Effect of Extracellular Calcium on the Intracellular Calcium Level of Newborn Rat Skin Basal Cells

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The effect of extracellular calcium on the intracellular calcium level of newborn rat skin basal cells was examined. The intracellular calcium level of basal cells was affected by the extracellular calcium concentration on culture of the cells on a collagen-coated filter, but not on a plate. The intracellular calcium ion concentration ([Ca$^{2+}$]) of cells on a collagen-coated filter with 0.05 mM external CaCl$_2$ was 15 nM and increased to 20–22 nM on culture with 2.0 mM extracellular CaCl$_2$. From these results we concluded that the culture matrix (e.g., culture plates, or collagen-coated filters) affects the response of the basal cells to changes in the extracellular calcium content.

The intracellular calcium level of Swiss 3T3 cells cultured both on plates and collagen-coated filters were affected by the extracellular calcium concentration. Their [Ca$^{2+}$], was determined as 49–50 nM in the presence of 0.05 mM external CaCl$_2$, and 54–56 nM in the presence of 2.0 mM CaCl$_2$. These different responses to extracellular Ca$^{2+}$ may be due to differences in the proliferative profiles of basal cells and fibroblasts.

Key words skin; calcium; basal cell; epidermal cell

Skin epidermis contains various types of cells, such as basal, spinous, granular and cornified cells. The mechanism of formation of the epidermis has not been clarified. Recently, an intercellular calcium gradient has been observed in the epidermal cell layer, where the Ca$^{2+}$ level of basal and spinous cells is below the serum Ca$^{2+}$ level, whereas the environment of granular cells and the stratum corneum has a high Ca$^{2+}$ level. These facts suggest that calcium ions play an important role in the formation of the epidermal layer. The importance of this intercellular calcium gradient has been suggested by in vitro studies of cultured murine keratinocytes in which the expression of basal or suprabasal markers of differentiation change when the extracellular Ca$^{2+}$ concentration is raised from 0.05 mM to above 0.10 mM. There have been several studies on signaling pathways involving Ca$^{2+}$. For example, there are reports that an increase in extracellular Ca$^{2+}$ is associated with an increase in intracellular Ca$^{2+}$ and graded stimulation of the metabolism of inositol phospholipids in cultured keratinocytes, resulting in changes in intracellular diacylglycerol (DAG) and inositol phosphate (InsP) that vary directly with the extracellular Ca$^{2+}$ level. We previously reported that the extracellular Ca$^{2+}$ concentration affects the proliferation and keratin synthesis of newborn rat skin basal cells. However, we found that their synthesis of keratin increased with an increase in extracellular Ca$^{2+}$ when they were cultured on a collagen-coated filter, but not when they were cultured on a plate. Thus, the culture matrix is an important factor in the response of basal cells to the extracellular Ca$^{2+}$ level. In the present study, we investigated the effect of the culture matrix on the intracellular Ca$^{2+}$ level of basal cells.

MATERIALS AND METHODS

Reagents The reagents used in this study were as follows: Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan), fetal bovine serum (FBS; Whittaker M.A. Bioproducts, Walkersville, MD, U.S.A.), dispase (Godo-Shusei Co., Tokyo, Japan), Millicell-CM (12 mm diameter; Millipore Products, Bedford, MA, U.S.A.), type I collagen (bovine skin dermis; Koken, Tokyo, Japan), and fura 2-AM (Dojin-do, Kumamoto, Japan). Media Eagle's MEM was supplemented with 20 mM HEPES, 24 mM sodium bicarbonate and 10% FBS (MEM-FBS). Then, it was treated with 2% Chelex 100 (Bio-Rad Hercules, CA, U.S.A.), and its calcium level was reduced by omitting Ca$^{2+}$. This Chelex-treated medium was found to contain 0.01 mM Ca$^{2+}$ using the Arsenazo III assay system described previously. MEM-FBS, containing various levels of Ca$^{2+}$, was prepared by the addition of CaCl$_2$ to the Chelex-treated medium.

Preparation and Culture of Skin Basal Cells Basal cells were prepared from the skin epidermis of 3-d-old Wistar rats by digestion with dispase and trypsin–EDTA as described previously. The cells were suspended in MEM-FBS, then seeded into 24-well culture plates or type I collagen-coated filters as described previously.

