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The effect of hydrocortisone and thyroxine on development of calcium homeostasis in embryonic intestinal epithelium

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Abstract. Cytoplasmic Ca^{2+} concentration of epithelial cells from 14-day embryonic chick duodena decreased during 72 h of organ culture to a value 54% of that found at 17 days in vivo. The ability of cells to maintain a constant Ca^{2+} concentration when challenged with high extracellular calcium was also significantly reduced. Addition of 1 μM hydrocortisone during culture restored both parameters of Ca^{2+} homeostasis to that of 16-day uncultured duodena, and rise in cytoplasmic Ca^{2+} was significant within 4 h of hormone treatment. Thyroxine influenced epithelial Ca^{2+} similarly, but to a lesser degree and only after 48–72 h of culture. These data indicate that glucocorticoids, and possibly thyroid hormones, influence the development of calcium homeostasis in intestinal epithelium.
Key words. Duodenum; cytoplasmic calcium; glucocorticoid; thyroxine; epithelial differentiation.

The growth and differentiation of epithelial tissues is strongly influenced by calcium. In epithelial tissue cultures, low extracellular calcium typically promotes cell proliferation whereas higher calcium elicits terminal differentiation[1]. This phenomenon has been most extensively studied in mammalian skin epithelium; elevation of extracellular calcium in epidermal cultures results in stratification and cornification[2] with a narrow range of calcium concentration required to elicit optimal expression of differentiation-dependent genes[3]. High extracellular calcium is required for epithelial redifferentiation of organ-cultured buccal mucosa, oral mucosa and foreskin[4] and is essential for prolactin-induced differentiation of mammary gland epithelium[5,6]. In both rat intestinal and chick kidney epithelium, expression of the vitamin D-dependent calcium binding protein is modulated by extracellular calcium concentration[7,8]. Moreover, elevation of cytoplasmic calcium by artificial means can induce cell differentiation in a variety of cell types[9,10], and a rise in cytoplasmic calcium may control rate of differentiation during normal development of skin epithelium[11]. Although a growing body of evidence suggests that changes in epithelial calcium homeostasis play a crucial role in cell differentiation, the factors which regulate calcium homeostasis during development are obscure.

The epithelium of embryonic chick intestine provides a good model for examining developmental changes in calcium homeostasis. During the third week of development, the intestinal epithelium undergoes rapid differentiation in preparation for the digestive and absorptive functions that will begin soon after hatching[12–15], and distinct changes in epithelial calcium homeostasis also occur during this period[16]. Intracellular Ca^{2+} rises significantly during the third week of development, increasing from 78 nM at 14 days to a maximum of 209 nM by one day posthatch. Concurrently, the epithelial cells acquire an enhanced ability to maintain constant cytoplasmic Ca^{2+} levels when challenged with high extracellular calcium in an in vitro assay system. It is well established that glucocorticoid and thyroid hormones regulate development of embryonic chick intestine; effects of hydrocortisone (HC) and thyroxine (T4) on epithelial differentiation during organ culture include increase in glucose active transport rate, modulation of glycogen stores, acceleration in development of brush border enzyme activities, and stimulation of microvillus growth[13–15,17]. The present study was designed to evaluate epithelial Ca^{2+} homeostasis in organ-cultured embryonic intestine and to determine whether HC and T4 also influence this parameter of epithelial differentiation.

Materials and methods

Chemicals. T4 (3,3′,5,5′-tetraiodothyronine) and HC (hydrocortisone 21-phosphate) were obtained from

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Sigma Chemical Co. (St. Louis, USA); fura-2 AM and tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) from Molecular Probes (Eugene, USA); and Medium 199 and penicillin/streptomycin from Gibco (New York, USA).

