to IGM in the presence of phloretin was significantly greater than BLECs switched to IGM alone (refer to Table 1), suggesting a direct blocking of inositol release. Alternatively, phloretin has been classified as a protein kinase C inhibitor.[14] However, the positive effect of phloretin to suppress inositol release compared with the negative effect by several other protein kinase inhibitors (Fig. 6) indicate that phloretin is likely not functioning as a protein kinase inhibitor. The collective data constitute a powerful argument that phloretin disrupts myo-inositol efflux by directly blocking a myo-inositol transport or channel

protein.

Inhibition of myo-[^{3}H]inositol release by anion channel blockers

Banderali and Roy[22] postulated that a volume-sensitive anion channel, described in MDCK cells, might account for the swelling-induced release of organic osmolytes, including sorbitol and myo-inositol. Cell volume regulation is the proposed function for the rapid release of organic osmolytes. When extracellular osmolality decreases, cells swell because of the influx of water by osmosis. As cells return to their normal volume, a rapid efflux of electrolytes, followed by a loss of organic solutes ensues. As there is no evidence for *active* transport of organic osmolytes out of the cell, the mechanism of accelerated efflux is likely an increase in permeability via channel (or carrier protein) activation. The ability of the anion channel blockers niflumic acid[23] and 1,9-dideoxyforskolin[24] to suppress myo-inositol release in response to extracellular medium osmolality reduction (i.e. tonicity-activated inositol efflux) indirectly supports this hypothesis and suggests a role for chloride channels (Fig. 7). Similar observations have been reported with C6 glioma cells.[15] With cultured nonpigmented ciliary process epithelial cells, the application of niflumic acid, likewise, slowed the regulatory volume decrease response after reduction of medium osmolality.[25] It is noteworthy to mention that, to our knowledge, ours is the first report (Fig. 5A) that an anion channel blocker effectively suppressed myo-inositol release responding to intracellular osmotic stress induced by polyol accumulation (i.e. polyol-activated inositol efflux). The precise mechanism by which niflumic acid treatment suppressed myo-inositol efflux cannot be established with this data. Preincubation with niflumic acid for 2 hours might have

inhibited a chloride channel, reducing anion efflux, potentially increasing the steady-state cell electrolyte content and cell volume. It is possible that the myo-inositol efflux pathway is responsive to the absolute cell volume rather than the degree of cell volume change. The observed reduction in myo-inositol release might be a secondary effect linked to total intracellular electrolytes and intracellular volume.

Recently, a role for chloride channels in the maintenance of lens clarity was established. It was reported that tamoxifen, a specific blocker of cell volume-regulated chloride channels, induced lens opacity in organ culture consistent with cataractogenesis.[26] Our data, and data from numerous other laboratories, manifests a relationship between hypertonicity, volume regulation, organic osmolyte efflux and chloride channels. We stress, however, that the present data does not permit a determination as to whether myo-inositol efflux and chloride movement are cotransported via a *shared* channel or, rather, are jointly fluxed via two independent pathways working in concord. Given the current data, we would not rule out the possibility that some aspect of the observed myo-inositol efflux pattern might be contributed to by a facilitated-diffusional carrier-mediated inositol transport protein as recently described in cultured renal epithelial cells.[27] That transport protein was reported to be bidirectional and phloretin-sensitive.

Inhibition of Myo-[^{3}H]inositol Release by Ion Substitution

Tonicity-activated myo-inositol efflux was inhibited partially by substitution of extracellular Cl^- but was unaffected essentially by substitution of extracellular Na^+. Myo-inositol efflux from cultured bovine lens epithelial cells is Na^+-independent and probably

proceeds via a passive gradient as has recently been reported for intact lens.[28] The mechanism by which Cl^- substitution inhibits myo-inositol efflux is unclear. Removal of Cl^- ions likely causes some cell shrinkage. Our experimental approach does not permit us to determine the degree to which Cl^--free medium affects the magnitude of cell swelling. Possibly the efflux pathway is sensitive to the removal of Cl^- by affecting intracellular pH, which, may in turn, regulate the efflux pathway. Nevertheless, these data lend further support to the notion that myo-inositol movement from cell to medium is associated with Cl^- movement. In summation, the results of this study argue that a common transport pathway mediates the efflux of myo-inositol from cultured bovine lens epithelial cells, regardless of whether release is stimulated by tonicity-activation or polyol-activation. Anion channel blocker and Cl^- substitution studies suggest that the common transport route is affiliated with chloride movement.

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Figure 1. Effect of decreasing medium osmolality on myo-[^{3}H]inositol efflux. Data points from left to right represent Eagle's minimal essential medium supplemented with decreasing incremental adjustments of NaCl. Data points represent mean ± SE of individual samples from triplicate flasks after a 4-hour efflux. Note that 480 mOsm typically is referred to in the text as sodium hypertonic medium, whereas a range of 255 to 285 mOsm is referred to as isotonic medium.

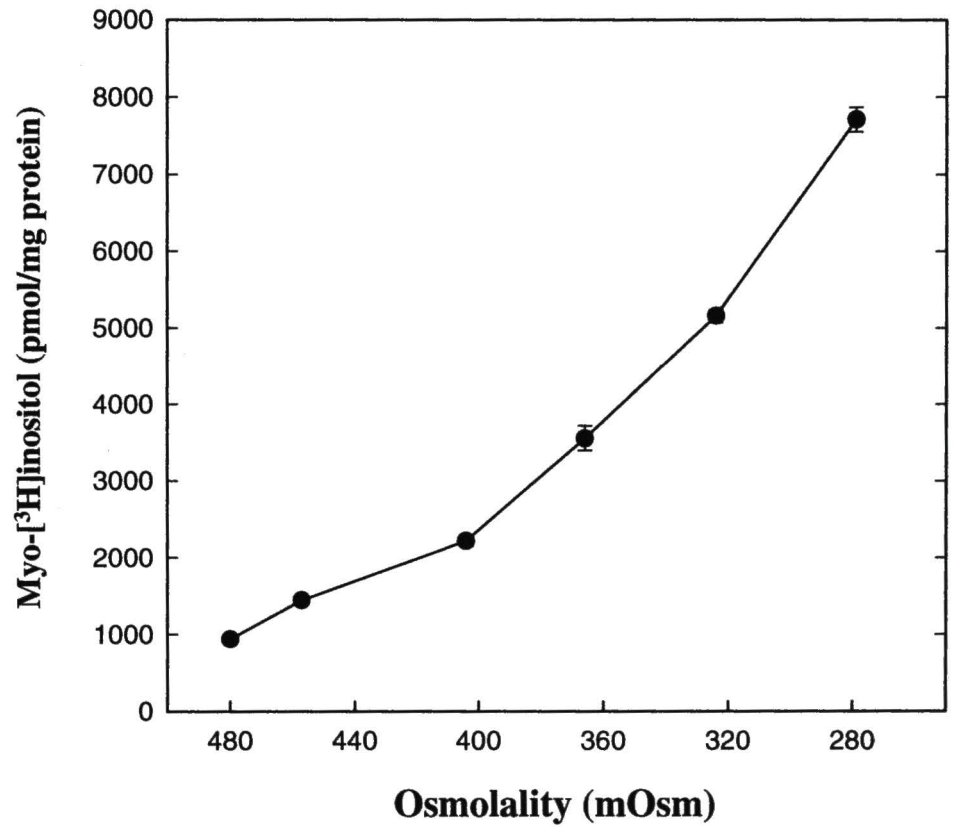
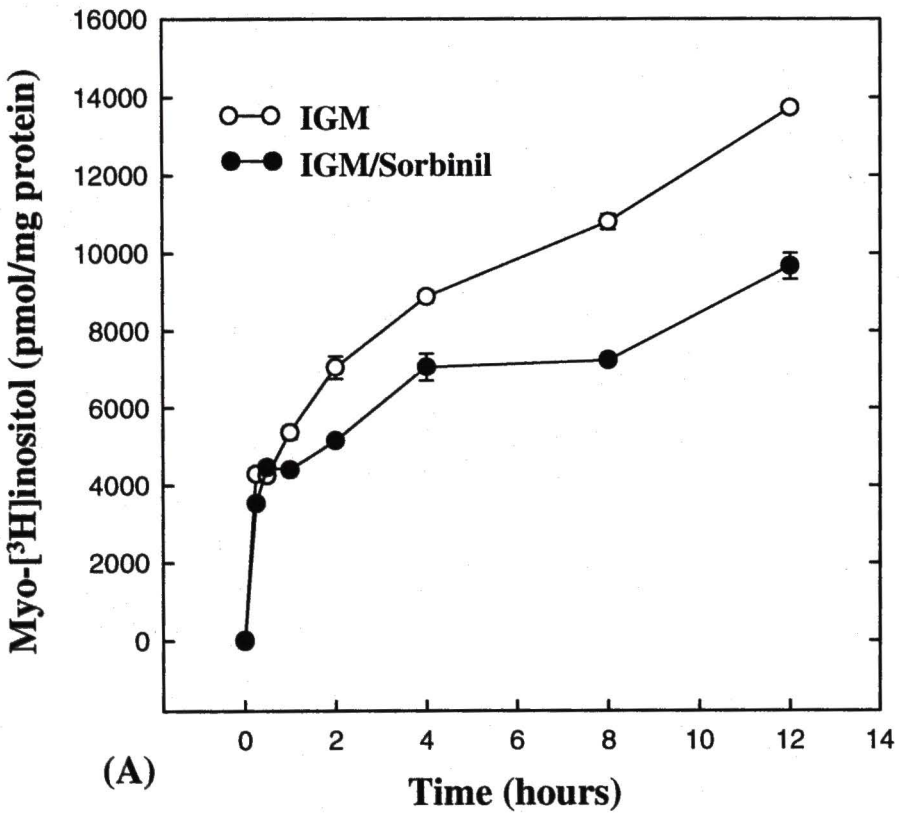

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Myo-[3H]inositol (pmol/mg protein)
480
440
400
360
320
280
Osmolality (mOsm)

Figure 2. Biphasic myo-[^{3}H]inositol efflux. (A) The hypertonic medium was replaced with isotonic medium containing 40 mM galactose (285 ± 4 mOsm) and myo-[^{3}H]inositol efflux monitored over a 12 hour efflux assay period. (B) The hypertonic medium was replaced with either galactose-free isotonic medium (257 ± 2 mOsm) or isotonic medium containing 40 mM galactose (285 ± 4 mOsm) ± Sorbinil and myo-[^{3}H]inositol efflux monitored at 2 and 4 hours. The data points (or bars) represent released myo-[^{3}H]inositol measured from preloaded cells collected at the designated time points. Data points represent mean ± SE taken from individual samples of triplicate flasks. Isotonic galactose medium is represented by IGM. MEM = galactose-free isotonic medium (minimal essential medium).


16000
14000
12000
10000
8000
6000
4000
2000
0
Myo-[3H]inositol (pmol/mg protein)
IGM
IGM/Sorbinil
0
2
4
6
8
10
12
14
Time (hours)
(A)

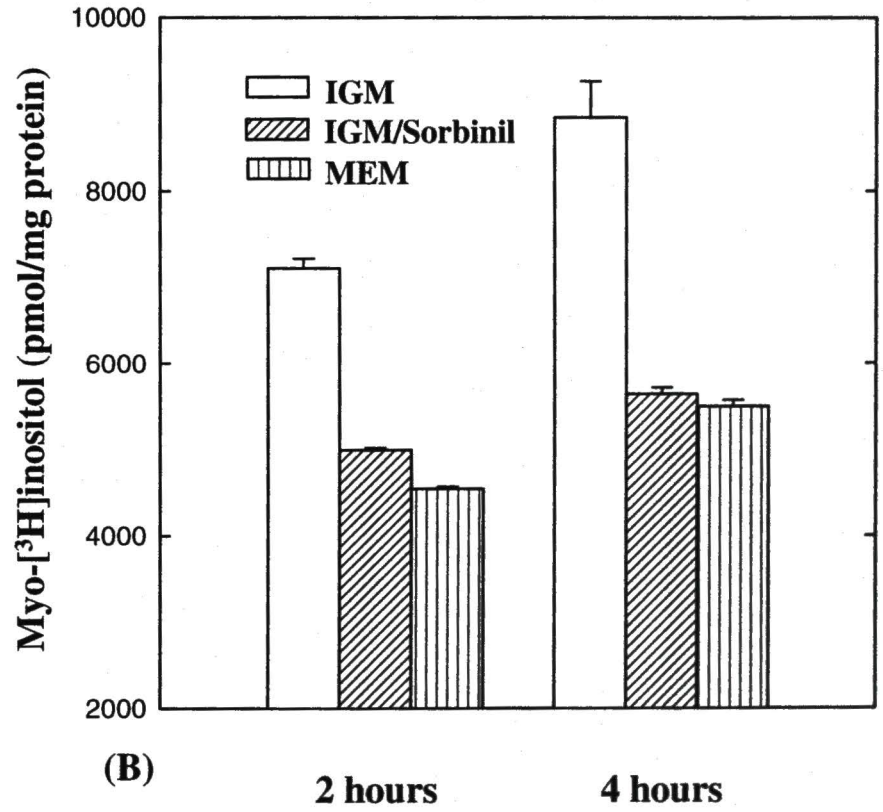

10000
8000
6000
4000
2000
Myo-[3H]inositol (pmol/mg protein)
IGM
IGM/Sorbinil
MEM
2 hours
4 hours
(B)

Figure 3. Influence of intracellular galactitol accumulation on myo-[^{3}H]inositol efflux. Bovine lens epithelial cells acclimated in sodium hypertonic medium (473 ± 6 mOsm) were transferred to fresh sodium hypertonic medium or hypertonic galactose medium. Data points represent released myo-[^{3}H]inositol measured from preloaded cells collected at the designated time points over an 8 hour period. Data points represent the mean ± SE of individual samples of triplicate flasks. SHM = sodium hypertonic medium; HGM = hypertonic galactose medium.

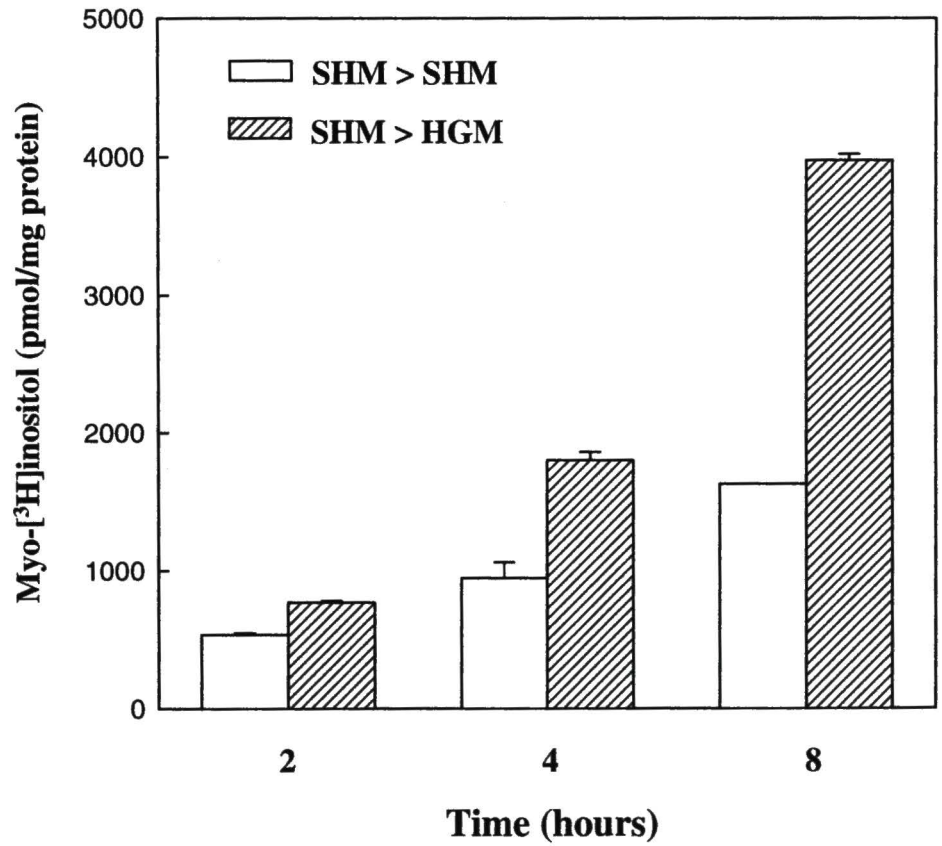

5000
4000
3000
2000
1000
0
SHM > SHM
SHM > HGM
Myo-[3H]inositol (pmol/mg protein)
2
4
8
Time (hours)

Figure 4. Effect of phloretin on tonicity-activated myo-[^{3}H]inositol efflux. Bovine lens epithelial cells acclimated for 2 hours in sodium hypertonic medium containing 0.2 mM phloretin were transferred to isotonic galactose medium in the continued presence of phloretin, and myo-[^{3}H]inositol efflux was monitored over a 12 hour period. Data points are mean ± SE and represent single determinations from triplicate flasks.

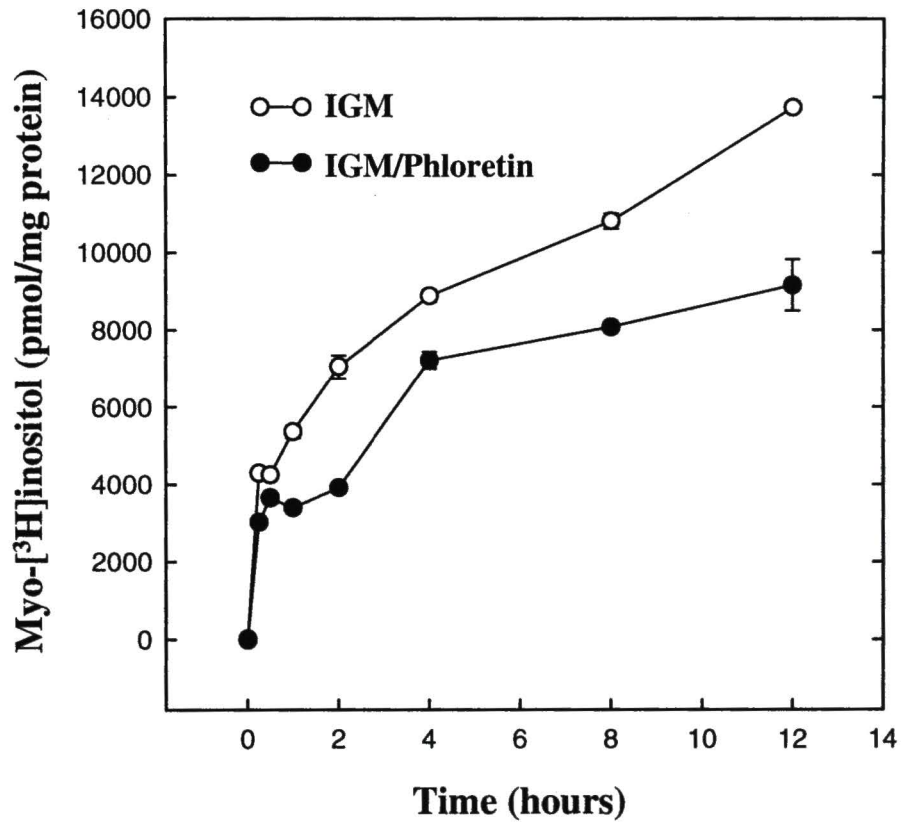
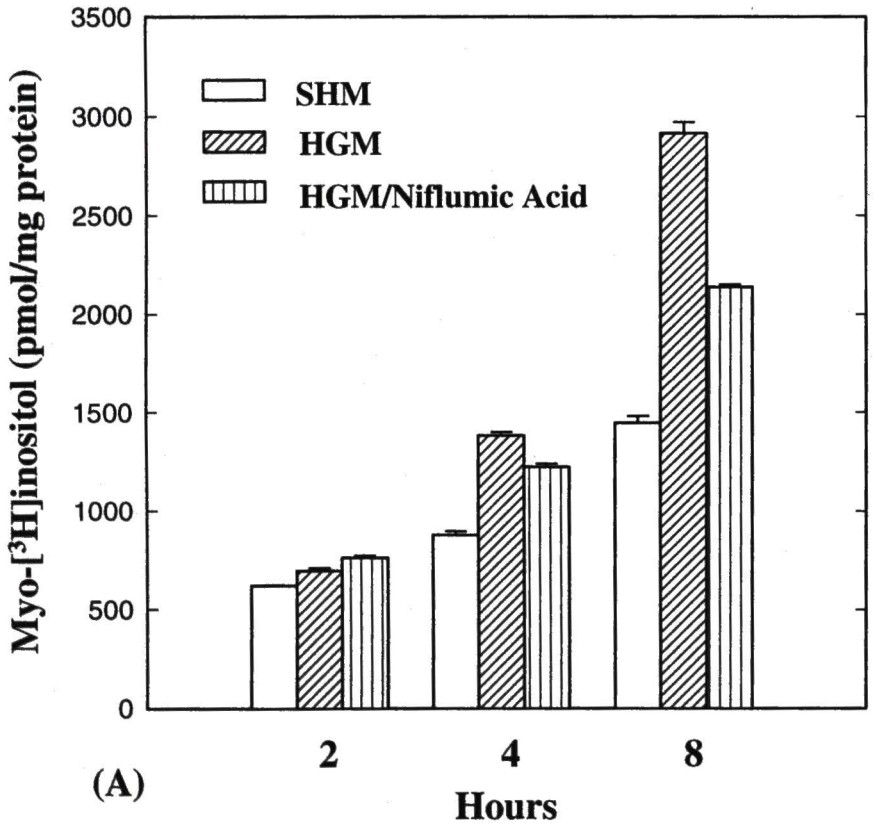

16000
14000
12000
10000
8000
6000
4000
2000
0
IGM
IGM/Phloretin
Myo-[3H]inositol (pmol/mg protein)
0
2
4
6
8
10
12
14
Time (hours)

Figure 5. Effect of niflumic acid and phloretin on polyol-activated myo-[^{3}H]inositol efflux. Bovine lens epithelial cells acclimated for 2 hours in sodium hypertonic medium containing either the chloride channel inhibitor, niflumic acid (0.5 mM, Fig. 5A) or phloretin (0.2 mM, Fig. 5B) were transferred to hypertonic galactose medium (HGM) in the continued presence of niflumic acid or phloretin, and myo-[^{3}H]inositol efflux monitored at 2, 4 and 8 hours. Data represents mean ± SE from individual samples taken from triplicate flasks.


