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

Francis H. Kruszewski,2 Henry Hennings, Robert W. Tucker, and Stuart H. Yuspa3

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-rasH* gene are resistant to Ca2+. In normal epidermal cells, the elevation of extracellular Ca2+ (Caoo) from 0.05 to 1.2 mM causes a biphasic intracellular Ca2+ (Ca2+) 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 Ca2+. The transient peak in response 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 Ca2+ response to an increase in Ca2+. 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 Ca2+. Furthermore, 308 cells remain sensitive to the serum-induced Ca2+ transient after 24 h in 1.2 mM Ca2+. To determine whether the activating c-rasH* mutation in 308 and SP-1 cells was responsible for the altered Ca2+ regulation, a v-rasH* 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 transient peak of the Ca2+ profile in response to 1.2 mM Ca2+ was identical in normal and v-rasH*-transduced keratinocytes, and these cells lost the serum-induced transient Ca2+ peak after 24 h. Thus, the activation of the c-rasH* gene in 308 or SP-1 cells is probably not solely responsible for the altered Ca2+ response in neoplastic cell lines. Sustained physiological elevation of Ca2+ may be relevant to the loss of proliferative potential in both normal and v-rasH*-transduced keratinocytes in 1.2 mM Ca2+. In addition, v-rasH*-mediated or activated c-rasH*-modified 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 Ca2+. 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-rasH* oncogene is introduced into normal keratinocytes by a retrovirus (5–7). Since activation of the c-rasH* protooncogene is a common initiating event in skin carcinogenesis (8), resistance to Ca2+-induced differentiation and changes in the regulation of Ca2+ could be direct consequences of a rasH* gene mutation. Indeed, neoplastic keratinocyte cell lines, which express a mutated c-rasH* allele, are Ca2+ resistant and have altered intracellular Ca2+ responses to a change in Ca2+ (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 Ca2+ 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+-induced differentiation (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-rasH*-transduced epidermal cells (7, 15).

Several studies have indicated that keratinocyte differentiation is associated with a rise in Ca2+. Sharpe et al. (16) reported that Ca2+ is elevated in human keratinocytes induced to differentiate by Ca2+. In mixed colonies of differentiating and basal-like human keratinocytes, the Ca2+ is higher in the differentiating cells (16). Maclaughlin et al. (17) demonstrated a rise in Ca2+ in keratinocytes induced to differentiate by 1,25(OH)2-vitamin D3. Our laboratory has utilized personalized computer-based videomicroscopy, digital image analysis, and a fluorescent intracellular calcium probe, Fura-2, to demonstrate a rapid increase in Ca2+ in normal mouse keratinocytes stimulated by increased Ca2+. Furthermore, the Ca2+ increase was sustained and graded to the level of Ca2+, 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 Ca2+ response of normal and neoplastic keratinocytes exposed to changes in Ca2+ 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 Ca2+ 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 oncogene, primary cultures on coverslips were infected with a replication-defective retroviral vector containing the v-ras²⁶ 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-ras²⁶ 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 neo resistance gene (neo') or sham-infected in the presence of polybrene (4 μg/ml). Replica infected plates with cells on coverslips were monitored for resistance to Ca²⁺-induced differentiation to ensure that v-ras²⁶ 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-ras²⁶ retrovirus (5–7, 18) 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 c-ras²⁶ 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, McGraw Park, IL), rinsed with water, and attached to the inner surface of the plastic dish using a molten mixture of paraffin: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 fluorescence 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 of Krebs/HEPES 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⁺ and the accentuated transient response observed in line 308 are also noted in cell line SP-1 (not shown). The second component of the biphasic response is a sustained plateau of elevated Ca₂⁺ which, in normal cells exposed to 1.2 mM Ca²⁺, equilibrated at about 60–70 nM or about 2½ times the basal value (Fig. 1). This plateau persists for days, is dependent on extracellular Ca²⁺, and is proportional to the level of extracellular Ca²⁺ (not shown (46)). In contrast, the plateau value for 308 cells (and SP-1 cells) is only transiently increased after a switch to 1.2 mM Ca²⁺ and returns to basal...
CALCIUM REGULATION IN EPIDERMAL NEOPLASIA

Normal Keratinocytes

Fig. 1. A. Ca₂⁺ increase is sustained in normal primary keratinocytes exposed to 1.2 mM Ca²⁺. Normal keratinocytes were cultured at confluence in 0.05 mM Ca²⁺ medium for 6 days, loaded with Fura-2, and challenged with 1.2 mM Ca²⁺ medium. The average Caᵦ of all cells in the microscope field is plotted as a function of time after elevation of external Ca²⁺ from 0.05 to 1.2 mM. The 22-h point is the Caᵦ of cells grown in serum-containing medium with 1.2 mM Ca²⁺ for 20 h and then loaded with Fura-2 for 2 h in 1.2 mM Ca²⁺; B. Caᵦ increase in neoplastic cell line 308 is not sustained in 1.2 mM Ca²⁺ medium. Experimental conditions were as described in A.

