Differences in the Regulation of Intracellular Calcium in Normal and Neoplastic Keratinocytes Are Not Caused by ras Gene Mutations

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ABSTRACT

The development of resistance to terminal differentiation is an early event in epidermal neoplasia. Altered differentiation can be detected in vitro since normal epidermal cells are induced to differentiate in medium with Ca²⁺ > 0.1 mM while neoplastic epidermal cells and keratinocytes transduced with a v-ras²¹⁶ allele are resistant to Ca²⁺. In normal epidermal cells, the elevation of extracellular Ca²⁺ (Caₑ) from 0.05 to 1.2 mM causes a biphasic intracellular Ca²⁺ ([Ca²⁺]ᵢ) response in which a transient (10 min) peak of 4–5-fold over basal values is followed by a sustained (>24 h) 2-fold increase in steady-state Caᵢ. The transient peak in Caᵢ is dependent on a serum component and independent of Caₑ, while the sustained plateau is directly dependent on Caₑ. The transient peak responding to a serum factor is lost in normal cells after 24 h in 1.2 mM Ca²⁺, a time when these cells are differentiating. Two neoplastic keratinocyte cell lines, SP-1 and 308, which produce benign tumors in vivo, also have a biphasic Caᵢ response to an increase in Caₑ. In these cells, the transient peak is also serum-dependent and amplified to 10-fold over basal values. However, the plateau value is not sustained and returns to basal values by 8 h, independent of Caₑ. Furthermore, 308 cells remain sensitive to the serum-induced Caᵢ transient after 24 h in 1.2 mM Ca²⁺. To determine whether the activating c-ras²¹⁶ mutation in 308 and SP-1 cells was responsible for the altered Caᵢ regulation, a v-ras²¹⁶ gene was introduced into normal keratinocytes by a defective retrovirus. This also produces the papilloma phenotype in vivo. Recipient cells were resistant to Ca²⁺-induced terminal differentiation although they did not proliferate in 1.2 mM Ca²⁺. The Caᵢ profile in response to 1.2 mM Ca²⁺ was identical in normal and v-ras²¹⁶ keratinocytes, and these cells lost the serum-induced transient Caᵢ peak after 24 h. Thus, the activation of the c-ras²¹⁶ gene in 308 or SP-1 cells is probably not solely responsible for the altered Caᵢ response in neoplastic cell lines. Sustained physiological elevation of Caᵢ may be relevant to the loss of proliferative potential in both normal and v-ras²¹⁶ keratinocytes in 1.2 mM Ca²⁺. In addition, v-ras²¹⁶-mediated or activated c-ras²¹⁶-mediated changes in a complementary pathway may contribute to the block in terminal differentiation in neoplastic cells.

INTRODUCTION

The earliest phenotypic change detected in mouse epidermal cells initiated by chemical carcinogens is an altered response to Caₑ. Ca²⁺ is a potent inducer of keratinocyte terminal differentiation (1, 2). Resistance to Ca²⁺-induced differentiation has provided methods to select for chemically initiated cells in vitro (3, 4) and is an acquired phenotype when the v-ras²¹⁶ oncogene is introduced into normal keratinocytes by a retrovirus (5–7). Since activation of the c-ras²¹⁶ protooncoprotein is a common initiating event in skin carcinogenesis (8), resistance to Ca²⁺-induced differentiation and changes in the regulation of Caᵢ could be direct consequences of a ras²¹⁶ gene mutation. Indeed, neoplastic keratinocyte cell lines, which express a mutated c-ras²¹⁶ allele, are Ca²⁺ resistant and have altered intracellular Ca²⁺ responses to a change in Caₑ (9). Furthermore, ras-related proteins Saccharomyces cerevisiae have been directly implicated in the control of Ca²⁺ transport (10).