Myo-[3H]inositol (pmol/mg protein)
3500
3000
2500
2000
1500
1000
500
0
SHM
HGM
HGM/Niflumic Acid
2
4
8
Hours
(A)

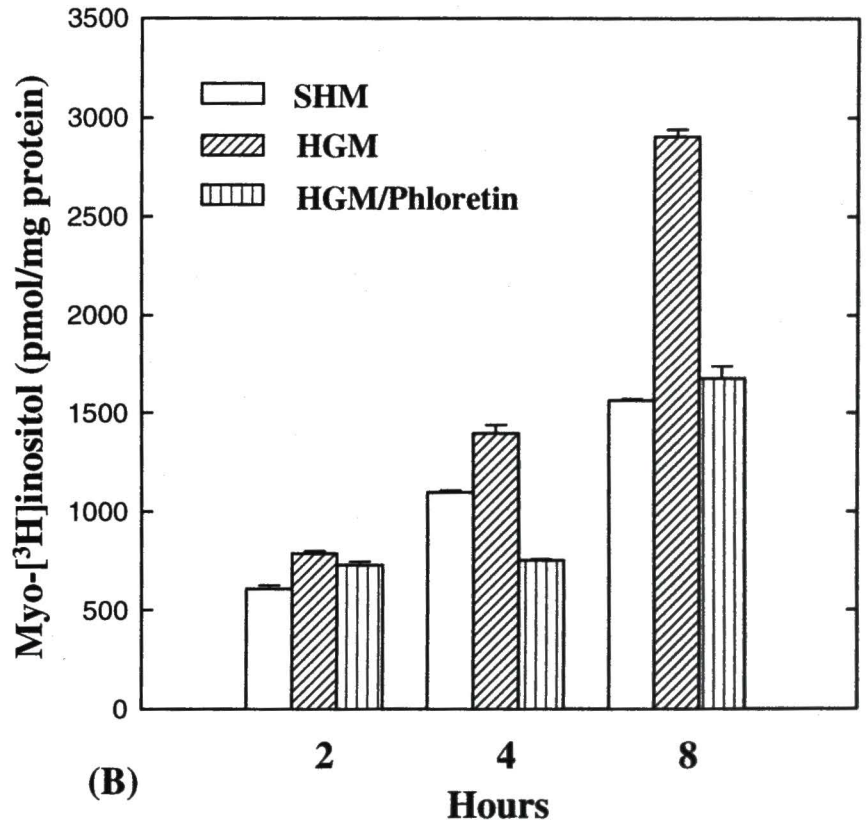

Myo-[3H]inositol (pmol/mg protein)
3500
3000
2500
2000
1500
1000
500
0
SHM
HGM
HGM/Phloretin
2
4
8
Hours
(B)

Figure 6. Effect of protein kinase inhibitors on myo-[^{3}H]inositol efflux. Bovine lens epithelial cells acclimated for 2 hours in sodium hypertonic medium containing 6 μM of the protein kinase inhibitors HA-1077, H-7, or hypericin were transferred to isotonic galactose medium (IGM), and myo-[^{3}H]inositol efflux monitored for a 2-hour efflux period. Phloretin (0.2 mM) was included with one set of cells as a positive control. Data are means ± SE from triplicate flasks for each condition.

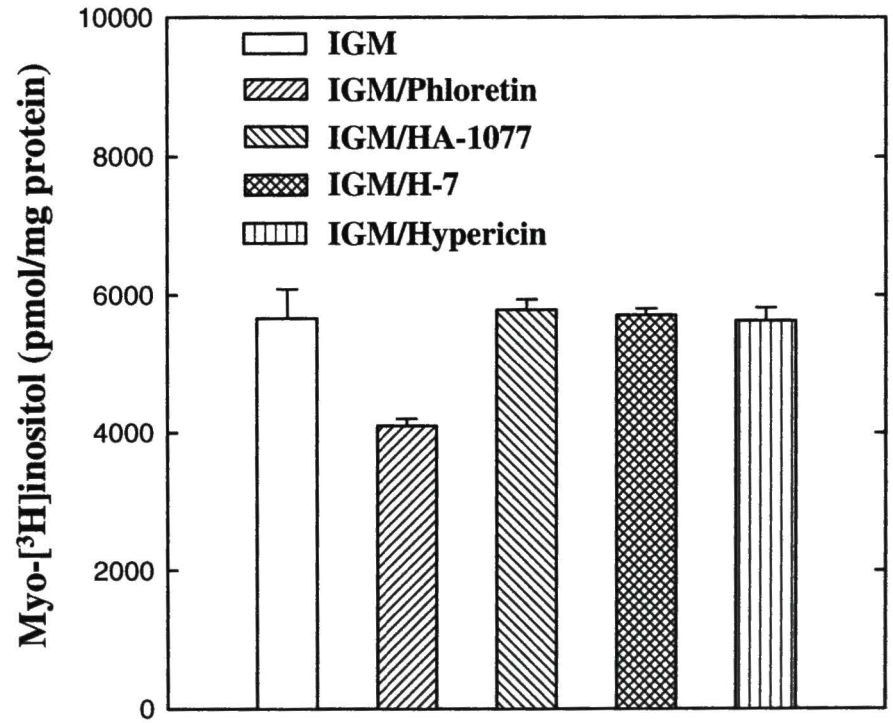

10000
8000
6000
4000
2000
0
Myo-[3H]inositol (pmol/mg protein)
IGM
IGM/Phloretin
IGM/HA-1077
IGM/H-7
IGM/Hypericin

Figure 7. Effect of chloride channel inhibitors on tonicity-activated myo-[^{3}H]inositol efflux. Bovine lens epithelial cells were acclimated for 2 hours in sodium hypertonic medium with the chloride channel inhibitors; IAA-94 (0.01 mM), SITS (0.5 mM), NPPB (0.1 mM), niflumic acid (0.5 mM) or 1,9-dideoxyforskolin (0.1 mM). Cells were transferred to isotonic galactose medium (IGM), in the continued presence of inhibitors and myo-[^{3}H]inositol efflux monitored for a 2 hour efflux period. Data are mean ± SE from individual samples taken from triplicate flasks.

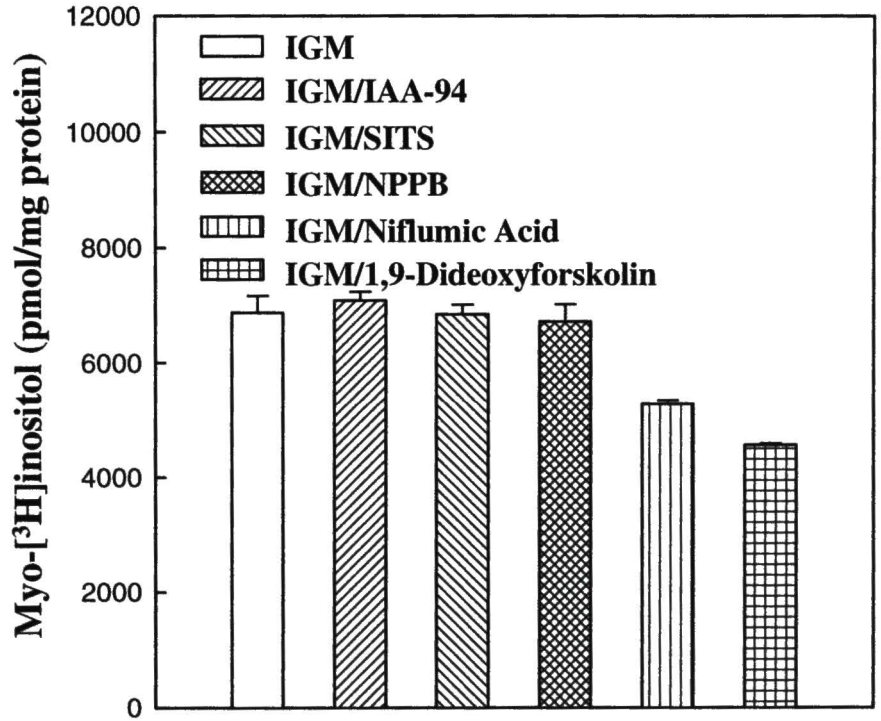
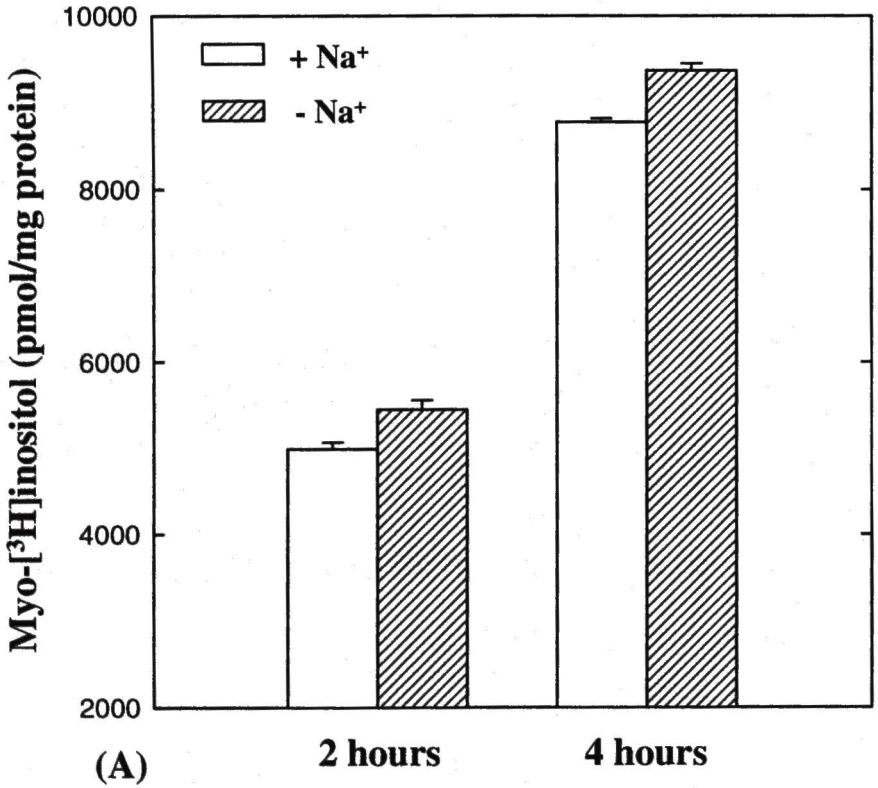

Myo-[3H]inositol (pmol/mg protein)
12000
10000
8000
6000
4000
2000
0
IGM
IGM/IAA-94
IGM/SITS
IGM/NPPB
IGM/Niflumic Acid
IGM/1,9-Dideoxyforskolin

Figure 8. Effects of ion substitutions on tonicity-activated myo-[^{3}H]inositol efflux. Bovine lens epithelial cells previously adapted to sodium hypertonicity for 48 hours were transferred to (A) Na^+-free medium substituted with N-methyl-D-glucamine or (B) Cl^--free medium substituted with gluconate. Myo-[^{3}H]inositol efflux measurements were monitored after 4 hours in each respective medium. Values are mean ± SE from individual samples from triplicate flasks.


10000
8000
6000
4000
2000
+ Na+
- Na+
Myo-[3H]inositol (pmol/mg protein)
2 hours
4 hours
(A)

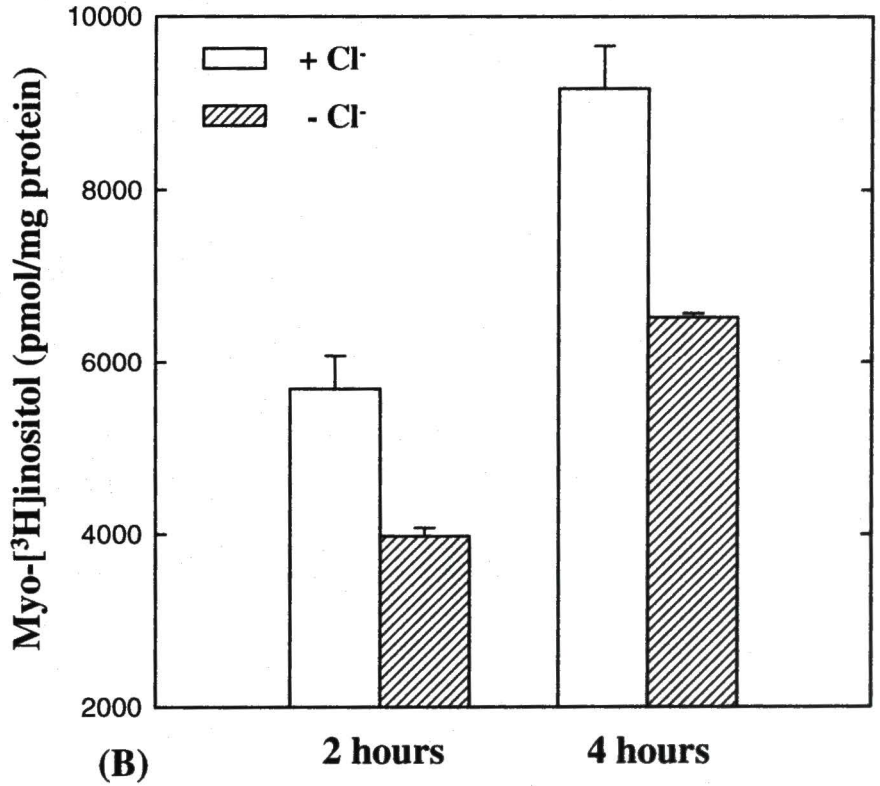

10000
8000
6000
4000
2000
+ Cl-
- Cl-
Myo-[3H]inositol (pmol/mg protein)
2 hours
4 hours
(B)

TABLE 1. Effect of Phloretin on Intracellular Myo-inositol, Galactose, and Galactitol Content

	Myo-inositol	Galactose	Galactitol
4 hours			
IGM	47.3 ± 1.1	496.9 ± 4.7	173.3 ± 8.2
IGM + phloretin	76.7 ± 5.5*	701.5 ± 14.6	183.2 ± 18.2
8 hours			
IGM	37.7 ± 5.2	431.7 ± 14.9	293.8 ± 14.1
IGM + phloretin	59.8 ± 1.1*	576.0 ± 22.5	276.6 ± 13.5
12 hours			
IGM	28.8 ± 1.8	339.0 ± 28.8	335.8 ± 12.6
IGM + phloretin	53.5 ± 0.3*	517.4 ± 15.6	342.4 ± 30.7

Values were acquired by anion exchange chromatography and are means ± SE expressed as nmol/mg protein. Data points represent duplicate determinations from triplicate flasks.
* Significant difference by independent *t*-test ($P < 0.05$).

Upon demonstrating a relationship between the pathways of chloride and osmolyte efflux in cultured bovine lens epithelial cells, the following study was undertaken to evaluate the mRNA expression of the protein, pI_{Cln}. This protein has been shown to be swelling-sensitive and is believed to be involved in cell volume regulation and anion/osmolyte movement in cells. The response of pI_{Cln} mRNA expression in relation to changes in cell volume and/or intracellular osmolality was evaluated.

CHAPTER III

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Expression of pI_{Cln} mRNA in Cultured Bovine Lens Epithelial Cells: Response to Changes in Cell Volume

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Swelling-activated myo-inositol efflux has recently been linked to anion channels and Cl^- movement in cultured bovine lens epithelial cells (BLECs). In this study, the relationship between fluctuations in cell volume and/or intracellular osmolality and mRNA expression of pI_{Cln} in BLECs was determined. To demonstrate the effect of cell volume changes on pI_{Cln} transcription, BLECs were exposed to either hypertonic or hypotonic medium conditions. For rapid cellular shrinkage, BLECs were maintained at confluence in physiologic medium (MEM, 257 ± 2 mOsm) then transferred to sodium hypertonic medium (SHM = MEM + 116 mM NaCl, 473 ± 6 mOsm) or MEM + 150 mM raffinose (Raf, 452 ± 2 mOsm). For rapid cellular swelling, cells were switched from SHM to MEM. Upon cell volume reduction (increasing intracellular osmolality), BLECs upregulate the expression of pI_{Cln} mRNA. Contrastly, when cell volume rapidly increases (decreasing intracellular osmolality), BLECs moderately downregulate pI_{Cln} mRNA, with expression levels reaching near physiologic control by 24 hours. These data suggest a converse relationship exists between pI_{Cln} mRNA expression and cell volume changes.

1. Introduction

Cells gain or lose osmolytes through distinct membrane transport/channel pathways which are spontaneously actuated by fluctuations in cell volume and intracellular osmolality. Exposure to hypertonicity rapidly shrinks cultured cells, initially concentrating internal solutes via the transitory uptake of inorganic ions but subsequently followed by the active accumulation from surrounding medium to cell of small organic molecules, collectively identified as, "organic osmolytes," which includes among others; myo-inositol, sorbitol, taurine, and betaine (Nakanishi et al., 1989). Conversely, hypotonicity causes rapid cell swelling and the loss from cell to medium (i.e., efflux) of organic osmolytes and inorganic ions, such as K^+ and Cl^- (Chamberlain and Strange, 1989; Sarkadi and Parker, 1991). This gain or loss of osmolytes in response to cell volume fluctuation constitutes an essential component of the volume regulatory mechanisms associated with cellular regulatory volume increase (RVI) and decrease (RVD).

Early studies regarding swelling-activated (hypotonic-induced) efflux of organic osmolytes from cell to medium suggested that osmolyte loss is mediated through anion channels (Kimelberg et al., 1990; Roy and Malo, 1992; Banderali and Roy, 1992; Kirk et al, 1992). A role for anion channels regarding volume regulatory functions associated with a swelling-activated Cl^--conductance mechanism (Yantorno et al., 1992; Civan et al., 1994) has been suggested in cultured human ciliary epithelial cells. Volume-sensitive anion channels have been associated with myo-inositol efflux in cultured C6 glioma cells (Strange et al., 1993; Jackson and Strange, 1993) and cerebellar astrocytes (Gonzalez et

al., 1995). We recently linked anion channels, chloride movement and myo-inositol efflux in cultured bovine lens epithelial cells [(BLECs), Reeves and Cammarata, 1996].

A channel classified as the volume-sensitive organic osmolyte-anion channel (VSOAC) has been associated with swelling-activated whole cell anion current and organic osmolyte efflux in rat C6 glioma cells (Jackson et al., 1994). VSOAC has been been compared to a regulator/channel protein, pI_{Cln}. While the two display striking similarities, verification that "pI_{Cln} is either the VSOAC channel, a functional subunit of the channel, or a channel regulator" remains to be demonstrated (Strange et al., 1996). A cDNA coding for, pI_{Cln}, has been cloned and overexpressed in *Xenopus* oocytes (Paulmichl et al., 1992). These experiments led to the conclusion that an outwardly rectifying anion conductance is: (1) blocked by anion channel inhibitors and external ATP, (2) inactivated by strong depolarization, and 3) anion selective in the order $SCN^- > I^- > Br^- > Cl^-$ (Paulmichl et al., 1992; Strange et al., 1996). The significant similarities between pI_{Cln} and VSOAC has sparked considerable controversy. What is the role of pI_{Cln} in the swelling-activated anion/osmolyte loss from cells? One school of thought suggests that pI_{Cln} is the VSOAC channel or a funcional subunit of the channel (Paulmichl et al., 1992; Strange et al., 1996). Another proposal (Krapivinsky et al., 1994) suggests that pI_{Cln} is a channel regulator instead of being the anion channel, itself. Studies with nonpigmented ciliary epithelial cells lends support to the latter proposal by suggesting that pI_{Cln} regulates volume-sensitive Cl^- movement, possibly through the Cl^- channel ClC-3 (Coca-Prados et al., 1996). Irrespective of interpretation, both groups of workers strongly imply that pI_{Cln} is involved in the cellular process of anion/osmolyte efflux and

cell volume regulation. With this in mind, the aim of the present study was to determine whether fluctuations in cell volume or intracellular osmolality altered mRNA expression of pI_{Cln} in cultured BLECs. Also, the effect of chloride channel inhibitors on pI_{Cln} mRNA expression was evaluated.