Organ culture. Fertile chicken eggs of a broiler strain (Arbor Acres, Gastonbury, USA) were obtained from the Poultry Science Department at North Carolina State University and incubated for 14–17 days at 38–39 °C. Duodenal loops were removed from 14-day embryos, cut into segments, and incubated in defined medium at 38 °C as previously described[14]. Duodena from 9–12 embryos were cut into 9–12 segments and distributed among 9–12 culture flasks with control and hormone-treated cultures always receiving one segment from each duodenum. The media of hormone-treated cultures contained a final concentration of 1 nM T4 or 1 μM HC, and paired control cultures contained an equivalent volume of hormone solvent (100 μl of 0.01 N NaOH or 0.9% NaCl, respectively). In some experiments the calcium concentration of culture medium was lowered from the standard 1.3 mM to 0.7 mM by addition of 0.75 mM EGTA. Ca^{2+} concentrations of all culture media were determined by use of an ICA1 ionized calcium analyzer (Radiometer).

Assay of cytoplasmic Ca^{2+}. Epithelial cells were isolated from the submucosa of cultured or uncultured duodena by shaking the tissue in EDTA-containing buffer[17]. Epithelial cell suspensions were loaded with fura-2 and assayed for cytoplasmic Ca^{2+} as previously described[16]. Briefly, cells were incubated with 5 μM fura-2 AM for 15 min, rinsed twice, and suspended in assay buffer containing 1.3 or 0.7 mM Ca^{2+}. Viability of the loaded cells was determined in aliquots of each cell suspension by ability to exclude 0.2% trypan blue. Assays of 1–20 min duration were performed in the 37 °C chamber of a computer-controlled spectrofluorometer with Ca^{2+} concentration calculated every 12 seconds from fluorescence ratio at alternating excitation wavelendths of 350 and 385 nm[16]. Unless otherwise indicated, results are expressed as an average of values obtained during the first minute of assay. Leakage of fura-2 from loaded cells was routinely monitored by filtering cell suspensions after 5 min of assay and expressing filtrate fluorescence as a percentage of total fluorescence at excitation wavelengths of 350 nm. In the calcium challenge protocols, fluorescence from extracellular fura-2 was quenched by adding 10 μl of 40 mM $MnCl_2$ to 2 ml cell suspension, and fluorescence ratios recorded 1–2 min later were used to calculate cytoplasmic Ca^{2+} concentration.

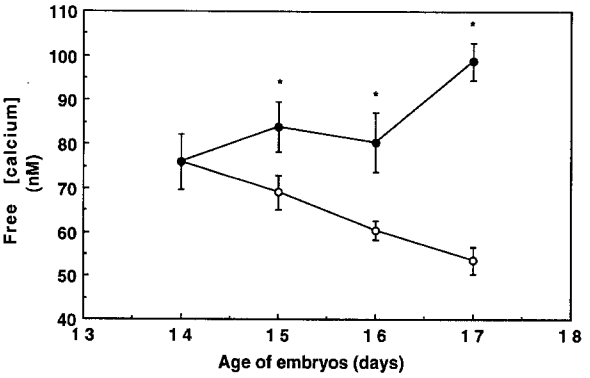

Figure 1. Development changes in cytoplasmic Ca^{2+} concentration of duodenal epithelium. Cells were isolated from uncultured duodena of embryos aged 14 through 17 days (●) or from 14-day duodena cultured in hormone-free media with 1.3 mM Ca^{2+} for 24, 48, and 72 h (○). Cell suspensions were assayed for cytoplasmic Ca^{2+} concentration in assay buffers containing 1.3 mM Ca^{2+}. * Value is significantly different from cultured tissue.

Statistical analysis. Results are expressed as the mean ± SE of values from 4–6 cell preparations. Comparisons between uncultured and cultured tissue at a given embryonic age (fig. 1) were evaluated for significance by using the unpaired t-test. Comparisons between homone-treated and paired control cultures utilized the paired t-test. Comparisons involving more than two groups (table 1) were made by one-way analysis of variance followed by the protected least-significant difference procedure. In all cases, $p > 0.05$ was considered not significant.