Biochemical data are consistent with a linkage among ras gene mutations, epidermal neoplasia, and altered Ca²⁺ metabolism. In both normal and neoplastic keratinocytes, an increase in Caₑ stimulates phospholipase C activity, raising intracellular inositol phosphates and diacylglycerol (11, 12). However, basal phosphatidylinositol metabolism is 2–3-fold higher in unstimulated neoplastic cells resulting in substantially elevated steady-state cellular content of inositol phosphates and diacylglycerol, second messengers which regulate Ca²⁺ (13). Pharmacological studies have demonstrated that agents which cause substantial elevations of inositol phosphates and diacylglycerol, such as ionomycin or aluminum fluoride, block the expression of Ca²⁺-induced differentiation-specific genes such as keratins 1 and 10, filaggrin and loricrin (14). The Ca²⁺-regulated expression of these genes is also inhibited in chemically initiated keratinocytes and v-ras²¹⁶-transduced epidermal cells (7, 15).

Several studies have indicated that keratinocyte differentiation is associated with a rise in Caₑ. Sharpe et al. (16) reported that Caₑ is elevated in human keratinocytes induced to differentiate by Ca²⁺. In mixed colonies of differentiating and basal-like human keratinocytes, the Caₑ is higher in the differentiating cells (16). Maclaughlin et al. (17) demonstrated a rise in Caₑ in keratinocytes induced to differentiate by 1,25(OH)₂-vitamin D₃. Our laboratory has utilized personal computer-based videomicroscopy, digital image analysis, and a fluorescent intracellular calcium probe, Fura-2, to demonstrate a rapid increase in Caₑ in normal mouse keratinocytes stimulated by increased Caₑ. Furthermore, the Caₑ increase was sustained and graded to the level of Caₑ, and this appeared to correlate to specific culture conditions which were permissive to express differentiation markers (9, 46). The current report uses these imaging methods to compare the Caₑ response of normal and neoplastic keratinocytes exposed to changes in Caₑ, and to explore the consequences of the expression of a ras oncoprotein in these responses.

MATERIALS AND METHODS

Chemicals. Fura-2/AM and Fura-2 free acid were purchased from Molecular Probes, Inc. (Eugene, OR); pluronic-F127 was from BASF Wyandotte Corp. (Wyandotte, MI); calcium chloride and EGTA were from Aldrich Chemical, Co., Inc. (Milwaukee, WI); and ionomycin was from Calbiochem (San Diego, CA).

Preparation of Cells for Culture and Caₑ Analysis. Primary BALB/c keratinocytes were isolated as previously described (1). Cells were
cultured at 36°C in a humidified incubator with 7% CO₂ in Eagle's minimal essential medium with 8% Chelex-treated fetal bovine serum (Intergen/Armour, Kankakee, IL) and 0.05 mM calcium. All cells used for Ca²⁺ determination were grown on glass coverslips in order to minimize the interference of background fluorescence in the accurate measurement of intracellular Fura-2 fluorescence. Confluent cultures of primary keratinocytes were analyzed 6 days after plating. To study the influence of the ras oncprotein, primary cultures on coverslips were infected with a replication-defective retroviral vector containing the v-ras⁴⁶ gene as described (18). Cell growth and the efficiency of viral infection were enhanced when coverslips were preconditioned by growth of keratinocytes for 3 days, lysis by 0.025 M ammonium hydroxide, 0.5% Triton X-100 in phosphate-buffered saline, and replating new keratinocytes on the remaining matrix (suggested by Dr. Tamar Tennenbaum, NCI). Keratinocytes transduced with the v-ras⁴⁶ gene are resistant to Ca²⁺-induced differentiation, express mutant p21 in all media Ca²⁺ concentrations, and produce benign squamous papillomas when grafted to nude mice (6, 18). Control cells were infected with the same viral vector containing the neomycin resistance gene (neo′) or sham-infected in the presence of polybrene (4 µg/ml). Replicate infected same viral vector containing the neomycin resistance gene (neo′) or sham-infected in the presence of polybrene (4 µg/ml). Replicate infected cells were exposed to 5-7, 18 and rendered differentiation defective.

An A→T transversion in the 61st codon. They also produce benign squamous papillomas when grafted to nude mice (6, 18). Control cells were infected with the same viral vector containing the neomycin resistance gene (neo′) or sham-infected in the presence of polybrene (4 µg/ml). Replicate infected plates with cells on coverslips were monitored for resistance to Ca²⁺-induced differentiation to ensure that v-ras⁴⁶ introduction was effective in each experiment. In previous studies using the identical infection protocol and time course of study, we have shown that virtually all keratinocytes in the population are transduced by the v-ras⁴⁶ retrovirus and rendered differentiation defective.

