ABSTRACT

MACK, CINA MARIE. EMBRYONIC EPITHELIAL DEVELOPMENT is MODULATED by EXTRACELLULAR CALCIUM. (Under the direction of Betty L. Black.)

This study investigated the effects of calcium on epithelial differentiation in organ-cultured embryonic chick duodenum. Alteration of calcium ion concentration of culture medium above or below the physiological Ca^{2+} concentration of embryonic chick serum strongly influenced gastrointestinal development. Culture of 16- or 18-day embryonic intestine in high Ca^{2+} resulted in elevated activity of alkaline phosphatase, a brush border digestive enzyme, and an increase in goblet cell number within the epithelium. Conversely, low Ca^{2+} reduced both indices of functional differentiation. Lead acetate, a disruptor of Ca^{2+} homeostasis, was as effective as low Ca^{2+} in impeding epithelial differentiation. The voltage sensitive Ca^{2+}-channel (VSCC) antagonists verapamil and nifedipine were used to selectively block Ca^{2+} influx into the cultured tissue. Both verapamil, a non-specific channel blocker, and nifedipine, which is highly selective for L-type VSCCs, significantly inhibited differentiation, but nifedipine gave more consistent results. Imaging of calcium within the epithelium of cultures was performed by confocal scanning laser microscopy, using the fluorescent calcium probe indo-1. Quantification of Ca^{2+} within enterocytes revealed that both verapamil and nifedipine reduced intracellular Ca^{2+} concentration. Together, these results indicate that extracellular Ca^{2+} influences epithelial differentiation by modulating intracellular Ca^{2+}-dependent signaling pathways, possibly mediated by VSCCs.

EMBRYONIC EPITHELIAL DEVELOPMENT IS MODULATED BY EXTRACELLULAR CALCIUM

by

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BIOGRAPHY

Cina Mack attended the University of North Carolina at Chapel Hill and received a B.S. degree in Biology. After participating in a summer science program, she decided to pursue a graduate education in the area of physiology. Next, she attended North Carolina State University where she received an M.S. in Physiology with a concentration in neurophysiology. Shortly thereafter, she entered the doctoral program in physiology at NCSU. A career as a research scientist studying the effects of environmental stresses on developmental neurophysiology is planned.

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Chapter 1

GENERAL INTRODUCTION

Cellular communication is an essential component of life. Cell signaling involves interactions among a complex network of macromolecules and ions. Free calcium (Ca^{2+}) is the single most important mediator of cellular communication. Calcium is a well-studied regulator of varied physiological cellular functions (Campbell, 1983). It is a ubiquitous second-messenger in diverse cell types. Identification of an intracellular Ca^{2+} storage site and the Ca^{2+}-binding protein, troponin (Ebashi and Lipmann, 1962; Ebashi, 1974), established a clear link between Ca^{2+}, binding proteins, and cellular responses (muscle contraction). Subsequently Ca^{2+}-binding proteins were found in other cells and the importance of Ca^{2+} in cellular signaling and regulation was increasingly recognized (Kretsinger, 1976). Growth and differentiation are two vital cellular processes that are Ca^{2+}-dependent (Campbell, 1983). Historically, attention has focused on intracellular Ca^{2+} as a mediator of these processes. However, the concentration of Ca^{2+} in extracellular fluid is vital to several physiological processes including membrane permeability, excitability, and as a regulator of cytoplasmic Ca^{2+} levels.

Study Objective

The objective of this study was to investigate the effects of extracellular Ca^{2+} on later stage (post 14D) embryonic chick epithelium development in cultured duodenum. The biochemical index of maturation was alkaline phosphatase (ALP), a microvillar brush-border enzyme sensitive to Ca^{2+} (Black and Rogers 1992). Goblet cell number was used as a morphological index of epithelial differentiation, since most goblet cells in embryonic intestinal epithelium arise by terminal differentiation of stem cells. The effects of a non-specific Ca^{2+}-channel blocker (lead acetate, Pb^{2+}) and two

voltage-sensitive Ca^{2+}-channel (VSCC) antagonists (verapamil and nifedipine) were used to determine if extracellular Ca^{2+}-influx is directly involved in Ca^{2+} effects on differentiation. Cytoplasmic Ca^{2+} was measured in embryonic epithelial cells cultured with Ca^{2+}-channel blockers to determine if extracellular Ca^{2+} influences differentiation by modulating intracellular free Ca^{2+} concentrations. Fluorescent imaging was used to detect possible changes in the subcellular localization as well as concentration of cytosolic free Ca^{2+} in epithelium.

STUDY JUSTIFICATION

The chick embryo has been used for scientific investigation since the age of Aristotle, 345 B.C. (Romanoff, 1960). Beginning in the 1900s, the avian embryo was used to study developmental biochemistry and physiology (Hart and DeLuca, 1985). The availability and relatively low maintenance and cost have contributed to the chick embryo becoming a widely used model of development. In addition, the gestational period is relatively brief and free of maternal physiology.

The intestinal epithelium of embryonic chick is characterized by a series of distinct morphological changes (Burgess, 1975). Thus, it offers numerous biochemical and structural indices of cellular differentiation and is an ideal model for studying Ca^{2+} as a developmental regulator. While intestinal development has been investigated extensively, the influence of Ca^{2+} on growth and functional differentiation of intestinal epithelium has not been as thoroughly researched. This study will attempt to further characterize the role of Ca^{2+} in these complex processes.

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LITERATURE REVIEW

INTESTINAL DEVELOPMENT

Intestinal epithelium begins as embryonic endoderm, with the mesenchyme giving rise to connective tissue and musculature. The morphogenesis and differentiation of intestinal epithelium is a programmed, multi-step, species-specific process. Overall, structural changes are similar, varying primarily in timing. For example, human duodenum develops early in gestation whereas rodent duodenum develops late in gestation (Louvard *et al.*, 1992). However, duodenal development follows a similar pattern in all species.

Morphogenesis of the intestine is initiated by the mesenchyme, which induces the endoderm to fold and form primitive villi. Postnatally, the intervillus epithelium forms crypts at the base of the villi as the epithelial cells penetrate the underlying mesenchyme. Stem cells proliferate until reaching the upper third of the crypt, where they become committed to a differentiation pathway. Cells differentiate into enterocytes, goblet cells, enteroendocrine or Paneth cells. Cellular differentiation continues as cells migrate from crypts to villi tips. Paneth cells are the exception, they undergo inverse migration and move to the inner crypt (Simon-Assmann *et al.*, 1995; Daniele and D'Agostino, 1996). Each cell type will maintain its differentiation pattern as a function of its location on the duodenal to ileal axis and crypt-to-villus incline (Simon and Gordon, 1995). Initially, progenitor cells yield descendants that yield multipotent stem cells of the adult. The stem cell hierarchy that creates the spatial-temporal patterning during intestinal morphogenesis is regulated by complex gene and transgene expression. The inherent activation of these genes and their gene products is both time and location dependent (Simon and Gordon, 1995; Daniele and D'Agostino, 1996).

In mature intestine, absorptive enterocytes change morphologically, biochemically and functionally as they move up the villus. A structural

polarization occurs, which distinguishes the cytoplasmic membrane into two zones separated by tight junctions, allowing a specialized apical membrane to form. The other domain is the basolateral membrane, which recognizes the basement membrane of the extracellular matrix and adheres to it creating the cellular polarization (Madara *et al*, 1981). The apical membrane develops a cytoskeleton that supports microvilli, thus giving the appearance of a "brush border". The structural changes are accompanied by expression of digestive enzymes and transporters for nutrient absorption.

Morphogenesis of the vertebrate intestine is a highly regulated, discontinuous process. It may consist of several phases varying by species. In general terms, it can be divided into two critical periods of functional significance. In embryonic chick, during the third week of development, duodenal epithelium undergoes an initial phase of accelerated biochemical and morphological differentiation. In the chick, this is characterized by spiking ALP activity, enzyme concentration into the enterocyte brush border and simultaneous appearance of columnar form (Moog, 1950; 1951). The development of intestinal epithelium in mammals, the rat for example, is more complicated. The fetal rat duodenum (age 15 day) is a small tube and lumen wrapped in a 2 to 3 cell layer thick stratified epithelium (Mamajiwalla *et al*., 1992; Daniele and D'Agostino, 1996). At this age, there are no microvilli. Two days later, the epithelium has doubled to 4 to 8 cells and a basal lamina underlies the basal layer of epithelial cells. Some small microvilli are now present, as well as numerous junctional complexes, which have secondary lumena. By age 18 day, the cells adjacent to the secondary lumena develop microvilli. Epithelial folds are created from invagination of the mesenchyme. Twenty-four hours later, short villi with a one-cell layer differentiated epithelium are noticeable (Mamajiwalla *et al*., 1992; Daniele and D'Agostino, 1996). Post birth, depressions in the intervillus epithelium form in the neonatal rat, which will become the intestinal crypts of the adult (Dunn, 1967). Immediately post hatching the chick intestine will complete its

intestinal maturation. In most mammals, the intestine undergoes a second phase of functional development, as the weaning period approaches (Moog, 1951).

Development of Embryonic Chick Duodenum

In early development of chick embryos (day 3), the duodenum is a tube of simple columnar epithelium. By day 6 the chick duodenum is lined with pseudostratified epithelium (Burgess, 1975). Around day 8, previllus ridges that run parallel to the length of the duodenum begin to form as cells become cuboidal and the epithelium folds. This precedes the appearance of definitive villi. Villus formation is asynchronous and occurs in two distinct periods (Grey, 1972). The initial phase begins on day 8 and concludes with the formation of 16 previllous ridges by day 13. By day 11 microvilli have emerged on the epithelial cells. Between embryonic day 14 and 16, the first set of previllous ridges folds into a clear zigzag pattern. This is followed by formation of definitive villi on day 17 as the ridge crests appear to swell, as the cells grow at the corner of each zigzag. Within 24 hours (day 18) the growth spreads across the length of each zigzag and by day 19 finger-like villi are present. The second phase of villus formation begins on day 16 and involves development of tongue like flaps between the existing previllous ridges from the intestinal floor. This phase continues through postnatal day 4. These ridges grow very rapidly and resemble mature villi by embryonic day 19. When the chick hatches, these late stage villi are shorter than those of phase 1. However, by postnatal day 4, all villi look the same.

The mechanism of previllous ridge formation is not clear. It is probably a function of both constriction of epithelial cells and mesenchymal induction (Mamajiwalla, *et al*., 1992). Microfilament mediated epithelial constrictions have been implicated in this process. Cytochalasin B, a microfilament disruptor, inhibits previllous ridge formation (Burgess, 1975). It should be noted that constriction might be actomyosin based. Actin, myosin, and

tropomyosin are present in the zonula adherens circumferential ring during late stage embryogenesis (Mamajiwalla, *et al*., 1992).

Other aspects of embryonic intestinal development include goblet cell differentiation (beginning on day 12–14). During the third week of development, goblet cells spread from the basal villus region toward the tip. By day 21 mature goblet cells are secreting mucin and are distributed from villus base to tip (Hinni and Watterson, 1963). Immature microvilli appear at day 11 and maintain their length while their core structure and physical orientation with the cell surface transform. In addition, microvillar diameter is also constant throughout the embryonic period. Height increases around hatching and will rise 6X by one day posthatch (days 18-22), while diameter will decrease by 25 % (Overton and Shoup, 1964). The terminal web appears at day 19, and crypt formation is evident on day 20. Chick embryos pip the shell on day 20 and hatch on day 20-21.

The timing of specific embryonic events in vertebrates differs somewhat between species. In mammals, the rat for example, distinctive villi also form late in embryonic life, and intestinal crypts do not form until after birth (Dunn, 1967). In the chick, mitotic cells are present throughout the duodenal epithelium through embryonic day 9. Between days 9 and 16 the mitotic cells gradually become restricted to the lower half of previllus ridges. After day 19 mitotic cells are restricted to the proximal villus region, and by postnatal day 2 all have been segregated into the crypts (Overton and Shoup, 1964). Thus, initially proliferating cells are evenly distributed across the epithelium and later become restricted to the basal epithelial region, as villi grow. Goblet cell development also differs between chicks and rats. In the chick, goblet cells are abundant and functional by day 21 when the embryos hatch. However, in fetal rats immature goblet cells do not appear until day 19. Over the next 48 hrs the number present triples, and increases slightly more by day 22. The population remains stable through postnatal day 3. A final phase of maturation and differentiation significantly increases goblet cell number

in the rat around postnatal day 23, as the duodenum prepares for a solid food diet (Morimoto *et al*, 1987). Thus, while the specific details of chick and rat intestinal morphogenesis vary, the general developmental pattern is similar.

Functional differentiation of the embryonic duodenum accompanies the morphological changes. The brushborder membrane develops as microvilli form and grow, and biochemical differentiation is indicated by the specific activity of the enzyme hydrolases that accumulate during this time (Moog, 1979; Smith *et al*, 1984). In addition, the microvillus membrane becomes capable of nutrient transport.

Early work on embryonic chick biochemical differentiation was performed by Moog, who discovered that alkaline phosphatase was present in tissues during initial development (Moog, 1944). Alkaline phosphatases (ALP) are a family of isoenzymes that hydrolyze organic phosphates in an alkaline pH environment, assisting their absorption (Deren, 1968). ALP is found across the apical membranes of embryonic duodenum. In addition, gradients of enzymatic activity exist across the brushborder (Calvert *et al*, 1981). ALP is activated by divalent cations that interact with choline, which shields the active site from endogenous phosphate (Seethram *et al*, 1985; Gilles-Baillen and Croux, 1992). Removal of choline leads to complete loss of enzymatic activity (Tiruppathi *et al*., 1987). Moog found low levels of active ALP in 7 day old embryos, with enzyme activity steadily rising from day 9 through 18. On day 18 activity sharply increases (up to 50X) and continues to rise through post-hatch day 1. From post hatch day 2-7 activity declines and remains stabilizes after several weeks at the adult level (Moog, 1950). Overton and Shoup (1964) indicated embryonic ALP activity increased significantly as microvillar surface area increased just prior to hatching. Later, in 1968, Deren reported functional differentiation and morphogenesis in embryonic duodenum were clearly correlated. This was followed by a report that measured a 7.8X increase in the specific activity of ALP between embryonic

days 14 and 19 in chicks (Black and Moog, 1978). In addition to ALP, disaccharidase enzyme activity increases during the third week of incubation. Maltase activity rises by 12X between embryonic day 17 and day 21, while the crypt to villus gradient increases approximately 4X (Siddons, 1969; Grimes and Black, 1987). Sucrase follows a similar developmental pattern rising by over 300% between gestational days 18 and 21, and exhibiting a crypt to villus tip gradient of over 4X (Siddons, 1969; Grimes and Black, 1987).

Nutrient transporters, which similarly are found in the microvillar membrane, are another index of functional differentiation in embryonic chick. Post hatching, glucose is essential for optimum growth and development. Initially it was reported that glucose active transport did not develop until hatching (Bogner and Haines, 1964). Follow up studies demonstrated active glucose transport did occur in embryos (Holdsworth and Wilson, 1967; Shehata *et al*, 1981) Later work by Black (1988) revealed glucose transport could be measured as early as day 12 in chick embryos, and the transport rate rose over time, most significantly between day 17 and hatching. This increase was reasoned to be a result of parallel increases in microvillus surface area (Black, 1988). Thus, embryonic chick duodenal development is a complex, multi-faceted process. In many respects, it resembles the morphogenesis of fetal mammalian duodenum. A review of the factors that regulate the development of duodenal epithelium is appropriate at this time.

Regulation of Epithelial Growth and Differentiation:
Immuno-Epithelial Communication, Mesenchymal-Epithelial Interactions and the Extracellular Matrix (ECM)

The mature intestinal mucosa is dependent upon a complex network of intercellular signaling. Molecular cross-talk between GI epithelial cells and closely juxtaposed lymphocytes is an essential component of normal

physiological function. The theory of immunological regulation of mammalian intestinal epithelial cell function was initially proposed by Castro twenty years ago (Castro, 1982). Lymphoepithelial communication is integral for mucosal development (Shanahan, 1999). Intestinal epithelium consists of functionally and phenotypically distinct sub-populations of lymphocytes. There are lymphocytes within the lamina propria, below the epithelial basement membrane and lymphocytes located peripherally. In the area immediately above the basement membrane are intraepithelial (IEL) lymphocytes (Brandtzaeg *et al*., 1989). Intercellular communication is required for development of both enterocytes and IEL. Watanabe (1995) used human intestinal cells and discovered IEL development is dependent upon growth factor and cytokine (interleukin 7) production in the intestinal epithelium. Conversely, intestinal epithelium requires immuno-interaction for its growth and development. The gamma-delta T cell receptor is associated with the gene for the interleukin 7-membrane receptor. When this gene is absent in mice, they do not develop the gamma-delta IEL subpopulation of T cells. Consequently, intestinal epithelium development is disrupted in these animals (Boismenu and Havran, 1994). Thus, mediators of the immune system, proinflammatory cytokines in particular, are directly involved in the proper development of the gut epithelium.

The extracellular matrix molecules (ECM) are known regulators of cellular growth and differentiation (Simon-Assmann *et al*., 1995). Chick and rat models, among others, have demonstrated that intestinal morphogenesis is influenced by epithelial-mesenchymal cell interactions (Haffen *et al*., 1983). There is increasing evidence that the basement membrane, formed from deposition of epithelial and mesenchymal products, influences the ontogeny of the epithelium (Paulsson, 1992). The basement membrane, also referred to as basal lamina, is composed of glycoproteins (laminin), collagen IV, proteoglycans and other molecules (Simon-Assmann *et al*., 1995). Basement membrane component molecules are present early in embryonic

development. Each family of molecules has multiple isoforms. Expression of the different isoforms creates specific molecular associations that are spatial and time-dependent. These combinations create signals for epithelial morphogenesis that are relayed via ECM receptors, the integrins (Hynes, 1992).

Laminin is the major component of the basement membrane. It is present early in gestation and exhibits maximal activity during fetal development. In fetal rat intestine, highest level of laminin synthesis occurs between embryonic days 16 and 18, which corresponds to villus formation (Simo *et al*., 1991). In addition, laminin is concentrated at the base of the emerging villi, whereas in the adult it is uniformly distributed from crypt to villus tip. Other matrix molecules, including collagen IV, perlecan, and fibronectin, are also found in the subepithelial basement membrane during early embryogenesis (Simon-Assmann *et al*., 1995). Basement membrane molecule receptors are expressed differentially as well during development. The combination of changing orientation and assembly of basement membrane molecules and receptors contributes to ECM regulation of intestinal growth and differentiation.

The significance of epithelial-mesenchymal communication is well established, but the precise mechanisms are not fully defined. Investigations have focused on three possible means of signal transmission. Interaction may be the result of cell-cell or cell-ECM contact or diffusion of secreted factors (Koike and Yasugi, 1999). Recent studies in fetal mice gut and embryonic Drosophilia midgut propose that differentiation is modulated by changes in the ECM composition, as epithelial cell surface receptors (E-cadherins and integrins) interact with ECM proteins (Hynes, 1992). Changes in these receptors have been linked to enterocyte differentiation in rat and human intestine (Rao *et al*., 1994; Basora et al, 1997). Three proteins have been suggested as possible cross-talk mediators. The fkh-6 factor is found in mesoderm and interacts with the c-met receptor (tyrosine kinase family)

(Kaestner *et al*., 1996). Smads proteins, part of the transforming growth factor-beta transduction pathway, are involved in embryonic epithelium development in mouse (Sirard *et al*., 1998). One of the more interesting proteins is sonic hedgehog (Shh), which is expressed in the endoderm during embryogenesis and influences development of the mesoderm (Apelqvist *et al*., 1997). Recent findings indicate Shh may actually facilitate bi-directional communication (Sukegawa, *et al*., 2000). Work by Kedinger *et al*. (1998) demonstrated that morphogenesis and differentiation of rat intestine requires physical contact between the epithelium and mesenchyme. They suggest, in addition to basement membrane molecules, soluble factors produced by the mesenchyme must be involved in epithelial behavior. Specifically, during the gestational and neonatal periods several factors that control epithelial growth, motility, and differentiation are expressed in a coordinated manner. These include hepatic growth factor/scatter factor (HGF/SF) and keratinocyte growth factor (KGF) which bind tyrosine kinase receptors primarily expressed in epithelial cells.

Digestive enzymes and fatty acid binding proteins exhibit proximo-distal axis characteristics that are a biomarker of functional differentiation in embryonic intestine. In Kedinger's study this positional gradient was in place by embryonic day 14 in the rat (Kedinger *et al*., 1998). These developmental changes were linked to variations in GI epithelial transcription factor homeobox gene expression. One of these genes is involved in the regulation of sucrase and lactase expression (Suh *et al*., 1994). The inference here is that epithelial-mesenchymal interactions mediated via basement membrane molecules (laminin according to Kedinger) regulate intestinal homeobox genes during development.

Taken together, these data demonstrate that the ECM, mesenchyme, and immune system act in conjunction to regulate embryonic intestinal epithelial development. This elaborate network of cellular interaction is controlled by cytokines and regulatory peptides (Shanahan, 1999). Previously it was stated

that proinflammatory cytokines are directly involved in immuno-regulation of intestinal epithelial growth. These molecules are also implicated in mesenchymal regulation and are produced by intestinal epithelia, immune and fibroblast cells. Ferretti *et al*. (1996) used rat duodenal cells (IEC-6) to investigate control of epithelial development. In their study morphological and functional differentiation were enhanced by fibroblast contact and cortisol. A few years earlier Montesano *et al*. (1991) discovered that fibroblasts produce a diffusible paracrine factor involved in kidney epithelial morphogenesis. Interleukin 1-beta (IL-1*b*) and tumor necrosis factor alpha (TNF-*a*) are cytokines that influence intestinal growth and differentiation in developing mice, rats and humans (Mengheri *et al*., Neumann *et al*., and Ziambaras *et al*., 1996). Transforming growth factor alpha (TGF-*a*) may be the major proliferative agent in intestinal epithelium. It is strongly expressed in the duodenum *in vivo* at weaning (Dvorak and Koldovsky, 1994; Hormi et al., 1995) and is suspected to be an intestinal maturation factor (Miettinen *et al*., 1995; Nsi *et al*., 1996). Fibroblast derived, multifunctional TGF-*beta* is the most potent growth inhibitor in most epithelial cells (Roberts *et al*., 1985;Barnard *et al*., 1990). TGF-*beta*, which elicits cellular effects through a serine-threonine receptor complex, induces differentiation in rat duodenum and jejunum (Kurokowa et al, 1987). Considering the results of several experiments conducted by Kedinger and others, a theory of integrated regulation of intestinal epithelial development has been postulated. It is suggested that early development is directed by mesenchymal influences mediated through basement membrane molecules, which conditions the epithelium for glucocorticoid-induced maturation (Kedinger *et al*., 1998). If this model is correct, hormones and exogenous factors control the immuno-mesenchymal-ECM integrated network responsible for embryonic intestinal epithelial development.