Table 1. Effect of calcium challenge on epithelial cells from 16-day uncultured and 14-day cultured duodena.

Culture treatment[a]	Initial cytoplasmic [Ca^{2+}] (nM)	Increase in cytoplasmic [Ca^{2+}] during challenge	
		(nM)	(% of initial value)
Uncultured	86.8 ± 8.9	71.6 ± 7.6	82.8 ± 3.2
HC solvent	48.5 ± 6.8[b]	128 ± 8.0[b]	278 ± 35.2[b]
1 μM HC	67.0 ± 5.4[c]	62.1 ± 7.4[c]	96.5 ± 17.4[c]
T4 solvent	57.9 ± 10.2[b]	100 ± 15.0	181 ± 22.5[b]
1 nM T4	55.9 ± 7.9[b]	77.1 ± 14.1[c]	140 ± 23.8[c]

[a] All cultured duodena were incubated for 48 h in media containing 0.7 mM Ca^{2+} plus the indicated hormone or hormone solvent.
[b] Significantly different from uncultured duodena.
[c] Significantly different from paired solvent controls.

Results

Effect of culture on development of Ca^{2+} homeostasis. Cytoplasmic Ca^{2+} concentration of epithelial cells was measured in cell suspensions from uncultured and cultured duodena using assay buffers containing 1.3 mM Ca^{2+}, the concentration of free Ca^{2+} present in sera of 14- through 16-day chick embryos[18]. Cytoplasmic Ca^{2+} concentration of duodenal epithelium during the 14–17 day period in vivo was approximately 80 nM between days 14 and 16, then rose significantly to 99 nM to day 17 ($p < 0.025$). When 14-day duodenal tissue was cultured in the absence of exogenous hormones for 24, 48, and 72 h, the Ca^{2+} concentration of the subsequently isolated epithelial cells decreased during the culture period (fig. 1). By 24 h of culture, cytoplasmic Ca^{2+} was 69.1 nM $\pm$ 3.9, significantly lower than the 15-day in vivo value of 83.9 nM $\pm$ 5.8 ($p < 0.05$). By 72 h of culture, cytoplasmic Ca^{2+} concentration was 53.6 nM $\pm$ 3.1, a decrease of 29.4% from the 14-day value of 75.9 nM $\pm$ 6.2 and only 54.1% of the 17-day value in vivo ($p < 0.0005$). When 14-day duodena were cultured and assayed in media containing 0.7 mM calcium, results were similar; cytoplasmic Ca^{2+} was 56–67% of in vivo values after 48 h of culture (table 1).

The reduced Ca^{2+} level in cells from cultured tissue was not due to changes in cell viability, altered handling of fura-2, changes in heavy metal concentration within the cells, or depletion of nutrients during culture. In 24–72 h cultures and in all uncultured tissue, viability of loaded cells was consistently >90%. Cells from cultured and uncultured duodena loaded fura-2 to a similar extent based on autofluorescence as a percentage of total fluorescence of loaded cells which averaged 2.4% for 16-day uncultured cells and 2.9% for cells from 48 h cultures. Leakage of fura-2 from loaded cells occurred in all cell preparations as previously reported[16], but degree of leakage was not altered by culturing. Extracellular ('leaked') fura-2 was found to represent 19.4–20.4% of total fluorescence in cell suspensions from both uncultured and cultured tissue. The extent to which heavy metals might interfere with Ca^{2+} measurements by fura-2 was tested by adding 25 µM TPEN (a membrane-permeant chelator of heavy metals) to cell suspensions during assay of Ca^{2+}. When TPEN was added to epithelial cells prepared from 14-day uncultured duodena and from 14-day duodena cultured for 48 h, the apparent cytoplasmic Ca^{2+} concentration increased by only 3.8% and 8.2%, respectively, after 5 min of treatment. The possibility that reduction of epithelial Ca^{2+} during culture was due to depletion of calcium or nutrients was tested by providing fresh culture medium at 24 h intervals. When epithelial Ca^{2+} was assayed in paired 72 h cultures, there was no significant effect of this procedure; the cytoplasmic Ca^{2+} concentrations were 51.1 nM $\pm$ 6.6 and 52.5 nM $\pm$ 5.3 in cells from cultures with changed versus unchanged media, respectively ($n = 3$).