Two neoplastic keratinocyte cell lines, 308 and SP-1 (19), which do not terminally differentiate in response to 1.2 mM Ca²⁺, were plated on coverslips as above and studied when confluent. Cell line 308 was derived from keratinocytes isolated from mouse skin initiated by 7,12-dimethylbenz[a]anthracene, cultured in medium with 0.05 mM Ca²⁺ medium, and selected in 1.4 mM Ca²⁺ medium. This cell line proliferates equally well in 1.2 and 0.05 mM Ca²⁺. Cell line SP-1 was derived from papillomas induced in SENCAR mice by initiation with 7,12-dimethylbenz[a]anthracene and promotion with 12-O-tetradecanoylphorbol-13-acetate using the same in vitro selection. This cell line grows preferentially in 0.05 mM Ca²⁺ but also proliferates at a slow rate in 1.2 mM Ca²⁺. These Ca²⁺-resistant cells contain a mutant v-ras⁴⁶ allele with an A→T transversion in the 61st codon. They also produce benign squamous papillomas when grafted to nude mice (19) and thus represent the benign phenotype. For studies of cornified envelopes, cells were plated on 100-mm plastic culture dishes and grown to confluence in 0.05 mM Ca²⁺ medium. After appropriate treatments with Ca²⁺ or ionomycin, cornified envelopes were quantified by the method of Rice and Green (20).

The culture chamber for Ca²⁺ studies was constructed from a plastic 35-mm tissue culture dish (Corning Glass Works, Corning, NY) in which a 14-mm hole bored in the plastic bottom was covered by a 22-

mm-square glass coverslip (VWR, San Francisco, CA). The coverslip was acid-washed (Dichrol; American Scientific Products, McGray Park, IL), rinsed with water, and attached to the inner surface of the plastic dish using a molten mixture of paraflin:white petroleum (3:1). The chambers were sterilized prior to use by exposure to 70% ethanol for 30 min and air dried. Analysis of Intracellular Free Ca²⁺. For the analysis of intracellular free Ca²⁺, cells were loaded with Fura-2/AM by the following procedure modified from Tsien et al. (21). Cells were washed three times with serum-free Krebs/HEPES buffer (pH 7.4) with 0.05 mM (or in some experiments 1.2 mM) Ca²⁺. Cells were then bathed in 2 ml of this buffer which also contained 25 µM Fura-2/AM, 0.03% pluronic F127, and 0.1% dimethyl sulfoxide. After 1 h at room temperature, cells were washed with serum-free Krebs/HEPES buffer (pH 7.4) with 0.05 mM (or 1.2 mM) Ca²⁺ and maintained in 2 ml of this medium for 1 h at room temperature prior to experimental analysis to allow intracellular deesterification of Fura-2/AM. Leakage of dye was not observed in any Ca²⁺ medium following loading. These loading procedures did not interfere with the differentiation response induced in keratinocytes by 8% fetal bovine serum plus 1.2 mM Ca²⁺. Fura-2-loaded cells were imaged in Krebs/HEPES media alone, for 1 min, then changed to medium containing the stimulator of interest. Cells were observed on an inverted epifluorescence microscope (Leitz Diavert) using a 40× Nikon UV-fluor (numeric aperture, 1.3) objective, quartz optics, and a direct current-stabilized 50-W mercury arc lamp. Throughout the period of fluorescent image collection in a given experiment, the temperature was ambient (23-27°C), and the chamber position, optical focus, and microscope alignment were not changed. Fluorescent images of cells, buffer, and Fura-2 free acid control solutions were excited at 340 and 380 nm using narrow-band (bandpass of 8 nm) interference filters (Ditric Optics, Hudson, MA), and emitted light was collected through a broad-band filter (500 nm; bandpass of 40 nm) (Ditric Optics). Experimental images at specific time intervals were collected from cells both in Krebs/HEPES buffer alone and following a medium change to 2 ml Krebs buffer (pH 7.4) containing specific concentrations of Ca²⁺ and/or Chelex-treated fetal bovine serum or other test agents. Measurements of Ca²⁺ in culture media were made with a Perkin-Elmer 4000 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT). To confirm the Ca²⁺ sensitivity of the Fura-2 within experimental cells, all imaging experiments were concluded by artificially raising cellular free Ca²⁺ levels by treatment with 1.2 mM plus 6.5 µM ionomycin, a calcium ionophore.

