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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.

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Signal Transduction for Proliferation and Differentiation in Keratinocytes

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INTRODUCTION

The analysis of epidermal growth and differentiation *in situ* and by the use of *in vitro* model analogues has been extraordinarily informative to cell and molecular biologists. These studies have revealed the relationship of growth factors to growth control,[1] the regulation of expression of keratins and other differentiation-specific genes[2–4] and aberrations in function associated with pathological states, especially neoplasia,[5] among other findings. These studies have also suggested a previously unappreciated function of epidermis as a secretory tissue. Secretory activity of keratinocytes has the potential to influence the behavior of the epidermis itself and to function in the maintenance of physiological homeostasis of the host or to modify the host response to pathological conditions.

Sufficient experience has accumulated to indicate that epidermal secretory activity for a particular effector is regulated by the differentiation state of the keratinocyte. Furthermore, the response of keratinocytes to exogenous effectors is often determined by the state of maturation. Grafted epidermis is being considered as a source for the expression of products for gene therapy, and expression of such products is likely to be modified by the keratinocyte differentiation state. For these reasons, an understanding of the signals which regulate epidermal differentiation is central to a full understanding of epidermal secretory activity.

The discovery that extracellular Ca^{2+} (Ca_o) regulates many aspects of epidermal differentiation *in vitro*[6] provided a model in which the major differentiation specific functions of epidermis could be modified. In this model, cells cultured in medium with Ca_o between 0.02 and 0.1 mM are phenotypically similar to basal epidermal cells[7] (FIG. 1). Elevation of Ca_o to >0.1 mM induced a rapid change in biochemistry and morphology in which the cells have many properties of the suprabasal phenotype *in vivo* (FIG. 1). Such cells become irreversibly committed to terminal differentiation after 48–72 hr of increased Ca_o and slough from the culture plate as mature squames.

Many aspects of this Ca_o-activated process of epidermal differentiation appear to be physiological. For example, at 1 mM Ca_o most maturing keratinocytes *in vitro* express pemphigus antigen,[8] form an extensive desmosomal network,[9] and produce

FIGURE 1. Schematic depiction of the terminal differentiation of mammalian epidermis *in vivo* and *in vitro*. Markers expressed in specific Ca_e of the culture medium are shown as + or −, but + does not necessarily indicate expression by all cells (see text). Low Ca^{2+} is 0.02–0.09 mM and high Ca^{2+} is >0.1 mM.

corneocytes analogous to those c
10–20% of keratinocytes in 1.0 mM
expressed by most or all supral
differentiation *in vivo* or at least m

Analysis of Ca^{2+} content in
exists.[13,14] Total and free Ca^{2+} are
relative to serum and dermal conte
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not clear. Nevertheless, the existe
potential importance of Ca_e as a ph
vivo.

Pathways Involved in C

Evidence supporting the valid
differentiation was provided by re
phorbol-13-acetate (TPA) could
keratinocytes cultured in 0.05 mM
associated with TPA responses are
C, a phospholipid and Ca^{2+} depe
multicellular organisms,[18,19] this en
tion response. A link to the Ca^{2+} pa
in epidermal protein synthesis and
two hr after exposure to either Ca
studies showed that exogenous bact
could mimic the action of TPA on
suggested a pathway regulating ep
increasing concentrations of extrac
pase C, a Ca^{2+} requiring enzyme.

Phospholipase C activation is
phosphatidylinositol (PI) yielding t
and inositol trisphosphate (IP3).[2
protein kinase C while IP3 mobiliz
determine if a change in Ca_e could
phospholipase C, we prelabeled cul
and added Ca^{2+} to 1 mM.[23] Inositol
phosphatidylinositol, increased witl
radiolabeled phosphoinositides sug
IPs. After 3 hr in 1 mM Ca^{2+} r
130–140% of control levels. Two
increased IP levels in cells maintain
important in the increased turnover
phosphatidylcholine metabolism bu
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suggesting that both protein kinase
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demonstrate that the second me
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To assess the Ca_i response to
employed by loading cells with a c
change in intracellular fluorescence

FIGURE 1. Schematic depiction of the terminal differentiation of mammalian epidermis *in vivo* and *in vitro*. Markers expressed in specific Ca_o of the culture medium are shown as + or −, but + does not necessarily indicate expression by all cells (see text). Low Ca^{2+} is 0.02–0.09 mM and high Ca^{2+} is >0.1 mM.

corneocytes analogous to those of the stratum corneum *in vivo*.[10] However, only 10–20% of keratinocytes in 1.0 mM Ca^{2+} express K1 or K10[11] and filaggrin,[12] markers expressed by most or all suprabasal cells *in vivo*. Thus Ca_o may not regulate differentiation *in vivo* or at least may not be the only signal to regulate the process.

