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An increase in intracellular free calcium is an early event during differentiation of cultured human keratinocytes

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The effect of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in cultured human keratinocytes was studied using dual-wavelength microspectrofluorometric techniques. The results show that increasing $[Ca^{2+}]_o$ from 70 μM to 1 mM causes an early rise in $[Ca^{2+}]_i$ complete by 2 h. Heterogeneity within cultures was demonstrated.The $[Ca^{2+}]_i$ in spontaneously differentiated cells of low Ca^{2+} cultures was similar to that of Ca^{2+} induced differentiated cells. The increase in $[Ca^{2+}]_i$ preceded the morphological changes and growth inhibition induced by increasing $[Ca^{2+}]_o$. These observations are consistent with an increase in $[Ca^{2+}]_i$ mediating differentiation of human keratinocytes.

Keratinocyte; Differentiation; Ca^{2+}, intracellular; Ca^{2+}, extracellular

1. INTRODUCTION

The extracellular calcium concentration ($[Ca^{2+}]_o$) is known to be a regulator of growth and differentiation of cultured keratinocytes [1]. An increase in $[Ca^{2+}]_o$ changes the balance between cell growth and maturation; differentiation is promoted whilst growth is reduced. Human keratinocytes may be grown as a proliferating monolayer in low calcium medium (70 μM). An increase in $[Ca^{2+}]_o$ to 1 mM causes flattening of the cells with increased cell-cell contact and granularity within 12–24 h and progressive stratification over the next 24–48 h [2]. These effects of $[Ca^{2+}]_o$ are largely reversible within 24 h but by 72 h many cells are committed to terminal differentiation. Cells in the suprabasal position express involucrin (a precursor of the insoluble cell envelope) and other markers of differentiation [3]. These suprabasal differentiated cells are shed into the medium if the $[Ca^{2+}]_o$ is changed from high to low levels [4].

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The molecular mechanisms involved in the $[Ca^{2+}]_o$ induced cellular changes are yet to be established. It has been tacitly assumed that a rise in intracellular calcium ($[Ca^{2+}]_i$) accompanies the change in $[Ca^{2+}]_o$ and thus may be the initial differentiation stimulus. This has not been confirmed by direct measurement. Microspectro-fluorometric techniques now permit the measurement of $[Ca^{2+}]_i$ within living cells. Using the Ca^{2+}-sensitive dyes fura-2 and indo-1 we now report changes in $[Ca^{2+}]_i$ in human keratinocytes after an increase in $[Ca^{2+}]_o$ of the culture medium. The results suggest an important link between $[Ca^{2+}]_i$ and regulation of growth and differentiation.

2. MATERIALS AND METHODS

Normal human epidermis was obtained from circumcisions or retro-aural skin removed during plastic procedures. The skin was trimmed of fat and incubated at 4°C in phosphate-buffered saline (PBS) containing dispase (2 mg/ml) for 12 h. The epidermis was removed, incubated for 10 min at 37°C with trypsin (0.05%) and EDTA (0.02%) and gently shaken to disaggregate the basal cells. After washing with medium the cells were plated onto 22-mm (BDH) glass cover slips for intracellular calcium studies or 35-mm wells for thymidine labelling studies. Serum-

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free medium MCDB153 was used with calcium at 70 μM. Medium MCDB151 was obtained from Sigma; trace elements, growth factors [5], and amino acid supplements [6] were added to obtain complete medium MCDB153. Cells were passaged with the addition of bovine hypothalamic extract to enhance cell attachment [5], and for these studies cells used from passage 1 to 4. The $[Ca^{2+}]_o$ in the media was measured using a calcium sensitive electrode. Cell proliferation was measured by labelling for 2 h in the presence of tritiated thymidine and corrected for the DNA content [7].

Cells were loaded with fura-2 or indo-1 by incubation with the acetoxymethyl esters of the dye (Molecular Probes, OR) at 1–5 μM for 60 min at 37°C. Measurement of fluorescence emitted from single cells was made using a Nikon Diaphot inverted microscope with either a dual excitation or dual emission photon counting system [8] (Newcastle Photometric Systems). For fura-2 the excitation wavelengths were 350 and 380 nm, emission 520 nm and for indo-1, excitation 355 nm and emission wavelengths 405 and 470 nm. Results are expressed as means ± SE and the difference between means was determined by analysis of variance, or Student's *t*-test.