2. MATERIALS AND METHODS

Cell Culture

Bovine (*Bos taurus*) eyes obtained from a local slaughterhouse were brought to the lab on ice and the anterior lens capsules with attached epithelial cells were aseptically removed from the lenses. Isolation and culture of bovine lens epithelial cells (BLECs) were performed as previously described (Cammarata et al., 1988). Briefly, cells were maintained in Eagle's minimal essential medium (MEM, 257 ± 2 mosm) supplemented with 10% adult bovine serum at 37°C in a humidified 5% CO_2-95% air atmosphere. Unless otherwise stated, the culture medium contained 15 μM myo-inositol. All Northern analysis, determination of intracellular polyol content, and myo-inositol efflux/influx studies were performed with confluent monolayers of 2nd-passage cells in either 25-cm^2, 75-cm^2 or 150-cm^2 culture flasks.

Northern Blot Analysis

Poly $(A)^+$ RNA from cultured BLECs was isolated as previously described (Zhou et al., 1994) by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Briefly, purified lens epithelial cell poly $(A)^+$RNA was seperated by electrophoresis in a 1.0% agarose-formaldehyde denaturing gel as described (Lehrach et al., 1979) and then blotted

to Hybond-N nylon membrane (Amersham Life Science, Arlington Heights, IL) in 20X SSC for approximately 16 hours. After blotting, the nylon membrane was baked for 1 hour at 80°C under vacuum and then cross-linked using a UV crosslinker (Fisher Scientific, Pittsburgh, PA). The blots were hybridized with either a α-[^{32}P]-labeled 456-bp pI_{Cln} cDNA or a 2.2 Kb rat brain ClC-3 cDNA. Then the blots were stripped and rehybridized with β-actin genomic DNA (Nudel et al., 1983). The probes were labeled with Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) using previously described procedures (Agarwal et al., 1990). Next, the blot was washed for two 20 minute periods in 2X SSC with 0.1% SDS at 42°C followed by two more 20 minute washes in 0.2X SSC with 0.1% SDS at 42°C in a hybridization oven. The blots were then subjected to fluorography using an intensifying screen and Hyperfilm (Amersham, Arlington Heights, IL) at -80°C. The relative densitometric intensity (RDI) of lens cell pI_{Cln} mRNA, normalized to β-actin mRNA, was determined by scanning densitometry using Diversity One software from Protein and DNA ImageWare (PDI) Systems (Huntington Station, NY).

Determination of Myo-[^{3}H]inositol Efflux

Myo-[^{3}H]inositol efflux was determined according to previously described procedures (Reeves and Cammarata, 1996). In brief, BLECs were grown to near confluence in physiological medium (MEM, 257 ± 2 mOsm) then switched to sodium hypertonic medium (MEM + 116 mM additional NaCl, 473 ± 6 mosm), hereafter referred to as SHM, for 48 hours with a concurrent preload of cells with 0·5 μCi/ml myo-[^{3}H]inositol (Amersham, Arlington Heights, IL) during the final 24 hours. Each flask was then rinsed

three times (5 ml/rinse) with isotope-free SHM to remove free myo-[^{3}H]inositol from the cells.

To determine the inhibitory effect of niflumic acid or tamoxifen (Sigma, St. Louis, MO) in a dose dependent manner on swelling-activated myo-[^{3}H]inositol efflux, hypertonically acclimated BLECs were maintained in SHM for a 2 hour incubation period in the presence of either drug. In order to correct for myo-inositol leakage during this 2 hour period, myo-[^{3}H]inositol efflux was monitored in cells transferred to fresh SHM for 2 hours and found to be negligible. Thereafter, to promote rapid cell swelling, the cells were switched to 5 ml of fresh MEM (257 ± 2 mOsm) in the continued presence of their respective treatment conditions for 2 hours. By exposing BLECs to hypotonic medium conditions, the effect of increasing concentrations of these drugs on swelling-activated myo-[^{3}H]inositol efflux was evaluated.

Efflux medium collected from individual flasks was placed into a 15 ml centrifuge tube and spun at 2500g for 10 minutes at room temperature. Duplicate aliquots of 1 ml were taken from each triplicate set of tubes for liquid scintillation counting (Packard Tri-Carb TR1600, Meriden, CT). The culture flasks were drained overnight at 4°C then 5 ml of 2% sodium carbonate in 0.1 N sodium hydroxide was added to each and left undisturbed for 24 hours to ensure cell lysis. Triplicate 50 µl aliquots were removed from each flask for protein determination by the Bradford method (Bradford, 1976). Radioactivity released into the medium is expressed as pmol myo-[^{3}H]inositol per total mg protein in each sample flask.

Tamoxifen and niflumic acid were dissolved in methanol or dimethyl sulfoxide

(DMSO) at concentrations no greater than 0.2% of solvent. As previously described (Reeves and Cammarata, 1996), control experiments were performed to monitor the effects of methanol or DMSO on myo-[^{3}H]inositol efflux from BLECs, resulting in no significant difference ($P > 0.3$) in efflux ± solvents. A Wescor Model 5500 vapor pressure osmometer (Wescor, Inc., Logan, UT) was used to measure the medium osmolalities for all experiments.

L-Glucose Depletion of Intracellular Myo-inositol

BLECs were grown to confluency in 150-cm^2 culture flasks containing 25 ml of either MEM + 40 mM D-fructose or MEM + 40 mM L-glucose for 1, 2, and 3 days. Triplicate sets of flasks were collected for both medium condtions at each of the three time points as previously described (Reeves and Cammarata, 1996). Cells were dispersed with trypsin in serum-free MEM and centrifuged at 2500*g* at 4°C for 8 min. The cells were resuspended in 0.9 ml of 0.3 N zinc sulphate (Sigma Chemical, St. Louis, MO). Cells were disrupted by rapid freezing in liquid nitrogen and thawing at 37°C for 3 consecutive times. The samples were then transferred to a 5-ml Dounce homogenizer in an ice bath and subjected to five strokes for each sample. The homogenate was centrifuged at 18,000*g* at 4°C for 20 min, and the cell pellet was saved for protein determination. The supernatant was adjusted to 1 ml with 0.3 N zinc sulphate followed by the addition of 1.0 ml of 0.3 N barium hydroxide (Sigma Chemical, St. Louis, MO). After centrifugation at 2,500*g* and 4°C for 8 min, the supernatant was removed and then stored at -20°C. Intracellular myo-inositol content was subsequently quantified by anion exchange chromatography and pulsed electrochemical detection using a Dionex BioLC chromatography system (Dionex,

Sunnyvale, CA) as previously described (Cammarata et al., 1992; Cammarata et al., 1994; Reeves and Cammarata, 1996).

Determination of Myo-[^{3}H]inositol Influx

To monitor influx, cells were grown to near confluency in MEM then switched to MEM containing 40mM L-glucose for 24 hours in order to deplete intracellular myo-inositol. This was followed by a 1 hour hypertonic incubation by placing BLECs into SHM + 40mM L-glucose and 0.05mM ouabain ± 1mM niflumic acid (533 ± 4 mOsm). The incubation medium was then replaced with MEM containing 40mM L-glucose, 150mM myo-inositol, 100*u*Ci/ml of myo-[^{3}H]inositol and 0.05mM ouabain ± 1mM niflumic acid (353 ± 2 mOsm) for a 1 hour hypotonic exposure. The cells were then rinsed 5 times in Earle's solution and the culture flasks were drained overnight at 4°C. Next, 5 ml of 2% sodium carbonate in 0.1 N sodium hydroxide was added to each flasks and left undisturbed for 24 hours to ensure cell lysis. Duplicate aliquots of 1 ml were taken from each triplicate set of flasks for liquid scintillation counting (Packard Tri-Carb TR1600, Meriden, CT). Radioactivity in cell lysate samples is expressed as pmol myo-[^{3}H]inositol per total mg protein in each sample flask. Triplicate 50 µl aliquots were removed from each flask for protein determination by the Bradford method (Bradford, 1976).

Statistical Analysis

Results are expressed as means ± SE. The statistical significance of these results, defined as $P < 0.05$, was analyzed using either one-tailed or two-tailed Student's *t* test where applicable.

3. RESULTS

Transcripts for pI_{Cln} and ClC-3 are Expressed in BLECs

Poly $(A)^+$ RNA extracted from BLECs maintained under physiologic conditions was probed with radiolabeled cDNAs for pI_{Cln} and ClC-3 (Fig. 1). Northern blot analysis utilizing a 2.2 Kb rat brain cDNA probe revealed two major transcripts for ClC-3 in BLECs, consistant with human retinal and ciliary body tissue and a cell line from human nonpigmented ciliary epithelial cells (Coca-Prados et al., 1996). A single transcript for pI_{Cln} was obtained utilizing a 456 bp cDNA probe hybridized to the same blot. It has recently been suggested that pI_{Cln} is, functionally, a regulatory protein for a chloride channel, that possibly being ClC-3 (Coca-Prados et al., 1996). The current data confirm the presence of both transcripts in cultured BLECs. Our data does not permit us to make the distinction as to whether pI_{Cln} is the chloride channel or a regulatory protein to the chloride channel.

pI_{Cln} mRNA Expression Upregulates in Hypertonicity

Given the previously demonstrated sensitivity to volume fluctuations by pI_{Cln} (Paulmichl et al., 1992; Krapivinsky et al., 1994), we deemed it necessary to evaluate transcription levels of this Cl^- channel/channel regulator, under conditions favorable to induce its expression. Initially, we examined the effect of hypertonicity (causing cell shrinkage), which has been shown to concentrate internal solutes and activate volume regulatory mechanisms such as the Na^+, K^+, Cl^- cotransporters (O'Donnel, 1993). BLECs initially maintained under physiologic conditions (MEM, 257 ± 2 mOsm) but subsequently

transferred to sodium hypertonic medium (SHM, 473 ± 6 mOsm) for up to 8 hours responded with the rapid induction of pI_{Cln} mRNA, as demonstrated by Northern blot analysis [Fig. 2(A)]. Upregulation of pI_{Cln} mRNA was evident within 1 hour (lane 2) of sodium hypertonic exposure and was maintained at a consistently higher level throughout the 8 hour time period as compared to physiologic control (MEM, lane 1). The relative densitometric intensity (RDI) showed nearly a 4-fold increase (lane 3) in pI_{Cln} mRNA with 4 hours of sodium hypertonic exposure. After hybridization using the 456 bp pI_{Cln} cDNA, the blot was stripped and hybridized with a genomic probe for β-actin in order to determine lane loading constancy of poly $(A)^+$ RNA.

In order to demonstrate that the induced upregulation of pI_{Cln} was not sodium specific, raffinose induced hypertonicity was also examined [Fig. 2(B)], by transferring BLECs from MEM (257 ± 2 mOsm) into MEM supplemented with 150mM raffinose (Raf, 452 ± 2 mOsm), again for an 8 hour period. A pattern of upregulation similar to that of hypertonic sodium was observed with hypertonic raffinose exposure. A 3.2-fold increase (lane 4) in the expression of pI_{Cln} mRNA was seen after 8 hours of exposure to 150 mM raffinose. By 24 hours, the expression of pI_{Cln} mRNA exposed to either sodium or raffinose hypertonicity had decreased to near physiologic levels (data not shown).

pI_{Cln} mRNA Expression Downregulates with Hypotonic Exposure

Hypotonicity causes cell swelling subsequently followed by rapid osmolyte movement from cell to medium (efflux), which may be impeded with chloride channel inhibitors (Reeves and Cammarata, 1996). To determine the effect of hypotonicity on pI_{Cln} mRNA expression (Fig. 3), BLECs were maintained in SHM (473 ± 6 mOsm) for 12 hours, in

order to first upregulate the message (Fig. 3, lane 2), and then switched into physiologic medium (MEM, 257 ± 2 mOsm), for a duration of 24 hours. As shown in Figure 3, exposure to SHM for 12 hours (hypertonic control, lane2) upregulates pI_{Cln} mRNA expression compared to BLECs maintained in physiologic medium (MEM, lane 1), consistent with the data shown in Figure 2(A). Greater than a 3-fold increase in pI_{Cln} mRNA was shown by RDI in hypertonically acclimated BLECs (SHM-12hr, lane 2), as compared to cells maintained under physiologic conditions. Subsequent transfer of BLECs from hypertonic medium into physiologic medium (lanes 3 through 6) uncovered a pattern of moderate downregulation of pI_{Cln} mRNA with expression levels reaching physiologic control by 24 hours (lane 6) of hypotonic exposure. Within 1 hour of switching BLECs from hypertonic to physiologic medium (SHM-12hr > MEM-1hr), the RDI for pI_{Cln} mRNA expression was reduced to 70% of the hypertonic control seen in lane 2. The RDI showed further downregulation of expression of pI_{Cln} mRNA to 50%, 40% and 30% of control at 4, 12 and 24 hours of hypotonic exposure, respectively. It is noteworthy to point out that this pattern of relatively gradual downregulation contrasts significantly with that of the Na^+-myo-inositol cotransporter mRNA which is particularly sensitive to hypotonicity and degrades almost immediately upon cell swelling (Zhou et al., 1994).

Inhibition of Myo-[^{3}H]inositol Efflux with Niflumic Acid and Tamoxifen

The inhibitory effects of tamoxifen and niflumic acid on swelling-activated chloride currents and volume-induced osmolyte efflux have been documented in numerous cell culture systems. (Strange et al., 1993; Jackson and Strange, 1993; Zhang et al., 1994;

Civan et al., 1994; Gonzalez et al., 1995; Reeves and Cammarata, 1996; Coca-Prados et al., 1996). Moreover, it has recently been demonstrated that tamoxifen influences lens opacification in whole lens studies of bovine eyes (Zhang et al., 1994; Zhang and Jacob, 1996). In an intact lens study, tamoxifen was reported to increase lens swelling by up to 13% and prevented volume regulation upon hypotonic insult (Zhang and Jacob, 1996). Furthermore, niflumic acid has been shown to prolonge cell swelling by inhibiting RVD in nonpigmented ciliary epithelial cells (Coca-Prados et al., 1996). As shown in Figures 4(A) and (B), swelling-activated myo-[^{3}H]inositol efflux is inhibited in a dose-dependent manner by the chloride channel inhibitors tamoxifen and niflumic acid, respectively. Hypertonically adapted BLECs were incubated in fresh sodium hypertonic medium (473 ± 6 mOsm) containing different concentrations of inhibitors for 2 hours, the medium was subsequently replaced with physiologic medium (257 ± 2 mOsm) at the same inhibitor concentrations for a 2 hour efflux period. The results indicate that 0.05 and 0.1mM tamoxifen inhibited myo-[^{3}H]inositol efflux by 48% ($P < 0.0005$) and 65% ($P < 0.0001$), respectively, [Fig. 4(A)], while niflumic acid effectively blocked swelling-activated efflux by 30% ($P < 0.0015$) at a concentration of 0.5mM and 51% ($P < 0.00015$) at 1.0mM [Fig. 4(B)].

Effect of Tamoxifen and Niflumic Acid on pI_{Cln} mRNA Expression

Given that tamoxifen and niflumic acid impede myo-inositol efflux while prolonging lens swelling, the following experiment was designed to determine the impact of these chloride channels inhibitors on the mRNA levels of pI_{Cln} in response to hypotonic insult. The effect of tamoxifen and niflumic acid was independently evaluated in BLECs

acclimated in sodium hypertonic medium for 6 hours and subsequently transferred to physiolologic medium for a 4 hour hypotonic exposure (Fig. 5). Lane 1 in figure 5 is representative of the characteristic upregulated state of pI_{Cln} mRNA after 6 hours in SHM, showing nearly a 3-fold increase in pI_{Cln} mRNA as determined by RDI measurements. Our hypotonic control, seen in lane 2, represents a typical pattern of downregulation of pI_{Cln} mRNA observed in cultured BLECs switched from hypertonicity into physiologic medium (i.e., hypotonicity, SHM-6hr > MEM-4hr). Lanes 3 and 4 reveal additional downregulation of pI_{Cln} mRNA when hypertonically acclimated cells were switched into physiologic medium in the presence of either 0.1 mM tamoxifen (TX) or 1 mM niflumic acid (NF). Compared to control (lane 2), the RDI showed a 50% and 40% reduction in pI_{Cln} mRNA expression of BLECs exposed to hypotonicity in the presence of TX (lane 3) and NF (lane 4), respectively. These data suggest that blocking osmolyte efflux under hypotonic conditions, which normally elicit cell volume increase and osmolytes loss from cell to medium, exacerbates the situation further with protracted cell swelling when retained intracellular osmolytes prevent regulatory volume decrease. Consistent with this increased intracellular osmotic stress (i.e. cell swelling), the cultured BLECs respond with amplified downregulation of pI_{Cln} mRNA expression.

Swelling-activated Myo-[^{3}H]inositol Influx is Inhibited by Niflumic Acid

Swelling-activated loss of myo-inositol from cell to medium is mediated via a passive transport system, driven by the concentration gradient of intracellular myo-inositol, and inhibited by niflumic acid [Reeves and Cammarata, 1996; Fig. 4(B) of the current study]. We hypothesize that efflux inhibition is achieved through the direct blockage of a

chloride channel which mediates osmolyte movement from cell to medium. In order to lend further credibility to this conjecture, it was necessary to demonstrate that the "channel" is bi-directional, i.e. myo-inositol can be made to move *passively* from medium to cell as well as from cell to medium. In order to demonstrate swelling-activated *influx* of myo-[^{3}H]inositol, it was first necessary to reverse the normal physiologic gradient of myo-inositol, such that the intracellular concentration was *reduced* relative to the external medium. Intracellular myo-inositol levels were first depleted with a 24 hour exposure of cells to physiologic medium containing 40mM L-glucose (Cammarata et al., 1992). Figure 6 illustrates the successful depletion of intracellular free myo-inositol, as determined by anion exchange chromatography, with the BLECs maintained in physiologic medium supplemented either 40 mM L-glucose or 40 mM D-fructose (as a control). Significant depletion of myo-inositol was observed after a 1 ($P < 0.01$), 2 ($P < 0.007$), and 3 ($P < 0.002$) days of exposure to L-glucose as compared to the fructose control.

Figure 7 illustrates the ability of niflumic acid to inhibit swelling-activated *influx* of myo-[^{3}H]inositol in BLECs. Cells grown to near confluency were maintained in physiologic medium and subsequently switched back into physiologic medium but containing 40 mM L-glucose for 24 hours to deplete intracellular myo-inositol. Following this 24 hour period, BLECs were switched to sodium hypertonic medium containing 40mM L-glucose *and 0.05 mM ouabain* ± 1 mM niflumic acid (533 ± 4 mOsm) for a 1 hour incubation. This hypertonic incubation period served two distinct purposes. First, to induce cell swelling, hypertonic exposure of BLECs must precede medium osmolality

reduction. Secondly, the inclusion of ouabain arrests the possibility of active, sodium-dependent myo-inositol uptake (Cammarata et al., 1991). In order to initiate cell swelling and reverse the myo-inositol concentration gradient, the incubation medium was replaced with physiologic medium in the continued presence of 40 mM L-glucose, but with the additional inclusion of 150 mM myo-inositol and 100 *u*Ci/ml of myo-[^{3}H]inositol with 0.05 mM ouabain ± 1 mM niflumic acid (353 ± 2 mOsm). The high specific activity of myo-[^{3}H]inositol in conjunction with an extracellular myo-inositol concentration gradient approximately ~90 fold higher than the intracellular myo-inositol level allowed for myo-[^{3}H]inositol influx to be monitored under hypotonic conditions. As shown in figure 7, the chloride channel inhibitor, niflumic acid (1 mM), significantly inhibited the swelling-activated *influx* of myo-[^{3}H]inositol at 5 ($P < 0.00015$), 20 ($P < 0.0006$), and 60 ($P < 0.0015$) minutes after hypotonic exposure.

4. DISCUSSION

Since the introduction of the anion channel hypothesis (Roy and Malo, 1992), substantial data has amassed to support the notion that osmolyte efflux from cell to medium is mediated through a swelling-activated anion (chloride) channel (Banderali and Roy, 1992; Kirk et al., 1992; Strange et al., 1993). A positive correlation between chloride movement and osmolyte efflux with cells exposed to hypotonic insult has been demonstrated (Jackson and Strange, 1993; Gonzalez et al., 1995). The molecular characterization of the protein(s) involved in the efflux process has been a subject of intense interest in recent years, with several candidate proteins rising to the forefront. The overexpression of the protein, pI_{Cln}, in *Xenopus* oocytes, strongly implicated its

involvement in cellular chloride movement and cell volume regulation (Paulmichl et al., 1992). Additional studies, have characterized pI_{Cln}, as an abundant, soluble protein, with a predominantly cytosolic localization, lending support to the notion that it is a channel regulator (Krapivinsky et al., 1994) rather than a channel-forming protein (Paulmichl et al., 1992). It was recently proposed that pI_{Cln} is a regulatory protein for the chloride channel protein, ClC-3, known to provide a pore for Cl^- translocation (Coca-Prados et al., 1996). In the present study, ClC-3 and pI_{Cln} were identified using cultured bovine lens epithelial cells (Fig. 1). The interrelationship between these two proteins remains to be determined.