All of the calculations and digitizations were performed using a microcomputer image-analysis system (BIAS; Loats Associates, Inc., Westminster, MD) based on IBM/AT. An Epyx board was used to digitize a central region, 128 horizontal x 240 vertical pixels. Each image was acquired at 1/30 s/frame, with 256 grey-level values (8 bits). Thirty-two frames were averaged in 15 s in an integral 1-Mbyte memory on the Epyx board. Ca²⁺ was calculated from the ratio of fluorescence (500 nm emission) at two excitation wavelengths, 340 and 380 nm, after background subtraction, as previously described (22). The results are presented as population averages of Ca²⁺ in 15–20 cells in a microscopic field over time. Data are illustrated for individual experiments, and all experiments were repeated at least twice with comparable results.

RESULTS

Normal Keratinocytes and 308 Cells Have Biphasic but Different Ca²⁺ Responses to an Increase in Ca²⁺. Previous studies on normal and neoplastic keratinocytes have revealed marked changes in Ca²⁺ when Ca²⁺ is changed from 0.05 to 1.2 mM (9). When normal cells are cultured for 6 days in 0.05 mM Ca²⁺, the individual cell responses are very uniform, although a variable response among cells was previously observed in 3-day cultures of normal keratinocytes (9). Individual cell responses varied by about 5% from the population average in 3 separate experiments totaling 31 cells examined in 6-day cultures (not shown). Likewise individual neoplastic cell responses correspond closely to the population average (9). In Fig. 1, the resting Ca²⁺ in normal cells (cultured in 0.05 mM Ca²⁺ medium) is 25–30 nM, whereas in neoplastic 308 cells, resting Ca²⁺ is higher, around 50 nM. Both cell types respond to 1.2 mM Ca²⁺ with a large transient spike in Ca²⁺ which increases approximately 4–5-fold in normal cells and approximately 10-fold in neoplastic cells. The resulting peak Ca²⁺ in the neoplastic 308 cells is 5 times higher than the peak in normal keratinocytes. The higher basal value of Ca²⁺ in the neoplastic 308 cells is 5 times higher than the peak in normal keratinocytes. The higher basal value of Ca²⁺ in the neoplastic 308 cells is 5 times higher than the peak in normal keratinocytes. The higher basal value of Ca²⁺ in the neoplastic 308 cells is 5 times higher than the peak in normal keratinocytes. The higher basal value of Ca²⁺ in the neoplastic 308 cells is 5 times higher than the peak in normal keratinocytes.

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in normal cells is as high or higher than the steady-state Ca$_i$ in 308 cells (Fig. 2). The transient Ca$_i$ peak in normal and neoplastic cells is dependent on serum concentration in the medium and independent of Ca$_o$, as shown in Fig. 3. A transient Ca$_i$ increase is observed only in normal cells stimulated with serum, but elevated plateau values for Ca$_i$ are identical in 1.2 mm Ca$^{2+}$ medium with and without serum (Fig. 3A). Redistribution of intracellular Ca$^{2+}$ accounts for the serum-induced transient Ca$_i$ peak (46). The accentuated transient peak in neoplastic cells is also dependent on serum (Fig. 3B). The magnitude of the peak suggests that 308 cells (and SP-1 cells) are more sensitive to the serum effects. Since differentiation proceeds in high-Ca$^{2+}$ medium in the absence of serum, the serum-induced transient increase in Ca$_i$ is not required for the differentiation response.