3. RESULTS AND DISCUSSION

As previously reported [9] progressive changes were seen in the morphology of keratinocytes after increasing $[Ca^{2+}]_o$ from 70 μM to 1 mM. Early changes were seen after 4 h with a reduction in the number of membrane ruffles; increased flattening and granularity of cells was observed after 8 h; and multilayered areas within the cultures after 1–2 days. The profiles of $[Ca^{2+}]_i$ in the cell populations maintained in 70 μM and 1 mM $[Ca^{2+}]_o$ are shown in fig.1a. Considerable variation was observed within one culture. The values in each condition are not normally distributed, but skewed to the right with a proportion of very high calcium cells. Morphological and biochemical heterogeneity in populations of cultured keratinocytes is well recognised [10]. When the data in fig.1a are separated on morphological criteria into edge cells and layered differentiated cells a clear difference is seen. This is confirmed by the differences in the profile of intracellular calcium between the centre and edge of a clone after 3 days at 1 mM calcium (fig.1b); subpopulations within the centre cells may also be present. The centre of the clone is stratified after calcium addition, but there are a number of cells (single cells or in small groups) in the suprabasal compartment in low calcium cultures. Such cells have spontaneously differentiated and express involucrin, but fail to form desmosomes and are released into the medium. The internal cell calcium

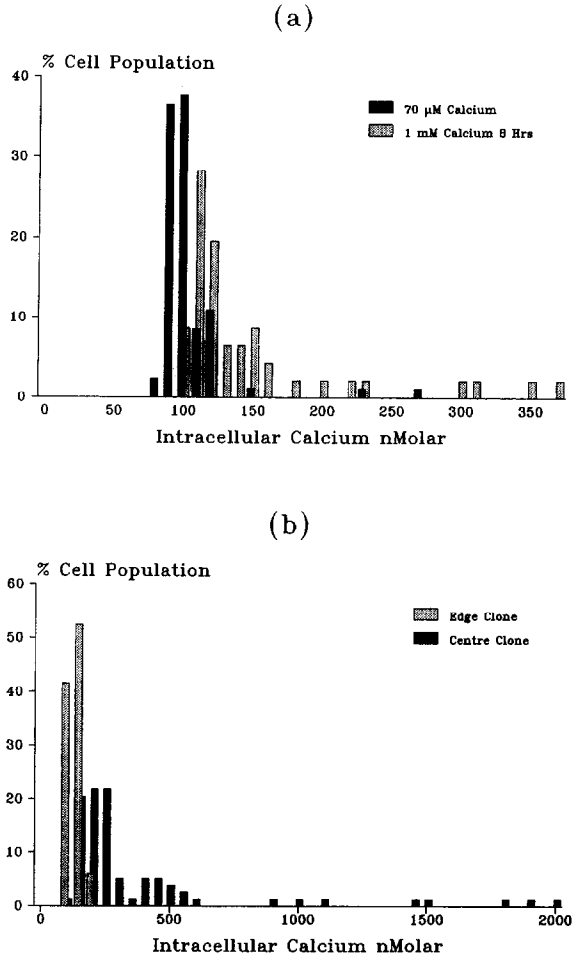

Fig.1. Population profiles of $[Ca^{2+}]_i$. (a) 70 μM and 1 mM $[Ca^{2+}]_o$. (b) Edge and centre of a clone after 1 mM $[Ca^{2+}]_o$ for 3 days.

in spontaneously differentiated cells (372 ± 19 nM) was much higher than basal cells (101 ± 3.5 nM, $p < 10^{-7}$), fig.2a. There was no difference between the intracellular calcium in spontaneously differentiated cells and the centre of a calcium induced stratified layer.

Withdrawal from the cell cycle has been shown to be an early event in differentiation, occurring while cells are still in the basal compartment [11]. The thymidine labelling studies showed no reduction in thymidine incorporation 8 h after the calcium was increased to 1 mM, but thereafter labelling decreased to 7% at 4 days (fig.2b). Our results confirm that, following a rise in $[Ca^{2+}]_o$,