Cells from cultured 14-day duodena also exhibited a reduced ability to maintain low cytoplasmic Ca^{2+} when subjected to the 'calcium challenge' protocol illustrated in fig. 2A. In this procedure, elevation of extracellular calcium during the assay was used as an alternative to calcium ionophore to test the ability of cells to maintain a constant cytoplasmic Ca^{2+} concentration under conditions of increased calcium influx. When cell suspensions from 48 h hormone-free cultures were exposed to a ten-fold increase in extracellular calcium, cytoplasmic Ca^{2+} increased by an average of 230% as compared to 83% in 16-day uncultured epithelial cells (table 1). Thus the function of calcium homeostatic mechanisms appears to be subnormal in epithelium from cultured duodena.

Effect of hormones on epithelial calcium homeostasis. The effect of HC and T4 on epithelial calcium homeostasis was initially tested by incubating 14-day duodena for 72 h with each hormone or its solvent (table 2). Cells from HC- or T4-treated tissue maintained cytoplasmic Ca^{2+} concentrations 44% and 18% higher, respectively, than cells from paired, hormone-free, control cultures ($p < 0.025$). Hormone-treated cultures showed no significant differences in cell viability and only small differences in fura-2 leakage as compared to controls (table 2).

Time course studies were performed to determine the lag period required for hormone action. HC increased

Table 2. Cytoplasmic Ca^{2+} concentration, cell viability, and fura-2 leakage of duodenal epithelium cultured in the absence or presence of hormones.

Culture treatment	72-hour cultures			48-hour cultures		
	cytoplasmic Ca^{2+} (nM)	cell viability (%)	filtrate fluorescence (% of total)	cytoplasmic Ca^{2+} (nM)	cell viability (%)	filtrate fluorescence (% of total)
HC solvent	47.9 $\pm$ 3.2	94.0 $\pm$ 1.4	23.1 $\pm$ 1.4	57.8 $\pm$ 2.3	95.3 $\pm$ 0.7	21.3 $\pm$ 1.7
1 µM HC	68.8 $\pm$ 2.1[a]	90.5 $\pm$ 2.5	20.8 $\pm$ 1.1[a]	82.3 $\pm$ 4.4[a]	91.5 $\pm$ 1.3	19.5 $\pm$ 1.0
T4 solvent	66.2 $\pm$ 2.8	97.0 $\pm$ 1.7	21.8 $\pm$ 1.0	60.5 $\pm$ 2.2	95.3 $\pm$ 0.7	21.3 $\pm$ 1.7
1 nM T4	78.2 $\pm$ 4.4[a]	95.2 $\pm$ 0.7	27.3 $\pm$ 1.7[a]	65.0 $\pm$ 4.6	91.7 $\pm$ 1.5	19.4 $\pm$ 0.5

[a] Significantly different from paired solvent control.