Volume-sensitivity of pI_{Cln} was demonstrated by Krapivinsky et al. (1994), who determined that this protein was critical for the activation of swelling-induced chloride currents in *Xenopus* oocytes. Moreover, antisense oligonucleotides to pI_{Cln} have been shown to supress cell volume-induced activation of chloride channels in NIH 3T3 fibroblasts (Gschwentner et al., 1995). If, indeed, a regulatory relationship exists between pI_{Cln} and the chloride channel ClC-3, that both protein's transcriptional control be volume-sensitve in order for the anion/osmolyte volume regulatory mechanism to be functional might not prove to be an essential requirement. In this study, we demonstrate that transcriptional levels of mRNA for the channel regulatory protein, pI_{Cln}, is regulated by a volume-sensitive mechanism. However, whether ClC-3 transcription is responsive to cell volume alterations remains to be demonstrated.

The upregulation of pI_{Cln} mRNA expression in BLECs, exposed to either hypertonic sodium [Fig. 2(A)] or raffinose [Fig. 2(B)], suggests a "preparatory" response,

via an as yet unidentified signaling mechanism, that "senses" the loss of cell volume and/or the gain in intracellular osmolality. As cells rapidly shrink, internal solutes concentrate. Moreover, we have previously demonstrated that hypertonic conditions prompt the upregulation of the Na^+-myo-inositol cotransporter and aldose reductase (Zhou et al., 1994), permitting the active uptake of myo-inositol and the enzymatic conversion of aldose sugars to sugar alcohols (i.e. polyol), ultimately resulting in the impending need to efflux these accumulated intracellular osmolytes from the cell to medium in order to maintain cellular osmotic homeostasis. We propose that under hypertonic conditions, the initial loss of cell volume and elevated intracellular osmolality provide the *stimulus* for upregulation of the efflux system, of which pI_{Cln} potentially plays an as yet undetermined pivotal role.

The opposing effect is achieved when the cells are exposed to hypotonic conditions (Fig. 3). In this case, rapid downregulation of pI_{Cln} mRNA expression suggests an "inhibitory" response, via an unknown signaling mechanism, that "senses" the gain in cell volume and/or the decrease in intracellular osmolality. Put in other terms, as cells rapidly swell, rapid efflux of intracellular osmolytes ensues (Reeves and Cammarata, 1996) negating the need for further osmolyte efflux. Thus, the need for the chloride (i.e. efflux) channel remaining open is alleviated which may in turn prompt pI_{Cln} downregulation. Taken together, these data are consistent with the suggestion that pI_{Cln} mRNA expression is responsive to cell volume alterations and/or the gain or loss of intracellular osmolytes.

Further supportive evidence for volume-sensitive transcriptional regulation of

pI_{Cln} was evident in Figures 5(A) and (B), which utilized tamoxifen and niflumic acid, respectively, to block swelling-activated osmolyte efflux. Under hypotonic conditions, tamoxifen and niflumic acid have been shown to increase cell swelling and prolong the swelling response by inhibiting RVD (Zhang and Jacob, 1996; Coca-Prados et al., 1996). That is, the addition of either tamoxifen or niflumic acid to the hypotonic-treated BLECs serves to further exacerbate cell swelling by effectively blocking osmolyte efflux [Fig. 4(A) and (B)]. The resulting magnified downregulation of pI_{Cln} mRNA expression with hypotonic exposure of cultured BLECs in the presence of tamoxifen or niflumic acid (Fig. 5) lends further strong support to the suggestion that transcriptional regulation of pI_{Cln} is directly associated with changes in cell volume. Other workers have provided equally compelling evidence which also suggests cells can sense the magnitude of changes in cell volume (Strange et al., 1996).

Efflux of myo-[^{3}H]inositol *from cell to medium,* as a result of hypotonic treatment, has been reported to be a passive, concentration-gradient dependent, occurrence (Strange et al., 1996). In the bovine lens epithelial cell culture system, medium myo-inositol levels of ~15-20 *u*M and intracellular myo-inositol levels ranging from 15-60 mM have been measured (data not shown). That is, a normal concentration gradient of up to 1000 fold exists between intracellular and extracellular myo-inositol content in cultured BLECs. In this study, we demonstrated that swelling-activated myo-inositol movement *from the medium to the cell* (i.e., influx) can also be blocked with chloride channel inhibitors. In order to achieve the passive flow of myo-inositol from medium to cell, we were obliged to reverse the normal myo-inositol concentration

gradient. This was accomplished by incubating the cells for 24 hours in physiologic medium containing 40mM L-glucose [a competitive inhibitor of myo-inositol uptake (Cammarata et al., 1992)] which has the resulting effect of depleting intracellular free myo-inositol levels. As shown in Figure 6, L-glucose treatment reduced intracellular myo-inositol content to well below normal physiologic levels. After a brief hypertonic exposure in the presence of ouabain (precludes the possiblity of *active*, sodium-dependent myo-inositol uptake), myo-inositol movement *from medium to cell* could be demonstrated upon transferrence to hypotonic medium. Hypotonic-induced influx of myo-inositol has previously been demonstrated with cultured Madin-Darby canine kidney (MDCK) and C6 glioma cells (Nakanishi and Burg, 1989; Strange et al., 1993); however, our study is the first to demonstrate the ability of a chloride channel inhibitor to effectively block the swelling-activated influx of myo-[^{3}H]inositol (Fig. 7). Furthermore, we stress that the evidence from this experiment is pharmacologic in nature. The current data do not permit us to distinguish whether myo-inositol movement and chloride movement are cotransported by a *shared* channel, or, rather, are fluxed jointly by two independent pathways working in concord. Nevertheless, this experiment convincingly confirms the passive bi-directional flow of myo-inositol across a "channel pathway."

In summation, pI_{Cln} transcriptional regulation in cultured BLECs appears to be responsive to the magnitude and mechanism of cell volume fluctuations. These data suggest a converse relationship exists between pI_{Cln} mRNA expression and cell volume reduction or expansion. Hypertonic-induced cell shrinkage upregulates pI_{Cln} mRNA expression while hypotonic-induced cell swelling downregulates pI_{Cln} mRNA expression.

When the effect of hypotonic-induced cell swelling is amplified by the use of chloride channel inhibitors, the cell culture system undergoes further intracellular osmotic stress and intensified downregulation ensues.

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FIGURE 1. Identification of pI_{Cln} and ClC-3 mRNA in cultured bovine lens epithelial cells (BLECs) maintained in physiologic medium. Northern blot analysis utilizing a 2.2 Kb rat brain cDNA probe revealed two major transcripts for ClC-3. A single transcript was obtained for pI_{Cln} using a 456 bp cDNA probe hybridized to the same blot. 5 *u*g of poly $(A)^+$ RNA were loaded per lane.

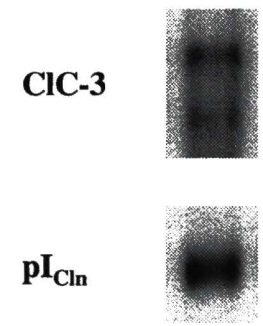
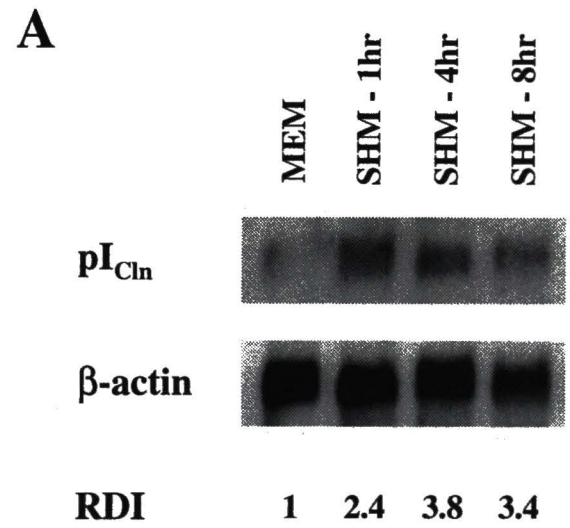

ClC-3
pI_{Cln}

FIGURE 2. Expression of pI_{Cln} mRNA in BLECs upregulates in both sodium [Fig. 2(A)] or raffinose [Fig. 2(B)] hypertonicity. Confluent monolayers of BLECs maintained in physiologic medium (MEM) were switched into sodium hypertonic medium (SHM = MEM + 116 mM NaCl, 473 ± 6 mOsm) or MEM + 150 mM raffinose (Raf, 452 ± 2 mOsm) for 1, 4, and 8 hours. The blots were probed with a cDNA for pI_{Cln} then stripped and reprobed with genomic β-actin. 5 *u*g of poly $(A)^+$ RNA were loaded per lane. RDI = relative densitometric intensity (normalized to β-actin)


A
MEM
SHM - 1hr
SHM - 4hr
SHM - 8hr
pI Cln
β-actin
RDI
1
2.4
3.8
3.4

B

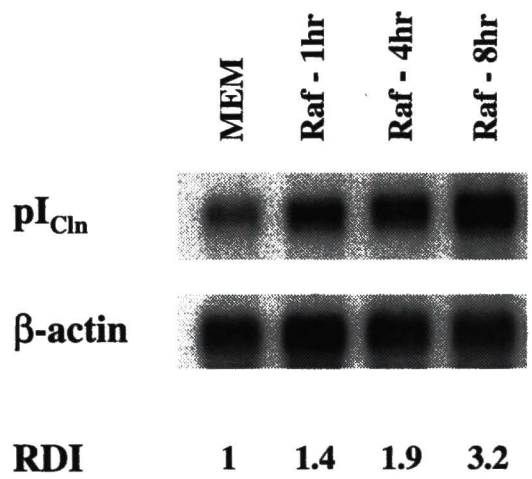


FIGURE 3. Hypotonic exposure of BLECs downregulates pI_{Cln} mRNA expression. BLECs were maintained in sodium hypertonic medium (SHM, 473 ± 6 mOsm) for 12 hours, in order to first upregulate pI_{Cln} mRNA, then switched into physiologic medium (MEM, 257 ± 2 mOsm), for 1, 4, 12, and 24 hours. Lane 1 represents the expression of pI_{Cln} mRNA in BLECs maintained throughout the experiment in physiologic medium for comparison with the hypertonic control (lane 2). 5 *u*g of poly $(A)^+$ RNA were loaded per lane. RDI = relative densitometric intensity (normalized to β-actin mRNA).

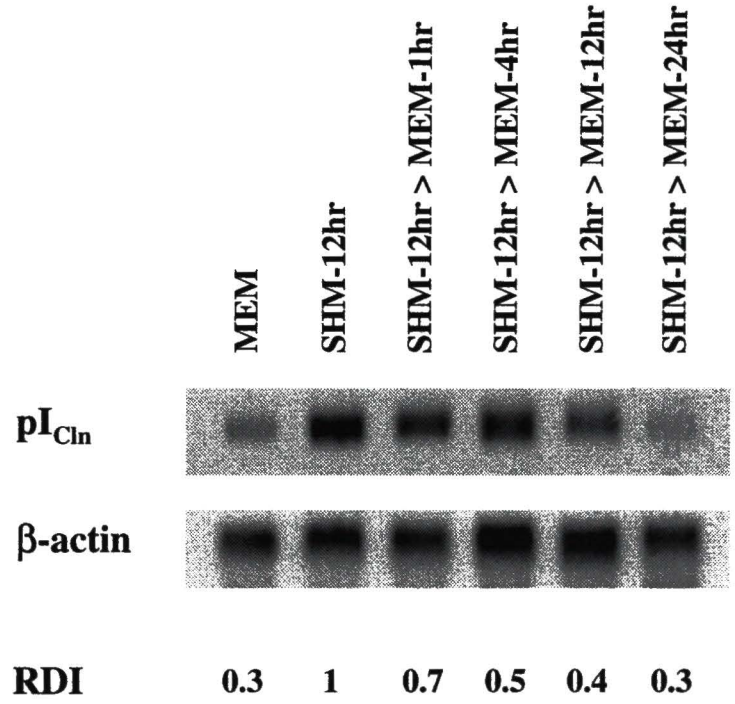
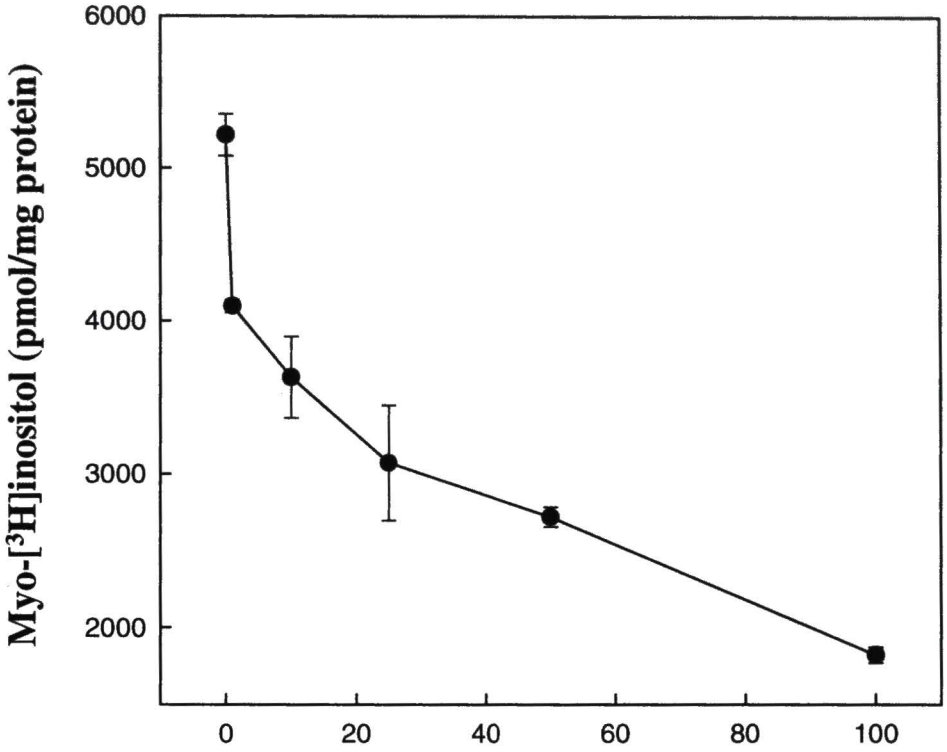
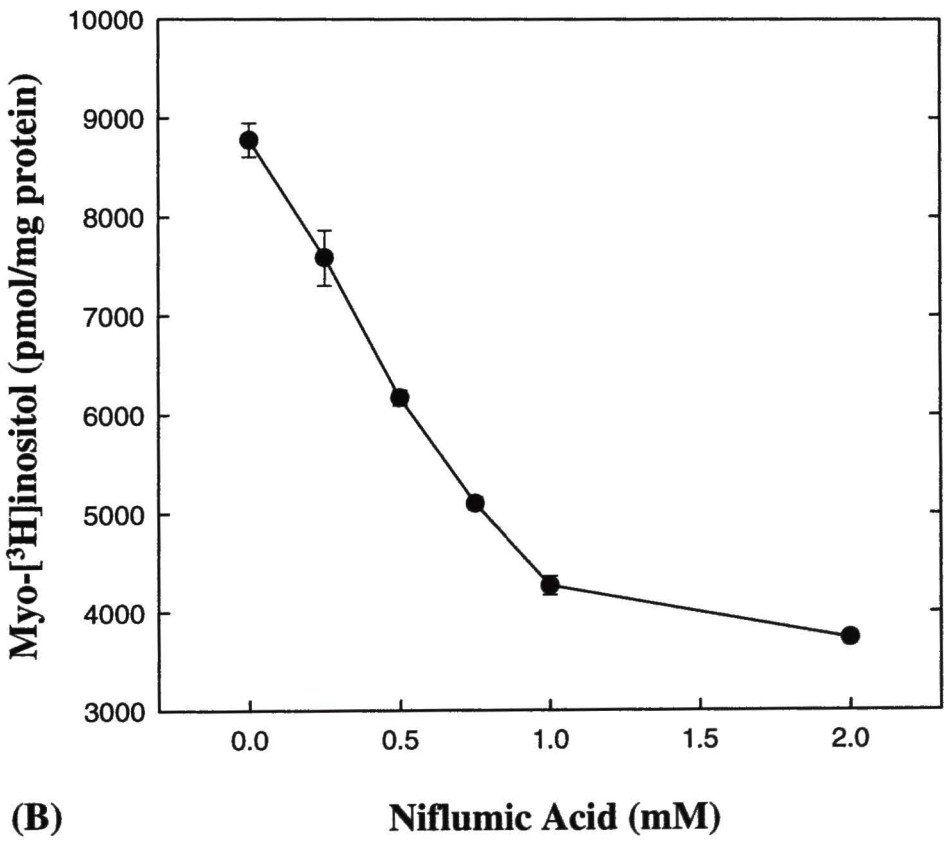

MEM
SHM-12hr
SHM-12hr > MEM-1hr
SHM-12hr > MEM-4hr
SHM-12hr > MEM-12hr
SHM-12hr > MEM-24hr
pI$_{Cln}$
β-actin
RDI
0.3
1
0.7
0.5
0.4
0.3

FIGURE 4. Dose responsiveness of tamoxifen [Fig. 4 (A)] and niflumic acid [Fig. 4 (B)] on swelling-activated myo-[^{3}H]inositol efflux. Cells were acclimate in SHM (473 ± 6 mOsm) for 48 hours for both conditions followed by a 2 hour incubation period in the appropriate inhibitor concentrations. Cells were then switched into the following medium conditions: MEM (257 ± 2 mOsm) containing tamoxifen over a concentration range of 0 to 0.100 mM [Fig. 4(B)], or MEM containing niflumic acid over a concentration range of 0 to 1.0 mM [Fig. 4(A)], for a 2 hour efflux period. Data are mean ± SE for duplicate samples from triplicate flasks.


6000
5000
4000
3000
2000
0
20
40
60
80
100
Myo-[3H]inositol (pmol/mg protein)
Tamoxifen (uM)

(A)


10000
9000
8000
7000
6000
5000
4000
3000
0.0
0.5
1.0
1.5
2.0
Myo-[3H]inositol (pmol/mg protein)
Niflumic Acid (mM)

(B)

FIGURE 5. Effect of tamoxifen and niflumic acid on pI_{Cln} mRNA expression in BLECs. Cells were acclimated in sodium hypertonic medium (SHM, 473 ± 6 mOsm) for 6 hours and subsequently transferred to physiologic medium (MEM, 257 ± 2 mOsm) ± tamoxifen (TX) or niflumic acid (NF) for a 4 hour hypotonic exposure. 5 *u*g of poly $(A)^+$ RNA were loaded per lane. RDI = relative densitometric intensity (normalized to β-actin mRNA).

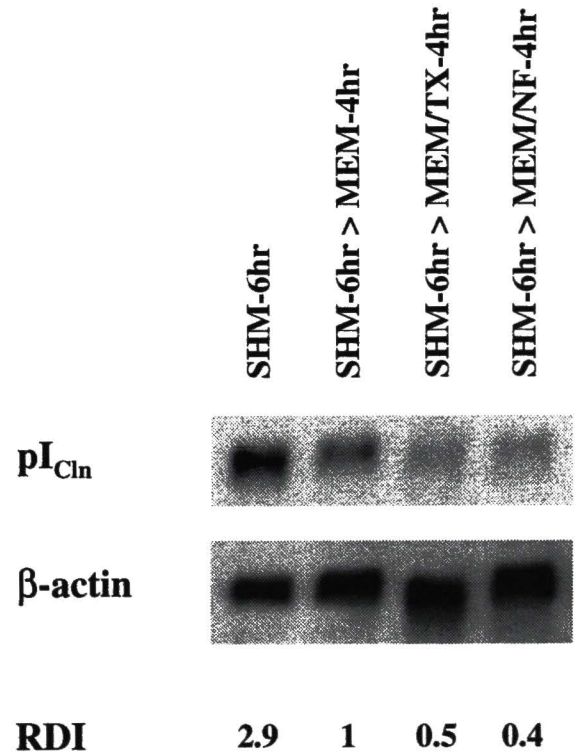

SHM-6hr
SHM-6hr > MEM-4hr
SHM-6hr > MEM/TX-4hr
SHM-6hr > MEM/NF-4hr
pI$_{Cln}$
β-actin
RDI
2.9
1
0.5
0.4

FIGURE 6. Depletion of intracellular myo-inositol with L-glucose. Bovine lens epithelial cells were maintained in physiological medium (MEM) until near confluency then switched to either MEM + 40mM L-glucose or MEM + 40mM D-fructose. Intracellular myo-inositol was measured by anion-exchange chromotography. The data represent triplicate samples of cells collected from individual flasks at 1, 2, and 3 days and the error bars indicate mean ± SE.