Analysis of Ca^{2+} content in epidermis *in vivo* indicates that a Ca^{2+} gradient exists.[13,14] Total and free Ca^{2+} are low in the basal cell and first suprabasal cell layers relative to serum and dermal content. Ca^{2+} in the granular cell layer is extraordinarily high. The processes by which a tissue can regulate and maintain a Ca^{2+} gradient are not clear. Nevertheless, the existence of this gradient in the epidermis supports the potential importance of Ca_o as a physiological regulator of epidermal differentiation *in vivo*.

Pathways Involved in Ca^{2+}-induced Epidermal Differentiation

Evidence supporting the validity of the cell culture model to study epidermal differentiation was provided by reports that the tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) could induce epidermal differentiation *in vivo* and in keratinocytes cultured in 0.05 mM Ca^{2+} medium.[15–17] Since the biological activities associated with TPA responses are mediated through the activation of protein kinase C, a phospholipid and Ca^{2+} dependent enzyme present in virtually all cells of all multicellular organisms,[18,19] this enzyme would appear to play a role in the differentiation response. A link to the Ca^{2+} pathway was implicated since the pattern of changes in epidermal protein synthesis and protein phosphorylation which occur within one or two hr after exposure to either Ca^{2+} or TPA were found to be similar.[20] Additional studies showed that exogenous bacterial phospholipase C and exogenous diacylglycerol could mimic the action of TPA on epidermal differentiation.[21] Together, these results suggested a pathway regulating epidermal differentiation which involves exposure to increasing concentrations of extracellular Ca^{2+} activating an endogenous phospholipase C, a Ca^{2+} requiring enzyme.

Phospholipase C activation is associated with the rapid metabolism of cellular phosphatidylinositol (PI) yielding two intracellular second messengers, diacylglycerol and inositol trisphosphate (IP3).[22] Diacylglycerol is the endogenous activator of protein kinase C while IP3 mobilizes intracellular Ca^{2+} (Ca_i) from bound stores. To determine if a change in Ca_o could stimulate PI metabolism by activation of cellular phospholipase C, we prelabeled cultures with [^{3}H]inositol in 0.05 mM Ca^{2+} medium and added Ca^{2+} to 1 mM.[23] Inositol phosphates (IPs), the water soluble metabolites of phosphatidylinositol, increased within 2 min. There was a corresponding decrease in radiolabeled phosphoinositides suggesting that these were the source of the increased IPs. After 3 hr in 1 mM Ca^{2+} medium, each of the IPs remained increased to 130–140% of control levels. Two Ca^{2+} ionophores, A23187 and ionomycin, also increased IP levels in cells maintained in 0.05 mM Ca^{2+}, suggesting that a rise in Ca_i is important in the increased turnover of phosphatidylinositol. Phorbol esters stimulated phosphatidylcholine metabolism but not phosphatidylinositol turnover.[23] In concert with ionomycin, phorbol esters became more potent inducers of differentiation suggesting that both protein kinase C activation, elevation of Ca_i and PI turnover were important components of the signal for epidermal differentiation. These results demonstrate that the second messenger system for Ca^{2+}-mediated keratinocyte differentiation may be through a direct effect on phospholipase C activity.

To assess the Ca_i response to a change in Ca_o, digital image technology was employed by loading cells with a calcium sensitive probe, Fura 2, and measuring a change in intracellular fluorescence.[24] When Ca_o is increased from 0.05 mM to 1.2

mM, there is a 10–20-fold increase in Ca_i which occurs within a few minutes and is sustained for at least 30 min. The magnitude of this response is greatest in the presence of serum, but individual cells show substantial heterogeneity in both time course and magnitude of response. In contrast, serum-free conditions reduce the Ca_i increase to <5-fold, but the cells respond homogeneously. Serum-free conditions also produce a sustained rise in Ca_i in response to an increase in Ca_o. Such results suggest that exogenous factors found in serum could influence the differentiation response in individual cells. Since both Ca_i and PI metabolism are increased simultaneously by a change in Ca_o and both are sustained and tightly linked to the differentiation response, a rise in Ca_i is likely to be an intracellular signal responsible for the induction of terminal differentiation in keratinocytes.

A Specific Ca_o Regulates the Expression of Certain Differentiation Markers

The sequential expression of differentiation-specific markers is characteristic of the terminal differentiation of the epidermis.[25] Keratin markers such as K1 (67 kd) and K10 (59 kd) are expressed early in the differentiation program as cells begin their

TABLE 1. Influence of Ca_o on the Expression of Specific Differentiation Markers in Cultured Mouse Keratinocytes

	Concentrations of Ca^{2+} in Culture Medium (mM)							
	Expression at 24 Hr				Expression at 48 Hr			
Marker	0.05	0.1	0.5	1.0	0.05	0.1	0.5	1.0
K1	–	+++	+	+	–	+++	++	+
K10	–	++	+	+	–	+++	+	+
Filaggrin	–	+	–	ND[a]	–	+++	+	ND
CE	–	+	–	ND	–	+++	+	ND

[a]Not determined.

maturation in the basal or first suprabasal layer.[25,26] Cornified envelope precursors and filaggrin are expressed later as cells enter the granular cell layer[27,28] (see Roop et al., in preparation). Monospecific antibodies have been made to unique sequences of K1, K10, filaggrin and the major protein component of the cornified envelope (here called anti-CE). These antibodies were used as probes to study the Ca^{2+} requirements for differentiation since they recognize maturation stage dependent markers. Both immunofluorescence analysis of cultured cells and Western blotting of cell extracts were used to evaluate the expression of these markers in cultured cells.