Hormones

Hormones profoundly effect postnatal, GI mucosal development (Henning, 1981; Blake and Henning, 1983). In most mammals, the intestinal epithelium must redifferentiate after birth to accommodate the adult diet (Moog, 1951). This second phase of differentiation begins at weaning and has been interpreted as a stress-induced effect. This was confirmed by adrenalectomy of suckling mice and administration of cortisone or ACTH (Moog and Kirsch, 1955).

The disaccharidase enzymes, which are primarily located at the microvillar membrane, are also developmentally influenced by hormones. In neonatal rodents, maltase is weakly active and sucrase-isomaltase is inactive. Just prior to weaning both enzymes display a rapid increase in activity. Sucrase activity can be prematurely induced with cortisol (Doell and Kretchmer, 1964). The importance of glucocorticoids to late fetal development is supported by additional evidence. Several species (cow, rodent, lamb) experience accelerated adrenocortical activity shortly before birth (Nathanielsz, 1976). Additionally, embryonic glucocorticoid receptor density is highest in concert with the rise in phosphatase activity that precedes birth (Lee *et al*., 1976). However, as was previously stated, precocial birds (chick) are functionally prepared for the adult diet at hatching. In embryonic chick, duodenum cultured in the presence of cortisol or ACTH expressed precocious elevations in ALP activity (Moog and Kirsch, 1955; Moog, 1961).

Endocrine regulation of intestinal development is not limited to glucocorticoids. Thyroid hormones influence epithelial maturation. Hypophysectomy of young rats (6D) results in incomplete enzymatic and morphological differentiation (Yeh and Moog, 1974). The epithelium in a 24D rat retains characteristics of neonatal tissue. These defects are reversible with separate administration of cortisol and thyroxine (Yeh and Moog, 1974, 1975a,b and 1977). Thus, thyroid hormone is required for complete

maturation of intestinal epithelium. Later work by Black found that both thyroxine and cortsol significantly increased ALP and maltase activity in cultured embryonic chick duodenum. Morphologically, cortisol was involved in preserving the epithelial and subepithelial structure. Thyroxine enhanced microvillar length and density and cell height (Black, 1978; Black and Moog, 1978). A recent study found that deletion of a gene coding for a thyroid hormone receptor produced delayed duodenal epithelial maturation (Fraichard *et al*., 1997). The synergistic effects of thyroid and adrenocortical hormones in promoting development of mammalian intestinal epithelium are likely an adaptive function that arose from changing circulating hormone levels arising during suckling (Daniels *et al*., 1973; Clos *et al*., 1974).

Epidermal growth factor (EGF) is a strong mitogen in adult intestine (Murphy, 1998) that also influences embryogenesis and neonatal development (Whitman and Melton, 1989). P.J. Mittinen (1993) found measurable mRNA for EGF and TGF-alpha in fetal human gastrointestinal tissue. Application of exogenous EGF resulted in EGF receptor-like immunoreactivity in the immature villi and crypts. Thus, Miettinen's results suggested a physiological role for EGF in fetal intestine. A more recent study using organ cultured embryonic Swiss-Webster mouse gut has illuminated the function of EGF in the developing gut Duh *et al*., 2000). Mouse gut cultured in exogenous EGF did not increase duodenal length, however epithelial proliferation and maturation were enhanced. The intestinal crypt/villus axis orientation was accelerated with treatment, as well as colonic goblet cell development. Clearly, EGF is an important regulator of embryonic intestinal maturation *in vitro*.

Hormones and growth factors are potent regulators of intestinal development. However, embryonic chick and fetal mouse intestine can be cultured in essentially hormone free medium and appropriate structural and biochemical differentiation will occur (Black, 1978; Black and Moog, 1978). The intestinal epithelium is programmed from an early stage to undergo a

pattern of self-differentiation, even when normal influences have been eliminated. In summary, hormones act to modulate and regulate the timing of programmed expression of developing duodenal epithelium.

Ca^{2+} as a Regulator of Epithelial Development

The exact mechanisms of endocrine regulation of intestinal epithelial growth and differentiation are unknown. One possibility is alteration of cellular function via a Ca^{2+}-mediated signal. This is plausible since intracellular free Ca^{2+} spikes in concert with peak glucocorticoid receptor density in week 3 of embryonic development (Lee *et al.*, 1976). In addition, intracellular Ca^{2+} levels during this developmental period are influenced by hydrocortisone and thyroxine. Rogers demonstrated cultured duodenal epithelial cells experienced an increase in cytoplasmic Ca^{2+} in response to HC (Rogers and Black, 1986).

Three pools of calcium in the chick embryo are derived from the albumin, eggshell, and yolk. Eighty percent of gestational Ca^{2+} comes from the eggshell (Tuan, 1987). Ca^{2+} is reabsorbed from the eggshell through the outer ectodermal layer of the chorioallantoic membrane (Romanoff, 1960),via a unidirectional, sodium and energy-dependent mechanism (Narbaitz, 1977). This system is functional by day 14. Prior to day 11 up to 95% of embryonic Ca^{2+} is reabsorbed from the yolk. This facilitates skeletal mineralization (beginning day 10-12) (Romanoff, 1960).

Chorioallantoic membrane Ca^{2+} transport consists of three units, carbonic anhydrase (cytosolic), a Ca^{2+}-specific binding protein (ectodermal surface) and a Ca^{2+}-ATPase membrane protein (Tuan, 1987). Expression of Ca^{2+}-binding protein (CBP) and carbonic anhydrase activity occurs around day 12. Carbonic anhydrase solubilizes calcite from the shell into Ca^{2+} for transport. CM transport can occur in the absence of carbonic anhydrase, therefore it seems to function as a facilitator. Ca^{2+} transport, carbonic anhydrase activity, and CBP expression levels are low during the first two

weeks of development (Tuan and Scott, 1977). However, all three measures peak between embryonic day 19 and 20 (Tuan and Knowles, 1984).. Chorioallantoic membrane Ca^{2+} transport is enhanced by 1,25-dihydroxy vitamin D3, which increases carbonic anhydrase activity (Narbaitz and Tolnai, 1978). Reported total serum Ca^{2+} concentration varies by method of measurement. The range is from 2.0-2.6 mM on embryonic day 16 to 2.5-3.2 mM in neonatal chicks (Taylor *et al*., 1975). Black and Smith (1989) reported serum Ca^{2+} ranging from 1.3 mM at embryonic day 14 to 1.5 mM by day 18.

Throughout embryonic development Ca^{2+} homeostasis is essential. The accelerated proliferation and differentiation during gestation presumably would be particularly sensitive intracellular Ca^{2+} concentrations. All cells, including intestinal epithelial cells are dependent upon the maintenance of the extracellular/intracellular free Ca^{2+} concentration gradient. The 10,000X difference between the extracellular environment and the cytoplasm ensures that external and internal stimuli will be transduced into a cellular signal. Cells have developed an elaborate system of buffers, intracellular storage, and ion pumps to maintain intracellular Ca^{2+} homeostasis. Ca^{2+} homeostasis in parathyroid cells is a function of recognizing and responding to minute changes in the extracellular fluid Ca^{2+} (Ca^{2+}o) concentration (Brown *et al*, 1998). Isolation of the cDNA that codes for the bovine parathyroid Ca^{2+}o receptor (CaR) has lead to its identification in many tissues. The G-protein-coupled receptor has been found in human kidney and parathyroid, rabbit kidney, rat kidney, brain and C-cell, and chicken parathyroid (Chattopadhyay and Brown, 2000). CaR is highly expressed throughout the entire GI tract (Brown *et al*, 1998). The presence of a membrane receptor sensitive to small changes in the Ca^{2+}o could theoretically allow extracellular Ca^{2+} to influence embryonic epithelial development and function. These actions may be mediated via plasma membrane mechanisms (i.e. G-protein signaling) involving modification of Ca^{2+}-channel activity.

Earlier work in the Black lab established that epithelial differentiation in cultured 14 day old embryonic duodenum was influenced by the Ca^{2+} concentration of the culture medium (Black and Smith, 1989). Extracellular Ca^{2+} levels of 1.1 mM or greater increased the rate of goblet cell differentiation in culture above that in vivo. The serum Ca^{2+} concentration in vivo was measured at 1.3 mM at embryonic day 14 and rose by 16 % to 1.52 mM on day 18. In culture, a corresponding 16 % increase in extracellular Ca^{2+} induced a 30 % increase in goblet cell number. Thus, as the culture environment neared (1.1 mM) and surpassed physiological Ca^{2+} levels (1.6-2.0 mM), goblet cell number in vitro rose significantly. Later work suggested that hormone-induced epithelial differentiation was mediated by cytoplasmic calcium (Rogers and Black, 1996). Addition of hydrocortisone or thyroxine to 14 day old embryonic duodenum cultured for 72 hours increased cytoplasmic Ca^{2+} concentrations by 44 and 18 % respectively. In separate experiments thyroxine and hydrocortisone also enhanced the ability of cultured cells to maintain a constant cytoplasmic Ca^{2+} level when challenged with non-physiological levels of extracellular Ca^{2+}.

As an extension of the previous work, this thesis study investigated how changes in extracellular Ca^{2+} influx and concentration effect embryonic intestinal epithelial differentiation. The first phase of the project concerned the effect of culture medium [Ca^{2+}] on epithelial ALP activity. Duodenal epithelium from 16 and 18 day old chick embryos were incubated in physiologically low (0.7 mM) or high (2.8 mM) extracellular Ca^{2+} .The published preliminary results reported significant increases in enzyme activity in 16 and 18 day old tissue cultured in high extracellular Ca^{2+} (Mack and Black, 2000). This finding focused attention on the potential pathways mediating the effects of the [Ca^{2+}] on embryonic development. In an effort to determine a mechanism of action, the remainder of this study investigated the following possibilities: 1). culture with agents that block or increase extracellular Ca^{2+} influx should produce effects that mimic those of low and

high extracellular [Ca^{2+}], 2). changes in extracellular [Ca^{2+}] influence epithelial morphology and function by altering normal cytoplasmic Ca^{2+} signals. Lastly, changes in epithelial development which result from alterations in the extracellular [Ca^{2+}] may interfere with signaling by modulating the intracellular distribution of free Ca^{2+}.

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Chapter 2. EXTRACELLULAR CALCIUM INFLUX ALTERS ALKALINE PHOSPHATASE ACTIVITY IN EMBRYONIC DUODENUM

ABSTRACT

Previous results indicate that epithelial cytoplasmic calcium (Ca^{2+}) concentration influences the activity of a microvillar enzyme, alkaline phosphatase (ALP), in 14-Day (D) embryonic chick duodenum. This study investigated the effect of extracellular Ca^{2+} in older (16D and 18D) developing chick intestine. Duodena from 16D and 18D chick embryos were cultured for 48 and 24 hours, respectively in media with high or low extracellular Ca^{2+}. ALP activity rose significantly higher in 16D (130 %) and 18D (182 %) tissue cultured in high Ca^{2+} than in paired tissue from low Ca^{2+} cultures. In separate experiments, 16D and 18D tissues were cultured in medium containing 1.3 mM (physiological) Ca^{2+} and a Ca^{2+}-channel blocker, nifedipine (0.01 mM) or verapamil (0.1 mM) or lead acetate (PB^{2+}) (1.05 mM). ALP in PB^{2+} treated tissue was markedly lower than paired controls at 16 and 18 days (56 % and 37 % of controls, respectively). Since tissue weight and protein concentration in control and treated groups were comparable, gross morphological change did not seem to be the basis of the decrease. Nifedipine also significantly reduced ALP values at 16D (46 % of control) and 18D (72 % of control) without affecting protein or weight. These findings indicate that reduction in extracellular Ca^{2+} influx results in decreased duodenal ALP activity during late stage embryonic development.

INTRODUCTION

Intestinal brushborder enzymes are recognized as markers of cellular differentiation (Yeh and Moog, 1975a; Quaroni *et al.*, 1999). Alkaline phosphatases (ALP) are a family of enzymes that bind and hydrolyze inorganic phosphate from their esters in the intestine (Posen, 1967).

Enzymatic activation requires the presence of a divalent cation (Wang and Gilles-Baillien, 1992). Prior studies have demonstrated that in early embryonic stages intestinal ALP enzyme activity is sensitive to extracellular Ca^{2+} modulation (Black and Rogers, 1992b). Inorganic lead is a commonly found toxic contaminant, which competitively acts to impair Ca^{2+} function. Lead has deleterious effects during gestational, neonatal and early developmental periods (Lockitch, 1993). Broiler chicks exposed to dietary lead sulfate or lead acetate at hatching experience depressed body weight gain and decreased delta-aminolevulinic acid dehydrase activity (Bakalli *et al*., 1995). Gestationally, Pb^{2+} injected into the yolk sac of chicks on embryonic day 7 produced stunted growth and deformities of the beak, legs and head (Anwer *et al*., 1987). After hatching, absorption of Pb^{2+} occurs primarily through the GI tract and the neonatal period is particularly susceptible to exposure. Young rats fed a diet containing Pb^{2+} experience decreased glucose, glycine, lysine and phenylamine absorption (Wapnir *et al*., 1977; 1979). In addition, Pb^{2+} reduces the activity of duodenal gamma-glutamyl transpeptidase, which catalyzes membrane transport of small peptides and amino acids (Wapnir *et al*., 1984). In a different model, teleost fish exposed to Pb^{2+} developed GI damage including inhibition of duodenal aminotripeptidase and glycylglycine dipeptidase activity and decreased gastric ALP (Sastry and Gupta, 1978a). It was not determined in these studies exactly how Pb^{2+}produced the reported effects.

This study was designed to determine if changes in extracellular Ca^{2+} concentration influence the functional differentiation of embryonic duodenum as reflected by the activity of the brushborder enzyme, ALP, in late stage (16D or older) cultured tissue. Since potential effects of extracellular Ca^{2+} are presumably mediated by alteration of intracellular Ca^{2+} levels, the effects of Ca^{2+}-channel blockers was also tested. The effects of Pb^{2+} are due in part to an ability to disrupt the influx of extracellular Ca^{2+}. (Lockitch, 1993). Although many reports demonstrate harmful effects of Pb^{2+}

on the digestive system in young and adult animals, there is little information on Pb^{2+} effects in embryos. Therefore, we examined the effects of Pb^{2+} and voltage sensitive Ca^{2+}-channel (VSCC) antagonists on ALP activity, a Ca^{2+}-sensitive parameter, in epithelium of cultured embryonic intestine. We hypothesize that interference with Ca^{2+} influx alters the cytoplasmic Ca^{2+} concentration, which theoretically will reduce late-stage increases in ALP activity.

MATERIALS and METHODS

Culture

Fertile chicken eggs (broiler strain) from the N. C. State Poultry Science Department were incubated in a forced-draft incubator at 38-39° C. At the appropriate age, (16-Day or 18-Day) duodenal loops were excised from embryos and prepared for culture as previously described (Black and Moog, 1978). Each duodenum (n=8-11 embryos) was cut in quarters with the adjacent middle segments divided between culture flasks with either: a.) high Ca^{2+} or low Ca^{2+} b.) Pb^{2+} vs. control (NaCl) or c.) Ca^{2+}-channel antagonist vs. control (See Table 2.1 note: free [Ca^{2+}] measured previously in this lab by Ca^{2+}-sensing electrode.). Prior to addition of any chemicals, culture media contained 1.3 mM free Ca^{2+} (physiological for chick embryos). EGTA was added to low Ca^{2+} cultures to reduce the Ca^{2+} in the culture medium to 0.7 mM, and $CaCl_2$ was added to the high $Ca2^{+}$ cultures to increase Ca^{2+} concentration of the culture medium to 2.8 mM (Black and Smith, 1989). The end pieces were immediately stored in 0.9% NaCl and labeled according to the corresponding treatment group of the middle section from which they had been attached. Duodena were placed in flasks, total volume 3 ml, with Medium 199 (GIBCO) and control or treatment agent. To adjust the pH of the medium, flasks were gassed with 95% 0_2 5 % CO_2 to 7.4. Tissue was then incubated for either 48 h (16D) or 24 h (18D) at 38°C (see Black and Moog, 1978). Chemical agents were obtained from Sigma

Chemical Co. and paired control cultures contained equivalent volumes of 0.9 % NaCl.

Table2.1. Culture medium additives

CHEMICAL	CONCENTRATION OF ADDITIVE IN MEDIUM	FREE $[CA^{2+}]$ OF MEDIUM
EGTA (low CA^{2+})	0.75 mM	0.70 mM
$CaCl_2$ (high CA^{2+})	1.60 mM	2.80 mM
Verapamil	0.10 mM	1.3 mM
Nifedipine	0.01 mM	1.3 mM
Lead Acetate	1.05 mM	1.3 mM
NaCl (control)	0.9%	1.3 mM

Measurements

The King-Armstrong technique described by Black and Moog (1978) was used to measure the nonspecific ALP activity in uncultured and cultured duodenal homogenates (assayed in duplicate), with disodium phenyl phosphate (Fisher Scientific) as the substrate. In addition, the protein content of the uncultured and cultured tissue homogenates (assayed in triplicate) was measured by the Lowry Method (1951). Bovine serum albumin served as the assay standard. Enzyme activities are expressed as micromoles of substrate hydrolyzed for 60 minutes per milligram of tissue weight, and protein concentrations are expressed as *u*g/mg tissue. Analysis of ALP activity of uncultured end segments from the same embryo were not statistically different from each other, therefore values were combined into an average baseline (BL) for each embryo for purposes of comparison to paired cultured duodena. This was also done for tissue weight and protein measurements.

Statistical comparisons between treated cultures and paired controls for tissue weight and protein and ALP were evaluated for significance by the paired student t-test ($p<0.05$ was considered significant).

RESULTS

Effects of culture on tissue integrity

Tissue weights (see Table 2.3) from all cultured segments were unchanged or increased as compared to uncultured (BL) values. Protein concentration was measured to detect gross deleterious effects on duodenal integrity from addition of treatment agent to the culture medium. Protein content was unaffected by treatment in 16 or 18 D duodenal cultures and was equivalent to or greater than BL values in each group (see Table 2.4).

ALP analysis

High and Low Ca^{2+}

The extracellular free Ca^{2+} concentration (1.3mM) of the culture medium was lowered by EGTA to 0.7 mM or increased with $CaCl_2$ to 2.8 mM. ALP activity in 16D old duodenum cultured in low Ca^{2+} was unchanged after 48 hours of incubation (Figure 2.1). Conversely, culture in high Ca^{2+} resulted in an increase in enzyme activity to 244% of BL value ($p<.001$). In older tissue, ALP activity tended to decrease after culture in lower extracellular Ca^{2+} concentration. Although not significant ($p=.0525$), activity decreased by 30 % after 24 hours of culture (Figure 2.1). ALP in 18D duodenum cultured in 2.8 mM Ca^{2+} increased significantly, by 206%, as compared to low Ca^{2+} cultures ($p<.02$).

In all of the experiments of this study, culture in control medium with 1.3 mM Ca^{2} effectively increased ALP activity over BL values (Figs. 2.2, 2.3, 2.4). When the ALP activity of control cultures from experiments using Ca^{2+} antagonists is compared to that of tissue cultured in low and high Ca^{2+}

(Figure 2.4), it appears that development of ALP activity is nearly maximal with an extracellular Ca^{2+} concentration of 1.3 mM.

Table 2.2. ALP activity vs. culture medium [CA^{2+}]

AGE	ALP ACTIVITY BL TISSUE (*u*mole/60min/mg)	FREE [CA^{2+}] OF CULTURE MEDIUM	ALP ACTIVITY OF CULTURES *(u*mole/60min/mg)
16D	.185 ± .018	0.7 mM	.160 ± .009
		1.3 mM	.505 ± .012
		2.8 mM	.551 ± .016
18D	.670 ± .110	0.7 mM	.365 ± .092
		1.3. mM	1.00 ± .001
		2.8 mM	1.12 ± .080

Pb^{2+}

In Figure 2.2 the effects of the heavy metal Pb^{2+} are summarized. The presence of inorganic Pb^{2+} in the culture medium of 16 D embryonic explants reduced ALP activity in comparison to control cultures ($p<.0001$). In 18 D duodenum the inhibitory effects of Pb^{2+} were exacerbated. Pb^{2+} reduced ALP activity below baseline values ($p<.02$). Thus, the effects of Pb^{2+} reduced the Ca^{2+}-evoked developmental enhancement associated with tissue culture.

Ca^{2+} -Channel Antagonists

Culture in control medium and verapamil both resulted in significantly higher ALP activity in 16D tissue than BL values ($p<.001$), but there was no difference between control and verapamil cultures (Figure 2.3). However, in

the 18D tissue, verapamil inhibited ALP activity by 53 % relative to controls and significantly reduced activity below baseline levels ($p<.02$). Unlike verapamil, nifedipine reduced enzyme activity in cultured 16D embryonic tissue ($p<.01$) (Figure 2.4). In addition, nifedipine abolished any significant increase in activity in cultured 18D explants.