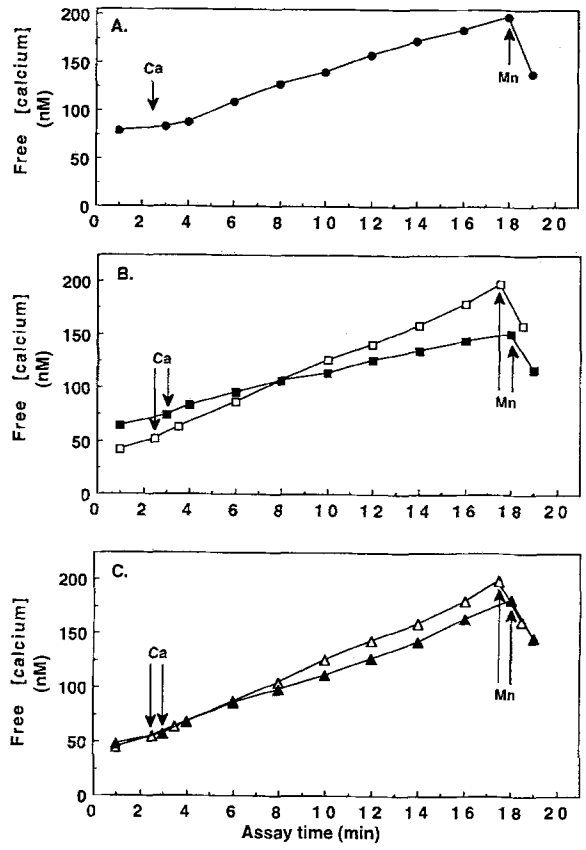

Figure 2. Change in cytoplasmic Ca^{2+} concentration of duodenal epithelial cells during calcium challenge. Cell suspensions were suspended in buffer containing 0.7 mM Ca^{2+} and assayed for 20 min with continuous recording of cytoplasmic Ca^{2+} concentration. $CaCl_2$ and $MnCl_2$ were added to cell suspensions at the indicated times to final concentrations of 7.0 mM and 0.2 mM, respectively. Each point represents an average of Ca^{2+} values obtained during the previous minute of assay. Representative experiments are shown using cells from: *A*) 16-day uncultured duodena, *B*) 14-day duodena cultured for 48 h with 1 μM HC (■) or its solvent (□), *C*) 14-day duodena cultured for 48 h with 1 nM T4 (▲) or its solvent (△).

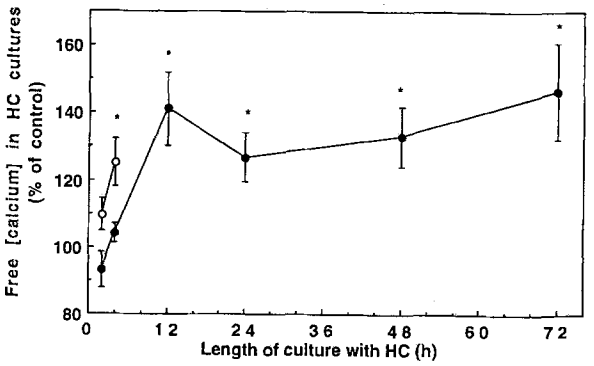

Figure 3. Time course of HC effect on cytoplasmic Ca^{2+} in duodenal epithelial cells. Cells were isolated from 14-day duodena cultured in media containing 1 μM HC or hormone solvent (paired controls) and assayed for cytoplasmic Ca^{2+} concentration in buffer containing 1.3 mM Ca^{2+}. Duodena were incubated for the indicated times following HC addition, with HC present at the beginning of culture (●) or added after 48 h of culture in hormone-free medium (○). HC-induced rise in epithelial Ca^{2+} is expressed as $[Ca^{2+}]$ of HC-treated cells/$[Ca^{2+}]$ of paired control cells × 100. * Values in HC-treated cells are significantly different from paired controls.

cytoplasmic Ca^{2+} levels by 25 nM (42% of control values) after 48 h of culture, whereas T4 did not have a significant effect (table 2). Neither cell viability nor fura-2 leakage were altered by hormone treatment for 48 h. Organ culture for 2–72 h with HC revealed that the HC effect on Ca^{2+} became significant between 4 and 12 h, with maximal response present by 12 h of hormone treatment (fig. 3). Addition of HC versus hormone solvent after 48 h of culture in hormone-free medium resulted in a 25.2% ± 7.2 increase of cytoplasmic Ca^{2+} over control values ($p < 0.025$) after only 4 h of additional culture (fig. 3). Addition of HC or T4 directly to cell suspensions followed by a 15 min assay did not alter cytoplasmic Ca^{2+} levels in epithelial cells from 14-day uncultured duodena or from 14-day duodena cultured for 48 h in hormone-free medium (data not shown).