Exposure to 1.2 mm Ca$^{2+}$ Medium Reduces the Stimulated Ca$_i$ Transient in Normal but not 308 Keratinocytes. Exposure to 1.2 mm Ca$^{2+}$ induces terminal differentiation in normal but not neoplastic keratinocytes (23). An early response associated with differentiation is the loss of proliferative activity in the Ca$^{2+}$-induced normal population within 24–48 h (1). Differentiating normal keratinocytes also lose the transient Ca$_i$ response to serum within 24 h (Fig. 4A), although the response is intact at 8 h. Since plateau values of Ca$_i$ are increased at both 8 and 24 h, a steady-state Ca$_i$, value after 30 min to 21 h (Fig. 1). When intermediate times were studied (Fig. 2), Ca$_i$ remained elevated in normal cells at 8 and 24 h while 308 cells were at basal values at both time points. In fact, by 8 and 24 h in 1.2 mm Ca$^{2+}$ medium, the Ca$_i$ concentration of 308 cells is as high or higher than the steady-state Ca$_i$ in normal primary keratinocytes but not in neoplastic line 308. Cells grown in serum-containing medium with 0.05 mm Ca$^{2+}$ (0 h) were switched to medium with 1.2 mm Ca$^{2+}$ for 8 or 24 h. Columns, mean Ca$_i$, averaged from 3 experiments; bars, SE. To determine steady-state Ca$_i$, cells were loaded with Fura-2 for 2 h in medium with appropriate Ca$^{2+}$ concentration. $\Delta$, primary keratinocytes; $\Box$, line 308.

Fig. 3. The early peak in Ca$_i$ in both normal and neoplastic keratinocytes is dependent on serum. Ca$_i$ concentrations (cell population average) are plotted as a function of time after changing the medium from 0.05 to 1.2 mm Ca$^{2+}$ in the presence (●) or absence (●) of 8% fetal bovine serum. $A$, normal primary keratinocytes; $B$, line 308 cells.

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Normal Keratinocytes

Fig. 4. Neoplastic 308 cells, but not normal keratinocytes, retain the transient 
Ca²⁺ response to serum after culture for 24 h in medium with 1.2 mM Ca²⁺. Normal 
cells (A) or 308 cells (B) were incubated either in medium with 0.05 mM Ca²⁺ 
(Φ) or in medium with 1.2 mM Ca²⁺ for 8 h (B) or 24 h (A) before exposure to 
serum-containing medium with 1.2 mM Ca²⁺. Points, Ca²⁺ cell population averages 
of all cells in a microscope field.

Line 308 Keratinocytes

Fig. 5. Incorporation of the v-ras¹⁶ gene does not affect the early Ca²⁺ response 
to increased Ca²⁺. When the v-ras¹⁶ gene is introduced 
into normal keratinocytes, recipient cells have similarities to 
308 and SP-1 cells including resistance to Ca²⁺-induced differ 
entiation, loss of suprabasal marker expression, and benign 
tumor formation when cells were grafted to nude mice (6, 18, 
19). Since both 308 and SP-1 cells have activating c-ras¹⁶ mutations (19), v-ras¹⁶ keratinocytes provided a model to test 
whether ras¹⁶ gene activation is responsible for altered Ca²⁺ 
regulation in benign neoplastic keratinocytes. When v-ras¹⁶, v-
neo', and mock-infected cells in 0.05 mM Ca²⁺ medium were 
switched to 1.2 mM Ca²⁺, the Ca²⁺ responses were identical (Fig. 
5). The three cell types had basal Ca²⁺ values of around 20–25 
nM, transient peaks measuring 5–6-fold over basal levels, and 
a plateau value of around 50–55 nM by 32 min. Furthermore, 
the plateau was sustained at the elevated level for at least 24 h 
for all groups (not shown). Thus v-ras¹⁶ transduction alone did 
not reproduce the changes in Ca²⁺ regulation characteristic of 
neoplastic keratinocyte cell lines.