DISCUSSION

In this experiment ALP activity was significantly increased in 16D and 18D embryonic duodenum cultured in high (2.8 mM) extracellular Ca^{2+} as compared to tissue cultured in low (0.7 mM) Ca^{2+}. In addition, a clear dose-dependent relationship was demonstrated between the extracellular [Ca^{2+}] and ALP activity in cultured duodena (Table 2.4). Lead effectively lowered the level of ALP in cultured embryos at 16 and 18 days of age. The non-specific Ca^{2+} -channel antagonist verapamil decreased ALP activity in 18D explants (vs. paired controls), but not in younger tissue. Lastly, nifedipine, a dihydropyridine (DHP) voltage-sensitive Ca^{2+} -channel (VSCC) blocker also significantly reduced ALP in both 16D and 18D tissue. Thus, disruption of the normal influx of extracellular Ca^{2+} by three different mechanisms resulted in reduced activity of a microvillar enzyme (ALP) during embryonic development. This strongly suggests that extracellular Ca^{2+} influences epithelial differentiation by modulating the cytoplasmic Ca^{2+} concentration, possibly via reduction in VSCC activity.

A Ca^{2+} mediated signal underlies embryonic ALP activity. However, it should be noted that this is not due to enzymatic activation of ALP by Ca^{2+}. The intestinal ALP is a dimeric metalloenzyme that is highly conserved across *E. coli* to man (Cioni *et al*., 1989; Xie and Alpers, 2000). X-ray crystallography has been used to solve the structure of *E. coli* ALP and approximate the structure of the enzyme found in higher order vertebrates (Kim and Wycoff, 1991; Stec *et al*., 2000). From these studies a better

understanding of the ALP mechanism has developed. The monomeric subunits do not possess independent enzymatic activity, thus the intact molecule is necessary for physiological function (Hoylaerts *et al*., 1997). The active site of the enzyme requires two Zn^{2+} and one Mg^{2+} in the pocket for activation (Hoylaerts *et al*., 1997; Stec *et al*., 2000). The active site affinity for Ca^{2+} is several orders of magnitude less than that of Zn^{2+} and Mg^{2+}. In addition, the underlying geometry of the active site complex and disruption of the hydrogen binding network would deactivate a Ca^{2+} substituted ALP molecule (Holtz and Kantrowitz, 1999; Stec *et al*., 2000). Therefore, increasing the concentration of Ca^{2+} in the extracellular and cytoplasmic pools would not lead to direct enzymatic activation of ALP.

Decreasing the rate of Ca^{2+}-influx during culture reduced brush border enzyme activity as compared to paired controls (Mack and Black, 2000). In an earlier study using younger tissue (14D), inhibition or alteration of extracellular free Ca^{2+} was highly correlated with reduced cytoplasmic Ca^{2+} concentration and ALP activity (Black and Rogers, 1992b). Thus, changes in the normal cytoplasmic Ca^{2+} signal in the 16 and 18 day old duodena are probably responsible for decreased ALP activity in the current study. However, the exact mechanism of the Ca^{2+} signal is still unknown.

Changes in ALP activity may reflect differences in the amount of catalyzed ALP within the brush border or altered activity of the enzyme protein, or both. During the 60 hours prior to hatching, ALP activity in the chick embryo spikes and remains high until shortly after hatching. During this critical period of development, cytoplasmic free Ca^{2+} increases and remains high (Black and Rogers, 1996). Thus, Ca^{2+} may serve as a signal for cellular maturation that must be completed for postnatal life.

Recent in vitro studies have explored how intracellular and extracellular Ca^{2+} influence digestive enzyme activity. Huang *et al*. (1996) used rat pancreatic acinar cells to investigate what signals are involved in amylase and cholesterol esterase secretion. Amylin stimulation triggered the release

of both enzymes. Using pharmacological challenges, they demonstrated that amylin induced an increase in the cytoplasmic Ca^{2+} concentration. Further, this result persisted in Ca^{2+} free medium, indicating an intracellular Ca^{2+} store-dependent mechanism. Thapsigarin, which inhibits microsomal Ca^{2+}ATPase, inhibited the amylin effect. Amylin was found to increase inositol 1,4,5-triphosphate (IP3), a second messenger critical to induce Ca^{2+} release from the ER-like intracellular Ca^{2+}. Huang concluded that in this model, digestive enzyme release was a function of increased cytoplasmic Ca^{2+} from IP3 sensitive stores activated through a G-protein-coupled mechanism. Rarity *et al*. (2000) had similar results after examining neural (A Ch) and endocrine (CCK) stimulated digestive enzyme release from the apical side of pancreatic acinar cells. Hyperstimulation of the acinar cells lead to excessive enzyme (trypsin) release that was transduced via a sustained rise in the cytoplasmic Ca^{2+} concentration. The microvillus cytoskeleton is composed of a core of bundled microfilaments. Villin, a major component protein, severs the membrane-microfilament linkages when activated by Ca^{2+} promoting membrane vesiculation, (Matsudaira, 1983). This may be the underlying mechanism, which leads to excess trypsin in the presence of increased intracellular Ca^{2+}. Ca^{2+} free medium did abolish this signal, indicating a dependence on extracellular Ca^{2+} influx. Thus, both studies found digestive enzyme release is the result of increased cytoplasmic Ca^{2+}.

A study by Perkins *et al*. (1997) focused on the relationship between digestive enzyme expression and cytoplasmic Ca^{2+}. Pancreatic rabbit lobules and rat acini cultured with microsomal Ca^{2+} ATPase inhibitors showed a sustained increase in cytoplasmic Ca^{2+} that is associated with intracellular Ca^{2+} store depletion and increased digestive enzyme polypeptide completion. However, by preventing intracellular Ca^{2+} stores from refilling through inhibition of extracellular influx, digestive enzyme expression decreased. Polysome size was reduced and indications were

translational initiation was reduced which produced a 30 % decrease in new protein synthesis (Perkins *et al*., 1997). These reports imply that a cytoplasmic Ca^{2+} signal affects pancreatic epithelial digestive enzyme expression. Pancreatic and duodenal enzymes clearly differ however their activation may be mediated through a similar mechanism, an increase in intracellular free Ca^{2+}.

Incomplete or compromised development of the subcellular network or extracellular matrix of embryonic duodenum may underlie the reduction of ALP activity of the current study. Kedinger *et al*. (1986) found that brush-border enzyme activity is influenced by the mesenchymal cell compartment. Rat IEC-17 (intestinal crypt cell line) cells and isolated progenitor cells from 14 day old rat embryonic endoderm were incubated with 14 day old rat mesenchyme (Kedinger *et al*., 1986). The cell cultures were grafted under adult rat kidney capsule and incubated for several days. After nine days the mesenchyme-endoderm recombinants had induced morphogenesis of the cells into villus epithelium which expressed ALP, lactase, sucrase and maltase. Further, Simo and associates (1992) have demonstrated that the extracellular matrix molecule, laminin is integrally involved in mesencyme induced embryonic intestinal morphogenesis and cytodifferentiation. Their work found laminin deposition at the epithelial-fibroblastic interface is associated with enhanced brush border enzyme expression in embryonic intestine. Since in the intestine digestive enzymes are embedded in the microvillus membrane, impaired structural development would likely result in decreased enzyme levels. Ca^{2+} is a primary component and regulator of the microvillar microstructure, which is maintained by an actin filament bundle. Villin and fimbrin, two actin-bundling proteins, form the core microvillus bundle, and villin is a calcium-regulated actin-bundling protein and is present in early endoderm (Mamajiwalla et al., 1992). Unlike fimbrin, it is thought to be integrally involved in early microvillus development. Therefore, abnormal Ca^{2+} levels during embryonic development may

destabilize aspects of the substructure and affect ALP expression by interfering with enzyme anchoring and insertion at the brush border membrane (Low and Saltiel, 1988).

Inorganic Pb^{2+} and other heavy metals disrupt Ca^{2+} function. In this study lead acetate substantially reduced ALP activity without producing any gross signs of tissue damage as determined by observation of tissue sections (see Chapter 3). Tomczok *et al.* (1991) found enterocyte ultrastructure from young rats orally exposed to Pb^{2+} for 2 days resembled that of controls. It was only after chronic administration (60 days) that damage appeared. Interestingly, Timm sulfide silver reaction sites were localized to the microvillar brushborder surface. This implies Pb^{2+} selectively damages the brushborder region of enterocytes after prolonged exposure.

The mechanism of action of Pb^{2+} in the current study could be due to direct inhibition of enzyme activity or an indirect result of Pb^{2+} being present in the culture medium. In an earlier study, Sastry and Gupta performed *in vitro* studies using teleost fish in which Pb^{2+} inhibited ALP activity (Sastry and Gupta, 1978a). The focus of the work was to determine the mechanism of intestinal Pb^{2+} poisoning in order to develop clinical treatments. Surprisingly, It was determined that Pb^{2+} directly interacted with the ALP molecule. The amount of inhibition was correlated to the concentration of Pb^{2+}. Applying the chelating agent EDTA completely restored ALP activity to normal levels (Sastry and Gupta, 1978b). However, in the current study, all duodena were rinsed in fresh phosphate buffer several times following culture before being assayed. Further, all cultured tissue was visually inspected using a stereomicroscope and there was no evidence of Pb^{2+} precipitate on any treated tissue. It is highly unlikely that Pb^{2+} was present in tissue homogenates during enzyme assays. Therefore, decreased activity is not the result of direct Pb^{2+} inhibition of ALP during assay.

A more plausible explanation of the results of this study is ALP activity is reduced due to indirect actions of Pb^{2+} at the level of both the enzyme itself

and normal cellular physiology. For example, chicks exposed to aluminum had reduced intestinal levels of the Ca^{2+} transport protein calbindin (Cox and Dunn, 2001). This was the result of aluminum-induced reductions in calbindin mRNA. Pb^{2+} could similarly reduce the overall supply of Ca^{2+} necessary for normal enzyme activity in this experiment. Further, although Pb^{2+} is not bound to the ALP molecule post culture, it does directly interact with the active site (Sastry and Gupta, 1978b). ALP activation is the function of a conformational change that occurs only with the binding of two zinc ions and a magnesium ion (Holtz and Kantrowitz, 1999). The resulting combination of molecular geometry, bonding and electrostatic potential are what induce the catalytic ability of the enzyme (Hoylaerts *et al*., 1997; Holtz and Kantrowitz, 1999). Deviation from the correct conformation destroys molecular stability and catalytic ability (Hoylaerts *et al*., 1997; Stec *et al*., 2000). Therefore, in this study one probable effect of Pb^{2+} is interaction with the ALP active site during the culture period, which possibly results in structural changes that reduce enzyme activity.

The Ca^{2+} channel antagonist verapamil reduced ALP activity in cultured duodenal epithelium, as did nifedipine. Previously, similar results were obtained with 14 day old embryos as verapamil reduced ALP activity in cultured duodena by 45 % (Black and Rogers, 1992b). The effectiveness of nifedipine, a DHP that is selective for L-type VSCCs, in the current study indicates that voltage-gated ion channels are present and physiologically functional in embryonic duodenum (Lomax *et al*., 1999). The findings of this experiment indicate ALP activity in embryonic duodenum is dependent upon extracellular Ca^{2+} influx. The ability of verapamil and nifedipine to reduce ALP activity infers involvement of VSCCs. It has been demonstrated in other tissue that Pb^{2+} targets voltage-sensitive Ca^{2+} channels (Hegg and Miletic, 1996). Therefore, in this experiment Pb^{2+} may have additionally reduced ALP activity by inhibiting VSCCs and lowering cytoplasmic Ca^{2+}, as well as by directly acting on the enzyme molecules. In conclusion, alteration of

available free Ca^{2+} in cultured embryonic duodenum via changes in extracellular Ca^{2+} concentration or influx significantly reduces the developmental increase in ALP activity.

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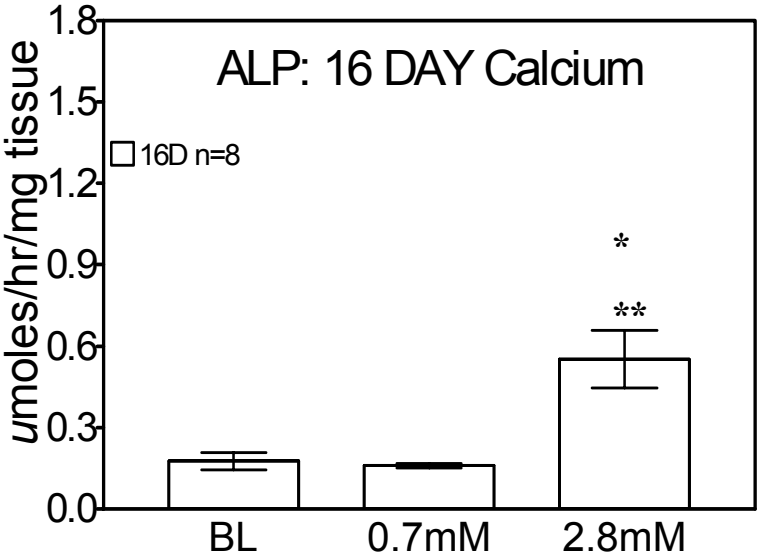
Table 2.3 Tissue weight (mg) pre and post culture

AGE	BASELINE	CULTURE	CULTURE
		0.7mM Ca^{2+}	**2.8mM Ca^{2+}**
16 D	4.24 ± .30 (n=16)	4.35 ± .25 (n=8)	4.34 ± .37 (n=8)
18 D	5.95 ± .47 (n=20)	7.49 ± .63 (n=10)	8.52± .55 (n=10)
		1.3mM Ca^{2+}	**Pb $^{2+}$ (1.3mM Ca^{2+})**
16 D	3.94 ± .12 (n=20)	4.43 ± .18 (n=10)	4.27± .21(n=10)
18 D	6.48 ± .56 (n=22)	7.87 ± .93 (n=11)	7.40 ±.84(n=11)
		1.3mM Ca^{2+}	**Verapamil (1.3mM Ca^{2+})**
16 D	4.95 ± .40 (n=20)	5.61 ± .48 (n=10)	5.79 ± 41(n=10)
18 D	6.54± .35 (n=22)	8.06 ± .71 (n=22)	8.11 ±.22(n=11)
		1.3mM Ca^{2+}	**Nifedipine (1.3mM Ca^{2+})**
16 D	4.40± .17 (n=20)	4.85± .19 (n=10)	5.29±.30(n=10)
18 D	5.99 ± .32 (n=20)	7.34± .70 (n=10)	7.28 ±.56(n=10)

Table 2.4 Tissue protein concentration (*u*g protein/mg tissue)

AGE	BASELINE	CULTURE	CULTURE
		0.7mM Ca^{2+}	**2.8mM Ca^{2+}**
16 D	40.0 ± 3.2 (n=16)	55.2 ± 6.9 (n=8)	56.1 ± 7.1 (n=8)
18 D	46.5 ± 3.3(n=20)	51.4 ± 5.9 (n=10)	53.3± 4.3(n=10)
		1.3mM Ca^{2+}	**Pb^{2+} (1.3mM Ca^{2+})**
16 D	39.8 ± 1.6(n=20)	51.3 ± 4.3(n=10)	55.4± 4.5(n=10)
18 D	47.6 ± 3.8 (n=22)	53.5 ± 4.9 (n=11)	51.0 ±4.4(n=11)
		1.3mM Ca^{2+}	**Verapamil (1.3mM Ca^{2+})**
16 D	46.7 ± 3.2 (n=20)	50.0 ± 7.8 (n=10)	47.8±3.4(n=10)
18 D	48.6± 2.7 (n=22)	49.9 ± 3.9 (n=22)	48.8 ±4.7(n=11)
		1.3mM Ca^{2+}	**Nifedipine (1.3mM Ca^{2+})**
16 D	41.8± 2.3 (n=20)	43.5± 6.9 (n=10)	47.2±3.5(n=10)
18 D	46.6 ± 1.8 (n=20)	52.6± 2.2 (n=10)	51.1 ±3.0(n=10)



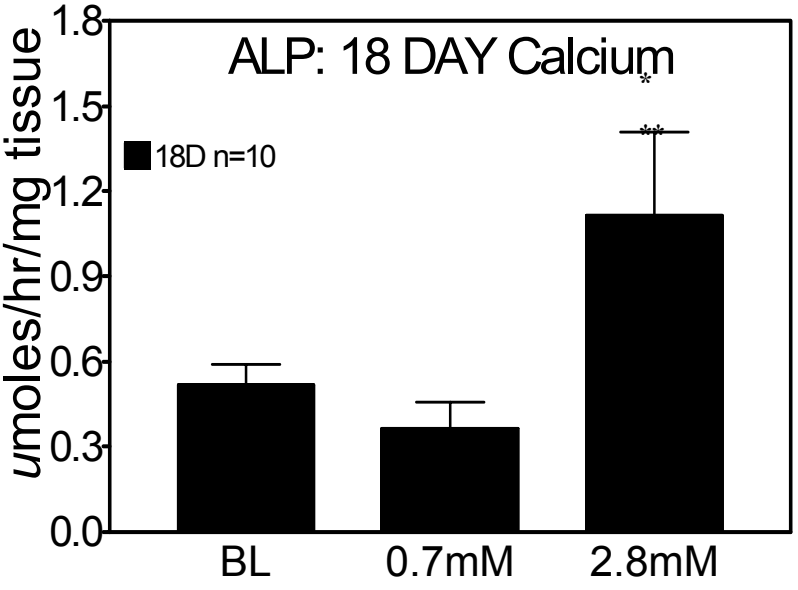
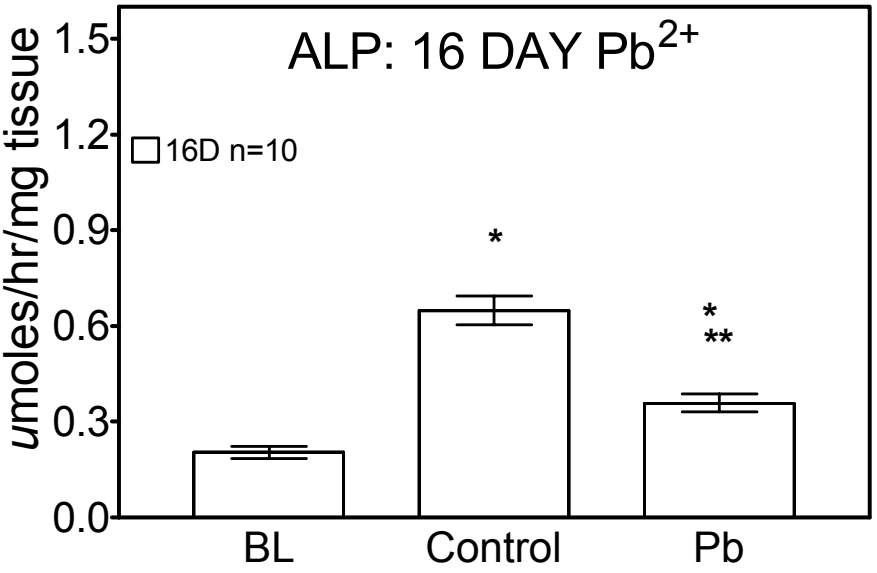


FIGURE 2.1. Effect of extracellular calcium on enzyme activity. ALP activity in tissue cultured in high Ca^{2+} compared to low Ca^{2+}, was higher in 16D and 18D embryos ($p<.02$). (*indicates significant difference from baseline (BL), **indicates significant difference from 0.7mM Ca^{2+}).



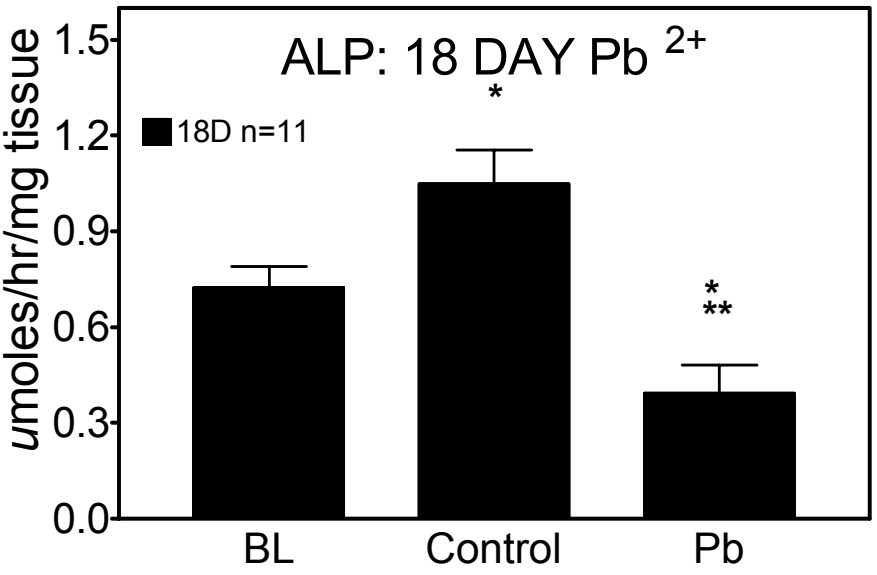
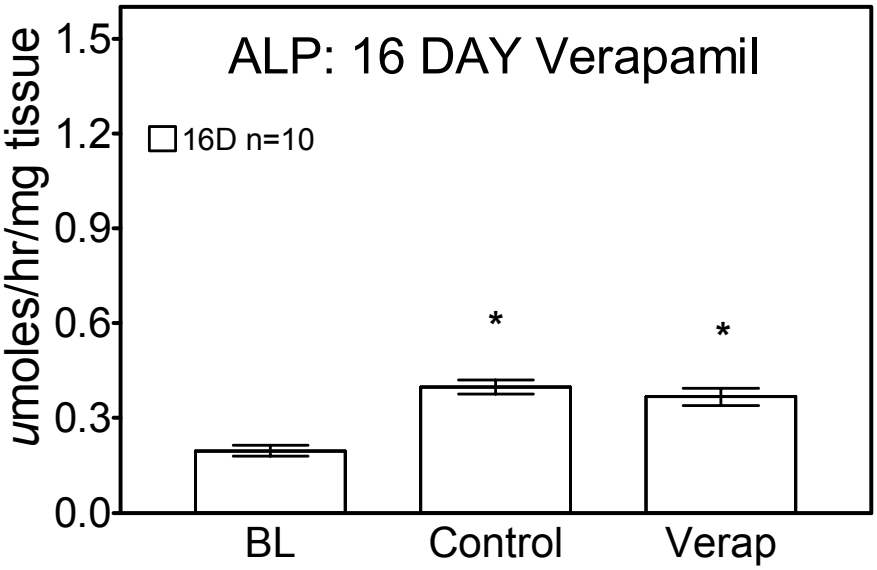


FIGURE 2.2. Effect of lead acetate on enzyme activity. Pb^{2+} significantly inhibited ALP in 16D and 18D embryos with no effect on weight or protein ($p<.001$). In addition, ALP activity in 18D cultures was significantly less than BL values ($p<.02$). (*indicates significant difference from BL, **indicates significant difference from control).