To further investigate the role of hormones in development of Ca^{2+} homeostasis, cells from 48 h cultures with 0.7 mM extracellular calcium were tested by the 'calcium challenge' protocol. Cells from duodena cultured in the presence of 1 μM HC had an initial cytoplasmic Ca^{2+} concentration of 67.0 nM ± 5.4 which increased to 129 nM ± 4.2 after addition of 7 mM calcium and correction for fura-2 leakage (table 1). The cells from paired control cultures had an initial cytoplasmic Ca^{2+} concentration of 48.5 nM ± 6.8 which increased to 177 nM ± 10. Thus the cytoplasmic Ca^{2+} concentration of cells from HC-treated duodena was initially higher than control values ($p < 0.005$), but dropped significantly below control values after 15 min in high extracellular calcium ($p < 0.01$) as illustrated by a representative assay (fig. 2B). T4 also enhanced the ability of cultured cells to resist a rise in cytoplasmic Ca^{2+}, but to a lesser degree than HC (fig. 2C, table 1). During 'calcium challenge', the increase in Ca^{2+} concentration of T4-treated cells was 77% of that occurring in paired control cultures, whereas the Ca^{2+} rise in HC-treated cells was less than 50% of control values.

Discussion

This work is the first to investigate the effect of glucocorticoid or thyroid hormones on Ca^{2+} concentration of intestinal epithelial cells. Results indicate that hydrocortisone influences Ca^{2+} homeostasis in differentiating intestinal epithelium. During culture in hormone-free medium, Ca^{2+} homeostasis of embryonic duodenal epithelium is abnormal; the concentration of cytoplasmic

Ca^{2+} assayed at either 0.7 mM or 1.3 mM extracellular calcium, as well as the ability of cells to maintain a constant cytoplasmic Ca^{2+} level, is reduced as compared to uncultured epithelial cells of the same chronological age. A similar decrease in concentration of cytoplasmic Ca^{2+} in vitro has been reported for chick embryo chondrocytes cultured in the absence of hormones[19]; Ca^{2+} decreased at an approximate rate of 4.2 nM per day during culture, as compared to 9.4 nM per day in duodenal epithelium. Addition of HC to duodenal cultures elevates cytoplasmic Ca^{2+} of 14-day tissue by approximately 40% relative to paired controls after 12–72 h of culture and restores Ca^{2+} values to those found at 14–16 days in vivo. Similarly, the ability of cells to resist a 'calcium challenge' is restored to that found at 16 days in vivo after 48 h of HC treatment. Reduction of the lag period for HC action on cytoplasmic Ca^{2+} to 4 h when tissue is precultured in hormone-free medium suggests that endogenous hormone dissipates during culture, although an alternate interpretation of increased sensitivity to HC due to development in vitro cannot be ruled out.

T4 has only a minor effect on epithelial Ca^{2+}, but the increases in cytoplasmic Ca^{2+} concentration and resistance to calcium challenge are statistically significant. Since the hormone concentrations utilized in these experiments were chosen for their optimal effects on development of enzyme activities in cultured duodena[14], dose-response studies of hormone action on Ca^{2+} homeostasis were not performed. Thus, it is possible that a different concentration of thyroxine or use of the more potent thyroid hormone triiodothyronine would produce greater effects. It should be noted that control values for cytoplasmic Ca^{2+} are unusually high in the 72 h T4 culture experiment (table 2). This may represent an effect of the T4 solvent, since the solvent control values in the 72 h HC experiment are significantly lower ($p < 0.05$, unpaired t-test) and are similar to those found in solvent-free cultures at 72 h (47.9 ± 3.2 versus 52.5 ± 5.3 nM). Hence, it is not valid to compare the Ca^{2+} values from T4 cultures to those of HC cultures at 72 hours.