To determine whether Ca²⁺ responses in v-ras¹⁶ cells would be 
altered when smaller changes in Ca²⁺ were tested, the three 
groups (control, v-neo', and v-ras¹⁶) were switched from 0.05 
to 0.12 mM Ca²⁺, a concentration of Ca²⁺ known to induce 
differentiation, suprabasal marker expression, and small in 
creases in Ca²⁺ in normal cells (24, 46). As shown in Fig. 6, all 
cells responded similarly, starting at a basal Ca²⁺ value of 20–25 
nM, rising transiently to 70–150 nM (the serum-induced peak), 
and reaching a plateau of around 40 nM by 32 min. The lower 
plateau value for these cells compared to cells in Fig. 1 indicates 
the dependence of the sustained plateau value on the concen 
tration of extracellular Ca²⁺ independent of the v-ras¹⁶ gene.

Neoplastic line 308 retained sensitivity to the serum-induced 
 transient Ca²⁺ peak after 24 h in 1.2 mM Ca²⁺, whereas normal 
keratinocytes lost this response. Fig. 7 indicates that v-ras¹⁶ 
keratinocytes mimic the normal cell response. In this experi-

h, Ca²⁺ is not directly reducing the serum response. Available 
intracellular stores of Ca²⁺ are similar at 8 and 24 h in normal 
cells in 1.2 mM Ca²⁺ (46), suggesting that the change is in the 
serum signaling pathway. In contrast to the normal cell re 
sponse, 308 cells do not lose the transient response to serum 
after 24 h in 1.2 mM Ca²⁺ (Fig. 4B), although the magnitude 
may be reduced. The persistence of the serum-stimulated Ca²⁺ 
transient in 308 cells is consistent with their continual proli 
feration in 1.2 mM Ca²⁺ medium and suggests that these cells 
maintain receptor-mediated signaling responses in 1.2 mM Ca²⁺ 
media. In multiple studies with SP-1 cells (not shown) the 
response is reduced or absent in most cells after 24 h in 1.2 mM 
Ca²⁺, consistent with their reduced growth rate in high Ca²⁺.

Normal and v-ras¹⁶ Keratinocytes Have Identical Ca²⁺ Responses to Increased Ca²⁺. When the v-ras¹⁶ gene is introduced 
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Fig. 6. Expression of the v-ras¹⁶ gene does not affect the early Ca²⁺ response 
to serum-containing medium with 0.12 mM Ca²⁺. Experimental conditions were 
identical to those described in Fig. 5 except that cells were switched from 0.05 to 
0.12 mM Ca²⁺. ■, v-ras¹⁶; ▲, v-neo'; Φ, mock-infected.
ment basal values of 40–55 nM were measured after 24 h in 1.2 mM Ca\(^{2+}\) (zero time). A switch to fresh medium with 1.2 mM Ca\(^{2+}\) did not cause a substantial transient rise in Ca\(_i\) for either of the virally transduced cells and did not change the plateau value. Thus for all of these parameters of Ca\(_i\) response, v-ras\(^{ha}\) keratinocytes were similar to normal cells and different from neoplastic line 308.

The normal profile of Ca\(_i\) response to v-ras\(^{ha}\) keratinocytes suggested that these cells might be blocked distal to the Ca\(_i\) response with regard to the induction of differentiation. To determine whether these cells could respond to an increase in Ca\(_i\), ionomycin was used to pharmacologically increase Ca\(_i\). Ionomycin (6.5 \(\mu\)M) causes a sustained increase in Ca\(_i\) of approximately 8–10-fold over basal values in either 0.05 or 1.2 mM Ca\(^{2+}\) media (not shown). Ionomycin (6.5 \(\mu\)M) in 1.2 mM Ca\(^{2+}\) medium caused a 4-fold increase in cornified cells in v-ras\(^{ha}\) keratinocytes compared to 1.2 mM Ca\(^{2+}\) alone after 24 h (3.5% versus 0.9% cornified cells in the total population). This is about the same magnitude increase in cornified cells produced by ionomycin after 24 h in normal keratinocytes cultured in 1.2 mM Ca\(^{2+}\) medium (1.7% versus 0.8%) or in 0.05 mM Ca\(^{2+}\) medium (11). Thus v-ras\(^{ha}\) keratinocytes will terminally differentiate in response to pharmacologically induced increases in Ca\(_i\). SP-1 cells also cornify in response to a 24-h exposure to ionomycin and 1.2 mM Ca\(^{2+}\) medium (4.2% versus 0.5%), but 308 cells are unresponsive (0.08% with ionomycin versus 0.06% in 1.2 mM Ca\(^{2+}\)) at 24 h and show limited cornification (1% versus 0.2% at 48 h in ionomycin-containing medium.