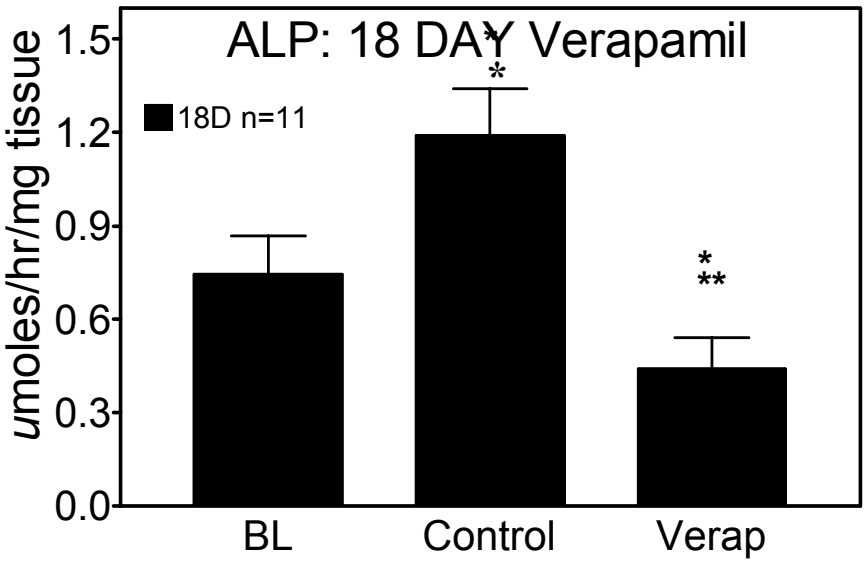


FIGURE 2.3. Effect of verapamil on enzyme activity. Cultured control and 16D verapamil epithelium had significantly more enzyme activity than BL tissue ($p<.001$). Verapamil effectively inhibited ALP in 18D embryos ($p<.02$). (*indicates significant difference from BL, **indicates significant difference from control).

Chapter 3. EXTRACELLULAR CALCIUM IS A MODULATOR OF GOBLET CELL DIFFERENTIATION IN EMBRYONIC DUODENUM

ABSTRACT

Epithelial differentiation is influenced by calcium (Ca^{2+}) in a variety of species and tissues. Previous results indicate extracellular Ca^{2+} modulates goblet cell differentiation in 14-day embryonic chick intestine. This study investigated the effects of extracellular Ca^{2+} concentration and influx in older embryonic duodenum. Fertile broiler chick eggs were incubated for 16 or 18 days (D). Duodenal loops were dissected and cultured in media containing 0.7 mM or 2.8 mM free Ca^{2+} to assess the effects of extracellular Ca^{2+} concentration on epithelial differentiation. Goblet cell (GC) counts (GC number per 100 previllus ridges/villi) were used as the index of differentiation. In a second experiment embryos were cultured in 1.3mM free Ca^{2+} (physiological plasma level) with an inhibitor of Ca^{2+} function, lead acetate (Pb^{2+} 1.05mM), verapamil (0.1mM), or nifedipine (0.01mM). GC numbers in duodena cultured with low extracellular Ca^{2+} (16D=96 ; 18D=337) were significantly lower than values of tissue cultured in high extracellular Ca^{2+} (16D=364; 18D=612). Thus, the extracellular Ca^{2+} concentration altered the functional differentiation of embryonic epithelium during a period of accelerated growth and development. Inhibition of extracellular Ca^{2+} influx by Pb^{2+}, verapamil and nifedipine reduced goblet cell numbers in cultured 16D duodena by 17–65 % and by approximately 44 % in 18D tissue. These results demonstrate that membrane Ca^{2+} entry is an important effector in late stage maturation of embryonic epithelium.

INTRODUCTION

Ca^{2+} acts as an intracellular regulator of numerous biological functions (Campbell, 1983) and has been reported to affect cellular differentiation. Most studies on Ca^{2} -induced differentiation have been performed in vitro, in many tissues including myoblasts (Shainberg *et al*., 1971; Schudt and Pete, 1978), epidermal cells (Hennings *et al*., 1980), human oral mucosa (Sacks *et al*., 1985), mammary gland (Bolander, 1985) and keratinocytes (Sharpe *et al*., 1989). In vivo, exposure of adult rat intestine to calmodulin resulted in increased ALP activity (Komoda *et al*., 1989). In vitro inhibition of calmodulin reduced production of milk proteins and RNA synthesis in cultured mammary gland tissue (Bolander, 1985).

The third week of development in embryonic chick duodenum is characterized by increased morphological differentiation. Earlier work by Black established that Ca^{2+} modulates the development of embryonic duodenal epithelium during the pre-hatching period. In 1989, Black and Smith demonstrated that goblet cell (GC) number and distribution were altered by changes in the extracellular Ca^{2+} ion content of cultured embryonic duodena. Increasing the extracellular Ca^{2+} concentration from 0.9 mM to 2.0 mM in 14D embryonic duodenum cultures caused elevations in GC numbers from 29 to 158 per 100 villus ridges in 48 hours.

In the embryonic chick intestine, epithelial cytoplasmic Ca^{2+} rises in the final days prior to hatching (Black and Rogers, 1992), a period of accelerated growth and differentiation. This pre-hatching period of development may be susceptible to the effects of inorganic lead (Pb^{2+}), which disrupts Ca^{2+} activity. The appearance of goblet cells in embryonic duodenum is a marker of cellular and functional differentiation. The objective of the following experiments was to determine whether disruption of extracellular Ca^{2+} influx inhibits the expression of GC differentiation in embryonic chick duodenum.

MATERIALS and METHODS

Culture

Fertile chicken eggs (broiler strain) from the N. C. State Poultry Science Department were incubated in a forced-draft incubator at 38-39^{o} C. At the appropriate age (16D or 18D), duodenal loops were excised from embryos (n=10-11) and prepared for culture (see chapter 2 methods). Adjacent middle segments of duodenum were divided between culture flasks containing: a.) 2.8 mM vs. 0.7 mM Ca^{2+}. b.) Pb^{2+} 1.05 mM vs. solvent (NaCl) or c.) Verapamil 0.1 mM or Nifedipine 0.01 mM vs. solvent. Duodena were cultured as described in chapter 2. The end pieces were immediately placed in fixative and served as uncultured, baseline tissue. Post culture explants were rinsed in buffer and placed in Carnoy's fixative for a minimum of 3 hours. After fixation tissue remained in 70 % ethanol until embedded with paraffin.

Histology and Goblet Cell Quantification

5-*u*m thick paraffin sections were cut and selectively stained for goblet cells using the periodic acid-Schiff (PAS) procedure. Removal of glycogen was accomplished by a 30 minute pretreatment with 0.5% amylase, which allowed identification of all mucin containing goblet cells. All PAS stained goblet cells on symmetrically sectioned (tip to base) tissue were tabulated, and counts were made on every third section of a specimen until 100 previllous ridges or villi were counted. Results are presented as total number of GC per 100 ridges/villi.

Statistics

In this experiment, baseline (BL) GC counts from each embryo were combined into an averaged value for each embryo for purposes of comparison to paired cultured tissue counts (see methods chapter 2). GC counts were analyzed for significance using the Student's paired t test, with $P < 0.05$ considered significant.

RESULTS

Culture of 16D and 18D embryonic explants (Figure 3.1) in control medium (1.3 mM Ca^{2+}) for 48 and 24 hours respectively, significantly increased GC counts compared to BL values ($p<.001$). Further, culture of 16D explants resulted in significant increases in GC numbers in all treatment groups relative to BL values. Similarly, culture of 18D embryonic duodenum increased GC appearance in all groups.

The Ca^{2+} ion content was adjusted by addition of EGTA to the culture medium below the normal 1.3 mM to 0.7 mM. Cultures supplemented with high $CaCl_2$ had a resultant extracellular Ca^{2+} concentration of 2.8 mM. A comparison of three different Ca^{2+} concentrations (0.7 mM, 1.3 mM, 2.8 mM) used in these experiments showed that GC numbers increased in a dose-dependent manner with increasing extracellular Ca^{2+} concentration (Figure 3.2A). GC expression in duodena cultured in low Ca^{2+} was reduced markedly as compared to paired tissue cultured in high Ca^{2+} at both stages of development. At 16D, counts were 26.4 % of paired high Ca^{2+} cultured explants and at 18D counts were 55 % (Figure 3.2B).

To establish whether goblet cell differentiation was directly modulated by extracellular Ca^{2+} influx, the effects of three calcium channel antagonists (Pb^{2+}, verapamil and nifedipine) were assessed. Pb^{2+} in the culture medium was a strong inhibitor of goblet cell differentiation (Figure 3.3). Cell counts in 18D Pb^{2+} cultured explants did not significantly increase from baseline values. GC counts in Pb^{2+} -containing cultures were 35 % of paired control values in 16D duodena and 48 % in 18D duodena. Contrary to Pb^{2+}, verapamil did not significantly reduce goblet cell numbers in cultured 16D explants compared to controls (Figure 3.4). Interestingly, in 18D cultured duodenum verapamil-induced goblet cell inhibition was equivalent to that of Pb^{2+}. Unlike verapamil, nifedipine effectively lowered goblet cell counts ($p<.001$) in the younger tissue (Figure 3.5) by 38 %. The 18D duodenal

explants cultured in nifedipine were similarly affected as those exposed to Pb^{2+} and verapamil; goblet cell counts in were 57 % of paired control values.

DISCUSSION

The results of this study demonstrate that alteration of the extracellular Ca^{2+} concentration and inhibition of extracellular Ca^{2+} influx influence GC expression in cultured 16 and 18D embryonic duodenal epithelium. Changing the culture medium Ca^{2+} concentration to 0.7 mM or 2.8 mM produced major effects on GC number. Additionally, using GC number as an index of embryonic development, duodenum cultured with Pb^{2+} did not mature to the same extent as paired control tissue. Tissue patches cultured in either Ca^{2+} -channel antagonist (verapamil or nifedipine) were inhibited. Clearly, disrupting the influx of extracellular Ca^{2+} in 16D and 18D chick embryos hindered epithelial GC differentiation. These results are similar to those reported by Black and Smith (1989) in 14D duodenum.

Black and Smith cultured 14D embryonic duodenum in extracellular Ca^{2+} concentrations ranging from 0.9 mM to 2.0 mM (Black and Smith, 1989). As extracellular Ca^{2+} increased, GC number rose from 29 to 158 per 100 previllus ridges. The effects of Ca^{2+} concentration on GC were maximal at 1.6 mM. However, counts in 2.0 mM Ca^{2+} could be significantly increased with the addition of small amounts of Ca^{2+} ionophore. In the present, experiment the counts per 100 villus ridges of explants cultured in 2.8 mM Ca^{2+} were 364 $\pm$ 18.8 at 16D. These numbers greatly exceed the 236 reported for the White Leghorn strain (Black and Moog, 1977). Conversely, GC counts of tissue cultured in 0.7 mM Ca^{2+} were only 96 $\pm$ 7 in 16D old embryos. However, in the current study, as in the Black and Smith work, GC numbers increased during culture at all Ca^{2+} concentrations tested.

In mammary gland and skin epithelial tissue cultures, maximal differentiation occurs at much lower Ca^{2+} concentrations, 0.1-0.2 mM, than

were used in the current experiment (Hennings *et al.*, 1983; McGrath and Soule, 1984). We were not able to test Ca^{2+} concentrations below 0.7mM, as tissue integrity is not maintained in chick intestine at lower concentrations (Black and Smith, 1989). However, in the current experiment, an enhanced extracellular Ca^{2+} concentration (2.8 mM) produced the greatest increase in goblet cell counts (364 at 16D and 611 at 18D). In vivo, the plasma Ca^{2+} concentration of a 16D chick embryo is 1.3 mM and increases to approximately 1.5 mM by day 18 (Black and Smith, 1989). Therefore, it is logical that levels above and below these concentrations (i.e., 0.7 and 2.8 mM) would be required to elicit changes from the normal expression of goblet cells during this period of embryonic development.

It is unlikely that the increased GC counts in this study are a by-product of Ca^{2+}-enhanced epithelial growth. It has been well documented that physiologically high levels of Ca^{2+} ions induce terminal differentiation, while low levels stimulate cellular proliferation (Carruthers and Suntzeff, 1944; Hennings *et al.*, 1978; Reese and Friedman, 1978; Swierenga *et al.*, 1978). The earlier work of Black and Smith (1989) with 14D embryos found no correlation between the effects of extracellular Ca^{2+} on GC count and epithelial integrity, surface area or mucin secretion. Although previllus ridges and villi lengthen during the third week of incubation, changes in extracellular Ca^{2+} did not significantly affect this process in 14D duodena compared to uncultured tissue of an equivalent age (Black and Smith, 1989). The resultant decreased GC counts of the present study post culture with Pb^{2+}, verapamil and nifedipine are not due to chemical toxicity, since tissue weight and protein concentration were not altered (see Chaper 2). Furthermore, Ca^{2+}-channel antagonists have been used effectively *in vitro* for many years without deleterious effects on embryonic intestine (Corradino, 1984; 1985).

Pb^{2+} can interfere with Ca^{2+} activity in three ways. It can compete for plasma membrane transporters effecting entry and exit at Ca^{2+}channels and

pumps. It can disrupt intracellular Ca^{2+} homeostasis by Pb^{2+}-Ca^{2+} interactions at mitochondria that inhibit mitochondrial uptake of cytoplasmic Ca^{2+}, and Pb^{2+} can obstruct Ca^{2+}-dependent signaling molecules (Simons, 1993). Determining specifically which of these Pb^{2+}-Ca^{2+} interactions attributed to the effects observed here is beyond the scope of this project. However, other Pb^{2+} studies may offer insight. Filerman and Berliner (1980) exposed an epitheliod cell line, RLC-GAI, to a growth-inhibiting dose of Pb^{2+}. Cells became rounded and were susceptible to osmotic shock, but microvilli were normal and microfilaments were able to polymerize. If cells were subsequently placed in Pb^{2+}-free medium rounding was reversed. Mitochondrial changes were only present after several days of Pb^{2+} exposure. In this study, duodenal explants were cultured for 24-48 hours. This would suggest that the effects of Pb^{2+} were not due to interactions with mitochondria. Pb^{2+} interference with normal Ca^{2+} transport at the plasma membrane in various types of tissues is well documented (Atchison and Narahashi, 1984; Rius *et al.*, 1986; Lal *et al.*, 1991). Atchison and Narahashi investigated the effects of Pb^{2+} on neurotransmitter release at the neuromuscular junction in rats. Direct application of Pb^{2+} to the bath significantly inhibited neurotransmitter release. They concluded Pb^{2+}'s actions were extracellular, the result of competitive antagonism with Ca^{2+} for influx through plasma membrane Ca^{2+} channels. Although Pb^{2+} can compete for carrier proteins and membrane receptors, it is only toxic after chronic exposure to high levels, which was not the case in this study (Lansdown, 1995; Hartwig, 1994). Therefore, reductions in GC counts by Pb^{2+}, verapamil and nifedipine were not due to chemical toxicity, but presumably by blocking the influx of extracellular Ca^{2+} at the plasma membrane.

Overall, the findings of the current study, in conjunction with early work by Black, demonstrate that extracellular Ca^{2+} has a regulatory effect on the rate of goblet cell differentiation in cultured embryonic chick intestinal epithelium. The precise mechanism of this effect is unknown. Other

regulators of intestinal development include growth factors (epidermal growth factor; fibroblast growth factor) and hormones (thyroid; glucocorticoid) influence late stage embryonic development (Rogers and Black, 1996; Bellusci *et al*., 1997).

Growth factors are well known modulators of enterocyte proliferation and differentiation in developing and mature vertebrates (O'Loughlin *et al*., 1985; Folzer-Jourdainne and Raul, 1990; Young *et al*., 1990; Podolsky, 1993). They are also active in embryonic development. Fibroblast growth factors (FGF) are differentially expressed in mesenchyme and epithelium from cultured mouse embryos during organogenesis (Peters, *et al*., 1992). Embryonic colonic fibroblasts from rats increase the survival of adult human and rat colonic epithelial cell cultures (Schorkhuber *et al*., 1998). In a recent study, embryonal stem cells, which lacked beta integrins, failed to differentiate into keratinocytes when cultured (Bagutti *et al*., 2001). However, addition of human dermal fibroblasts to the medium induced differentiation. Epidermal growth factor (EGF) has also been implicated in embryonic differentiation. EGF has been isolated in the amniotic fluid of gestational rats and in the small intestine of their fetuses (Weaver *et al*., 1990). This suggests EGF is active in the development of fetal duodenum. In fetal rabbits, gestational infusion of human EGF resulted in increased intestinal length and increased lactase and maltase activity (Buchmiller et al., 1993). The most interesting effect of EGF was a study by Morikawa *et al*. (1994). They reported that an EGF-like factor present in the amniotic fluid of pregnant rats increased the density of goblet cells in the colon of rat fetuses during late stage gestation. The findings of these studies demonstrate that growth factors have a physiological function in embryonic epithelial development.

Hinni and Watterson (1963) found hypophysectomy of chick embryos lead to incomplete duodenal development, establishing a link between hormones and epithelial differentiation (Bellware and Betz, 1970). Normal GC differentiation can be restored to the compromised embryos by grafting

pituitary tissue to the chorioallantois, therefore pituitary hormones such as TSH are involved in regulating development (Hart and Betz, 1972). Later work directly correlated calcium and hormones in cultured duodenum. Chick intestine cultured in hormone free medium for 48 hours underwent accelerated goblet cell differentiation when thyroxine was added to the culture medium, while hydrocortisone had the opposite effect (Black and Moog, 1977). A couple of years later, Corradino established that glucocorticoids decrease Ca^{2+} uptake (Corradino, 1979). This implied hydrocortisone inhibits GC differentiation by decreasing available cytoplasmic Ca^{2+}. Thus, hormones influence embryonic differentiation in part by modulating Ca^{2+}.

It has been proposed that increases in cytoplasmic Ca^{2+} concentration underlie extracellular Ca^{2+}-influenced epithelial differentiation (Black and Smith, 1989). Using isolated epithelial cells from cultured 14D duodena, Black and Rogers (1992) determined cytoplasmic Ca^{2+} does increase as the Ca^{2+} concentration of the medium is increased. Next Rogers and Black (1996) demonstrated that hydrocortisone and thyroxine influence Ca^{2+} homeostasis in epithelial cells isolated from duodenal cultures of 14D chick embryos. These effects occur with a lag period (hours to days) that suggests a nuclear site of action. They concluded that other effects of hormone on epithelial differentiation are likely mediated by cytoplasmic Ca^{2+}. In addition, a Ca^{2+}-glucocorticoid interaction has also been implicated in mammary gland epithelial development (McGrath and Soule, 1983; 1984). Human mammary epithelial cells growing in groups on culture dishes were induced to undergo terminal differentiation when cortisol was added to the cells (McGrath and Soule, 1983). In a separate study McGrath and Soule (1984) found that with reduced extracellular Ca^{2+} cells remained in a growth state and lived longer, as there was no cellular differentiation. They concluded Ca^{2+} acted as a trigger in mammary epithelium that committed cells to the differentiation pathway in a permissive hormonal environment.

It is still unclear exactly how Ca^{2+} regulates GC differentiation, but prostaglandins seem to be involved. Black and colleagues (1994) measured Prostaglandin E2 (PGE2) in 12, 14, 17 and 19D cultured chick duodena. The levels of PGE2 rose significantly over that time, as did GC numbers. The PGE2 antagonists indomethican and nordihydroguaiaretic acid were added to the culture medium of 14D duodenum and reduced GC counts respectively by 78 % and 67 %. Addition of PGE2 reversed this inhibition. PGE2 stimulation of GC differentiation was directly correlated with the Ca^{2+} ion concentration of the culture medium. PGE2 rose by 45 % as extracellular Ca^{2+} increased from 0.7 mM to 2.8 mM (Black *et al*., 1994). Therefore, it was concluded that high extracellular Ca^{2+} likely increases the cytoplasmic Ca^{2+} ion concentration, which stimulates PGE2 synthesis and induces GC differentiation. Thus, Ca^{2+}, Pb^{2+}, verapamil and nifedipine-induced changes in GC differentiation in the current study are probably due to changed levels of cytoplasmic Ca^{2+}, resulting from altered extracellular Ca^{2+} influx, which modulates Ca^{2+} signaling pathways involved in epithelial differentiation.