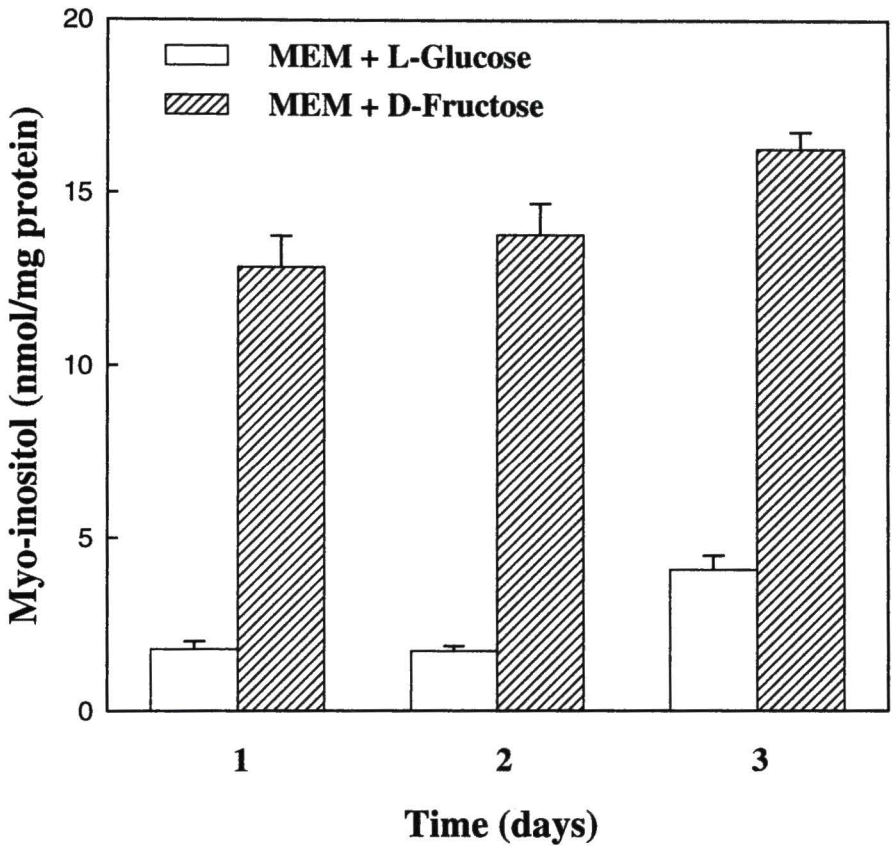
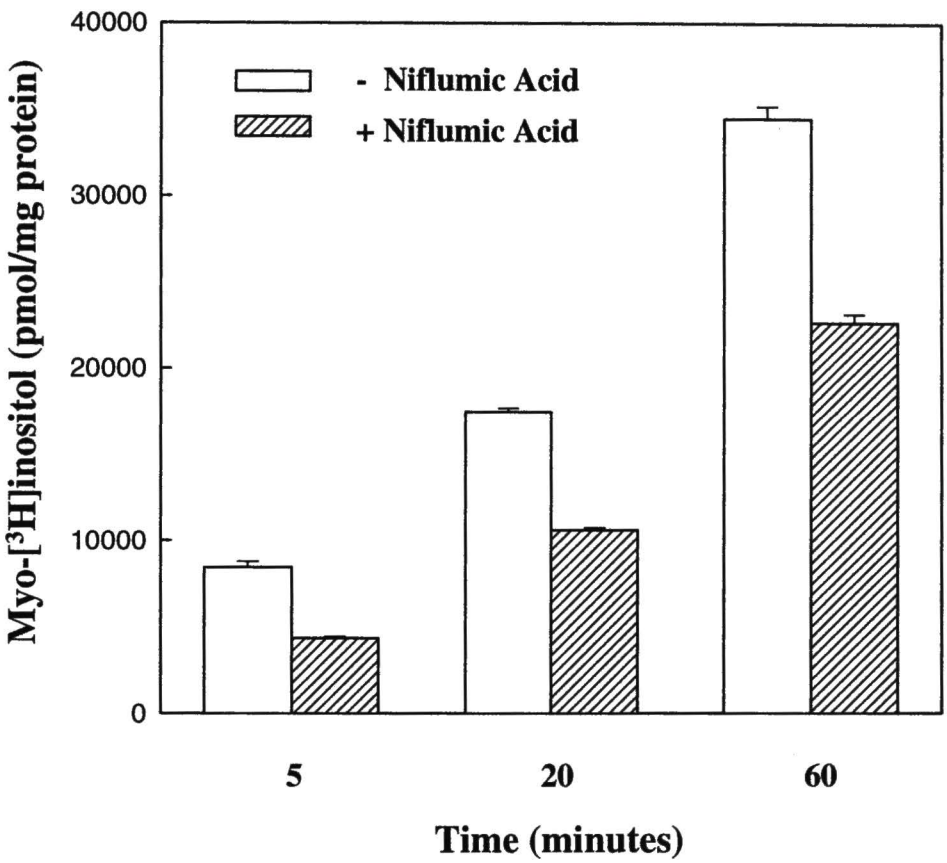

MEM + L-Glucose
MEM + D-Fructose
Myo-inositol (nmol/mg protein)
20
15
10
5
0
1
2
3
Time (days)

FIGURE 7. Swelling-activated influx of myo-[^{2}H]inositol is inhibited by niflumic acid. Bovine lens epithelial cells were maintained in MEM + 40mM L-glucose for 24 hours to deplete intracellular myo-inositol levels (refer to Fig. 6). Culture medium was replaced with sodium hypertonic medium, SHM (MEM + 116mM NaCl, 473 ± 6 mOsm) containing 40mM L-glucose plus 0.05mM ouabain ± 1mM niflumic acid for a one hour incubation period. For swelling activation, cells were then switched into 5 milliliters of physiologic medium (MEM, 257 ± 2 mOsm) containing 40mM L-glucose plus150mM myo-inositol ± 1mM niflumic acid for 5, 20, and 60 minutes, with each medium condition containing 100*u*Ci/ml of myo-[^{3}H]inositol and 0.05 mM ouabain. Data are mean ± SE for duplicate samples from triplicate flasks.


40000
30000
20000
10000
0
- Niflumic Acid
+ Niflumic Acid
Myo-[3H]inositol (pmol/mg protein)
5
20
60
Time (minutes)

This chapter will demonstrate the effect of polyol accumulation on intracellular myo-inositol levels in BLECs exposed to both galactose and glucose medium. Intracellular measurements of myo-inositol and polyols were determined by anion exchange chromotography. These results will establish why BLECs were exposed to galactose instead of glucose medium in the experiments in Chapter II, which were the first to demonstrate the polyol-activated efflux mechanism. Continuing the characterization of pI_{Cln} mRNA expression in BLECs, which was extensively investigated in the previous chapter, I determined the effect of rapid intracellular polyol accumulation on the expression of pI_{Cln} mRNA. Transcript levels of pI_{Cln} were determined by Northern analysis.

CHAPTER 1V

THE INFLUENCE OF INTRACELLULAR POLYOL ACCUMULATION ON MYO-INOSITOL EFFLUX AND pI_{Cln} mRNA EXPRESSION IN CULTURED BOVINE LENS EPITHELIAL CELLS

INTRODUCTION

Comparitive studies regarding myo-inositol efflux have been demonstrated in a wide variety of cell types, including cultured Madin-Darby canine kidney (MDCK) cells (Nakanishi and Burg, 1989; Yamauchi et al., 1991), C6 glioma cells (Strange et al., 1993), and bovine lens epithelial cells (Reeves and Cammarata, 1996). The effects of intracellular polyol formation and accumulation on the onset and development of sugar cataracts has been investigated for nearly 30 years; however, only recently has the role of polyols been conclusively demonstrated in the activation of an osmolyte efflux mechanism (i.e. polyol-activated myo-inositol efflux, Reeves and Cammarata, 1996). This mode of efflux has been linked to hypotonicity-induced myo-inositol efflux through studies with chloride channel inhibitors. Apparently, the polyol-activated efflux pathway serves an osmoregulatory function in the lens to alleviate intracellular osmotic stress observed in galactose exposed lenticular tissue.

In this study, we document the difference in myo-inositol efflux and intracellular polyol accumulation in BLECs exposed to a variety of experimental manipulations. Cell

swelling from polyol accumulation, hypotonicity, and ouabain-induction were compared and their influence on myo-inositol efflux was evaluated. The swelling-activated chloride channel inhibitor, tamoxifen, was used to further varify the involvement of chloride channels in osmolyte (myo-inositol) loss from cell to medium from either a polyol- or swelling-activated source. Also, the mRNA expression for pI_{Cln}, a protein sensitive to cell swelling and believed to be involved in cell volume regulation and chloride/osmolyte movement, was determined under conditions favorable for polyol-activated myo-inositol efflux in BLECs.

MATERIALS AND METHODS

Cell Culture

Bovine (*Bos taurus*) eyes obtained from a local slaughterhouse were brought to the lab on ice where the lenses were aseptically removed. Isolation and culture of bovine lens epithelial cells (BLECs) were performed as previously described (Cammarata et al., 1988). Briefly, cells were maintained in Eagle's minimal essential medium (MEM, 257 ± 2 mosm) supplemented with 10 % adult bovine serum at 37°C in a humidified 5% CO_2-95% air atmosphere containing 15 μM myo-inositol. All Northern analysis, determination of intracellular polyol content, and myo-inositol efflux studies were performed with confluent monolayers of 2nd-passage cells in either 25-cm^2 or 150-cm^2 culture flasks.

Determination of intracellular myo-inositol, galactitol and sorbitol content

Intracellular myo-inositol, galactitol and sorbitol content were quantified by anion exchange chromatography and pulsed electrochemical detection using a Dionex BioLC chromatography system (Dionex, Sunnyvale, CA) as previously described (Cammarata et

al., 1992; Cammarata and Chen, 1994). Bovine lens epithelial cells grown to confluency in their respective medium before dispersion with trypsin in serum-free physiological medium and centrifuged at 2500*g* at 4°C for 8 min. The cells were resuspended in 0.9 ml of 0.3 N zinc sulphate (Sigma Chemical, St. Louis, MO) and cell disruption was accomplished by rapid freezing in liquid nitrogen and thawing at 37°C. This process was repeated three times. Thereafter, the samples were transferred to a 5-ml Dounce homogenizer and subjected to five strokes while being maintained in an ice bath. The homogenate was centrifuged at 18,000*g* at 4°C for 20 min, and the cell pellet was saved for protein determination. The supernatant was adjusted to 1 ml with 0.3 N zinc sulphate and 1.0 ml of 0.3 N barium hydroxide (Sigma Chemical, St. Louis, MO) was added. The supernatant was centrifuged at 2,500*g* at 4°C for 8 min and then stored without further modification at -20°C for subsequent myo-inositol, galactitol and sorbitol analysis.

Northern Blot Analysis

Poly $(A)^+$ RNA from cultured BLECs was isolated as previously described (Zhou et al., 1994) by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Briefly, purified lens epithelial cell poly $(A)^+$ RNA was seperated by electrophoresis in a 1.0% agarose-formaldehyde denaturing gel as described (Lehrach et al., 1979) and then blotted to Hybond-N nylon membrane (Amersham Life Science, Arlington Heights, IL) in 20X SSC for approximately 16 hours. After blotting, the nylon membrane was baked for 1 hour at 80°C under vacuum and then cross-linked using a UV crosslinker (Fisher Scientific, Pittsburgh, PA). Next, the blots were hybridized with either a ^{32}P-oligolabeled 456-bp pI_{Cln} cDNA, followed by hybridization with DNA probes of genomic β-actin

(Nudel et al., 1983) made with Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) using previously described procedures (Agarwal et al., 1990). Next, the blot was washed for two 20 minute periods in 2X SSC with 0.1% SDS at 42°C followed by two more 20 minute washes in 0.2X SSC with 0.1% SDS at 42°C in a hybridization oven. The blots were then subjected to fluorography using an intensifying screen and Hyperfilm (Amersham, Arlington Heights, IL) at -80°C. The relative densitometric intensity (RDI) of lens cell pI_{Cln} mRNA, normalized to β-actin mRNA, was determined by scanning densitometry using Diversity One software from Protein and DNA ImageWare (PDI) Systems (Huntington Station, NY).

Determination of Myo-[^{3}H]inositol Efflux

To determine the effect of polyol accumulation on myo-[^{3}H]inositol efflux under isotonic medium conditions, BLECs maintained to confluence in physiologic medium (MEM, 257 ± 2 mOsm) were preloaded with 0.5 *u*Ci/ml myo-[^{3}H]inositol (Amersham, Arlington Heights, IL) for the final 24 hours. The cells were then rinsed three times (5 ml/rinse) with isotope-free MEM to remove free myo-[^{3}H]inositol. After the rinse, cells were transferred into MEM ± 0.1 mM sorbinil or 0.2 mM phloretin for a 1 hour incubation period. The incubation medium was removed and replaced with MEM containing 40 mM galactose ± 0.1 mM sorbinil or 0.2 mM phloretin for 2, 4, 8, 12, and 24 hours. The efflux medium was collected and processed as mentioned below.

Myo-[^{3}H]inositol efflux in hypertonically acclimated BLECs was determined according to previously described procedures (Reeves and Cammarata, 1996). In brief, BLECs were grown to near confluence in MEM, then switched to sodium hypertonic

medium (MEM + 116 mM additional NaCl, 473 ± 6 mosm), hereafter referred to as SHM, for 48 hours with a concurrent preload of cells with 0·5 μCi/ml myo-[^{3}H]inositol (Amersham, Arlington Heights, IL) during the final 24 hours. Each flasks was then rinsed three times (5 ml/rinse) with isotope-free SHM to remove free myo-[^{3}H]inositol from the cells. After these initial preparatory steps, the following protocols were used for individual efflux experiments.

The inhibitory effect of tamoxifen (0.1mM) on both tonicity-activated and polyol-activated myo-[^{3}H]inositol efflux was evaluated over a 4 hour time period. Following the preparatory steps mentioned above, BLECs from both experiments were transferred to 5 ml of fresh SHM (for controls) or SHM containing 0.1mM tamoxifen (for treatment conditions) for an incubation period of 2 hours. For initiation of swelling-activated efflux, cells were then switched into fresh MEM ± 0.1mM tamoxifen. To promote polyol-activated myo-[^{3}H]inositol efflux, BLECs were switched into SHM containing 40 mM galactose (513 ± 10 mosm), hereafter referred to as hypertonic galactose medium (HGM) ± 0.1mM tamoxifen. Cells were maintained in their respective treatment conditions for 30 minutes and 4 hours. The efflux medium was collected as mentioned below.

To determine the effect of ouabain on myo-[^{3}H]inositol efflux, BLECs acclimated in SHM according to the preparatory steps mentioned above were switched into MEM containing 40 mM galactose (Gal) ± 0.1 m ouabain for 2, 4, 8, and 12 hours. To evaluate the inhibitory effect of extracellular medium myo-inositol on myo-[^{3}H]inositol efflux, BLECs were switched from SHM into MEM containing 0.015, 0.30, 3.00 or 30.0 mM myo-inositol for a 2 hour efflux period. Samples of efflux medium were collected in

accord with the protocol mentioned below.

For each of the abovementioned experiments, the efflux medium collected from individual flasks was placed into a 15 ml centrifuge tube and spun at 2500g for 10 minutes at room temperature. Duplicate aliquots of 1 ml were taken from each triplicate set of tubes for liquid scintillation counting (Packard Tri-Carb TR1600, Meriden, CT). The culture flasks were drained overnight at 4°C then 5 ml of 2% sodium carbonate in 0.1 N sodium hydroxide was added to each and left undisturbed for 24 hours to ensure cell lysis. Triplicate 50 µl aliquots were removed from each flask for protein determination by the Bradford method (Bradford, 1976). Radioactivity released into the medium is expressed as pmol myo-[^{3}H]inositol per total mg protein in each sample flask. A Wescor Model 5500 vapor pressure osmometer (Wescor, Inc., Logan, UT) was used to measure the medium osmolalities for all experiments.

Statistical Analysis

Results are expressed as means ± SE. The statistical significance of these results, defined as $P < 0.05$, was analyzed using either one-tailed or two-tailed Student's *t* test where applicable.

RESULTS

Effect of Galactose and Hypertonic Medium on Intracellular Myo-inositol

Kawaba et al. (1986) demonstrated that myo-inositol concentrations in the lens are affected by the increase in polyol accumulation. In Figure 1, intracellular myo-inositol and polyol levels were determined by anion exchange chromatography in bovine lens epithelial cells (BLECs) maintained in physiologic medium (MEM, 257 ± 2 mOsm), Gal

(MEM + 40 mM galactose, 285 ± 4 mOsm), or sodium hypertonic medium (SHM = MEM + 116 mM NaCl, 473 ± 6 mOsm). Figure 1(A) demonstrates the loss of intracellular myo-inositol in (BLECs) maintained in Gal for a 3 day period. As early as day 1 of exposure to Gal, intracellular myo-inositol was significantly reduced by 48% ($P < 0.05$) as compared to physiologic control (MEM). By the end of day 2, intracellular myo-inositol in Gal exposed cells was reduced by 84% ($P < 0.04$) as compared to control, and by day 3, intracellular myo-inositol in Gal exposed cells was reduced to levels not detectable (ND). In addition, BLECs maintained in sodium hypertonic medium (SHM, 473 ± 6 mOsm) showed a significant increase in the accumulation of intracellular myo-inositol with a 4.9-fold increase at the end of day 1 ($P < 0.002$), a 4.2-fold increase at the end of day 2 ($P < 0.02$), and a 4.7-fold increase after day 3 ($P < 0.008$), as compared to control (MEM).

The results illustrated in Figure 1(B) help to explain the depletion of intracellular myo-inositol seen in BLECs maintained in Gal for 3 days, as previously mentioned in Figure 1(A). Intracellular galactitol accumulation was enhanced significantly in cells maintained in Gal medium with increases of 6.1-fold at the end of day 1 ($P < 0.0008$), 6.3-fold at the end of day 2 ($P < 0.009$), and 9.0-fold by day 3 ($P < 0.003$), compared to sorbitol levels in BLECs maintained in physiological medium (MEM). Also, hypertonicity prompted approximately a 2.3-fold increase in the intracellular sorbitol accumulation as compared to control (MEM), which was maintained throughout the 3 day period. Furthermore, sorbitol accumulation in hypertonically exposed BLECs peaked near 200nmol/mg protein by day 2, with no further significant increase seen at day 3.

Depletion of Intracellular Myo-inositol and Accumulation of Polyols Differ with Galactose and Glucose Exposure

In Figure 2, BLECs were maintained in sodium hypertonic medium (SHM, 473 ± 6 mOsm) for 48 hours then switched into Gal (MEM + 40 mM galactose, 285 ± 4 mOsm) or Glc (MEM + 40 mM glucose, 281 ± 6 mOsm) for an 8 hour hypotonic exposure. Intracellular myo-inositol and polyol levels were determined by anion exchange chromatography. The measured intracellular myo-inositol concentration after the 48 hour hypertonic acclimation period and prior to switching into hypotonicity for 4 hours was 72.4 ± 4 nmol/mg protein. As seen in Figure 2(A), BLECs switched from SHM into Gal medium showed a significantly greater efflux of intracellular myo-inositol as those switched into Glc medium. In Gal exposed cells, myo-inositol levels dropped 68% from 72.4 ± 4 to 23.1 ± 4.2 nmol/mg protein over the 4 hour efflux period. In BLECs switched from SHM into Glc, MI levels fell only 41% from 72.4 ± 4 to 42.4 ± 2.6 nmol/mg protein during the same time period. After the 4 hour hypotonic exposure [Fig. 2(A)], the intracellular myo-inositol level in cells transferred to Gal medium was only 54% of that found in cells transferred to Glc medium ($P < 0.002$).

Galactose is a preferred substrate over glucose for the enzyme aldose reductase and the data in Figure 2(B) shows that galactitol (GalOH), the metabolite of galactose via the polyoly pathway, accumulates at a much faster rate under these conditions compared to sorbitol (SorOH), the polyol metabolized from glucose via the same pathway. BLECs accumulated approximately twice the intracellular polyols when switched to Gal medium compared to Glc medium for a 4 hour efflux period. Intracellular galactitol peaked at a

significantly higher level of 412.8 ± 24.7 nmol/mg protein in cells switched into Gal medium while sorbitol levels reached only 209.7 ± 11.7 nmol/mg protein in cells switched into Glc medium ($P < 0.0005$).

Intracellular Galactitol Accumulation Enhances Myo-[^{3}H]inositol Efflux in BLECs Maintained Under Isotonic Conditions

It has recently been shown that rapid intracellular polyol accumulation enhances myo-[^{3}H]inositol efflux (polyol-activated efflux) in hypertonically acclimated BLECs switched into sodium hypertonic medium containing 40 mM galactose (Reeves and Cammarata, 1996); however, it has yet to be shown that polyol accumulation directly enhances efflux in BLECs maintained in isotonic galactose medium (Gal = MEM + 40 mM galactose). Unlike previous experiment, BLECs were not exposed to hypertonicity or hypotonicity, but were maintained throughout the entirety of the experiment in isotonic medium. In Figure 3, BLECs were maintained to confluence in physiologic medium (MEM) then switched into MEM containing 40 mM galactose (Gal) ± 0.1 mM sorbinil or 0.2 mM phloretin. Cells switched into Gal medium containing sorbinil (Gal/Sorbinil), an aldose reductase inhibitor, showed a significant reduction in myo-[^{3}H]inositol efflux as compared to those switched into Gal medium (control) at 8 ($P < 0.0007$), 12 ($P < 0.0004$), and 24 ($P < 0.0002$) hours. Furthermore, even greater inhibition of myo-[^{3}H]inositol efflux was seen in BLECs switched into Gal medium containing phloretin (Gal/Phloretin), a known inhibitor of sugar transport (Kimmich and Randles, 1975) which has previously been shown to inhibit swelling-activated myo-[^{3}H]inositol efflux in hypotoniclally exposed BLECs (Reeves and Cammarata, 1996). Approximately 75% of

myo-[^{3}H]inositol efflux was inhibited at 8 ($P < 0.00001$), 12 ($P < 0.000001$), and 24 ($P < 0.00002$) hours.