The mechanism(s) by which hormones elicit changes in calcium homeostasis remain to be elucidated. It is unlikely that HC acts by increasing epithelial Ca^{2+} influx, since glucocorticoids are known to decrease the calcium absorption of mammalian intestine[20,21], to lower the rate of Ca^{2+} transport by microvillus membrane vesicles[22], and to reduce $^{45}Ca^{2+}$ uptake by 40% during culture of 20-day embryonic chick intestine[23]. The effects of HC on calcium homeostasis could be explained by decreased Ca^{2+} influx through the epithelial microvilli coupled with a decrease in Ca^{2+} efflux via the basal-lateral membranes. The former action would expalin the enhanced resistance of HC-treated cells to 'calcium challenge', whereas the latter action could produce a higher cytoplasmic Ca^{2+} concentration under physiological conditions. Resistance to 'calcium challenge' could also reflect an increased uptake of Ca^{2+} by cell organelles. In mammalian intestine, the V_{max} of calcium uptake into endoplasmic reticulum vesicles increases during epithelial differentiation, and this organelle is thought to play a major role in regulating calcium homeostasis during early development[24]. The small increase in cytoplasmic Ca^{2+} produced by T4 could be explained by increased Ca^{2+} influx, although this hormone has little effect on calcium uptake into 15- or 20-day embryonic chick jejunum[25]. The enhanced ability of T4-treated cells to exclude calcium during 'challenge' may be due to increased epithelial respiration; glucose oxidation rate of 14 day embryonic duodenum is elevated 300% by T4 relative to hormone-free cultures which presumably increases the ATP pool available for ATP-dependent calcium pumps[17].

Hormones which increase cytoplasmic Ca^{2+} concentration of other cell types typically act at the plasma membrane to produce a rapid, transitory rise over baseline Ca^{2+} values[26]. In intestinal epithelium, however, hormone action requires a lag period of hours (HC) or days (T4), and the increase in Ca^{2+} is modest but sustained (for at least 60 h in HC cultures). This time course is more consistent with a nuclear site of hormone action and seems appropriate for a developing system in which relatively small changes in basal cytoplasmic Ca^{2+} might permanently alter the state of cell differentiation. Indeed, such small increases in the cytoplasmic Ca^{2+} concentration are thought to regulate the expression of differentiation-specific genes during development of skin epithelium[3,27]. The hormone-induced resistance to 'calcium challenge' after 48 h of culture may reflect an important aspect of epithelial differentiation, since mammary gland epithelium is also reported to become more resistant to rise in cytoplasmic Ca^{2+} after differentiation[28].

The possibility that glucocorticoid and thyroid hormones regulate epithelial Ca^{2+} homeostasis in vivo is supported by the rise in circulating levels of both hormones during the third week of development[29–31] in concert with a continued increase in cytoplasmic Ca^{2+} concentration and ability to resist 'calcium challenge' through 22 days of development in vivo[16]. The ability of HC and T4 to elevate epithelial Ca^{2+} suggests that other actions of these hormones on epithelial differentiation are influenced, or perhaps mediated, by cytoplasmic Ca^{2+}. Consistent with this hypothesis, varying the calcium concentration of culture medium in the millimolar range or adding calcium ionophore to hormone-free medium promotes several hormone-sensitive parameters of intestinal development including increase in alkaline phosphatase activity, differentiation of goblet cells, and

induction of calbindin-D_{28K}[18,32,33]. Other tissues in which calcium is thought to interact with glucocorticoid hormones during development include skin and mammary gland epithelium[4,34].

Glucocorticoids (and to a lesser extent thyroid hormones) have been shown to influence the development of calcium homeostasis in intestinal epithelium. Further evaluation of intracellular Ca^{2+} in cultured embryonic intestine should be of value in determining the mechanisms by which hormones alter epithelial calcium homeostasis, as well as in clarifying the role of Ca^{2+} in hormonal regulation of development.

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