**DISCUSSION**

We have detected aberrations in Ca\(_i\) metabolism in the benign neoplastic keratinocyte cell lines 308 and SP-1. First, these cells are particularly sensitive to serum-induced Ca\(_i\) transients, a response dependent on agonist-induced release of intracellular Ca\(^{2+}\) stores (46). In many cell types this response is mediated via phospholipase C activation and generation of inositol trisphosphate (25). Indeed, phosphatidylinositol metabolism is rapidly stimulated in both normal and neoplastic keratinocytes by a switch from 0.05 to 1.2 mM Ca\(^{2+}\) and by serum (11, 12). Furthermore, basal levels of inositol phosphates are 2–3-fold higher in 308 and SP-1 cells compared to normal keratinocytes, and stimulation by Ca\(^{2+}\) causes even further increases in an additive manner. These higher concentrations of inositol phosphates after Ca\(^{2+}\) or serum induction in neoplastic cells could be related to the higher Ca\(_i\) in neoplastic cells stimulated by serum. It is possible that the intracellular effector site responsible for release of Ca\(_i\) in response to a change in inositol phosphates is sensitized in neoplastic cells (26).

The Ca\(_i\) response to serum was maintained in 308 cells after 24 h in 1.2 mM Ca\(^{2+}\) medium, but the serum-induced Ca\(_i\) transient was lost in normal cells after 24 h. SP-1 cells were variable with some cells retaining the serum response and most cells losing the response. This corresponds to the growth potential of each cell type in 1.2 mM Ca\(^{2+}\). Several reports have indicated that differentiation of normal keratinocytes is associated with the loss of epidermal growth factor binding in vivo and in vitro (27–29). In studies to be reported separately, we have shown that epidermal growth factor is not the serum factor responsible for the transient stimulation of Ca\(_i\) by serum in normal cells. However, Ca\(_i\)-mediated changes in epidermal growth factor receptors may reflect other modifications in cell surface receptors associated with normal keratinocyte differentiation which may be altered in some neoplastic cells (30).

A third alteration in Ca\(_i\) regulation measured in 308 and SP-1 cells is the inability to sustain an elevated steady-state Ca\(_i\) plateau and above basal values when Ca\(_i\) is increased. Increased Ca\(_i\) could be important in terminal differentiation since ionomycin plus 1.2 mM Ca\(^{2+}\) raises Ca\(_i\) substantially and enhances differentiation in normal cells and v-ras\(^{ha}\) cells. SP-1 cells and to a lesser extent 308 cells are also sensitive to ionomycin-induced terminal differentiation as measured by an increase in cornified cells. In all of these cell types, the pharmacological elevation of Ca\(_i\) probably activates transglutaminase activity to cause the cornification response (20). Ionomycin may have other effects which are relevant to the keratinocyte differentiation response aside from or derived from its Ca\(^{2+}\)-mobilizing action. For example, ionomycin is a potent stimulator of phosphatidylinositol metabolism in keratinocytes (11) raising intracellular inositol phosphates and diacylglycerol rapidly and for a long period. These observations suggest that ionomycin or other Ca\(^{2+}\) ionophores could have antitumor activity in a squamous tumor model through the activation of transglutaminase or associated pathways.

Since plateau phase Ca\(_i\) is dependent on Ca\(_o\), the inability of 308 and SP-1 cells to sustain an increased Ca\(_i\) suggests an alteration in Ca\(^{2+}\) transport (influx and efflux) across the plasma membrane has occurred in neoplastic cells. Virtually nothing is known of the channels which regulate Ca\(^{2+}\) flux in keratinocytes. Most studies on Ca\(^{2+}\) transport have been performed on excitable cells, fibroblasts, or hematopoietic cells (31). Several studies have indicated that intracellular Ca\(^{2+}\) binding proteins, some of which are unique to tumor cells, may regulate Ca\(_i\) in addition to transport mechanisms (32, 33). However, binding proteins have not been identified in normal or neoplastic keratinocytes. Using calcium channel blockers, our recent studies suggest that verapamil can inhibit epidermal differentiation and Ca\(^{2+}\) transport (not shown). Future work will be directed to the study of verapamil-sensitive channels in neoplastic cells.