Elevated Extracellular Medium Myo-inositol Inhibits Myo-inositol Efflux

The volume regulatory efflux of myo-inositol in the lens epithelium is swelling-activated, polyol-sensitive, Na^+-independent and concentration-gradient driven. In this experiment we demonstrate the latter characteristic by showing an inhibitory effect on myo-[^{3}H]inositol efflux by increasing the medium myo-inositol levels. In Figure 4, BLECs maintained in sodium hypertonic medium (SHM) for 48 hours and preloaded with myo-[^{3}H]inositol in the last 24 hours of this period were switched into fresh MEM containing 0.015 mM (normal medium myo-inositol concentrations), 0.030 mM, 3.0 mM or 30.0 mM medium myo-inositol for a 2 hour efflux period. No significant effect on myo-inositol efflux was observed with additional extracellular medium myo-inositol levels up to 3.0 mM. However, as seen in Figure 4, at a concentration of 30.0 mM extracellular medium myo-inositol, myo-[^{3}H]inositol efflux was significantly inhibited by 34 % ($P < 0.002$). Almost identical results were seen at 4, 8 and 12 hours efflux periods under these same experimental protocol (data not shown).

Tamoxifen Inhibits Myo-[^{3}H]inositol Efflux

Previous data from our lab have shown that both swelling-activated (tonicity-activated) and polyol-activated myo-inositol efflux can be effectively inhibited with the chloride channel inhibitor, niflumic acid (Reeves and Cammarata, 1996). These results established a relationship between organic osmolyte efflux and chloride channels in BLECs. It was recently reported that tamoxifen, a specific blocker of swelling-activated chloride

channels, induces the formation of cataracts in lens organ culture systems (Zhang et al., 1994) As seen in Figure 5, we tested the effect of tamoxifen on both swelling- and polyol-activated myo-[^{3}H]inositol efflux in BLECs. In Figure 5(A), cells were maintained in SHM (473 ± 6 mOsm) for 48 hours with a concurrent preload of myo-[^{3}H]inositol in the last 24 hours. This was followed by a 2 hour incubtion in fresh SHM ± TX (0.10 mM), then cells were switched into MEM (257 ± 2 mOsm) ± TX for efflux periods of 30 minutes and 4 hours under hypotonic conditions. Tamoxifen significantly inhibited swelling-activated myo-[^{3}H]inositol efflux by 53% ($P < 0.0002$) and 47% ($P < 0.00005$) at these respective time points [Fig. 5(A)].

Rapid polyol accumulation (independent of hypotonic insult) promotes myo-[^{3}H]inositol efflux in BLECs (Reeves and Cammarata, 1996), a process which has operationally been called polyol-activated efflux. Figure 5(B) represents cells maintained in sodium hypertonic medium (SHM, 473 ± 6 mOsm) for 48 hours with myo-[^{3}H]inositol preloaded in the cells during the latter half of this period. This hypertonic acclimation period was followed by a 2 hour incubation period in fresh SHM ± 0.10 mM tamoxifen. Cell medium was then replaced with SHM + 40mM Gal, hereafter referred to as hypertonic galactose medium or HGM (510 ± 10 mOsm) ± 0.10 mM tamoxifen, without reducing the medium osmolality. As illustrated in Figure 5(B), cells switched into HGM + tamoxifen (TX) showed a significant reduction in polyol-activated myo-[^{3}H]inositol efflux as compared to controls (SHM > HGM). Efflux was inhibited by 71% ($P < 0.0001$) at 30 minutes and 39% ($P < 0.0002$) after 4 hours.

Ouabain Exposure Enhances Myo-[^{3}H]inositol Efflux

Ouabain, an inhibitor of Na^+/K^+ ATPase, has been reported to increase lens sodium levels and enhance lens swelling (Kinoshita, 1974). In Figure 6, cultured bovine lens epithelial cells were maintained in sodium hypertonic medium (SHM, 473 ± 6 mOsm) for 48 hours, with myo-[^{3}H]inositol preloaded in the last 24 hours of this period. After 48 hours of hypertonic treatment, cells were transferred into fresh SHM ± 0.1 mM ouabain for an 1 hour incubation period. Cells were then switched from hypertonic medium into physiologic medium supplemented with 40 mM galactose (Gal, 285 ± 4 mOsm) ± 0.1 mM ouabain and myo-[^{3}H]inositol efflux monitored for 2, 4, 8 and 12 hours after medium osmolality reduction. Significant enhancement of myo-[^{3}H]inositol efflux was seen at each time point (Fig. 6), with a 1.7-fold increase ($P < 0.00001$) after 2 hours. Further enhancement was seen at 4, 8 and 12 hours with a 1.8-fold ($P < 0.00007$), 2.0-fold ($P < 0.003$), and a 2.6-fold ($P < 0.000004$) increase, respectively.

The Effect of Galactose Exposure and Polyol Accumulation on pI_{Cln} mRNA Expression

The chloride channel/channnel regulatory protein, pI_{Cln}, has sparked recent interest in its involvement with chloride movement, osmolyte efflux and cell volume regulation. The expression of pI_{Cln} mRNA has been linked to changes in cell volume and/or intracellular osmolality (Paulmichl et al., 1992; Krapivinsky et al., 1994; Reeves et al., 1997). With the knowledge that intracellular polyol accumulation, resulting in cell swelling, activates efflux pathways for organic osmolytes, it was our intent to determine if rapidly accumulating intracellular polyol levels affect pI_{Cln} mRNA expression. Figure 7 illustrates

the effect of polyoly (galactitol) formation and accumulation on pI_{Cln} mRNA expression in BLECs resulting from exposure to hypertonic galactose medium (HGM = SHM + 40mM galactose). In Figure 7, BLECs were acclimated in SHM for 24 hours to enhance galactitol formation and accumulation from the upregulation of aldose reductase gene expression (Carper et al., 1990; Zhou et al., 1994). Cells were then transferred into fresh SHM or SHM + 40mM Gal (referred to as hypertonic galactose medium, HGM) ± 0.1mM sorbinil. With elevated polyol accumulation in cells switched to HGM (lanes 2 and 5), no significant difference in mRNA expression of pI_{Cln} was observed at 8 or 24 hours (Fig. 4B). As shown by relative densitometric intensity (RDI), there is no significant difference in pI_{Cln} mRNA expression in BLECs switched into SHM or HGM ± 0.1 mM sorbinil at either time point. These results indicate BLECs exposed to intracellular polyol accumulation (SHM > HGM), a condition known to enhance osmolyte efflux, or galactose exposure alone with blockage of polyol formation (SHM > HGM/S), does not significantly influence the levels of expression of pI_{Cln} mRNA over control (SHM > SHM)

DISCUSSION

Broekhuyse (1968) originally reported the inability of whole rat lenses exposed to galactose medium to accumulate intracellular myo-inositol. As galactitol accumulates in the lens, water imbibition and lens swelling occurs (Kinoshita et al., 1968). Furthermore, the intracellular accumulation of galactitol has an inhibitory effect on the uptake of myo-inositol and also promotes the activation of swelling-induced osmolyte efflux mechanism(s) associated with passive concentration-gradient driven loss of myo-inositol

(Cammarata et al., 1992; Reeves and Cammarata, 1996). As seen in Figure 1(B), intracellular galactitol levels in BLECs cultured in galactose medium (Gal, MEM + 40 mM galactose) reached extremely high levels, which resulted in the depletion of intracellular myo-inositol [Fig. 1(A)]. BLECs exposed for 3 days in Gal exhibited severely depleted intracellular myo-inositol levels within 1 day of exposure, with levels not detected by the third day [Fig. 1(B)]. This observed depletion of the intracellular myo-inositol pool in BLECs results from the concomitant inhibition of myo-inositol uptake and activation of the myo-inositol efflux pathway.

An increase in the Na^+/myo-inositol cotransporter uptake activity and accumulation of intracellular myo-inositol has been reported in primary cultures of both human lens epithelial cells and bovine lens epithelial cells exposed to hypertonicity (Yokoyama et al., 1993; Cammarata and Chen, 1994). Zhou et al. (1994) demonstrated that hypertonicity also increases the Na^+/myo-inositol cotransporter mRNA expression, with maximal induction by 8 hours. As seen in Figure 1(A), hypertonic exposure (SHM) significantly enhanced the accumulation of intracellular myo-inositol in BLECs by nearly 5-fold after 1 day of exposure, as compare to physiologic control (MEM). Also, enhanced sorbitol formation and accumulation from the upregulation of aldose reductase gene expression was evident from elevated (2.3-fold) intracellular sorbitol levels as compared to BLECs maintained in physiologic medium (MEM). Intracellular polyol accumulation differed by nearly 4-fold in cells exposed to Gal medium as compared to those maintained in SHM for 3 days. The additional accumulation of galactitol in BLECs can possibly be explained because of certain characteristics of the polyol pathway with regards to

substrate preference. Besides galactose being a preferred substrate over glucose for the enzyme aldose reductase, sorbitol is further metabolized by sorbitol dehydrogenase to fructose which slowly permeates out of the cell. Galactitol, on the other hand, is not a substrate of sorbitol dehydrogenase allowing it to accumulate intracellularly at much higher concentrations than sorbitol. These elevated intracellular levels of galactitol in BLECs exposed to galactose medium provide the necessary "stimulus" to activate the efflux pathway and promote significant myo-inositol loss.

Measurments of intracellular levels of myo-inositol [Fig. 2(A)] and polyols [Fig. 2(B)] in BLECs maintained in sodium hypertonicity (SHM, 473 ± 6 mOsm) then transferred into Gal (MEM + 40 mM galactose, 285 ± 4 mOsm) or Glc (MEM + 40 mM glucose, 281 ± 6 mOsm) clearly demonstrates that cells switched into Gal medium experience greater "efflux pressure" from additional polyol formation compared to those switched into Glc medium. Under hypotonic medium conditions (and isotonic medium conditions seen in Figure 1), BLECs are capable of accumulating significantly greater intracellular polyol levels of galactitol as compared to sorbitol [Fig. 2(B)], due to certain previously mentioned reasons regarding characterisitcs of aldose reductase and the polyol pathway. These results strongly support the decision to use galactose instead of glucose in our attempt to demonstrate and characterize polyol-activated myo-inositol efflux in BLECs (Reeves and Cammarata, 1996).

The leakout (efflux) of various substance have been reported in galactose-exposed lens (Broekhuyse, 1968; Kinoshita et al., 1968; Kinoshita, 1965). Kawaba et al. (1985) also demonstrated the increase in myo-inositol run-out (efflux) in lens maintained in

galactose medium. These experiments presumed that myo-inositol efflux in galactose-exposed lens was due to changes in membrane permeability as a direct result from intracellular polyol accumulation. These results were inferred but not conclusive; however, BLECs maintained in physiologic medium and subsequently transferred into Gal medium, as shown in Figure 3, display a significantly greater efflux of myo-[^{3}H]inositol than cells exposed to Gal medium in the presence of the aldose reductase inhibitor, sorbinil (Gal/Sorbinil). Here, we conclusively demonstrate that inhibition of polyol formation and accumulation with sorbinil, which subsequently protects the cell from unnecessary water imbibition and cell swelling, prevents osmolyte loss (efflux) from the cell to medium. We have previously reported similar results in BLECs maintained in hypertonic medium then switched to either physiologic or hypertonic medium containing 40 mM galactose, both in the absence and presence of sorbinil (Reeves and Cammarata, 1996).

Phloretin, a known sugar transport and PKC inhibitor (Kimmich and Randles, 1975; Tanaka et al., 1986) has been shown previously to block swelling- and polyol-activated myo-inositol efflux (Reeves and Cammarata, 1996). These data determined that the mechanism of efflux inhibition by phloretin was not associated with reduced sugar uptake or PKC inhibition. In this study, efflux was demonstrated in isotonically maintained BLECs instead of cells exposed to hyptonic insult (as previously demonstrated). However, regardless of the experimental protocol or the mechanism of blockage, phloretin exhibited substantually greater inhibition of myo-[^{3}H]inositol efflux than sorbinil. Because of this significant difference in the inhibitory effect of phloretin

over sorbinil, these data suggests that the two drugs are blocking efflux in different manners. Phloretin has no effect on polyol formation or sugar uptake mechanisms (Reeves and Cammarata, 1996), but could possibly be interacting directly with the anion/osmolyte channel responsible for myo-inositol efflux, or alternatively, through an unidentified indirect secondary effect..

The inhibitory effect of niflumic acid, a chloride channel inhibitor, has been demonstrated on both swelling- and polyol-activated efflux of myo-[^{3}H]inositol in BLECs (Reeves and Cammarata, 1996; Reeves et al., 1997). In Figures 5(A) and (B) the effect of the chloride channel inhibitor, tamoxifen, is clearly visible on both swelling- and polyol-activated myo-[^{3}H]inositol efflux, respectively. The ability of these inhibitors to effectively block swelling-induced osmolyte efflux in many culture systems (Banderali and Roy, 1992; Jackson and Strange, 1993; Gonzalez et al., 1995; Reeves and Cammarata, 1996) lends further support to our proposal that swelling-activated osmolyte loss in BLECs, from either a hypotonic- or poylyol-induced source, is mediated through a chloride channel. Furthermore, the data obtained from these experiments strongly supports the view that both swelling- and polyol-activated myo-[^{3}H]inositol efflux in BLECs is mediated through a common anion pathway thought to be a swelling-activated chloride channel.

The rapid efflux of osmolytes, especially that seen in hpotonic-exposed cells, appears to have a cell volume regulatory function. When the extracellular osmolaltiy decreases, lens epithelial cells initially swell because of osmotic water uptake. The rapid efflux of chloride and potassium associated with concomitant water uptake and osmolyte

loss has been found to mediate volume regulatory decrease in many different types of cells (Nakanishi and Burg, 1989). This volume regulatory function could similarly explain the purpose behind myo-inositol efflux in galactose exposed cells experiencing polyol-induced swelling from water uptake.

The Na^+/K^+ ATPase inhibitor, ouabain, has been shown to promote swelling in the lens because of elevated intracelluar Na^+ levels once the pump mechanism is blocked. Kinoshita (1974) showed that exposure of lens to galactose medium or medium containing ouabain caused significant water gain compared to control. In Figure 6, amplified cellular swelling and water uptake could explain the amplification of myo-[^{3}H]inositol efflux seen in hypertonically acclimated BLECs switched into galactose medium in the presence of ouabain (SHM > Gal/Ouabain) as compared to those cells switched into galactose medium (SHM > Gal). Under these experimental circumstances, multiple swelling "stimuli" are present for both medium conditions including hypotonic exposure and polyol accumulation; however, cells transferred into Gal/Ouabain have an additional swelling effect from this inhibitor. It appears that activation of the myo-inositol efflux mechanism is responsive to the magnitude of cell swelling.

Cell volume changes, such as cell-swelling, not only activate myo-inositol efflux, but have recently been shown to regulate the mRNA expression of pI_{Cln}, a protein believed to be directly involved in osmolyte efflux, anion movement and cell volume regulation (Reeves et al., 1997). There is good evidence which suggests cells can sense both the magnitude and mechanism of changes in cell volume (Strange et al., 1996). Hypotonic-induced swelling can be a very rapid and intense occurance as compared to

cell swelling associated with intracellular polyol accumulatin, which tends to be a more gradual and prolonged event. As intracellular solutes (polyols) accumulate in cells exposed to galactose medium, gradual cell swelling occurs. Studies involving experimental animal models have shown that intracellular polyol accumulation results in swelling from increased lens hydration (Kinoshita, 1974), as recently demonstrated by the increase in lens wet weight of galactose-fed rats (Beyer-Mears, 1991).

In this study, we were interested if the mRNA expression of pI_{Cln} was affected by gradual cell swelling and hydration ensuing from intracellular polyol accumulation, an event associated with myo-inositol efflux in BLECs. If indeed the polyol-induced depletion of intracellular myo-inositol is a causative factor or contributor to diabetic complications as proposed (Finegold et al., 1983; MacGregor and Matschinsky, 1985; Winegrad, 1987; Greene et al., 1987; Cammarata et al., 1990; Hohman et al., 1991; Lin et al., 1993), then a polyol-promoted increase in transcription of pI_{Cln} mRNA might encourage the further decline of intracellular myo-inositol pools, resulting in even greater cellular damage. In Figure 7, BLECs were maintained in SHM for 24 hours to increase the activity of aldose reductase causing rapid and amplified intracellular polyol accumulation. However, conditions favoring rapid polyol formation (SHM-24hr > HGM-8 or 24hr) resulted in no significant difference of pI_{Cln} mRNA expression compared to cells switched into fresh sodium hypertonic medium for 24 hours (SHM-24hr > SHM-8 or 24hr). We cannot rule out the possibility that mRNA expression of pI_{Cln} in BLECs switched into hypertonic galactose medium was not affected by the initial 24 hour

hypertonic exposure. Sodium hypertonicity has been shown to upregulate pI_{Cln} mRNA expression in BLECs, with maximum induction occuring within 4 to 8 hours (Reeves et al., 1997); however, by 24 hours the expression of pI_{Cln} mRNA had returned to near physiologic levels (data not shown). Whereas there was no significant difference observed in mRNA expression of pI_{Cln} between the varying medium conditions, these results suggests that enhanced MI efflux from intracellular polyol-accumulation is not a result of increased transcription and the subsequent protein expression of pI_{Cln}.

In summary, polyol-induced myo-inositol efflux in BLECs favors intracellular galactitol accumulation simply because this polyol is capable of accruing at much greater intracellular concentrations than sorbitol. This property of galactose exposed cells results in greater intracellular osmotic stress which activates volume regulatory control mechanisms to counter cell swelling. Apparently osmolyte loss along with ion movement from cell to medium both play a functional role in volume regulation by releaving intracellular osmotic stress, initiating a gradual yet inevitable volume regulatory decrease.

Similar volume regulatory events, such as osmolyte loss, occur in hypotonically exposed BLECs, which are effectively inhibited by numerous anion channel blockers including tamoxifen, a potent inhibitor of swelling-activated chloride channels. Tamoxifen's inhibitory effect on myo-inositol efflux is extremely convincing; moreover, these data suggests a common pathway (probably a chloride channel) exists for polyol- or swelling-induced myo-inositol loss. Inhibition of Na^+/K^+ ATPase with ouabain exacerbates myo-inositol efflux in response to water imbibtion and cell swelling elicited

by rising intracellular Na^+ levels. This strongly supports previous results concerning the role of cell swelling, regardless of the source, in osmolyte loss (efflux) from cell to medium. Besides activating osmolyte efflux mechanisms, fluctuations in cell volume regulate the mRNA expression of the chloride channel/regulatory protein, pI_{Cln}. In previous studies (Reeves et al., 1997), the mRNA expression of this protein has been shown to respond to the magnitude of cell swelling; however, these data suggests that gradual cellular swelling associated with intracellular polyol accumulation does not exert the needed stimulus to have any appreciable effect on expression levels.