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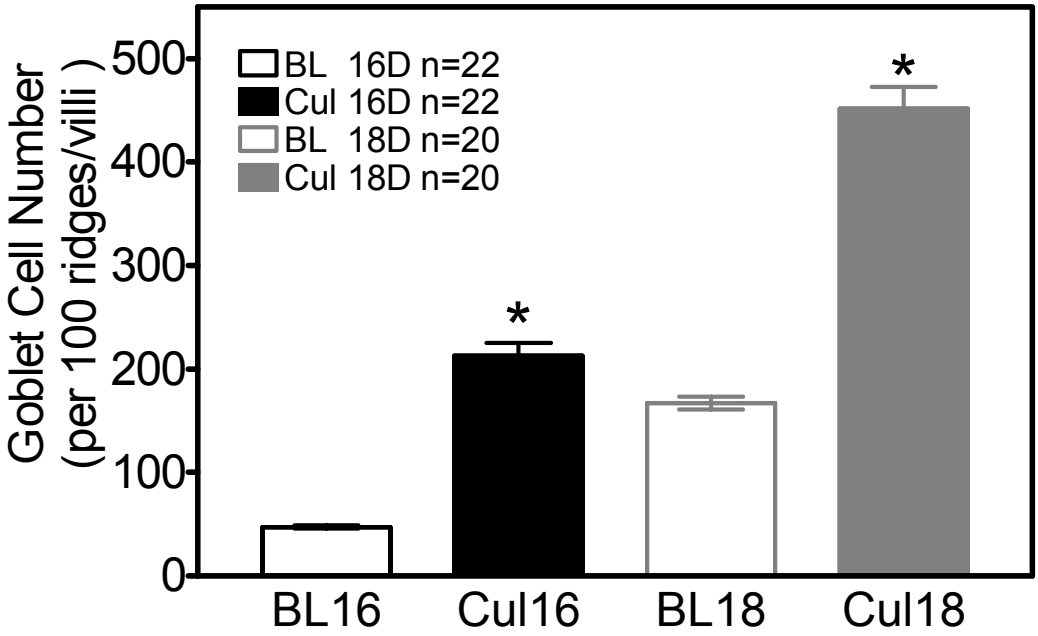
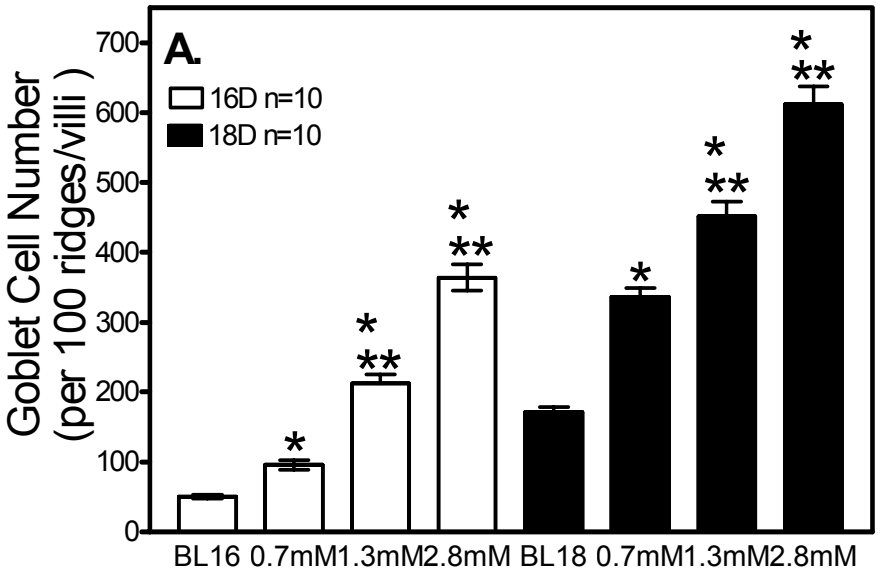


FIGURE 3.1. Embryonic goblet cell number. Developmental goblet cell counts in paired baseline (BL) and cultured duodenum (Cu l) in medium containing 1.3 mM Ca^{2+}. Goblet cell number in 16D and 18D tissue increases significantly during culture ($p<.001$). (*indicates significant difference from BL.)



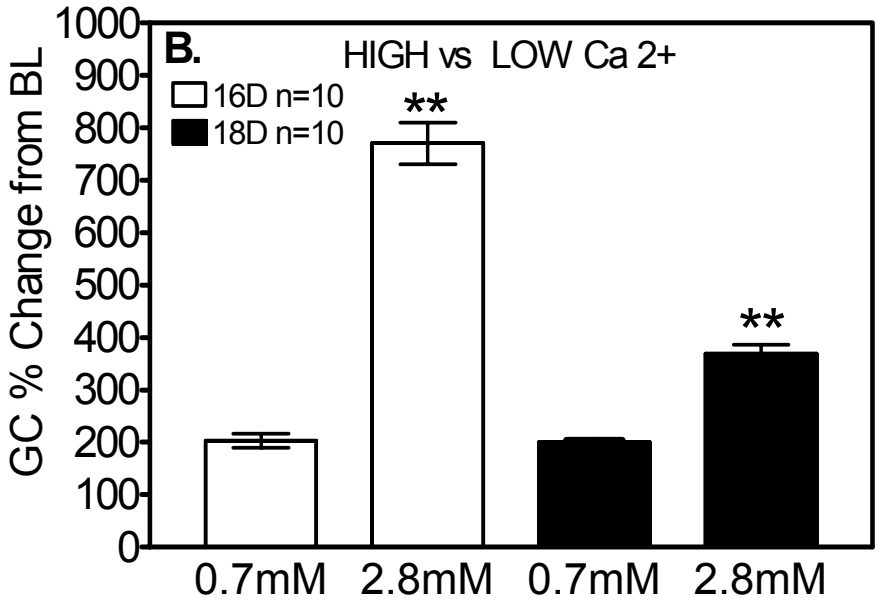


FIGURE 3.2. Effects of alteration of culture medium Ca^{2+} concentration on goblet cell (GC) number development in embryonic enterocytes. **A.**. GC number increased in duodenum cultured in low Ca^{2+} (0.7mM) and high Ca^{2+} (2.8mM) at 16D and 18D of age ($p<.001$) compared to BL. **B.** Incubation in high Ca^{2+} (2.8mM) significantly increased the number of GCs in 16D and 18D tissue ($p<.001$). (*indicates significant difference from BL, **indicates significant difference from 0.7mM Ca^{2+}.)

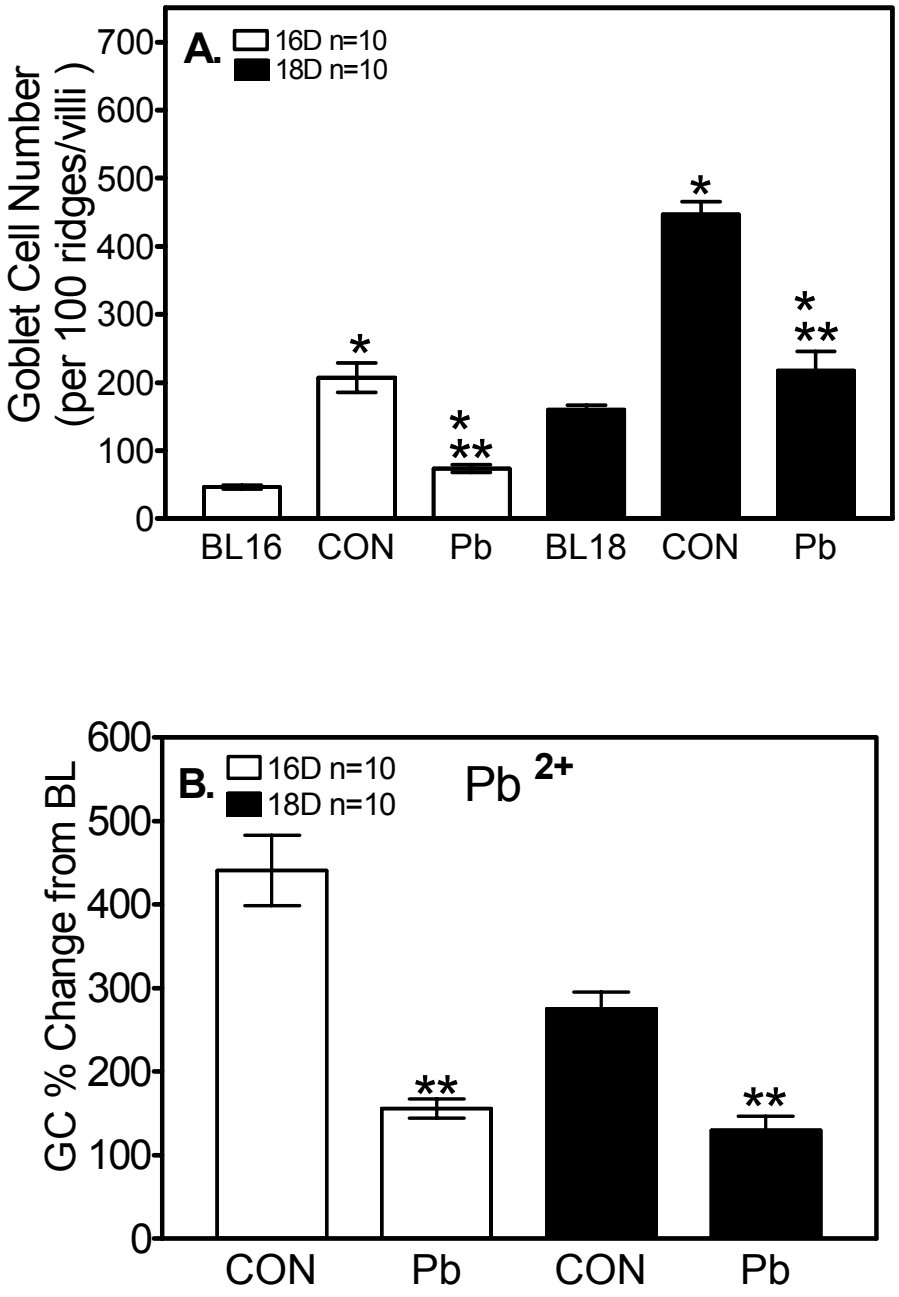
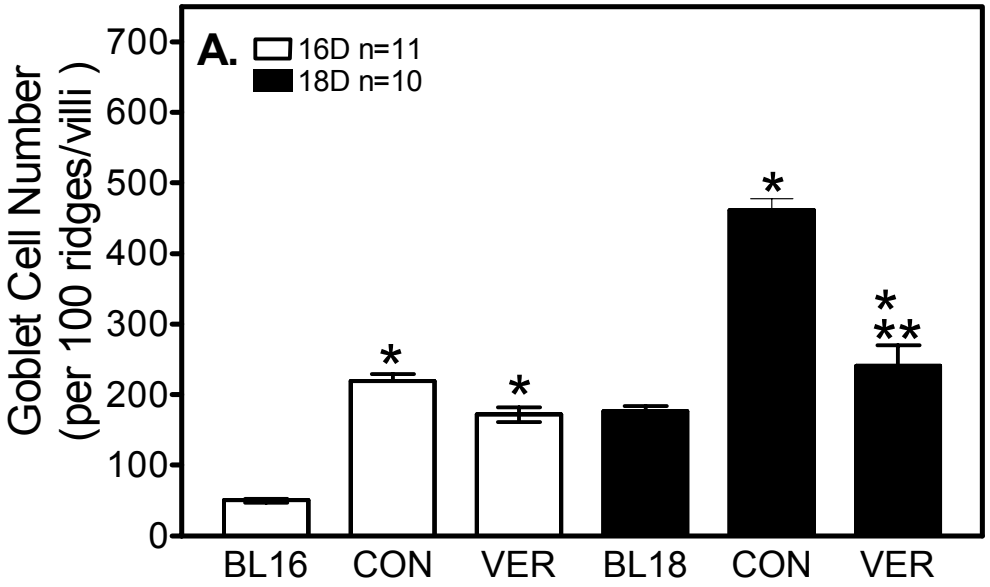


FIGURE 3.3. Effects of extracellular Pb $^{2+}$ on goblet cell number. A. Control (16D, 18D) and 16D Pb $^{2+}$ cultured epithelium have higher GC counts than BL values ($p<.05$). B. The presence of inorganic lead in culture medium is a potent inhibitor of GC differentiation in 16D and 18D tissue ($p<.001$). (*indicates significant difference from BL, **indicates significant difference from control.)



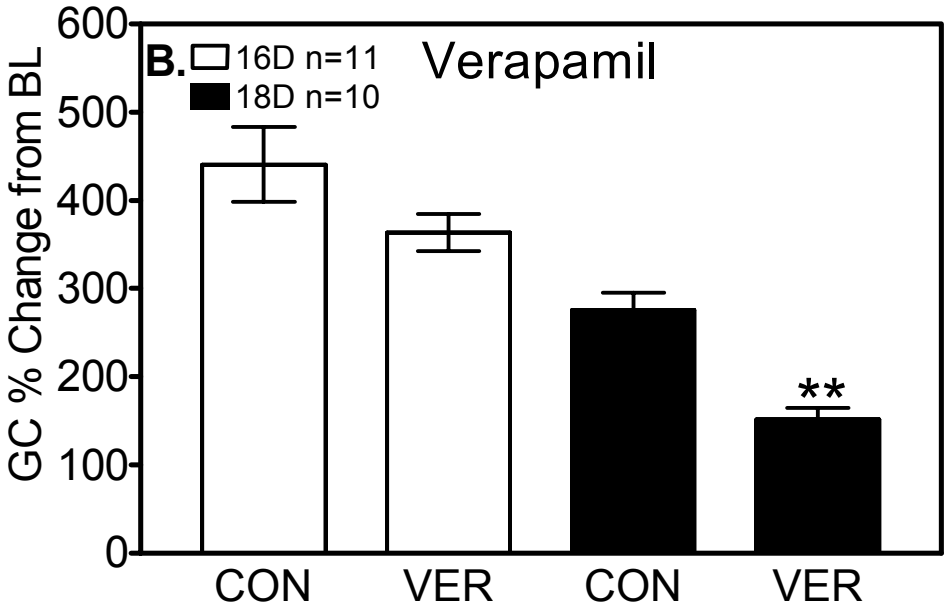


FIGURE 3.4. Effects of extracellular verapamil on goblet cell number.
A. Control and verapamil treated cultures have higher GC counts than BL values ($p<.01$). B. The addition of verapamil to culture medium decreases goblet cell expression significantly in 18 D duodenum ($p<.001$). (*indicates significant difference from BL, **indicates significant difference from control).

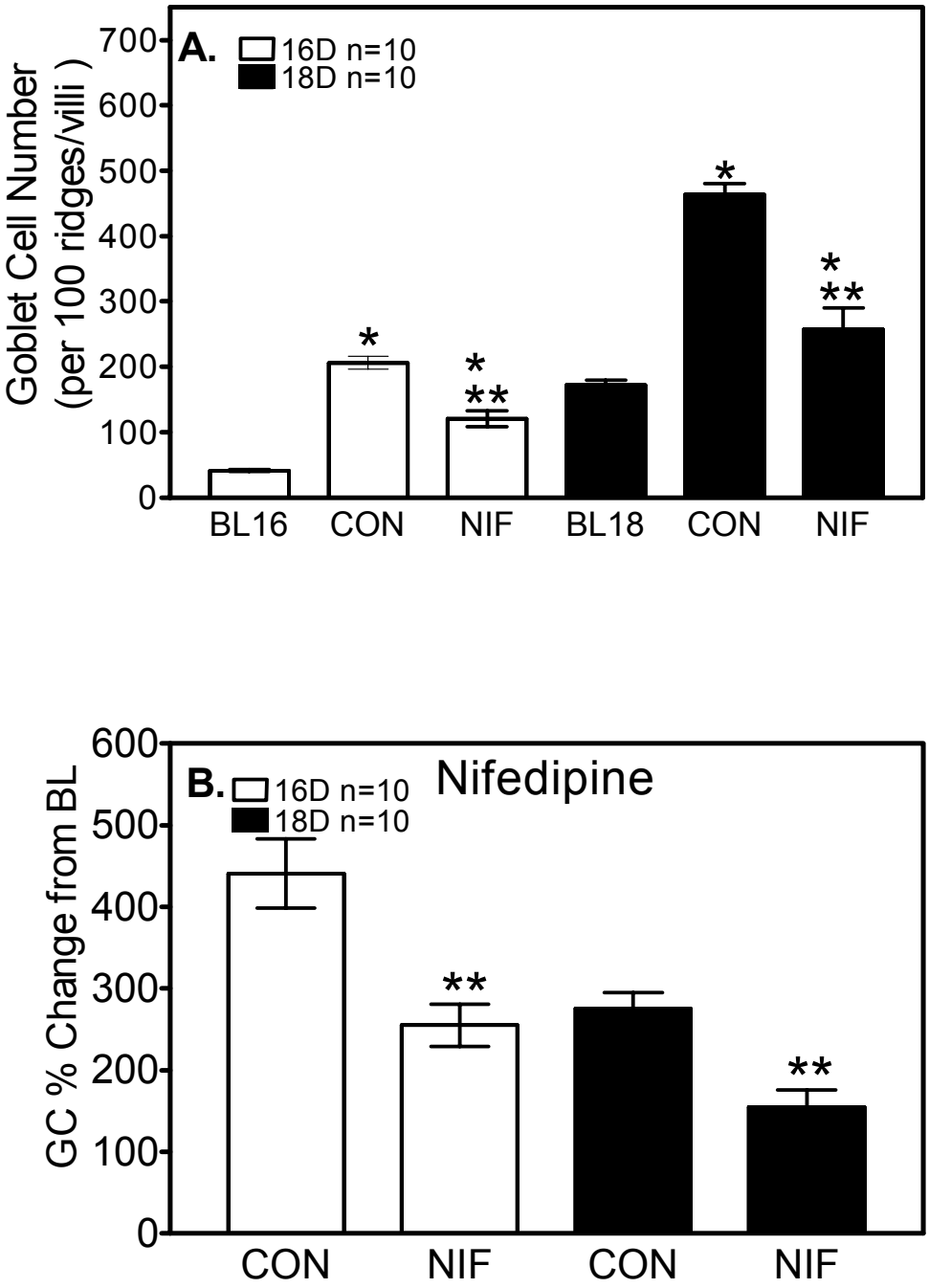


FIGURE 3.5. Effects of extracellular nifedipine on goblet cell number.
A. GC numbers increase significantly in control and nifedipine treated tissue compared to BL ($p<.01$). B. Nifedipine in culture medium decreases goblet cell numbers in 16D and 18D duodenum relative to paired controls ($p<.02$). (*indicates significant difference from BL, **indicates significant difference from control).

Chapter 4. VERAPAMIL AND NIFEDIPINE REDUCE EPITHELIAL CYTOPLASMIC CALCIUM

ABSTRACT

Epithelial differentiation (enzymatic and morphological) in embryonic chick duodenum is inhibited in the presence of Ca^{2+} blocking chemicals. The functionality of voltage-sensitive Ca^{2+} channels (VSCC) was investigated in cultured 18D embryos. Duodenal explants were cultured for 24 hours in medium treated with verapamil (1.0 mM) or the dihydropyridine (DHP) nifedipine (10 *u*M), or 0.9% saline control. Post incubation explants were loaded with the fluorescent Ca^{2+} probe, Indo1 and subcellular Ca^{2+} changes were measured using confocal microscopy imaging. In paired controls, the fluorescent intensity was greatest along enterocyte brushborders. In tissue exposed to the Ca^{2}-channel antagonists, fluorescence was diminished at the brushborder as well as within the cytoplasm. In the apical membrane region of verapamil cultured tissue Ca^{2+} concentration was reduced by 18.4 %. Nifedipine reduced Ca^{2+} by 21.6 %. In all groups, the Ca^{2+} concentration of the cytoplasmic region was lower than that of the apical region. However, the cytoplasmic concentration was lowest in the VSCC-antagonist treated explants. The effects of nifedipine cannot be attributed to non-specific effects at the plasma membrane, as it is highly selective for L-type Ca^{2+} channels. These results suggest that VSCC's are present and functional in late stage embryonic chick duodenum.

INTRODUCTION

The original concept of confocal microscopy was developed in 1957 by Marvin Minsky as a non-invasive method for high-resolution imaging of fixed biological samples (Paddock, Cullander, 1999). During the 1970's, improvements were made to the confocal design and laser-based confocal microscopes were developed. These provided optical sections and three-

dimensional reconstruction of sections that were not possible with traditional wide-field bright light microscopy. In addition, thick sections of fixed biological specimen were imaged as well as living tissue and cells. This has lead to the current explosion in the use of confocal laser scanning microscopy (CLSM) (Stricker and Whitaker, 1999; Perez-Terzic *et al.*, 1998).

Previous work by Black and Rogers (1992) used spectrofluorometry with the Fura-2 indicator to assay intracellular Ca^{2+} in isolated cells from embryonic chick duodena (14D embryo- 12D posthatch). Cytoplasmic Ca^{2+} concentration increased with age, peaking at 1D posthatch. In addition, cells developed an enhanced intracellular buffering ability when cultured in high levels of extracellular Ca^{2+}. However, lowering the culture medium from 1.3 mM Ca^{2+} to 0.7 mM yielded a decrease in intracellular Ca^{2+} in 14D-16D embryos.

In this study (Chapter 2 & 3), lowering extracellular Ca^{2+} to 0.7 mM and inhibiting extracellular Ca^{2+}-influx (via verapamil and nifedipine) resulted in decreased brushborder enzyme activity and decreased goblet cell differentiation in 16D and 18D cultured duodenum. The spectrophotometric analysis of Fura-2 fluorescence in isolated epithelial cells is limited to quantitative measurements of cytoplasmic or whole cell Ca^{2+}. CLSM technology can provide additional spatial information about intracellular Ca^{2+} levels and distribution. CLSM was therefore used to investigate changes in Ca^{2+} concentration and subcellular localization in 18D cultured epithelium.

MATERIALS and METHODS

Culture

Fertile chicken eggs (broiler strain) from the N. C. State Poultry Science Department were incubated in a forced-draft incubator at 38-39^{o} C. At 18-days of age duodenal loops were excised from embryos and prepared for

culture as previously described (Black and Moog, 1978). Each duodenum was dissected and the middle loop segment was cut into three pieces, which were divided between culture flasks with a Ca^{2+} -channel antagonist (verapamil, 0.1mM, or nifedipine, 0.01 mM) or with untreated culture medium (Control) (see previous chapters). Duodena were cultured in Medium 199 (GIBCO) (free [Ca^{2+}] =1.3 mM), pH=7.3, for 24 h at 38 ^{o}C. Drugs were obtained from Sigma Chemical Co. and prepared in 0.9 % NaCl solvent. Control cultures contained equivalent volumes (0.1 ml) 0.9 % NaCl. In separate experiments, uncultured 18D and 19D duodena were immediately stored in buffer containing glutamine and butyrate and served as baseline (uncultured) tissue for qualitative inspection.

Indo-1AM Loading

Post culture tissue segments were removed from medium and rinsed in buffer (NaCl, Na_2HPO_4, KCl, $CaCl_2$, KH_2PO_4, $MgSO_4$, hepes) with glutamine and butyrate (GTB), which provided energy for tissue during imaging. Next tissue was placed in flasks containing the fluorescent probe Indo1-AM (Molecular Probes) dissolved in 1mM dimethyl sulfoxide (Sigma) and GTB buffer ([Ca^{2+}] =1.3mM), total volume 3mls, final Indo1-AM concentration, 55 mM. The flasks were placed in a shaking water bath set at 37 ^{o}C and incubated for 45 minutes. After loading, tissue sections were rinsed in GTB and stored in fresh buffer for the duration of the experiment. Confocal microscopy imaging took place immediately thereafter. All imaging was completed within 45 minutes of final placement in GTB.