Measurement of Intracellular Calcium The intracellular Ca$^{2+}$ levels in basal cells cultured on plates or collagen-coated filters were measured by fluorescence video microscopy with a PC-based digital image analyzing system (IMT-2 (Olympus) and ARGUS-50/CA (Hamamatsu Photonics, Tokyo, Japan). Basal cells were cultured on each culture matrix in MEM-FBS with 0.05 or 2.0 mM CaCl$_2$ for 24 or 48 h. After culture, the medium was removed and the cells were washed with Hepes–KOH buffer (pH 7.4; 20 mM Hepes, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, and 13.8 mM glucose) with 0.05 or 2.0 mM CaCl$_2$. Cells were loaded with 5 µM fura 2-AM in Hepes–KOH buffer with 0.05 or 2.0 mM CaCl$_2$ for 1 h at 37°C in a CO$_2$ incubator, then the medium was changed to Hepes–KOH containing 0.05 or 2.0 mM CaCl$_2$. The fluorescence intensity at excitation wavelengths of 349 nm ($F_{340}$) or 380 nm ($F_{380}$) were measured at an emission
wavelength of 550 nm. The fluorescence intensity ratio of fura 2 with various amounts of calcium ions was determined to be \( F_{549}/F_{380} \) (Fig. 1). The results are reported as the mean of 50 cell values, and the experiments were repeated three times.

RESULTS

Effect of Extracellular Calcium Concentration on the Intracellular Calcium Level of Basal Cells

We examined the effect of the extracellular calcium concentration on the intracellular calcium ion level ([Ca\(^{2+}\)]\(\text{in} \)) of basal cells. Basal cells were cultured on plates with 0.05 or 2.0 mM CaCl\(_2\) for 24 h, and the change in [Ca\(^{2+}\)]\(\text{in} \) was measured. In these cultures, the [Ca\(^{2+}\)]\(\text{in} \) level varied at a level of about 31 nm during the 30 s observation period (open and closed circles in Fig. 2A). The levels of [Ca\(^{2+}\)]\(\text{in} \) were measured after culture for 48 h. The [Ca\(^{2+}\)]\(\text{in} \) level of the basal cells was not affected by the extracellular calcium concentration, and the [Ca\(^{2+}\)]\(\text{in} \) remained at about 31 nm (open and closed circles in Fig. 2B). As shown in Fig. 2C, the mean values of [Ca\(^{2+}\)]\(\text{in} \) were similar in the two culture conditions (0.05 and 2.0 mM calcium) and the two culture periods (24 and 48 h).

On culture on a collagen-coated filter with 0.05 mM CaCl\(_2\) for 24 or 48 h, the [Ca\(^{2+}\)]\(\text{in} \) level varied at a level of about 15 nm during the 30 s measurement period (open circles in Figs. 3A and B). When the cells were cultured with 2.0 mM extracellular calcium for 24 h, the [Ca\(^{2+}\)]\(\text{in} \) level was increased to 20 nm during the 30 s analysis period (closed circles in Figs. 3A). After culture for 48 h, the [Ca\(^{2+}\)]\(\text{in} \) level in the presence of 2.0 mM external calcium was 22 nm (closed circles in Fig. 3B). The mean [Ca\(^{2+}\)]\(\text{in} \) levels are shown in Fig. 3C: the [Ca\(^{2+}\)]\(\text{in} \) level changed 5 nm (after 24 h—7 nm (after 48 h) as a result of changes in the extracellular calcium concentration.

On the incubation of basal cells with various concentrations of extracellular calcium (0.05—2.0 mM) on collagen-coated filters for 24 h (open circles) or 48 h (closed circles), a change in [Ca\(^{2+}\)]\(\text{in} \) with the external calcium concentration was observed (Fig. 3D). Thus, the [Ca\(^{2+}\)]\(\text{in} \) of basal cells on collagen-coated filters depends on the extracellular calcium concentration.

Effect of Extracellular Calcium on [Ca\(^{2+}\)]\(\text{in} \) of Swiss 3T3 Cells

Swiss 3T3 cells were cultured on culture plates with 0.05 mM extracellular CaCl\(_2\) for 24 h; their [Ca\(^{2+}\)]\(\text{in} \) was about 50 nm during a 30 s observation period (Fig. 4A, open circles). An increase in the external calcium concentration to 2.0 mM, the [Ca\(^{2+}\)]\(\text{in} \) of the fibroblasts changed to 56 nm (Fig. 4A, closed circles). When 3T3 cells were cultured on collagen-coated filters, their [Ca\(^{2+}\)]\(\text{in} \) was about 49 nm during the observation period (Fig. 4B, open circles). The [Ca\(^{2+}\)]\(\text{in} \) of fibroblasts changed to 54 nm by an increase of the external calcium concentration to 2.0 mM (Fig. 4B, closed circles). In culture on plates, a 6 nm difference between the levels in the two types of culture conditions (with 0.05 mM (condition 1) or 2.0 mM (condition 2) external CaCl\(_2\)) was found in 3T3 cells (Fig. 4C). When 3T3 cells were cultured on collagen-coated filters, a difference of 5 nm was observed between the two culture conditions (with 0.05 mM (condition 3) or 2.0 mM (condition 4) external calcium, Fig. 4C).