When basal cells grown in 0.05 mM Ca^{2+} were switched to 1.0 mM Ca^{2+}, only 10–15% of cells expressed keratins K1 and K10[11] and fewer expressed CE and filaggrin by immunofluorescence analysis. Western blots of such cell extracts do not reproducibly detect K1 and K10 expression. When basal keratinocytes (0.05 mM Ca^{2+}) were switched to 0.10 mM Ca^{2+}, K1 was readily detected on Western blots by 24 hr and K10 staining was intense by 48 hr (TABLE 1). These marker proteins were reduced or not detectable when cells were switched instead to 0.5 mM or 1 mM Ca^{2+}. Western blots using antibodies to detect expression of filaggrin and CE showed a similar requirement for Ca_o of approximately 0.1–0.12 mM, although these bands were not seen prior to 48 hr. The expression of these differentiation specific genes diminished at approximately

0.2–0.3 mM Ca^{2+}. The number of p
escence analysis. When transcript
message was abundant at 18 hr in 0
1.0 mM for that time. Assuming
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From our studies, we can conclu
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a psoriatic-like skin disease. Chroni
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In mouse and human epidermi
substantially below serum Ca^{2+}, wh
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Certain Differentiation Markers

specific markers is characteristic of
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ıtiation program as cells begin their

Specific Differentiation Markers

n Culture Medium (mM)			
Expression at 48 Hr			
0.05	0.1	0.5	1.0
−	+++	++	+
−	+++	+	+
−	+++	+	ND
−	+++	+	ND

[5,26] Cornified envelope precursors and
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.5 mM or 1 mM Ca^{2+}. Western blots
and CE showed a similar requirement
these bands were not seen prior to 48
ic genes diminished at approximately

0.2–0.3 mM Ca^{2+}. The number of positive cells was greatly increased by immunofluorescence analysis. When transcripts for K1 were analyzed by RNA slot blots, K1 message was abundant at 18 hr in 0.1 mM Ca_o but not detectable in cells maintained in 1.0 mM for that time. Assuming these specific changes in Ca_o reflect analogous changes in Ca_i, the intracellular Ca^{2+} milieu may dictate the expression of the differentiation phenotype of normal keratinocytes and this may be under transcriptional control. These studies also demonstrate the importance of maintaining a Ca^{2+} gradient *in vivo* for the proper regulation of epidermal homeostasis.

From our studies, we can conclude that one function for Ca_o is to control epidermal inositol lipid metabolism. A primary role of inositol lipid metabolism for keratinocyte differentiation could have direct clinical significance for some skin diseases. For example, patients chronically treated with Li^+ for manic-depressive illness suffer from a psoriatic-like skin disease. Chronic Li^+ treatment causes a decrease in brain inositol levels, but skin levels of inositol have not been examined.[22,29] Psoriasis is a hyperproliferative disease in which keratinocyte differentiation is impaired. Further studies seem warranted to assess the possible relationship between agents which are known to influence cellular inositol metabolism and impaired responsiveness to endogenous regulators of keratinocyte differentiation.

SUMMARY

In mouse and human epidermis, the Ca^{2+} environment of the basal cell layer is substantially below serum Ca^{2+}, while that of the granular cell layer is unusually high. Reduction of extracellular Ca^{2+} concentration (Ca_o) in the medium of keratinocyte cultures maintains a basal cell phenotype while serum Ca^{2+} concentrations induce terminal differentiation. Measurements of intracellular Ca^{2+} (Ca_i) by the use of Fura 2 and digital imaging technology reveal that Ca_i increases 10–20-fold in response to an increase in Ca_o and remains elevated. Concomitant with the rise in Ca_i is an increase in the metabolism of phosphatidylinositol (PI) to yield inositol phosphates and diacylglycerol. PI metabolism is also stimulated by calcium ionophores suggesting that a rise in Ca_i is directly responsible. The consequent increase in diacylglycerol and Ca_i would activate protein kinase C, an event known to trigger epidermal differentiation. Specific Ca_o and Ca_i determine the expression of individual markers of keratinocyte differentiation *in vitro*. These findings may account for the importance of the Ca^{2+} gradient for maintaining regulated growth and differentiation of the epidermis *in vivo*.

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Purification Procedures

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