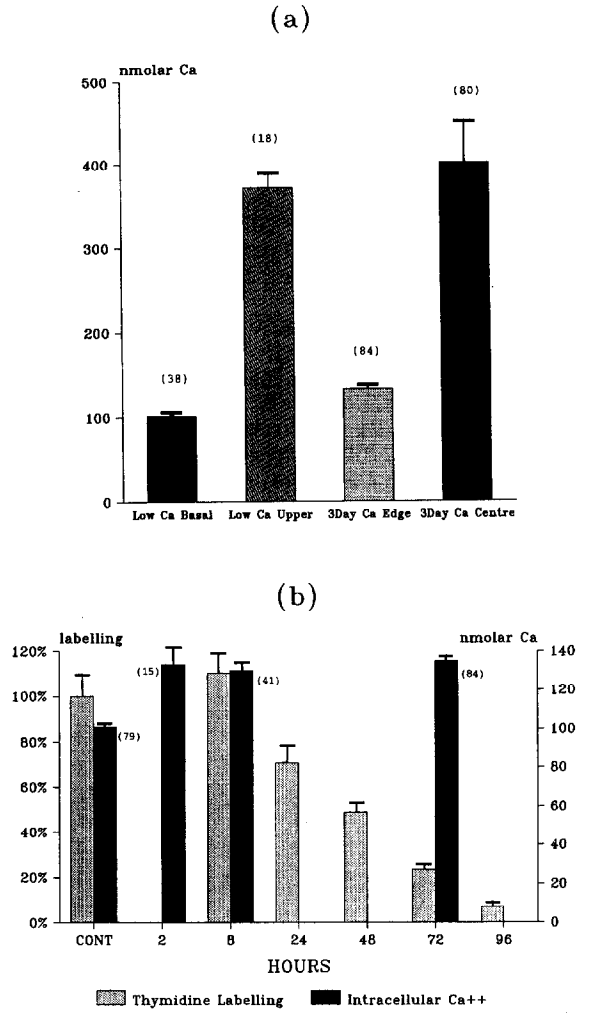

Fig.2. (a) $[Ca^{2+}]_i$ positional differences within a clone at 70 μM and 1 mM $[Ca^{2+}]_o$. (b) Time course of thymidine labelling (% of control culture) and $[Ca^{2+}]_i$ after increasing $[Ca^{2+}]_o$ from 70 μM to 1 mM. Mean ± SE, number of $[Ca^{2+}]_o$ observations in parentheses.

$[Ca^{2+}]_i$ is increased prior to the reduction in thymidine incorporation (100 ± 1.3 to 130 ± 4.8 nM at 8 h, $p < 10^{-7}$). This change may be important in mediating the shift towards differentiation. Cells which have sufficiently progressed in differentiation to move to the upper layer invariably have a high $[Ca^{2+}]_i$. We propose that the increase in $[Ca^{2+}]_i$ following calcium shift may induce withdrawal from the cell cycle. The further increase to very high $[Ca^{2+}]_i$ is associated with differentiation and movement of the cell to the suprabasal position.

Desmosome formation begins within 5–10 min of the addition of calcium and is complete by 2–5 h [12]. Calcium is known to be required for desmosome formation, but $[Ca^{2+}]_o$ is important for desmosome competence. A recording from a single cell after an increase in $[Ca^{2+}]_o$ is shown in fig.3 (one of 8 records). No rapid rise in $[Ca^{2+}]_i$ was detected within 10 min after the solution change. There was however a slow rise which increased dramatically when $[Ca^{2+}]_o$ was increased to 5 mM. The changes observed in $[Ca^{2+}]_i$ observed after increasing $[Ca^{2+}]_o$ over a longer period of time is shown in fig.2b. $[Ca^{2+}]_i$ was significantly increased to 133 ± 11 nM after 2 h ($p < 10^{-6}$), but no further increase was seen in basal or edge cells up to 3 days. Keratinocytes grown in 0.1 mM calcium have numerous large surface microvilli, suggesting a high rate of membrane activity; within 15 min of adding calcium membrane ruffling is decreased as cells flatten and spread [13]. This group also report the appearance of clusters of involucrin positive suprabasal cells at 8 h and sheets by 14 h. The early changes in membrane ruffling may be due to the $[Ca^{2+}]_o$ initiation of desmosome formation. A decrease in growth factor induced membrane ruffling has been demonstrated in epidermal cell lines when $[Ca^{2+}]_i$ is raised [14]. However the results show that an increase in $[Ca^{2+}]_i$ precedes other induced changes. Calcium is known to activate several cellular enzyme systems involved in cell proliferation and differentiation: protein kinases,

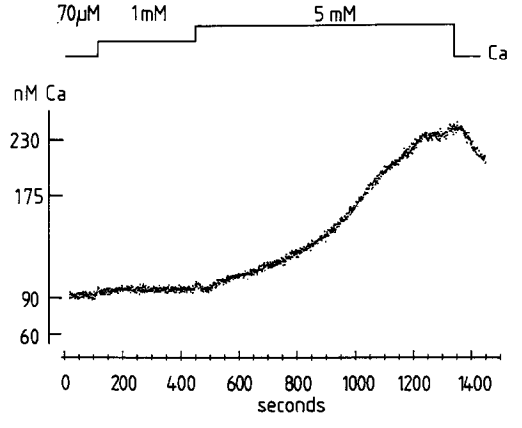

Fig.3. $[Ca^{2+}]_i$ recording in a single keratinocyte after increasing $[Ca^{2+}]_o$ from 70 μM to 1 and then 5 mM.

phospholipases, phosphodiesterases and adenylate cyclases. Changes in inositol phosphate levels and protein kinase C activity observed in keratinocytes after calcium shift have been proposed as regulatory mechanisms [15].

We have demonstrated that keratinocytes are heterogeneous with respect to intracellular free calcium and higher concentrations occur in differentiated cells. After addition of extracellular calcium there is an early but slow increase in intracellular calcium. This new observation has important consequences for our understanding of the control of calcium mediated differentiation of human keratinocytes in vitro.

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