Measurements and calculations

Fluorescence was viewed with a Leica TCS SP2 laser scanning spectral confocal microscope equipped with the appropriate optics and filter modules. Leica Confocal Software (LCS) was used to perform digital imaging and free Ca^{2+} ion measurements. Standards from the indo-1 dextran calcium calibration buffer kit (Molecular Probes) ranging from 0 *u*M to 40 *u*M free

Ca^{2+} were prepared prior to scanning and measured at the beginning and end of each experiment. Tissue segments were placed upside down in a well containing GTB (1.3 mM Ca^{2+}). Villi were perpendicular to the microscope and cross-sectional scans were performed. Initially, bright field images were inspected and clearly fluorescing tissue was scanned under the 20X objective (area=500 *u*mX500 *u*m). Next using the zoom function random areas measuring 250 *u*mX250 *u*m were scanned starting from the villus tip surface. Measurements were conducted on all epithelial cells with measurable fluorescence (see Figure 4.3, range indicator), generally made at a depth of 13.5 *u*m below villus tip surface. Thus, every cell around the periphery of a given villus was measured. End to end rectangular intracellular sections along the interior apical membrane (BB) and the central interior cytoplasm (CY) of individual epithelial cells were sampled once per second and averaged 4 times each. Section area varied ranging from 85 *u*m to 125 *u*m, and care was taken to exclude fluorescence from the basolateral membrane in CY scans.

The ratiometric measurements are represented by the equation: R=Channel2/Channel1, where R=fluorescence ratio, Channel2=fluorescence of probe and bound calcium, and Channel1=fluorescence of probe alone. The Leica imaging software was programmed to excite the tissue at 350 nm with the UV laser and fluorescence emission was alternately detected at 405 nm and 485 nm. The DIC (correlative bright field image) picture was simultaneously recorded with the fluorescence confocal images (see Figure 4.1). All measurements were made from the overlay image (R panel, Figure 4.1). Figures 4.2-4.8 are overlay (R) images. The R value was used to calculate the intracellular Ca^{2+} concentration with a K_d (see Scheeneen *et al*., 1998) of 0.23 *u*m with the following equation (Grynkiewicz *et al*., 1985).

$$[Ca^{2+}]_I = K_d \times (Q) \times \frac{R - Rmin}{Rmax - R}$$

R is the ratio of the absorption/emission at the two wavelengths (see above), K_d is the dissociation constant, Q is the ratio of the fluorescence intensity of the calibration standard at zero free Ca^{2+} divided by the calibration standard at saturating free Ca^{2+}, Rmax is the ratio of indo-1 fluorescence in saturating Ca^{2+} and Rmin is the minimal indo-1 fluorescence ratio in Ca^{2+}-free (EGTA containing) buffer, calculated post experiment using calibration values. Statistical comparisons between controls and treated cultures were evaluated for significance by one-way analysis of variance (ANOVA).

RESULTS

Uncultured 19D duodenum (chronological equivalent for 18D tissue cultured for 24 hours) was imaged for visual comparisons to cultured 18D control tissue (Figure 4.2) Inspection revealed no apparent gross morphological damage from the culture procedure. The indo-1 (AM) ester probe penetrated the epithelium of all incubated tissue effectively, uncultured and cultured. Fluorescence was visualized by color-coded magnitude of intensity (see Figure 4.3) from minimal Ca^{2+} to saturation levels (i.e., fluorescence exceeds standards parameters, 40 *u*M free Ca^{2+}). Therefore, quantitative Ca^{2+} measurements were not made from saturated sections of tissue. Ca^{2+} concentrations were generated from overlay images. This ratiometric analysis compensates for artifacts produced by uneven take up of probe, photobleaching, and path length differences. Qualitative inspection of bright field images revealed no gross tissue damage from culture procedure or chemical treatment. Clear fluorescent signals were obtained from epithelial cells, which were prominently illuminated compared to the dark villus cores (Figure 4.4).

Epithelial cells from cultured tissue displayed vivid fluorescence (Figure 4.5). ALP and other digestive enzymes, as well as a variety of Ca^{2+}-binding

proteins, are located along the microvillus lined apical membrane of enterocytes. Indo-1 fluorescence was most intense in this region of the cell (Figure 4.6). There was also prominent fluorescence along the basal region of the epithelium.

The structural integrity of duodenum cultured in verapamil and nifedipine appeared to be un-compromised compared to control tissue. However, tissue cultured in verapamil (Figure 4.7a, 4.7b) and nifedipine (Figure 4.8a,4.8b) did not display the same intensity of fluorescence as the cultured control tissue. The epithelium of these explants had a more uniform fluorescence from apical to basolateral membrane. Ca^{2+} was not concentrated at the apical end of the cell as it was in the controls. This suggested a reduction in the Ca^{2+} concentration of the brush border region, and perhaps the overall concentration within the cells. Quantification of the ratiometric fluorescence data (n=7 embryos) validated these observations (Figure 4.9). Enterocytes of verapamil and nifedipine treated duodena had lower Ca^{2+} concentrations than paired control cells. In the apical region, brushborder (BB) verapamil and nifedipine decreased the free ion concentration by 18.4 % (p=.0078) and 21.6 % (p=.0128), respectively. The inner cytoplasmic Ca^{2+} (CY) concentration was 11.4 % lower in verapamil treated tissue (p=.0350) and 13.6 % less with nifedipine (p=.0276). However, the overall ratio of brushborder to cytoplasmic Ca^{2+} was not significantly different from controls after verapamil and nifedipine treatment (p=.067).

DISCUSSION

Disruption of the influx of extracellular Ca^{2+} by two pharmacologically different antagonists decreases goblet cell numbers and activity of the digestive enzyme ALP during embryonic development in vitro (see Chapters 2 and 3). This suggests that intracellular Ca^{2+} levels are critical to embryonic epithelial differentiation. From the earlier results, we suspected that Ca^{2+}

might be concentrated within the interior apical membrane region under normal conditions. This was in fact a correct assumption. The measured intracellular Ca^{2+} concentration was higher in the vicinity of the apical membrane in all epithelial cells. The intense brushborder fluorescence seen in control and uncultured epithelium was reduced after culture in verapamil and nifedipine, VSCC antagonists. Although the visual representation of this change in Ca^{2+} localization appeared to be dramatic, the apparent decrease in the brushborder/cytoplasmic ratio relative to controls did not reach statistical significance. Thus, both brushborder and cytoplasmic intracellular Ca^{2+} diminished after culture with a Ca^{2+}-channel antagonist. Functionally, this reduction could impact processes triggered by a cytoplasmic Ca^{2+} concentration signal. For example, the insertion of ALP into the brushborder is probably influenced by local Ca^{2+} oscillations. ALP is attached to the apical membrane of enterocytes by a glycosylphosphatidylinositol (GPI) linkage (Engle *et al*., 1995). In vitro work with rat brain tissue found plasma membrane binding of GPI-anchored proteins was a Ca^{2+}-dependent process (Orito et al., 2001). Komoda previously demonstrated ALP activity in rat ileum was enhanced by calmodulin and inhibited by flunarizine, a Ca^{2+}-channel inhibitor (Komoda et al., 1989). Further, Barcelo *et al.* have reported that colonic mucin secretion in rats is dependent upon extracellular Ca^{2+} influx across the plasma membrane, specifically through L-type Ca^{2+} channels (Barcelo et al., 2001). In addition, signals responsible for other terminal differentiation pathways could be modulated by the cytoplasmic Ca^{2+} concentration.

Confocal microscopy represents a significant technological advancement in cell biology. Clear three-dimensional images of the internal cellular environment of living cells are readily obtainable. The reliability of quantification of ion and pH levels however is still debatable. In this study, the intracellular [Ca^{2+}] was measured at 74.5 nM along the apical membrane and at 64.1 nM in the interior cytoplasmic region of cultured duodenal

epithelium of 18D chick embryos. Black and Rogers (1992a) reported a fura-2 measured cytoplasmic [Ca^{2+}] range of 92-98 nM in isolated duodenal epithelial cells from 17D to 20D old embryos. At best, the values differ by 20 nM. These differences likely reflect the methods used to obtain the measurements (i.e., isolated cells vs. tissue segments and fura-2 vs. indo-1). Therefore, the confocal measurements seem similar enough to the reported values to be considered representative.

Epithelial cells are dependent upon cellular signaling for control of physiological functions. The free cytoplasmic Ca^{2+} concentration is the most common signaling mechanism in all vertebrate cells. In epithelia, cell growth and differentiation are two vital functions controlled by the cytoplasmic Ca^{2+} concentration. Signal transduction involves transient increases in intracellular Ca^{2+} due to release of Ca^{2+} from intracellular storage sites or from extracellular influx, or a combination of both. Control of this process is highly regulated by Ca^{2+} pumps, transporters and buffering proteins. Another regulator of cytoplasmic calcium, integral to plasma membrane influx and intracellular release, is the ion channel.

Epithelial intracellular Ca^{2+} signals can be generated by G-protein-coupled membrane receptors, tyrosine kinase receptors or ion-receptor complexes (Wickman and Clapham, 1995). G-proteins activate phospholipase C (PLC), which hydrolyzes phosphatidylinositol bisphosphate ($PtInsP_2$) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3 receptor (IP3R) Ca^{2+} channel stimulating intracellular Ca^{2+} store release. DAG stimulates the protein kinase C (PKC) cellular cascade. Tyrosine kinase receptor activation also leads to IP3 and DAG stimulation, but via a different PLC molecule. These pathways transiently increase the intracellular Ca^{2+} concentration. The cytoplasmic Ca^{2+} increase also activates extracellular influx across the epithelial membrane. This produces a sustained cytoplasmic increase, which signals Ca^{2+} sensitive cellular functions (i.e.,

growth and differentiation). Thus, extracellular influx and intracellular store release are Ca^{2+} ion channel-dependent.

Epithelium is considered "non-excitable" compared to neural and muscle cells. The presence of Ca^{2+} ion channels in epithelium is well established (Saunders *et al*., 1990). In addition, voltage-sensitive channels have been identified in intestinal muscle. However, the existence of VSCCs in epithelium in general and duodenum in particular is unclear. The focus of this study was to clearly demonstrate that extracellular Ca^{2+} modulates cultured epithelial differentiation via alteration of the cytoplasmic Ca^{2+} concentration. Another objective was to determine whether VSCCs were involved in this process. Prior to the last decade, VSCCs were believed to be limited to excitable cells. With advancements in technology and pharmacology, evidence is emerging that refutes this idea.

Saunders *et al*. (1990) published one of the earliest reports of possible VSCC regulation of epithelial function. They investigated intracellular Ca^{2+} modulation of prairie dog gallbladder ion transport. Verapamil disrupted epithelial ion transport through reversible inhibition of plasma membrane Ca^{2+} channels. Two studies using different eye tissues found cellular function could be influenced by VSCC-antagonist alteration of intracellular Ca^{2+}. The first report suggested verapamil and nifedipine modulate cytoplasmic Ca^{2+} in rabbit cornea via changes in apical junction permeability (Green *et al*., 1994). A separate study identified verapamil and nifedipine sensitive Ca^{2+} membrane currents in cultured pigment epithelial cells from rats (Strauss and Weinrich, 1994). Kidney cortical epithelial uptake of Ca^{2+} at the apical membrane is enhanced by parathyroid hormone (PTH). Results from Matsunaga *et al*. (1994) indicate this effect is insensitive to verapamil or omega-conotoxin. However, the DHP agonist BAY K8644 and antagonist nifedipine effectively regulated this process. This suggests PTH induced Ca^{2+} uptake in renal epithelium is through an L-type VSCC. Boitano *et al*., (1995) exposed cultured airway epithelial cells to nifedipine and BAY K8644,

which altered the intracellular Ca^{2+} concentration. They concluded VSCCs were present in airway epithelia and these channels influenced cytoplasmic Ca^{2+} levels in a manner that is dependent upon extracellular Ca^{2+} influx. In 1996, Zhang and O'Neil performed patch-clamp measurements in cultured rabbit kidney epithelium. They were able to definitively identify a nifedipine sensitive, L-type VSCC in the apical membrane of proximal tubule cells (Zhang and O'Neil, 1996 a&b).

Finally, in 1998 it was reported that voltage-gated Ca^{2+} channels (L-type) were detected in Atlantic cod intestinal cells (Larsson *et al*., 1998; Lomax *et al*, 1999). Fluorescence imaging of cultured enterocytes in a Ca^{2+}-free buffer with fura-2 and a DHP probe for L-type Ca^{2+} channels found the greatest intensity along the apical (brushborder) membrane. The Ca^{2+} probe Indo-1 used in this experiment also indicated concentrated fluorescence along the apical membrane of the embryonic duodenal epithelium. It should be noted that functional VSCCs have previously been discovered in cultured embryonic chick cardiomyocytes and retinal cells (Tohse and Sperelakis, 1990; Uchida and Iuvone, 1999). In the current experiment verapamil, an earlier generation, non-specific Ca^{2+}-channel blocker reduced the intracellular Ca^{2+} concentration of epithelial cells in cultured duodenum. Furthermore, nifedipine, a DHP that is selective for L-type VSCCs was equally effective in this model. The results of this study infer that voltage-gated ion channels are present and physiologically functional in embryonic duodenum.

Earlier work by Black and Smith (1989) demonstrated changes in cytoplasmic Ca^{2+} levels are correlated with increased epithelial differentiation (goblet cell expression) in embryonic duodenum. A later study demonstrated [Ca^{2+}] and ALP activity are directly related. Black and Rogers (1992b) measured cytoplasmic Ca^{2+} in isolated epithelial cells from 14D duodena cultured in varying extracellular Ca^{2+} concentrations (Black and Rogers, 1992b). As Ca^{2+} levels rose from 0.7 mM to 1.3 mM, the intracellular

[Ca^{2+}] rose from 42 nM to 60 nM. ALP activity increased by 91 % as extracellular Ca^{2+} increased from 0.7 mM to 2.8 mM. A comparison of ALP activity and cytoplasmic Ca^{2+} were highly correlated (0.95, r=0.91) over embryonic days14 through 21 (Black and Rogers, 1992b). The results of the current study further support the hypothesis that functional differentiation is influenced by extracellular Ca^{2+} modulation of the cytoplasmic Ca^{2+} concentration.

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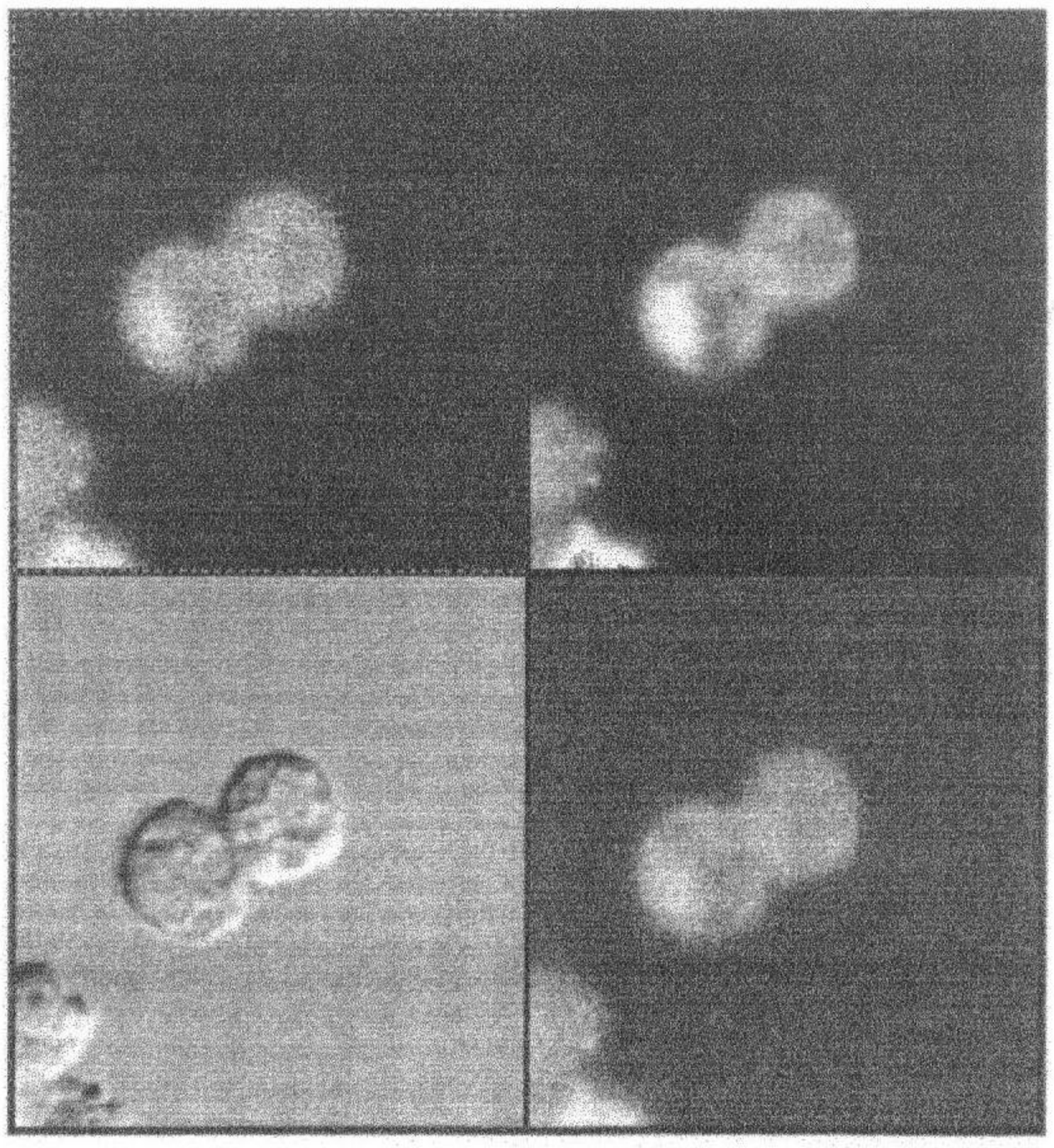
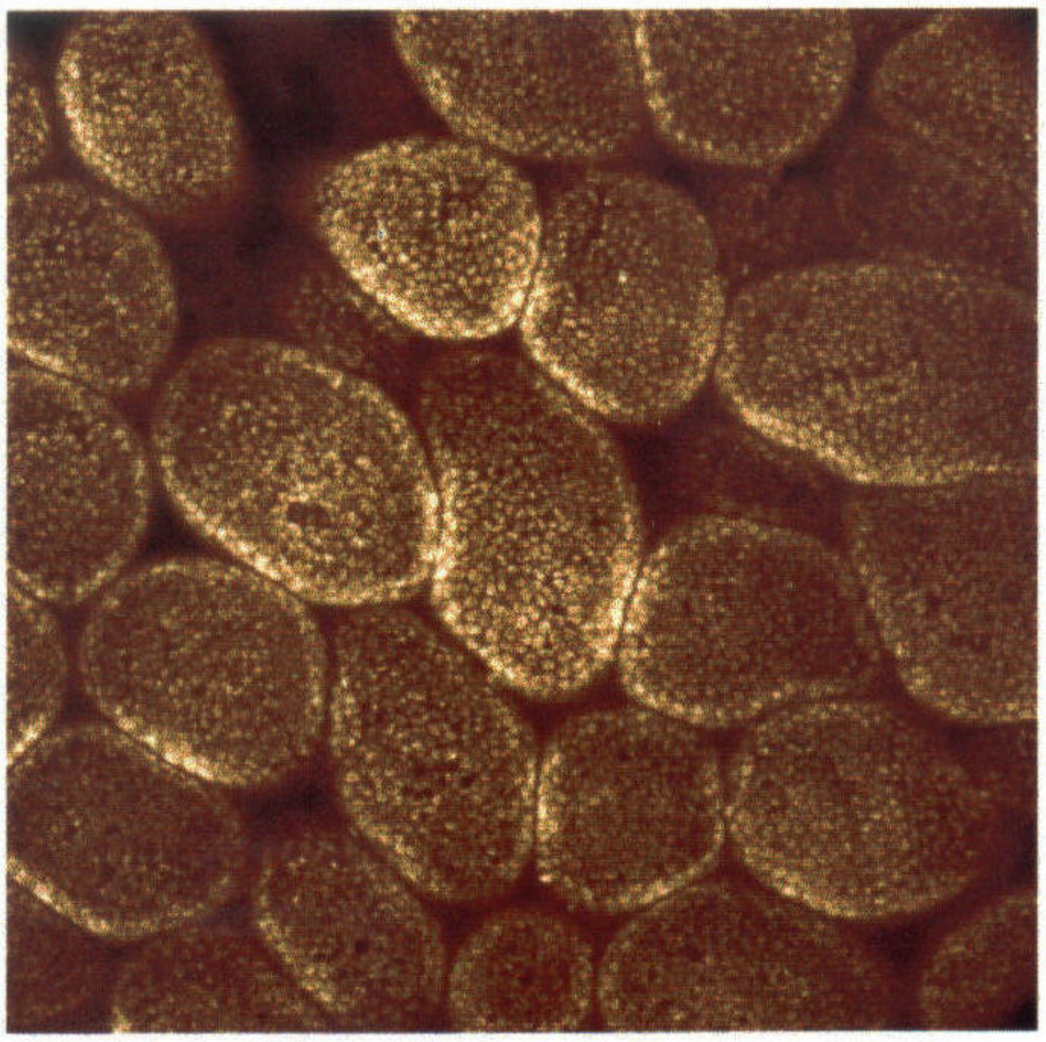


FIGURE 4.1. Leica confocal scanning laser microscope (CSLM) image of epithelial cell suspension from 18 day old duodenum. Top left panel is image of indo-1 unbound probe (channel1). Top right panel is image from probe bound to calcium (channel2). The bottom left panel is the DIC (bright field) image. The bottom right corner is an overlay of channel 1 and channel 2, the ratio (R) measurement.