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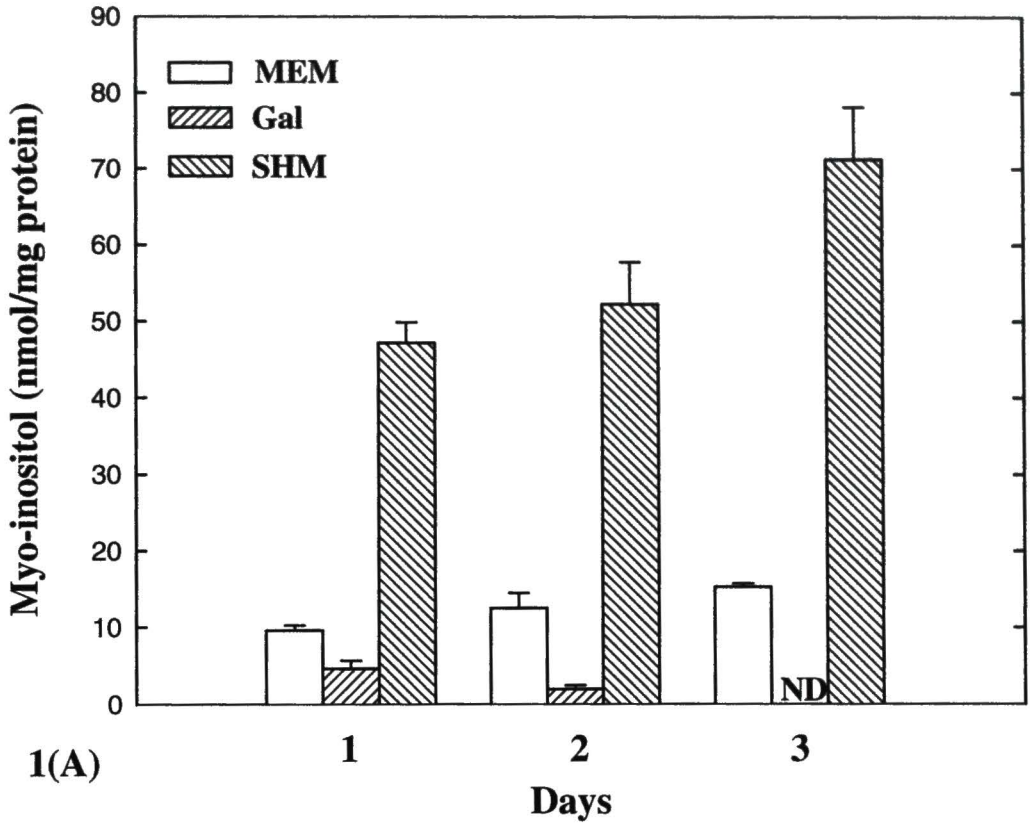
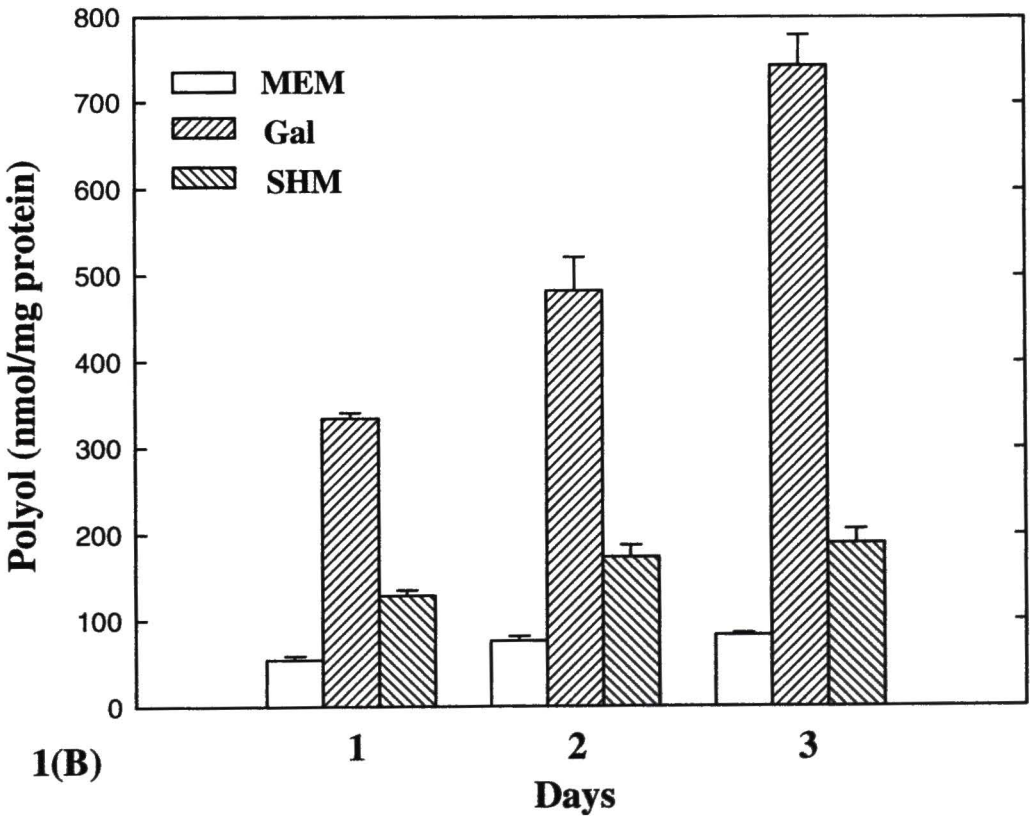
Figure 1. Intracellular myo-inositol and polyol accumulation in BLECs. Bovine lens epithelial cells were maintained in physiologic medium (MEM, 257 ± 2 mOsm), Gal (MEM + 40 mM galactose, 285 ± 4 mOsm), or SHM (sodium hypertonic medium = MEM + 116 mM NaCl, 473 ± 6 mOsm) for 3 days. Intracellular myo-inositol (A) and polyol (B) levels were determined by anion exchange chromotography. The data represent samples of cells collected from individual flasks at 1, 2, and 3 days. The error bars indicate mean ± SE.

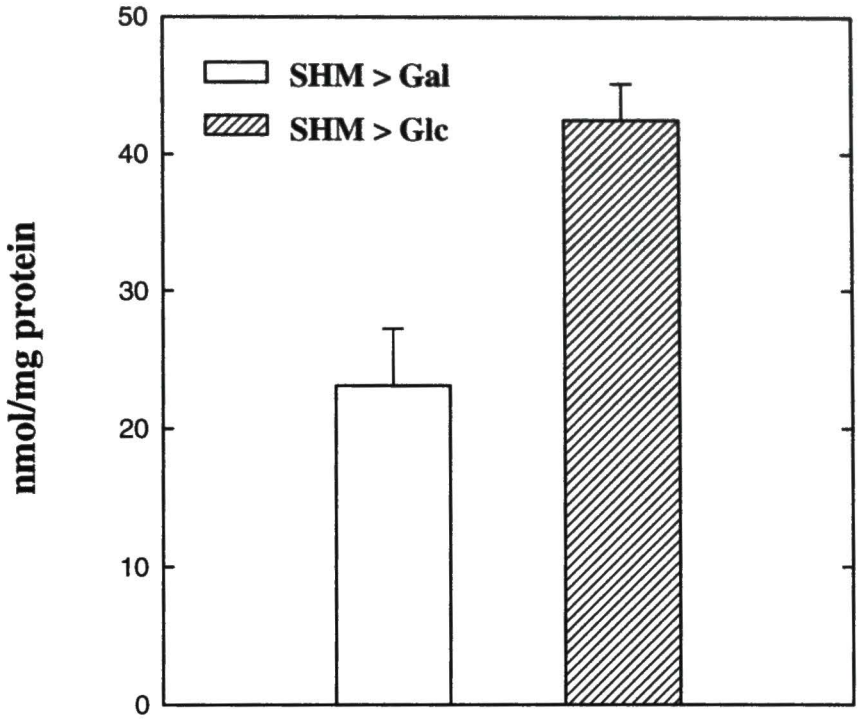

MEM
Gal
SHM
Myo-inositol (nmol/mg protein)
90
80
70
60
50
40
30
20
10
0
ND
1
2
3
Days

1(A)

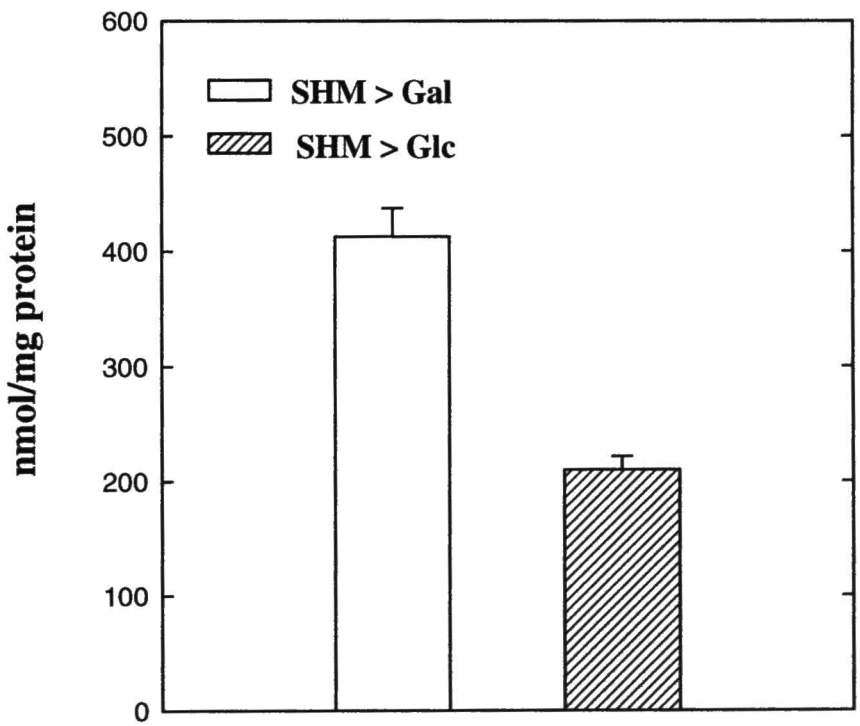

MEM
Gal
SHM
Polyol (nmol/mg protein)
800
700
600
500
400
300
200
100
0
1
2
3
Days

1(B)

Figure 2. Accumulation of intracellular myo-inositol and polyols differ with galactose and glucose exposure. Bovine lens epithelial cells were maintained in SHM (sodium hypertonic medium, 473 ± 6 mOsm) for 48 hours then switched into Gal (MEM + 40 mM galactose, 285 ± 4 mOsm) or Glc (MEM + 40 mM glucose, 281 ± 6 mOsm) for an 8 hour hypotonic exposure. Intracellular myo-inositol and polyol levels were measured by anion exchange chromotagraphy. The data represent triplicate samples of cells collected from individual flasks after 8 hours of medium osmolality reduction and the error bars indicate mean ± SE.


50
40
30
20
10
0
nmol/mg protein
SHM > Gal
SHM > Glc
MI
MI

(A)


600
500
400
300
200
100
0
nmol/mg protein
SHM > Gal
SHM > Glc
GalOH
SorOH

(B)

Figure 3. Intracellular galactitol accumulation enhances myo-[^{3}H]inositol efflux in bovine lens epithelial cells maintained under isotonic conditions. Cells were maintained to confluence in MEM then switched into fresh MEM containing 40 mM galactose ± 0.1 mM sorbinil or 0.2 mM phloretin and efflux monitored at 8, 12, and 24 hours. The data points (or bars) represent released myo-[^{3}H]inositol measured from preloaded cells collected at the designated time points. Data are mean ± SE for duplicate samples from triplicate flasks.

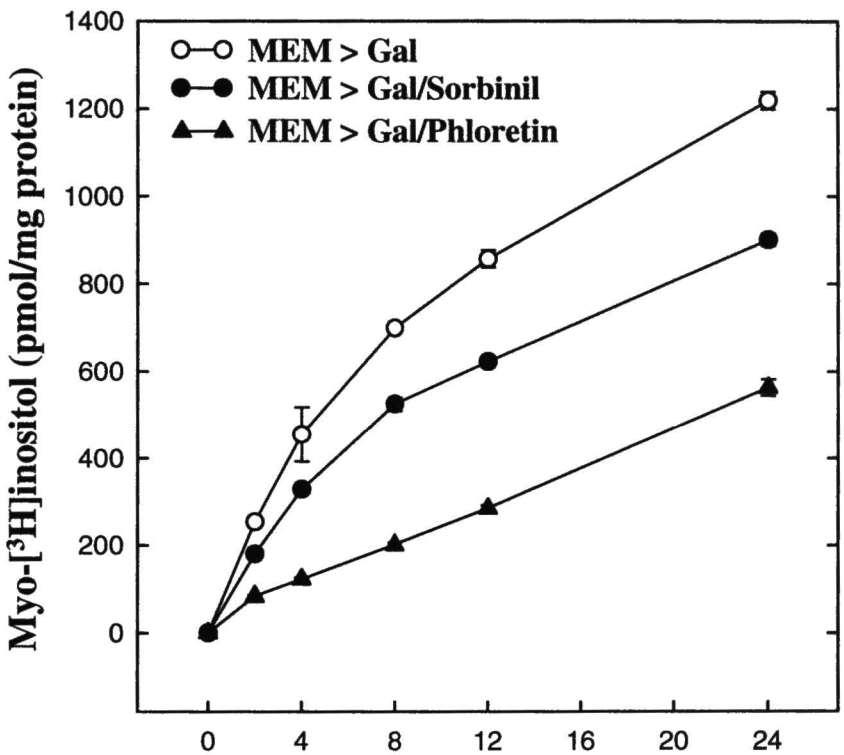

MEM > Gal
MEM > Gal/Sorbinil
MEM > Gal/Phloretin
Myo-[3H]inositol (pmol/mg protein)
1400
1200
1000
800
600
400
200
0
0
4
8
12
16
20
24

Figure 4. Elevated extracellular medium myo-inositol inhibits myo-[^{3}H]inositol efflux. Bovine lens epithelial cells maintained in sodium hypertonic medium (SHM) for 48 hours were switched in physiologic medium (MEM) containing 0.015, 0.030, 3.0 or 30.0 mM medium myo-inositol for a 2 hour efflux period. The data represent mean ± SE for duplicate samples from triplicate flasks.

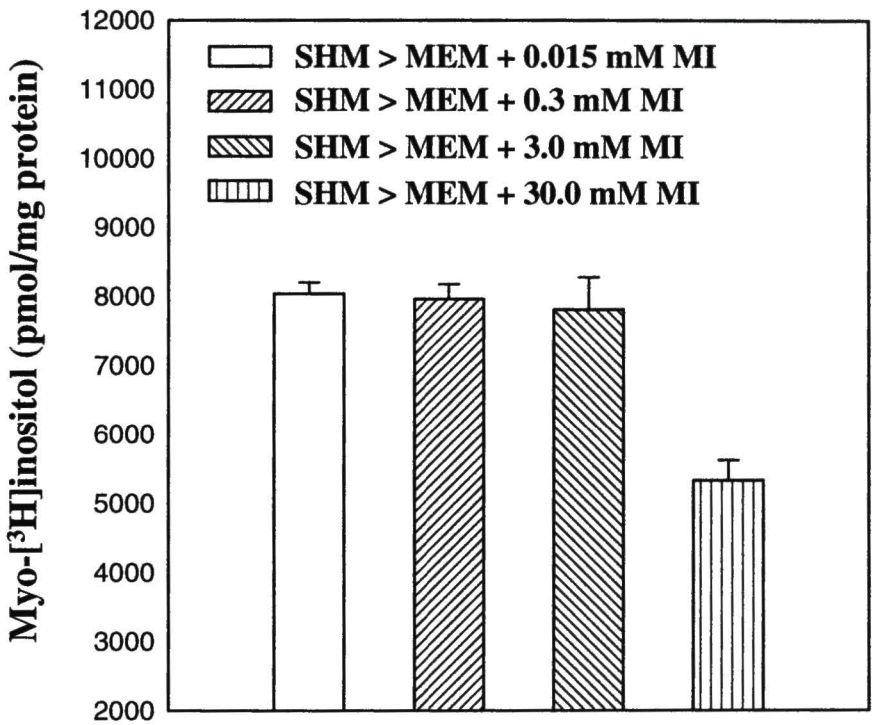
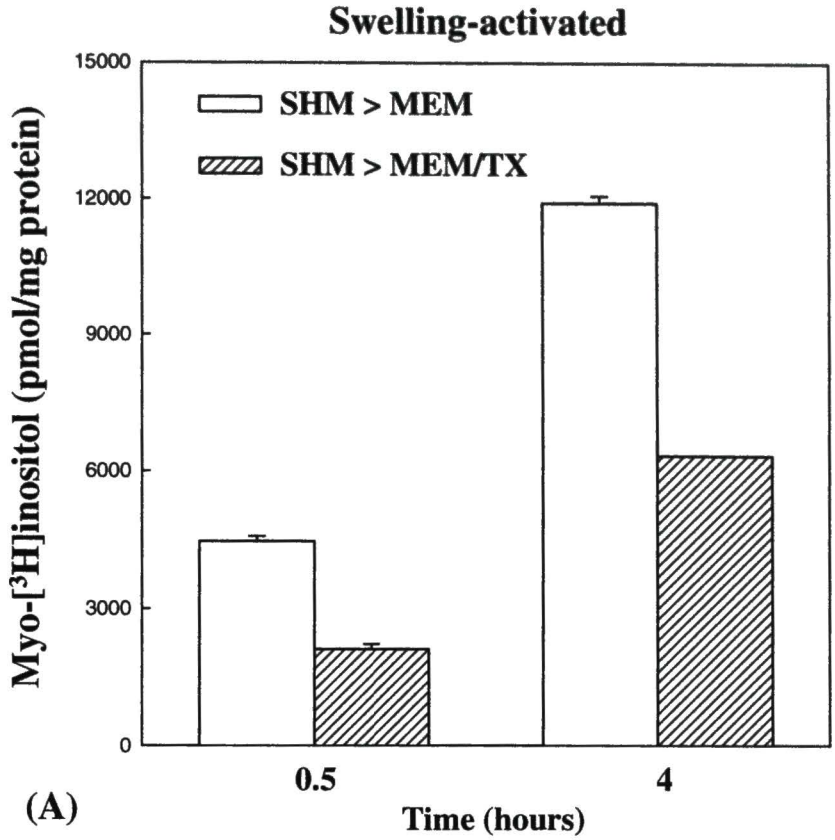
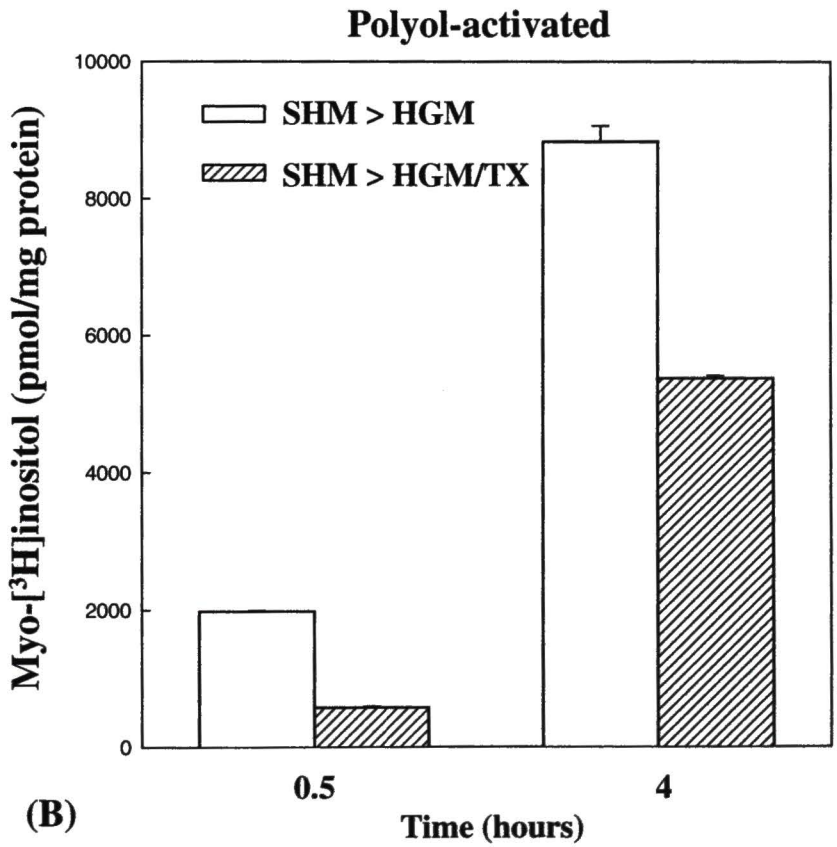

Myo-[3H]inositol (pmol/mg protein)
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
SHM > MEM + 0.015 mM MI
SHM > MEM + 0.3 mM MI
SHM > MEM + 3.0 mM MI
SHM > MEM + 30.0 mM MI

Figure 5. Tamoxifen inhibits myo-[^{3}H]inositol efflux. Bovine lens epithelial cells were maintained in SHM (473 ± 6 mOsm) for 48 hours followed by a 2 hour incubation in fresh SHM ± 0.1 mM tamoxifen (TX). For inhibition of swelling-activated efflux [Figure 5 (A)], cells were switched into MEM (257 ± 2 mOsm) ± 0.1 mM tamoxifen. For inhibition of polyol-activated efflux [Figure 5 (B)], cells were transferred to fresh SHM ± 0.1 mM tamoxifen. Data are mean ± SE for duplicate samples from triplicate flasks taken at 30 minutes and 4 hours of the efflux period.


Swelling-activated
SHM > MEM
SHM > MEM/TX
Myo-[3H]inositol (pmol/mg protein)
15000
12000
9000
6000
3000
0
0.5
4
Time (hours)

(A)


Polyol-activated
SHM > HGM
SHM > HGM/TX
Myo-[3H]inositol (pmol/mg protein)
10000
8000
6000
4000
2000
0
0.5
4
Time (hours)

(B)

Figure 6. Ouabain exposure enhances myo-[^{3}H]inositol efflux. Bovine lens epithelial cells (BLECs) were maintained in SHM (473 ± 6 mOsm) for 48 hours. Cells were then transferred into fresh SHM ± 0.1 mM ouabain for a 1 hour incubation period. After the incubation period, BLECs were switched into Gal (MEM + 40 mM galactose, 285 ± 4 mOsm) medium and efflux was measured at 2, 4, 8, and 12 hours. The data represent mean ± SE for duplicate samples from triplicate flasks.