DISCUSSION

Previously, we reported that cultured skin basal cells showed a culture matrix-dependent response to extracellular...
Fig. 3. Effect of Extracellular Calcium on the [Ca^{2+}] of Basal Cells and Dose-Response Curves of Intracellular Calcium Levels of Basal Cells

Basal cells were cultured on collagen-coated filters with 0.05 mM (open circles) or 2.0 mM (closed circles) CaCl₂ for 24 h (A) or 48 h (B), and the [Ca^{2+}] during 30 s were measured with an ARGUS-50/CA image analyzing system. Basal cells were cultured for 24 or 48 h with 0.05 mM (open columns) or 2.0 mM CaCl₂ (closed columns), and the mean changes of [Ca^{2+}], were determined, as for Fig. 2C. Basal cells were cultured on collagen-coated filters with various amounts of external calcium for 24 h (open circles) or 48 h (closed circles), and the mean values of [Ca^{2+}] were determined using an ARGUS-50/CA image analyzing system.

calcium,\textsuperscript{12,13} In this case, basal cells flattened and grew larger after only 2 d of culture on plates.\textsuperscript{12} This is inconsistent with the fact that a change of basal cells to a flattened-stratum corneum normally takes 30 d in intact skin.\textsuperscript{15} When the cells were cultured on a collagen-coated filter, they did not flatten after only 2 d of culture.\textsuperscript{12} Thus, the stage of differentiation seems to be maintained normally on collagen-coated filters. In the present study, we found that the [Ca^{2+}] of basal cells depended on the extracellular calcium concentration in cultures on collagen-coated filters, but was not affected by the extracellular calcium concentration in cultures on a plate (Fig. 2 vs. 3). From these results we considered that the morphological change in the cells is related to the intracellular calcium level. Indeed, when basal cells were cul-
tured on plates, the [Ca²⁺], level was not affected by the extracellular calcium level, being about 31 nm (Fig. 2), and the cells were flattened to 40–100 μm diameter. In contrast, when basal cells were cultured on collagen-coated filters with 0.05 mM CaCl₂ for 24 or 48 h, the [Ca²⁺] level was about 15 nm (Fig. 3). Furthermore, when the cells were cultured with 2.0 mM extracellular calcium for 24–48 h, the [Ca²⁺] level increased to 20–22 nm (Fig. 3) but no flattening of the cells was observed during the culture period. Thus, the [Ca²⁺] level of basal cells in culture on plates was higher (31 nm) than that in cultures on collagen-coated filters (15–22 nm), so we consider that the [Ca²⁺] level is related to the formation of a flattened stratum corneum.

On culture on collagen-coated filters with 0.05 mM CaCl₂ for 24–48 h, the [Ca²⁺] level increased from 15 to 20–22 nm when extracellular calcium was increased to 2.0 mM (Fig. 3). In this case, the meaning of the [Ca²⁺] increase (5–7 nm) during the initiation of the differentiation of basal cells has not yet been clarified. In nerve cells, the intracellular calcium level increases temporally, and this rise in [Ca²⁺], forms calcium waves in the cells. In the present study, it is possible that the temporal rise of [Ca²⁺], occurred immediately after an increase in the external calcium concentration, which triggered initiation of basal cell differentiation. We are now investigating the temporal rise in [Ca²⁺] in basal cells.

In culture both on plates and collagen-coated filters, the [Ca²⁺] level of 3T3 cells was affected by the extracellular calcium concentration, and the [Ca²⁺] level of fibroblasts increased from 49–50 nm to 54–56 nm on an increase in the external calcium content (Fig. 4). In general, fibroblasts (such as 3T3 cells) grow directly on a culture plate, while skin basal cells need a clearance under their basal side for proliferation; they are maintained on a basement membrane in vivo. Basal cells can’t obtain nutrition containing Ca²⁺ from a basal side in culture on plates, while they can take it from a basal side in culture on collagen-coated filters. These differences in culture environment seems to be related to the sensitivity of the cells to extracellular calcium concentration. In the present study, basal cells responded to changes in the external calcium content by maintaining a clearance under their cell layer as intact skin epidermis (culture on a collagen-coated filter), but the cells did not respond to changes in external calcium concentration in culture on a culture plate. From these results we consider that basal cells have a different proliferative profile from fibroblasts, and this peculiar property of the basal cells may play an important role in the differentiation of skin basal cells. From this point of view, we are now investigating the differentiation mechanism of skin basal cells, e.g. the relationship between the sensitivity to extracellular Ca²⁺ and the polarity of the cultured basal cell layer.

REFERENCES