19 day old uncultured duodenum



18 day old cultured duodenum

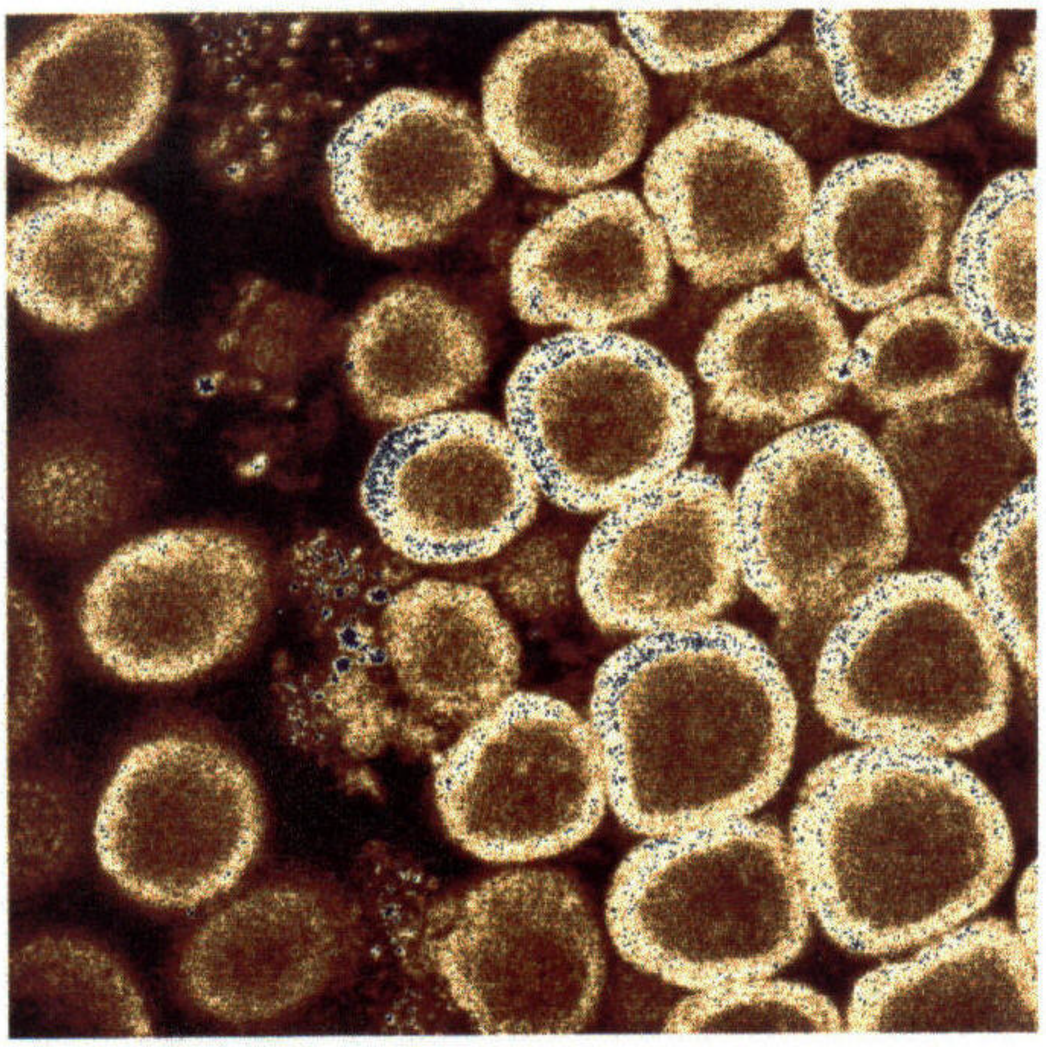


FIGURE 4.2. 19 day old uncultured and 18 day old cultured duodenum. Cultured tissue appears healthy after 24 hours of incubation. (area=500umx500um)

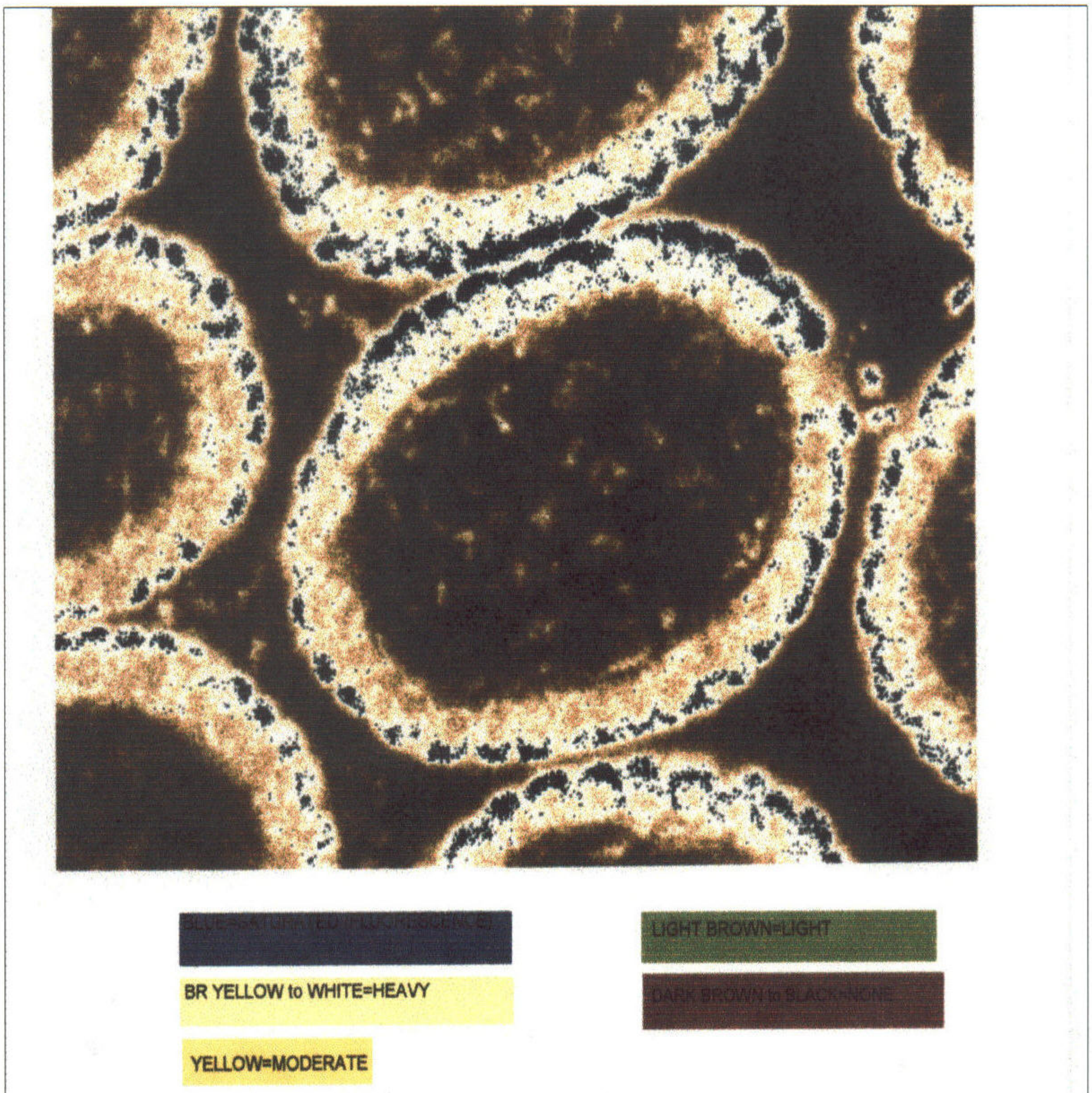


FIGURE 4.3. Calcium indo-1 fluorescence color scale. Measurements range from no fluorescence (dark brown) to complete fluorescent saturation (blue). Image from 18 D uncultured duodenum. (area=250umX250um)

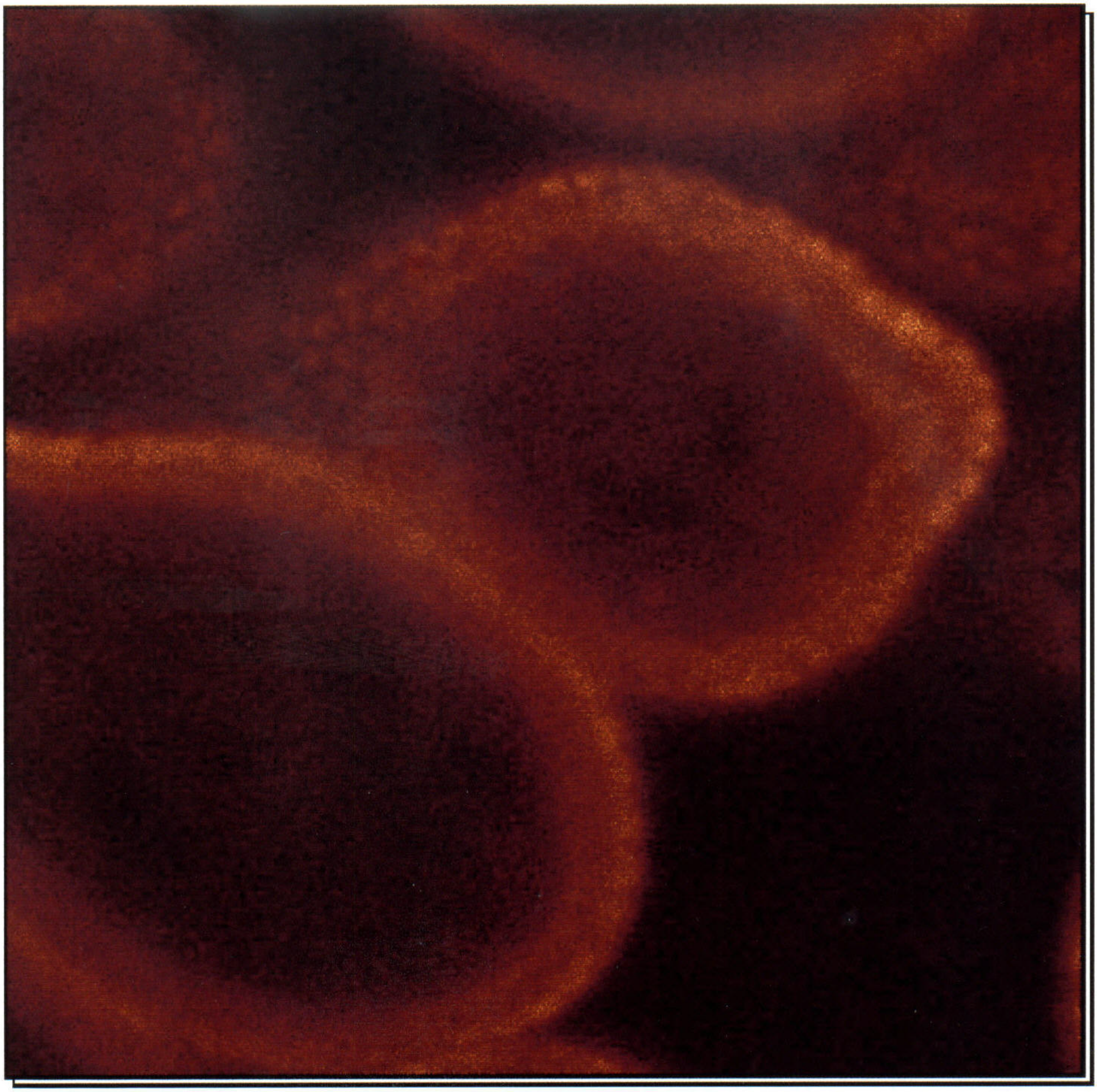


FIGURE 4.4. Villus cores do not fluoresce. Side view of 18D uncultured villi. The calcium probe indo-1 effectively penetrates villus epithelial cells. Core regions are without measurable fluorescence. (area=175umX175um)

FIGURE 4.5. 18 D CONTROL culture. Villi from explants cultured in control medium display a uniform pattern of fluorescence and epithelial cells are clearly differentiated. (area= 250*u*mX250*u*m)

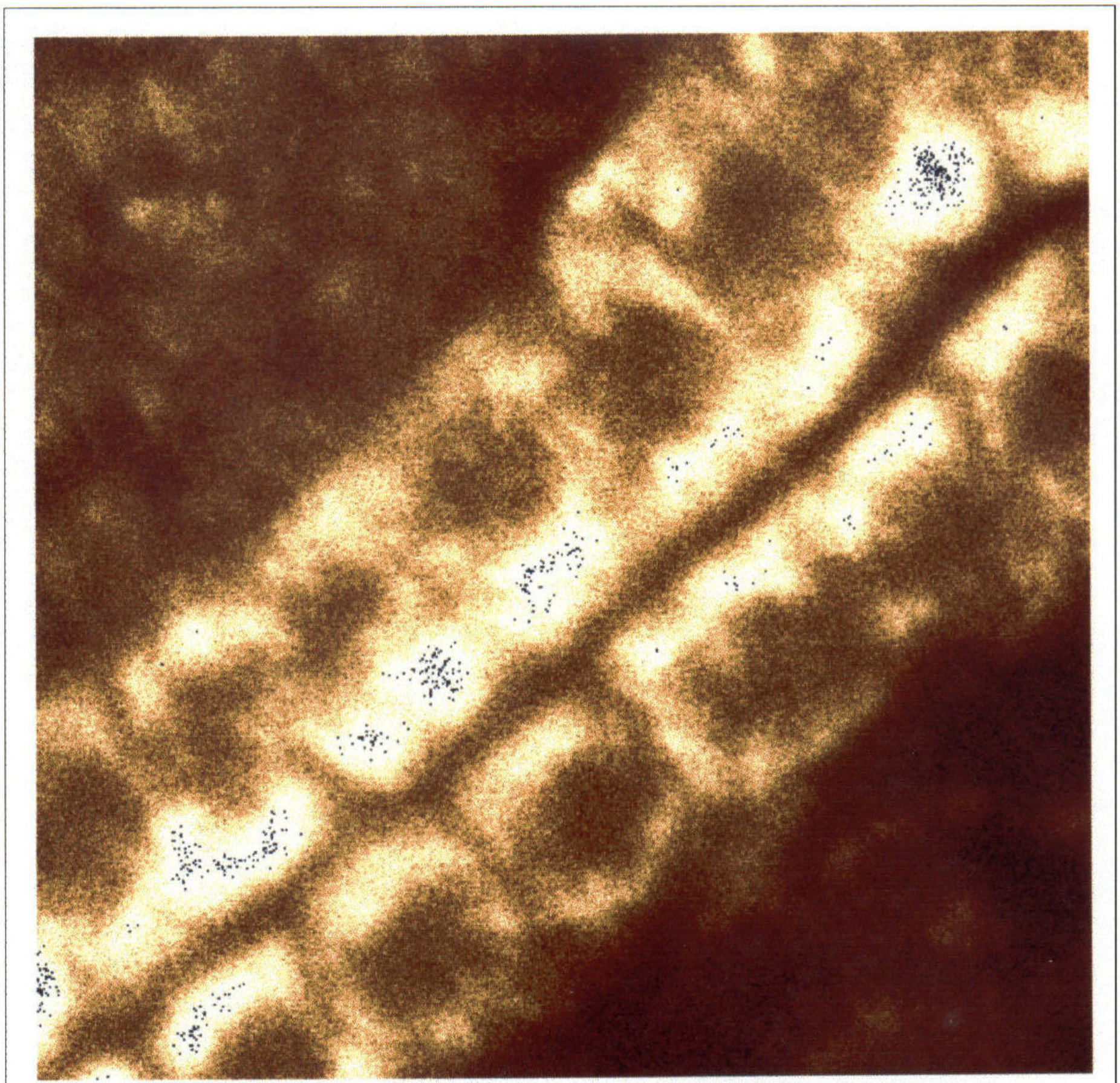


FIGURE 4.6. Calcium localization in 18 D CONTROL cultured duodenum. Center of image is of adjacent epithelial layers from 2 separate villi. Indo-1 fluorescence is most intense along the apical (brushborder) membrane region (where blue areas of saturation appear) of epithelial cells. Note: basolateral membrane adjacent to dark villus core area also has strong fluorescence. (area= 39*u*mX39*u*m)

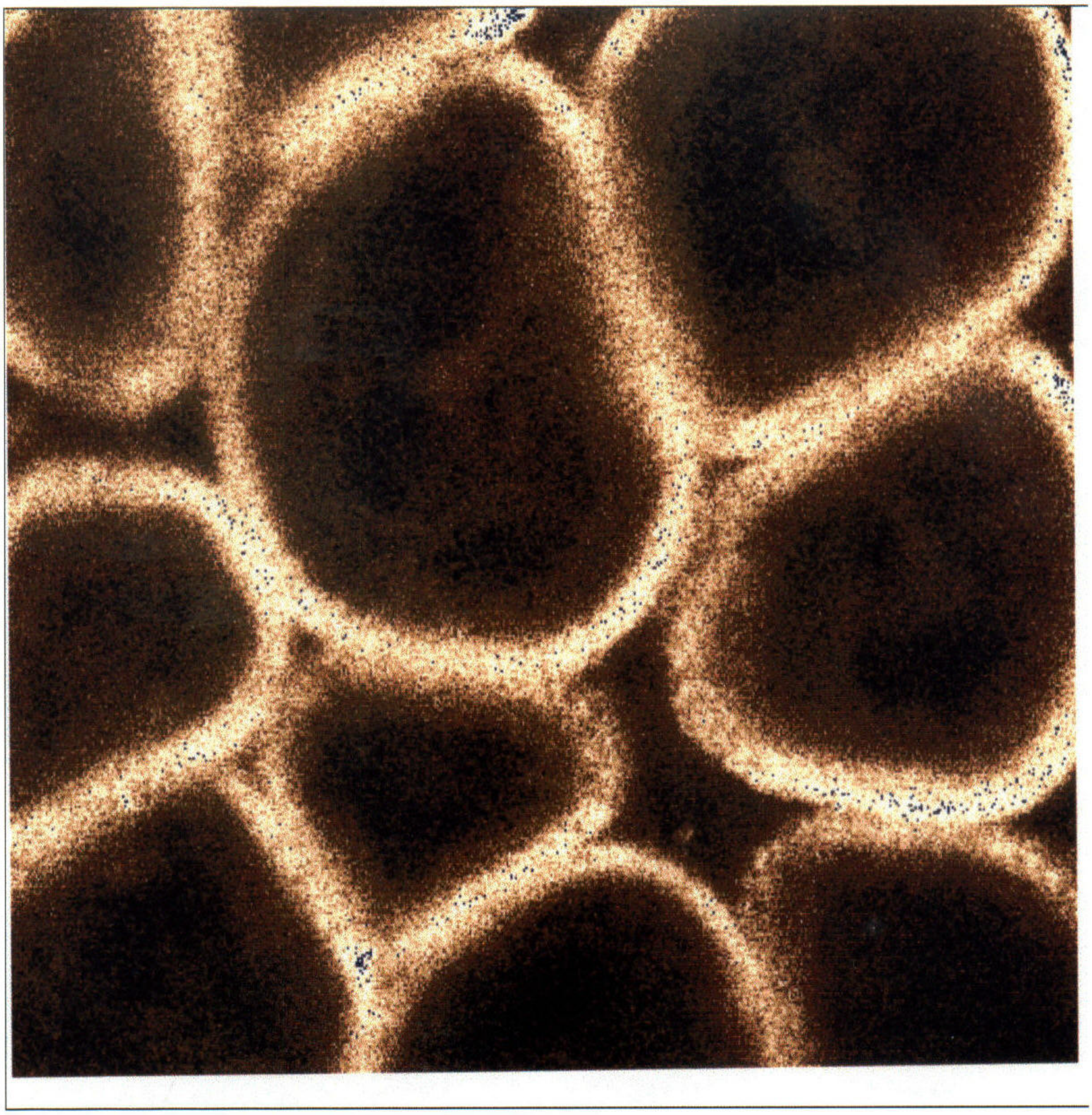


FIGURE 4.7.a. VERAPAMIL treated 18 D cultured duodenum. (area= 250*u*mX250*u*m)

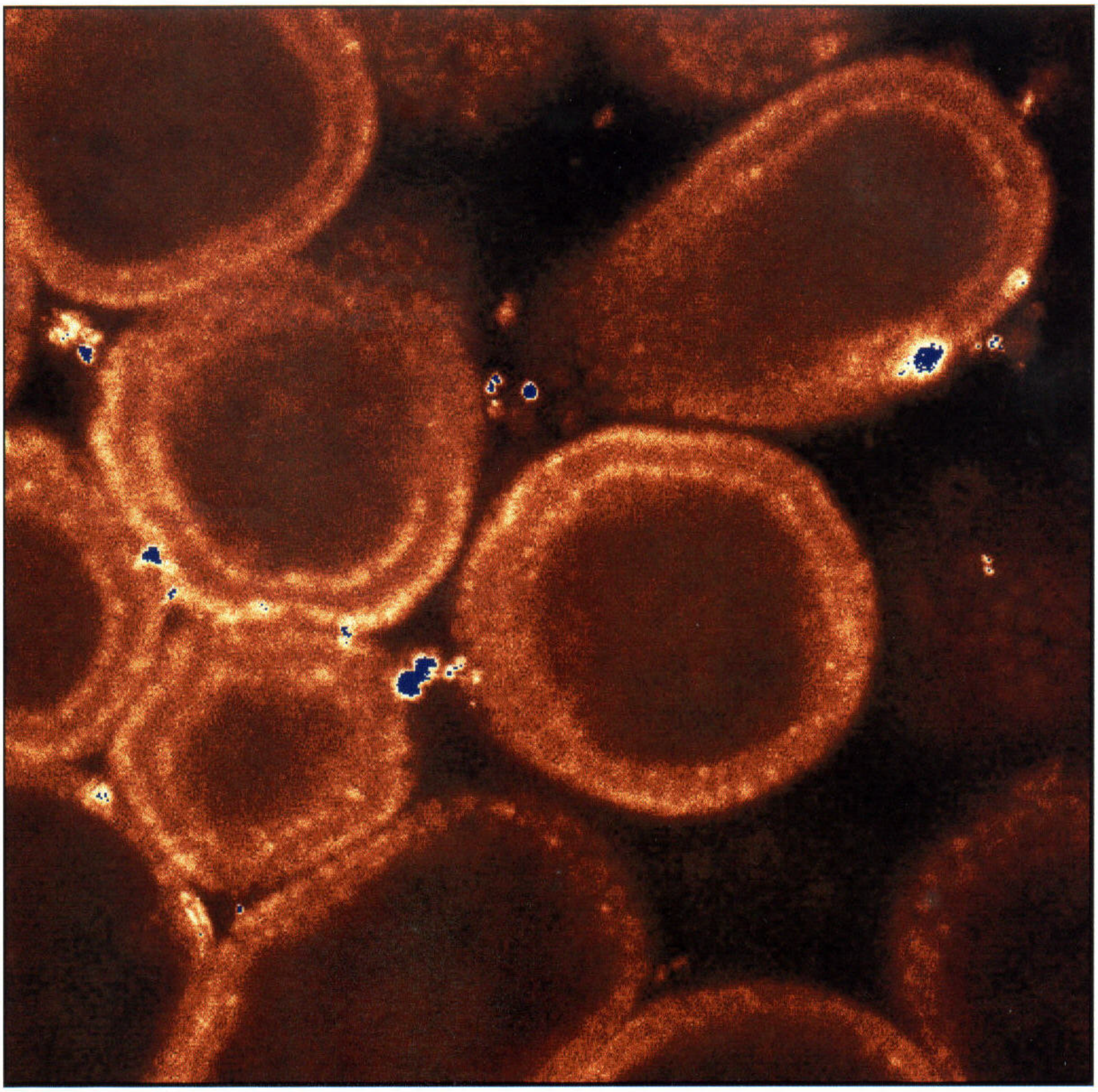


FIGURE 4.7.b. Paired Control for verapamil treated 18 D cultured duodenum. (area= 250*u*mX250*u*m)