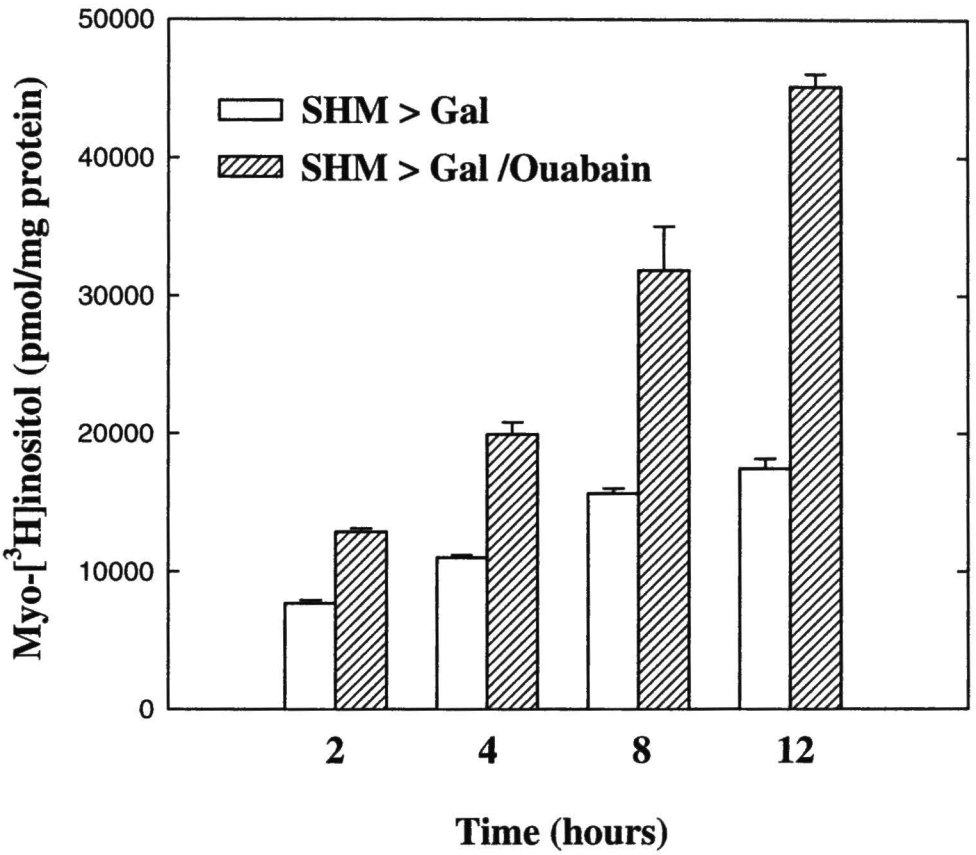
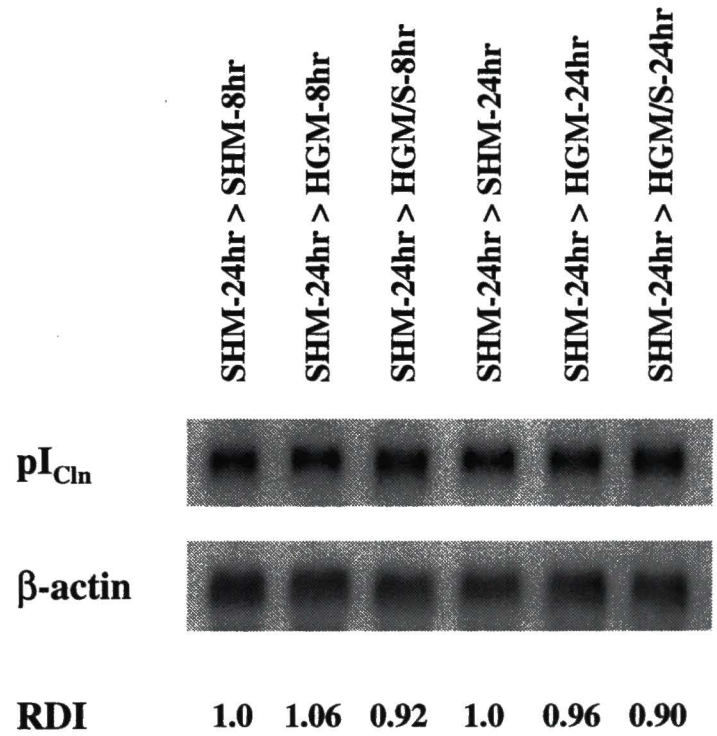

50000
40000
30000
20000
10000
0
SHM > Gal
SHM > Gal /Ouabain
Myo-[3H]inositol (pmol/mg protein)
2
4
8
12
Time (hours)

Figure 7. The effect of galactose exposure and polyol accumulation on pI_{Cln} mRNA expression. Bovine lens epithelial cells were acclimated in SHM for 24 hours then switched into fresh SHM or SHM ± 0.1 mM sorbinil for 8 and 24 hours. Lane 1 and 4 represent controls for each respective time period. 5 *ug* of poly $(A)^+$ RNA were loaded per lane. RDI = relative densitometric intensity (normalized to β-actin).


SHM-24hr > SHM-8hr
SHM-24hr > HGM-8hr
SHM-24hr > HGM/S-8hr
SHM-24hr > SHM-24hr
SHM-24hr > HGM-24hr
SHM-24hr > HGM/S-24hr
pI$_{Cln}$
β-actin
RDI
1.0
1.06
0.92
1.0
0.96
0.90

CHAPTER V

SUMMARY

Many different cell types, including the bovine lens epithelial cells, actively accumulate organic osmolytes, such as myo-inositol, taurine, betaine, and sorbitol, in order to fulfill cellular osmoregulatory requirements. These nonperturbing compounds can accumulate at relatively high intracellular concentrations without disrupting normal cellular and enzymatic processes. The lens, an avascular structure, requires a balance of these osmolytes as a protective mechanism against water stress. The depletion of osmolytes associated with disorders of sugar metabolism have been investigated for many years as causitive factors for pathological complications, such as sugar cataract formation and loss of nerve conductance (in diabetics).

Elevated extracellular sugar levels have been shown to increase the activity of aldose reductase, a key enzyme in the polyol pathway (Akagi et al., 1987). In the lens, disorders like diabetes and galactosemia can cause the depletion of intracellular myo-inositol by several different mechanisms. The intracellular depletion of myo-inositol in cultured lens epithelial cells begins with the elevation of intracellular polyols which in turn inhibit the Na^+/myo-inositol cotransporter (Cammarata et al., 1992), suppress Na^+/myo-inositol cotransporter mRNA expression (Zhou et al., 1994), and activate the myo-inositol efflux pathway (Reeves and Cammarata, 1996). Using cultured bovine lens epithelial cells (BLECs), the identification and characterization of the myo-inositol efflux

pathway, the least understood of the three avenues of intracellular myo-inositol depletion, was investigated in this study.

The initial requirement, regarding the understanding of this pathway, was to develop an assay in which the efflux of myo-inositol in the lens epithelial cells could be easily monitored. This was achieved by culturing cells under a variety of experimental media mimicing conditions such as hyperglycemia (diabetes), hypergalactosemia, hypertonicity and hypotonicity. Intracellular levels of myo-inositol, sorbitol, and galactitol, as well as glucose and galactose, were evaluated by anion exchange chromatography from this array of medium conditions.

Unique variations in intracellular content of these sugars and polyols were found between differing medium properties. In BLECs, sodium hypertonicity upregulates aldose reductase and the Na^+/myo-inositol cotransporter mRNA expression, in addition to inreasing the Na^+/myo-inositol cotransporter protein's uptake activity (Zhou et al., 1994; Cammarata and Chen, 1994). Verification of these events, determined by anion exchange chromatography, showed elevated intracellular myo-inositol and sorbitol levels in hypertonically maintained cells. What emerged from these initial observations was a method in which the intracellular levels of myo-inositol could be artificially elevated while simultaneously introducing labeled myo-[^{3}H]inositol into the cells. Cultured BLECs could then be transferred to different experimental media, depending on the desired method of efflux induction, to evoke myo-inositol loss from cell to medium. The characteristic upregulation of aldose reductase and the Na^+/myo-inositol cotransporter mRNA expression associated with sodium hypertonicity, which increased intracellular

myo-inositol content and aldose reductase activity, proved to be critical in confirming polyol-activated efflux.

Switching BLECs from hypertonic (473 ± 6 mOsm) to physiologic (257 ± 2 mOsm) medium caused a biphasic efflux (loss) of myo-inositol from the cell to medium. A very rapid loss occured within the first 30 minutes followed by a slower yet significant loss over the next 24 hours. This hypotonic-induced loss, referred to as swelling-activated efflux (or tonicity-activated), was not affected by the activation or inhibition of intracellular protein kinasce C (Reeves and Cammarata, 1996). Recently, swelling-activated efflux of sorbitol and taurine have been demonstrated in renal papillary cells (Furlong et al., 1991) and C6 glioma cell (Strange et al., 1993), respectively, with essentially identical efflux characteristics that have been described for myo-inositol efflux. These results suggests that these three osmolytes may share a common transport pathway for efflux.

Previous studies with C6 glioma cells and Madin-Darby canine kidney (MDCK) cells suggested an association with anion movement and intracellular osmolyte loss (Strange et al., 1993; Banderali and Roy, 1992). The chloride channel inhibitors niflumic acid, 1,9-dideoxyforskolin, and tamoxifen significantly inhibited swelling-activated myo-inositol efflux in hypotonically exposed BLECs. These data, combined with the observed inhibition of myo-inositol efflux with substitution of extracellular Cl^-, lend strong support to an interrelationship between anion (chloride) movement and intracellular myo-inositol loss in BLECs, possibly through the same channel or pathway.

The depletion of intracellular myo-inositol in galactose-exposed lenticular tissue

has been observed previously in numerous studies (Broekhuyse, 1968; Kawaba et al., 1986; Reddy et al., 1992). They assumed myo-inositol loss occured from intracellular accumulation of polyols with resulting hydration and increased cell permeability; however, these data did not conclusively demonstrate the specific activation of a polyol-induced efflux mechanism in the lens. Data presented in these studies positively confirmed polyol-activation of the myo-inositol efflux pathway in BLECs cultured in both hypertonic and isotonic medium conditions. Slight modification of the efflux assay combined with the use of the aldose reductase inhibitor, sorbinil, showed intracellular polyol formation and accumulation significantly amplified myo-inositol loss from cell to medium. Furthermore, BLECs were able to accumulate significantly higher intracellular levels of galactitol than sorbitol which resulted in enhanced myo-inositol efflux in galactose exposed cells. Specific chloride channel inhibitors which had previously been shown to block hypotonic-induced efflux showed an analogous effect on polyol-activated myo-inositol efflux. Tamoxifen and niflumic acid, both inhibitors of swelling-activated anion channels, in all likelihood, block myo-inositol loss from cell to medium by inhibiting channel conducted chloride movement. These results suggests a common efflux pathway exists for swelling- or polyol-induced myo-inositol loss which further imply that efflux is a reflexive osmoregulatory event responding to swelling "stimuli" and directly linked to chloride movement from cell to medium..

Replacement of $Na^+$with NMDG had no effect on swelling-activated myo-inositol efflux in BLECs; however, increasing the gradient of extracellular myo-inositol significantly inhibited efflux under hypotonic conditions. Further characterization of this

pathway was achieved by reversing the normal concentratioin gradients of myo-inositol in cultured BLECs. This was accomplished by culturing cells in L-glucose supplemented medium to deplete intracellular myo-inositol followed by a hypotonic exposure with significantly elevated medium myo-inositol concentrations. Once swelling was induced, rapid influx (from medium to cell) of myo-inositol occured which was significantly blocked with the chloride channel inhibitor niflumic acid. This experiment confirmed the passive bi-directional flow of myo-inositol across a channel pathway. Taken together, these data suggest a swelling-sensitive, Na -independent, concentration-gradient driven, non-specific anion/osmolyte channel as the major participant in the loss of osmolytes (i.e. myo-inositol) from the cell to medium. The characteristics of this pathway are summarized in Figure 1.

A functional relationship has been suggested for the channel regulatory protein, pI_{Cln}, and the chloride channel, ClC-3. It has been proposed that the volume sensitive protein, pI_{Cln}, regulates chloride movement through the chloride channel ClC-3 (Coca-Prados et al., 1996). The expression of mRNA for ClC-3 was identified in cultured bovine lens epithelial cells, maintained under physiologic conditions, utilizing Northern blot analysis of poly $(A)^+$ RNA. Also, the pattern of induction of pI_{Cln} mRNA levels in BLECs, cultured under conditions conducive to osmolyte efflux, were investigated using similar Northern blot techniques.

In cultured BLECs, the transcriptional regulation of pI_{Cln} mRNA appears to be responsive to the magnitude and mechanism of cell swelling. Hypertonic exposure (cell shrinkage) upregulates the expression of pI_{Cln} mRNA while hypotonicity (cell swelling)

downregulates pI_{Cln} mRNA expression. Amplification of cell swelling with the use of chloride channel inhibitor elicits an intensified downregulation of message. These data suggests that a converse relationship exists between changes in cell volume and pI_{Cln} mRNA expression. The volume-sensitivity of the protein, pI_{Cln}, coupled with its transcriptional characteristics, lend stong support to the proposal that this protein plays a regulatory role in both anion and osmolyte loss from cell to medium and cell volume management.

In summation, the myo-inositol efflux pathway functions as a "relief valve" in the cellular volume regulatory control mechanism by providing the cell a conduit to alleviate intracellular osmotic stress resulting from hypotonic exposure or intracellular polyol accumulation. A single swelling-activated channel may mediate not only the transport of myo-inositol from cell to medium, but also many other organic solutes (osmolytes) that are structurally unrelated. However, it is intriguing to postulate that the mechanism which evolved to function under normal cellular circumstances in relief of excessive accumulation of intracellular osmolytes (i.e. polyols), may, by design, inadvertently promote the loss of essential intracellular volume and nonvolume regulatory organic solutes. Ironically, under certain pathological conditions such as hyperglycemia and hypergalactosemia, this mechanism, designed to protect the cell from intracellular osmotic stress, may be detrimental to the cell by promoting the excessive loss of osmolytes essential for normal cell function.

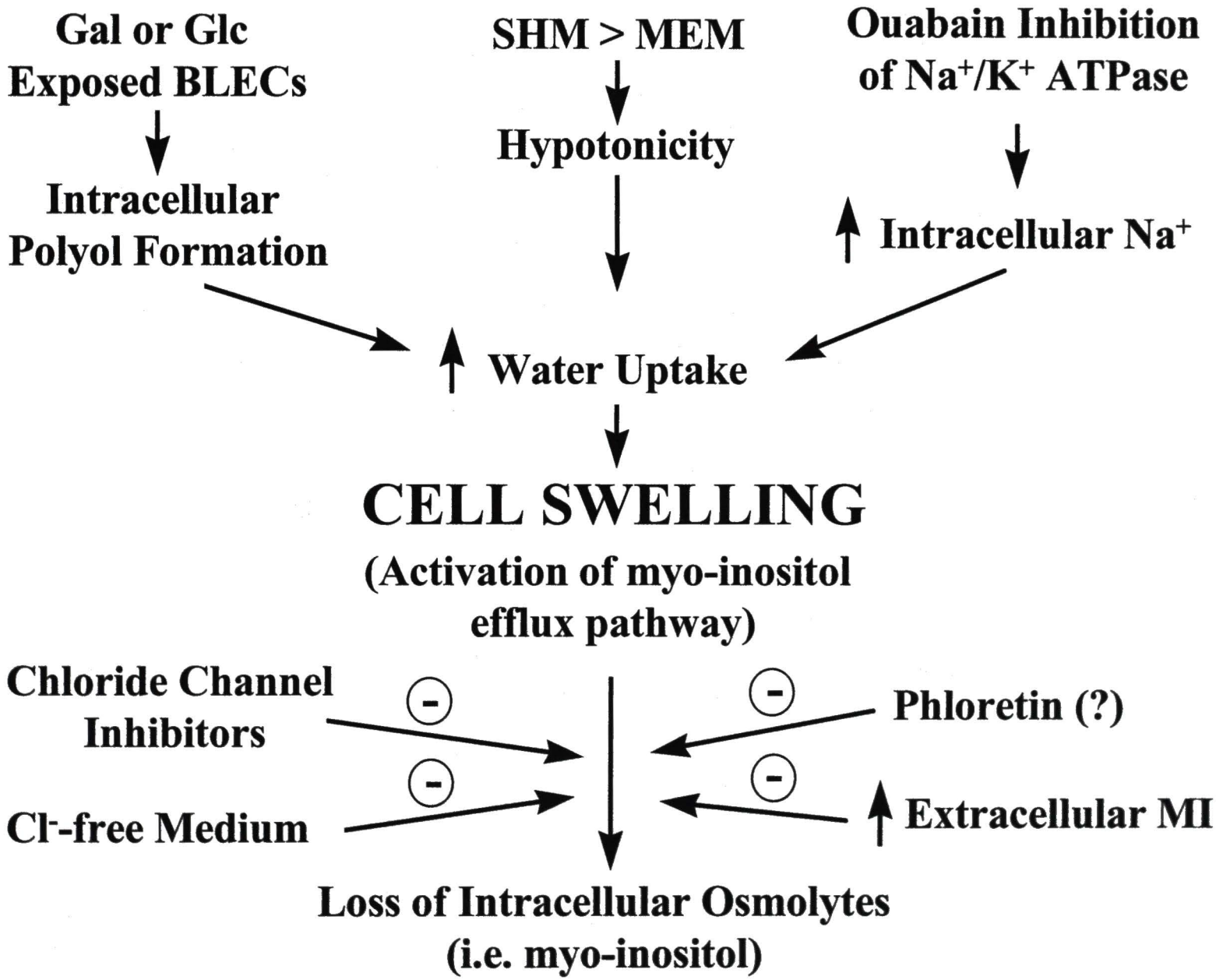


Figure 1. Myo-inositol Efflux Pathway in BLECs

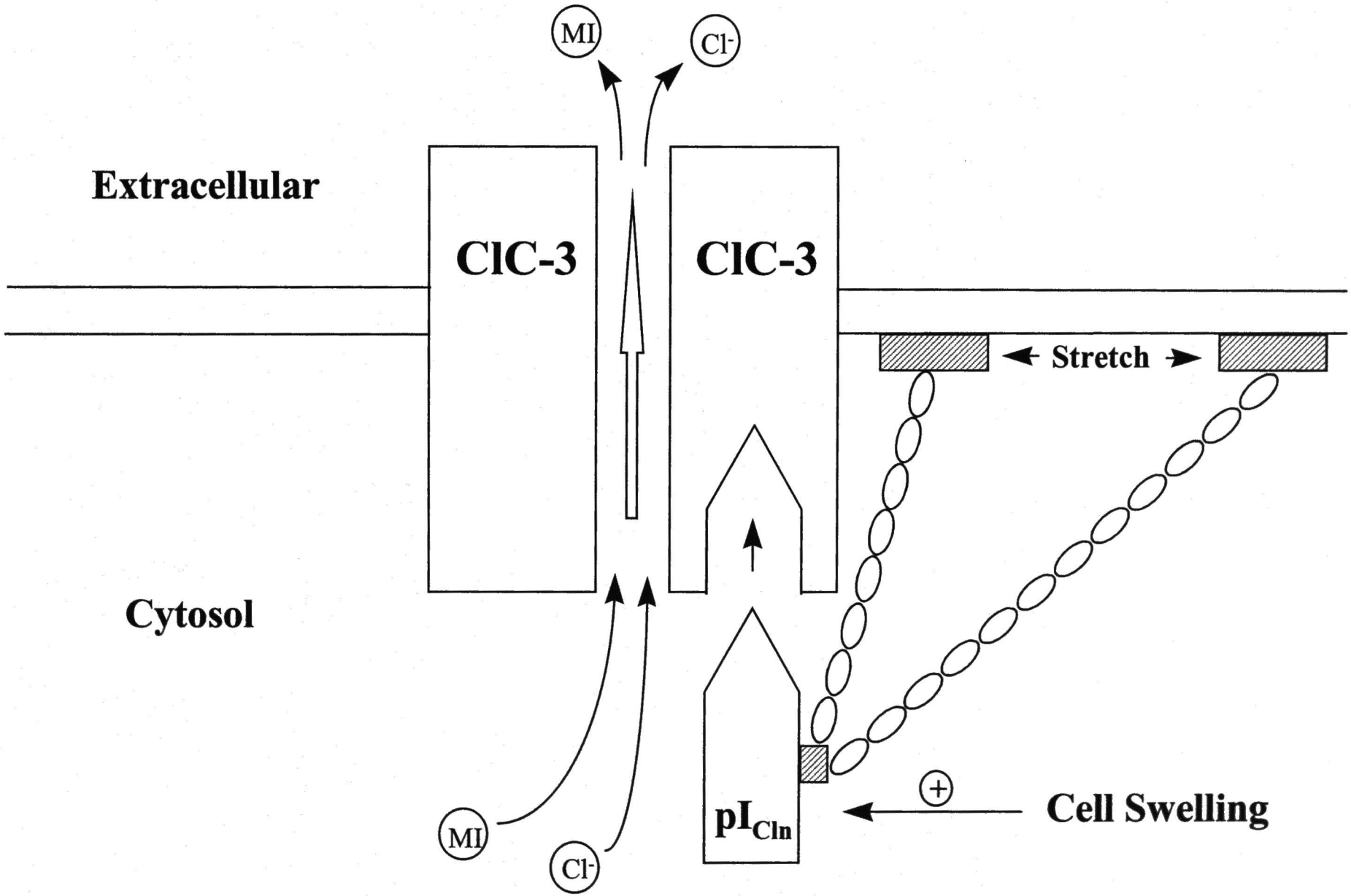


Figure 2. Model of pI_{Cln} regulated anion/osmolyte efflux in BLECs (Modified from Coca-Prados et al., 1996)

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