Bovine parathyroid cells, like keratinocytes, are very sensitive to Ca\(_o\) (34, 35). In response to graded increases in Ca\(_o\), parathyroid cells incrementally increase Ca\(_i\) and regulate secretion of parathyroid hormone (34). Strict Ca\(_i\) responsiveness is a logical characteristic for parathyroid cells which regulate serum Ca\(^{2+}\). The reasons for the similarities in the Ca\(_i\) response of parathyroid and epidermal cells to changes in Ca\(_o\) are unknown; nevertheless, the study of Ca\(_i\) responses in parathyroid adenoma
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Cell lines in comparison to SP-1 and 308 cells might establish a common profile for changes in Ca2+ regulation which are characteristic of neoplastic change in Ca0-sensitive cells. The mutated ras allele present in 308 and SP-1 cells is believed to be central in their neoplastic phenotype. ras gene mutations can alter Ca0 metabolism in 3T3 cells. For example, the ras oncogene blunts the Ca0 transient seen after exposure of 3T3 cells to serum or platelet-derived growth factor (36, 37). Expression of the ras oncogene also stimulates phosphatidylinositols turnover in 3T3 cells, generating inositol phosphates (38–40). However, our studies with v-ras ras keratinocytes suggest that the ras oncogene is not responsible for the changes in Ca0 responses of neoplastic cell lines 308 and SP-1. v-ras ras keratinocytes do not differ from normal cells in the magnitude of the serum-induced transient, the sustained plateau phase of the Ca0 response, or the loss of serum responsiveness after 24 h in 1.2 mM Ca0. Yet these cells are neoplastic and resistant to Ca0-induced differentiation. v-ras ras keratinocytes also have elevated basal phosphatidylinositol metabolism equivalent to the values of 308 and SP-1 cells (13). Thus, sustained increases in phosphatidylinositol metabolism alone do not explain altered Ca0 regulation in neoplastic keratinocyte cell lines.

There are biological differences between v-ras keratinocytes and 308 or SP-1 cells. For example v-ras ras keratinocytes do not terminally differentiate in 1.2 mM Ca0, but their proliferation rate is reduced to very low levels, a response also characteristic of normal cells (6). In contrast, both 308 and SP-1 cells continue to proliferate in 1.2 mM Ca0, although the labeling index is reduced for SP-1 cells and increased for 308 cells (19). These results suggest that the loss of the transient serum response and a sustained increase in plateau phase or steady-state Ca0 correlates to the loss of proliferation in normal epidermal cells (41, 42), and this is not changed by ras ras oncogene alone. Therefore, the development of cell lines such as 308 or SP-1 through a high-Ca0 selection protocol (19) would require an alteration in Ca0 regulation in addition to the ras gene mutation. This combination would provide both resistance to Ca0-induced terminal differentiation and sustained proliferative capability to Ca0 > 0.1 mM.

We conclude that the major alteration in Ca0 metabolism in 308 and SP-1 cells is not a direct consequence of ras gene mutation and may have been acquired in vitro. Alternatively, a subpopulation of variant cells may have existed in the original isolates from initiated skin or tumors used to develop 308 and SP-1 cells. Such variants, which could not sustain elevated Ca0 during 1.2 mM Ca0 selection, would have a growth advantage. In any case it is interesting to note that the biochemical differences among 308, SP-1, and v-ras ras keratinocytes with regard to the regulation of Ca0 are not associated with malignant progression. All three neoplastic cell types studied were benign. The expression of the v-fos oncogene in v-ras ras keratinocytes and in 308 and SP-1 cells is sufficient to cause malignant conversion (43, 44). This model could be useful to determine whether further changes in Ca0 metabolism can distinguish benign from malignant keratinocytes.

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REFERENCES

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