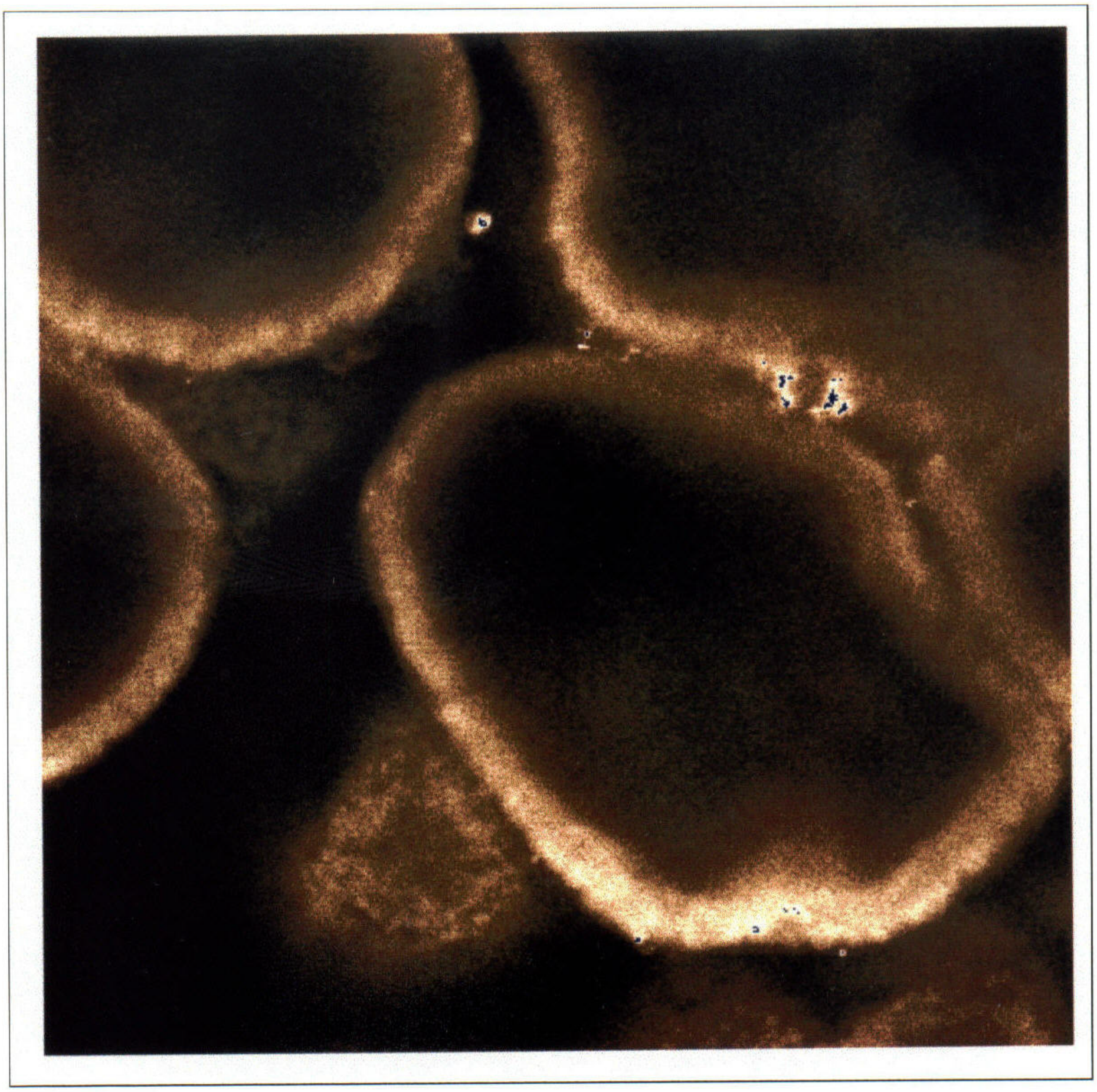


FIGURE 4.8.a. NIFEDIPINE treated 18 D cultured duodenum. (area= 200*u*mX200*u*m)

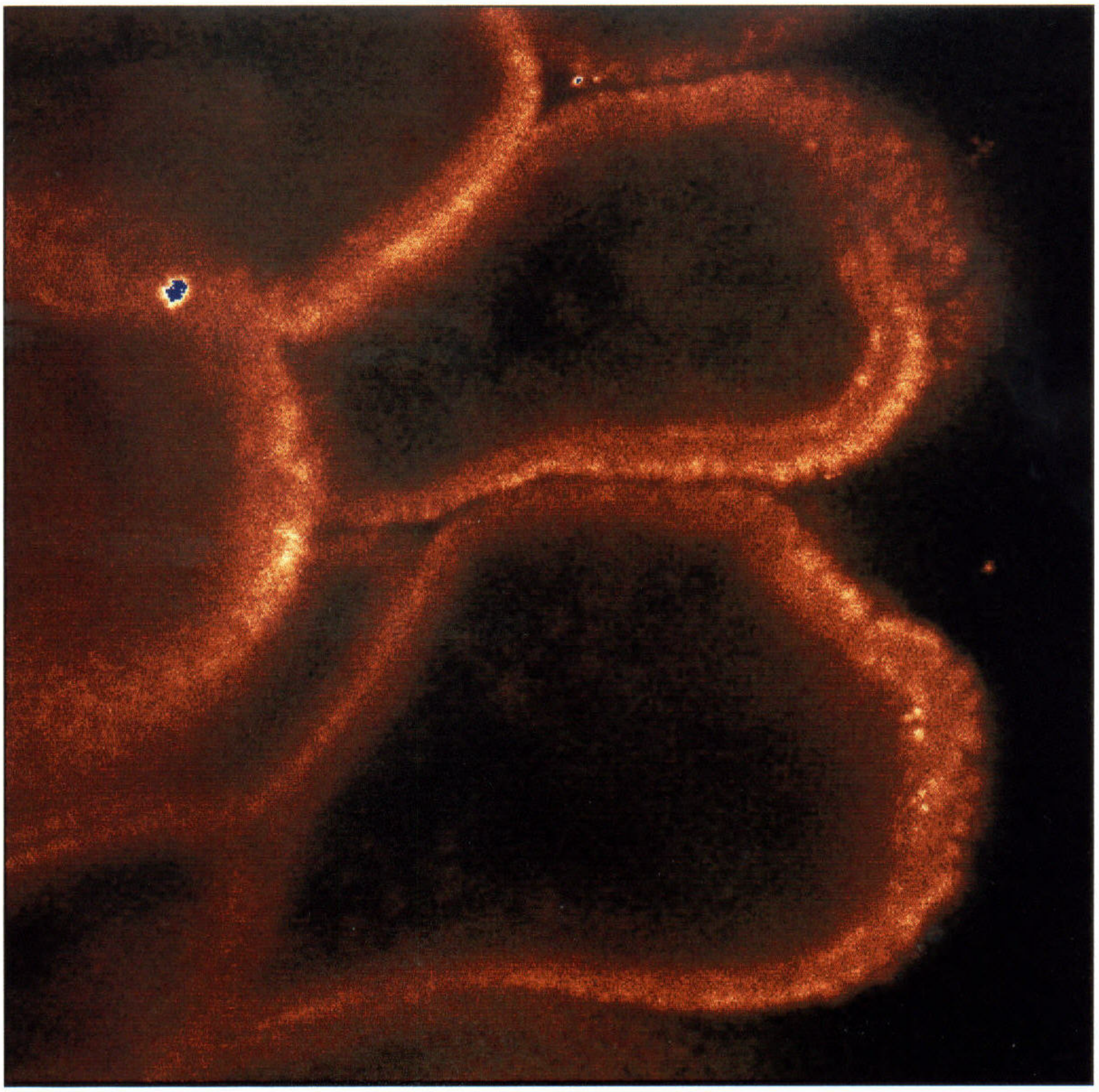


FIGURE 4.8.b. Paired Control for nifedipine treated 18 D cultured duodenum. (area= 200*u*mX200*u*m)

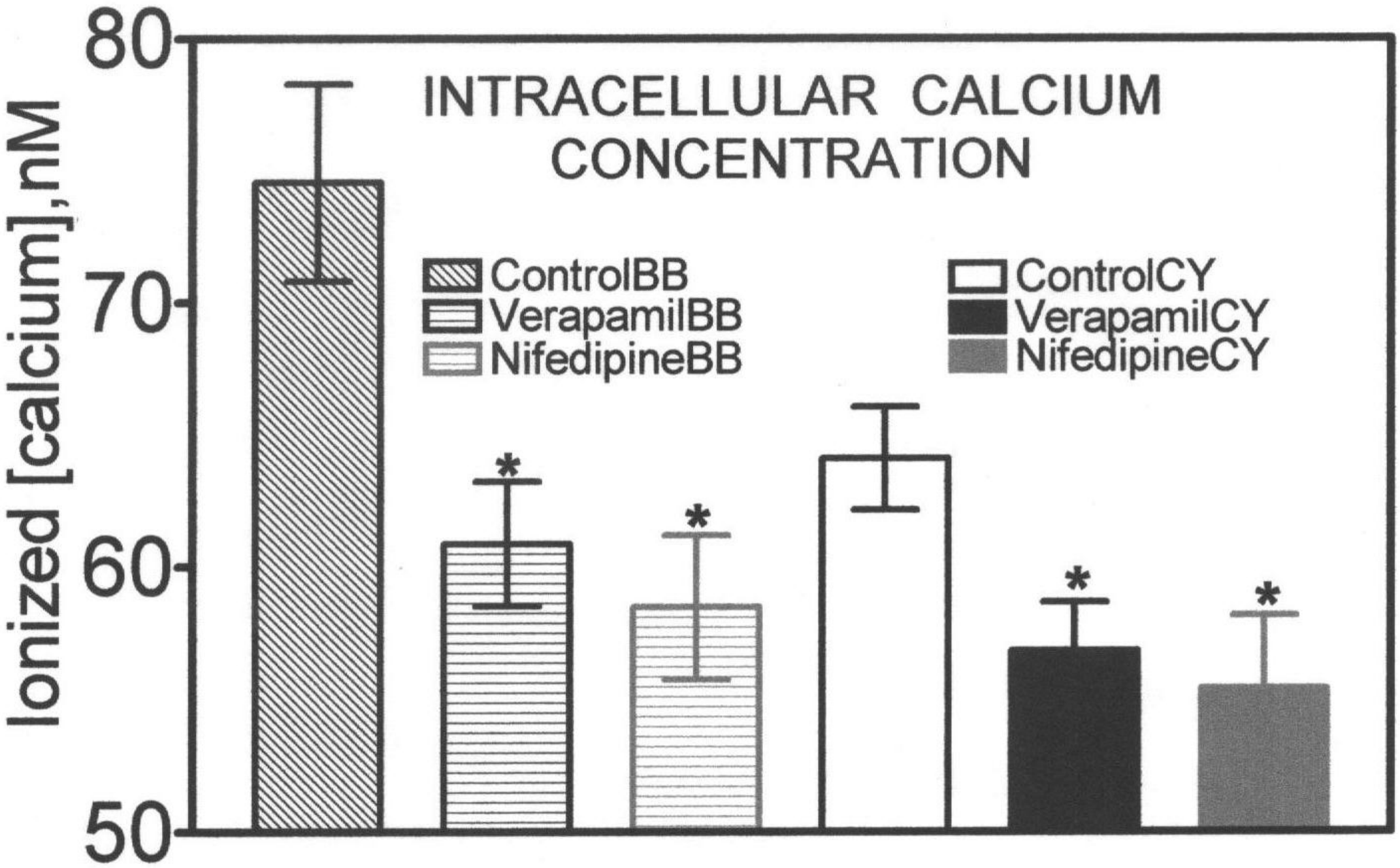


FIGURE 4.9. Intracellular calcium concentration measured in 18 D cultured duodenal epithelial cells from 7 embryos.

Ca^{2+} in all treatment groups was higher along the apical membrane (BB) than the internal cytoplasm (CY). Verapamil and nifedipine significantly reduced Ca^{2+} along the membrane ($p<.02$) and the inner CY region ($p<.05$). (*indicates significant difference from control value.)

GENERAL SUMMARY

In this study, embryonic duodenal ALP activity and GC differentiation were increased by culture in physiologically high extracellular Ca^{2+}. The serum Ca^{2+} concentration of 16D-18D chick embryos starts at 1.3 mM and rises to 1.5 mM. As the Ca^{2+} concentration of the culture medium rose from 0.7-1.3-2.8 mM, GC number and ALP activity progressively increased. Verapamil, nifedipine and Pb^{2+} interfere with normal Ca^{2+} function. In this study, each agent significantly inhibited ALP and GC expression, suggesting the direct involvement of extracellular influx through Ca^{2+} ion channels. Since we suspected decreased functional differentiation in the treated cultured tissue was the result of modulation of the cytoplasmic Ca^{2+} concentration, the fluorescent, ratiometric Ca^{2+} probe indo-1 was used to quantify intracellular ionized Ca^{2+} concentrations. Verapamil, a non-specific VSCC blocker, and nifedipine, an L-type selective VSCC blocker, significantly reduced cytoplasmic and apical membrane Ca^{2+} of 18D enterocytes. The presence of functional, VSCCs in cultured embryonic chick epithelium is a novel finding.

In addition, the Ca^{2+} concentration of the apical region was consistently higher than that of the interior cytoplasmic region of the epithelium in all imaged cells (control and treated). This is significant, as digestive enzymes, nutrient transporters, ATPase pumps, and ion-selective channels are located in the brushborder/apical membrane region. Thus, a relative concentration of Ca^{2+} in this area to support these systems is logical. This is the first report of subcellular localization of Ca^{2+} in embryonic intestinal epithelium.

Previous work in this lab investigated the influence of the extracellular Ca^{2+} concentration on cultured epithelium in younger tissue. Goblet cell differentiation was inhibited in 14 day-old embryonic duodena cultured in hormone free low, 0.7 mM Ca^{2+} (Rogers *et al.*, 1995). Increasing the calcium

concentration to 2.8 mM induced significant GC differentiation. In the current study, GC counts of older 16D and 18D duodena cultured in hormone free low Ca^{2+} increased significantly relative to paired baseline values. This would suggest a changing sensitivity to Ca^{2+} as the tissue matures.

In the Rogers study, addition of thyroxine, but not hydrocortisone, to low Ca^{2+} cultures of 14D duodena stimulated GC differentiation and shifted GC distribution out to the distal halves of previllous ridges. Conversely, an earlier study found opposite effects on ALP and maltase activity in cultured 14D embryonic duodenum. Addition of hydrocortisone to cultures with low (0.88 mM) extracellular Ca^{2+} enhanced maltase and ALP activity by 20-30 % above control values (Rogers and Black, 1986). Whereas, thyroxine stimulated enzyme activity decreased by 35 % compared to controls after culture in low Ca^{2+}. In a different study, ALP activity in cultured 14D old embryonic duodena increased significantly as the extracellular Ca^{2+} concentration increased (Black and Rogers, 1992). The cytoplasmic Ca^{2+} concentration was measured (using Fura-2) in epithelial cell suspensions from cultured 14D tissue and found to increase (42 nM to 60 nM) as extracellular Ca^{2+} increased from 0.7 mM to 2.8 mM. In addition, increase in cytoplasmic Ca^{2+} from uncultured cells of embryonic duodenum 14-21 days old was significantly correlated with the rise in ALP activity. Therefore, Ca^{2+} concentration clearly influenced in vitro epithelial development and the data implied a similar function in vivo.

In these earlier studies, goblet cell differentiation was stimulated by high levels of extracellular Ca^{2+}. Work in 1994 by Black *et al.* suggests a mechanism for Ca^{2+}-induced GC differentiation. In cultured 14D embryonic epithelium GC differentiation was stimulated by prostaglandin E2 (PGE2) and inhibited by two inhibitors of PGE2 synthesis. The effects of PGE2 were sensitive to the Ca^{2+} concentration of the culture medium, and increasing the Ca^{2+} from 0.7 mM to 2.8 mM increased PGE2 levels by 45 %. It is important to note that hydrocortisone, which inhibits GC differentiation, also inhibits

PGE2 synthesis (Santini *et al.*, 2001) Thus, it was concluded that extracellular Ca^{2+} modulates PGE2 synthesis and PGE2 induces goblet cell differentiation in embryonic duodenum.

The results of the present study support and advance previous work in this lab. Rogers and Black (1996) established that glucocorticoids, and to a lesser degree thyroid hormones, modulate the development of Ca^{2+} homeostasis in isolated epithelial cells from cultured embryonic duodenum. Addition of hydrocortisone or thyroxine to culture medium significantly increased the cytoplasmic Ca^{2+} concentration. This suggests that hormonal influence on late stage embryonic epithelial enzyme activity and cellular differentiation is mediated directly or indirectly via Ca^{2+} signaling processes.

Hormonal stimulation of epithelial cells induces a series of actions, which may involve cytoplasmic Ca^{2+} store release and extracellular Ca^{2+} influx. The resulting increase in intracellular Ca^{2+} concentration stimulates a cascade that activates signaling pathways leading to a particular physiological effect. G-protein or tyrosine kinase receptor activation lead to diacylglycerol stimulation of protein kinase C (PKC). Therefore, PKC is a Ca^{2+}-dependent second messenger capable of transducing signals for cellular proliferation, differentiation, function and apoptosis (Musashi *et al.*, 2000). Recent data (Frey *et al.*, 1997) implicates PKC mediated signal transduction in the regulation of cell cycle withdrawal and terminal differentiation in intestinal epithelium. Immature rat In situ IEC-18 cells revealed changes in PKC isoform expression and subcellular distribution during cessation of proliferation. Frey and associates (2000) postulate a complex model of PKC regulation of initiation of cell cycle exit in intestinal epithelial cells. They suggest PKC inhibits the cyclin-cdk (cyclin-cyclin-dependent kinase) complexes necessary for cell cycle progression through changes in cyclin (cell cycle regulatory protein) expression and accumulation of CKIs (cdk inhibitory proteins). The result is hypophosphorylation of pocket protein growth suppressors, which inhibit transcription factors (E2F) necessary for

genes involved in DNA synthesis. Further supporting their theory, results in culture paralleled developmental PKC activity. Lastly, a procedure was used that selectively produced the PKC-alpha isoform in the immature IEC-18. With this model, they demonstrated that over-expression of PKC-alpha in intestinal epithelial cells enhances phorbol ester-mediated growth arrest. Therefore, cytoplasmic Ca^{2+} activates PKC, which induces cell cycle withdrawal and terminal differentiation in intestinal epithelium.

The findings of other studies seem to support this theory of epithelial differentiation. In a human mammary epithelial cell culture line, epidermal growth factor (EGF) signal transduction is required for normal growth (Stampfer and Yaswen, 1993). Transforming growth factor-beta (TGF-beta) inhibits EGF dependent growth in mammary epithelium. TGF-beta inhibits epithelial growth through effects on cell cycle progression, and modulation of cyclin and cdk expression that activate terminal differentiation (Ravitz and Wenner, 1997). As was stated in an earlier chapter, keratinocyte differentiation is influenced by extracellular Ca^{2+} concentration (Hennings *et al*., 1983). Alani *et al*. (1998) report cultured human keratinocyte differentiation is a function of cdk and CKI function. Finally, a connection between cyclin regulated cellular differentiation and hormones has been reported. Work by Perez-Juste and Arnada (1999) demonstrated thyroid hormone controls neuroblastoma differentiation through increased levels of cyclin kinase inhibitor.

The results the current study, previous work in this lab and recent reports suggest a possible mechanism for the effects of extracellular Ca^{2+} on embryonic epithelial development. Hormones or growth factors stimulate epithelial differentiation via effects on cell cycle progression mediated by a Ca^{2+} transduced signal in the following manner. Activated G-proteins or tyrosine kinase receptors stimulate cytoplasmic Ca^{2+} store release and extracellular Ca^{2+} influx. The extracellular Ca^{2+} wave creates local changes in the intracellular Ca^{2+} concentration which activate PKC. The PKC cascade

induces withdrawal from the cell cycle by inhibiting DNA synthesis transcription factors. The result is Ca^{2+} stimulated terminal differentiation. Investigation of this theory may lead to better understanding of the underlying mechanisms involved in Ca^{2+}-dependent embryonic epithelial differentiation.

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