Fig. 2. Steady-state Caᵦ values remain elevated in primary keratinocytes but not in neoplastic line 308. Cells grown in serum-containing medium with 0.05 mM Ca²⁺ (0 h) were switched to medium with 1.2 mM Ca²⁺ for 8 or 24 h. Columns, mean Caᵦ averaged from 3 experiments; bars, SE. To determine steady-state Caᵦ, cells were loaded with Fura-2 for 2 h in medium with appropriate Ca²⁺ concentration. ●, primary keratinocytes; ○, line 308.

Fig. 3. The early peak in Caᵦ in both normal and neoplastic keratinocytes is dependent on serum. Caᵦ concentrations (cell population average) are plotted as a function of time after changing the medium from 0.05 to 1.2 mM Ca²⁺ in the presence (●) or absence (○) of 8% fetal bovine serum. A, normal primary keratinocytes; B, line 308 cells.

value after 30 min to 21 h (Fig. 1). When intermediate times were studied (Fig. 2), Caᵦ 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 Ca²⁺ medium, the Caᵦ value after 30 min to 24 h (Fig. 1). When intermediate times were studied (Fig. 2), Caᵦ 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 Ca²⁺ medium, the Caᵦ in normal cells is as high or higher than the steady-state Caᵦ in 308 cells (Fig. 2).

The transient Caᵦ peak in normal and neoplastic cells is dependent on serum concentration in the medium and independent of Caᵦᵦ, as shown in Fig. 3. A transient Caᵦ increase is observed only in normal cells stimulated with serum, but elevated plateau values for Caᵦ are identical in 1.2 mM Ca²⁺ medium with and without serum (Fig. 3A). Redistribution of intracellular Ca²⁺ accounts for the serum-induced transient Caᵦ 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-Ca²⁺ medium in the absence of serum, the serum-induced transient increase in Caᵦ is not required for the differentiation response.

Exposure to 1.2 mM Ca²⁺ Medium Reduces the Stimulated Caᵦ Transient in Normal but not 308 Keratinocytes. Exposure to 1.2 mM Ca²⁺ induces terminal differentiation in normal but not neoplastic keratinocytes (23). An early response associated with differentiation is the loss of proliferative activity in the Ca²⁺-induced normal population within 24–48 h (1). Differentiating normal keratinocytes also lose the transient Caᵦ response to serum within 24 h (Fig. 4A), although the response is intact at 8 h. Since plateau values of Caᵦ are increased at both 8 and 24
CALCIUM REGULATION IN EPIDERMAL NEOPLASIA

Normal Keratinocytes

Line 308 Keratinocytes

Fig. 4. Neoplastic 308 cells, but not normal keratinocytes, retain the transient Cai 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⁺ (○) or in medium with 1.2 mM Ca²⁺ for 8 h (□) or 24 h (▲) before exposure to serum-containing medium with 1.2 mM Ca²⁺. Points, Cai cell population averages of all cells in a microscope field.

Normal and v-ras²⁶ Keratinocytes Have Identical Cai Responses to Increased Ca²⁺. When the v-ras²⁶ gene is introduced into normal keratinocytes, recipient cells have similarities to 308 and SP-1 cells including resistance to Ca²⁺-induced differentiation, 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-ras²⁶ mutations (19), v-ras²⁶ keratinocytes provided a model to test whether ras²⁶ gene activation is responsible for altered Ca²⁺ regulation in benign neoplastic keratinocytes. When v-ras²⁶, v-neo', and mock-infected cells in 0.05 mM Ca²⁺ medium were switched to 1.2 mM Ca²⁺, the Cai responses were identical (Fig. 5). The three cell types had basal Cai 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-ras²⁶ transduction alone did not reproduce the changes in Cai regulation characteristic of neoplastic keratinocyte cell lines.

To determine whether Cai responses in v-ras²⁶ cells would be altered when smaller changes in Ca²⁺ were tested, the three groups (control, v-neo', and v-ras²⁶) were switched from 0.05 to 0.12 mM Ca²⁺, a concentration of Ca²⁺ known to induce differentiation, suprabasal marker expression, and small increases in Cai in normal cells (24, 46). As shown in Fig. 6, all cells responded similarly, starting at a basal Cai 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 concentration of extracellular Ca²⁺ independent of the v-ras²⁶ gene.

Neoplastic line 308 retained sensitivity to the serum-induced transient Cai peak after 24 h in 1.2 mM Ca²⁺, whereas normal keratinocytes lost this response. Fig. 7 indicates that v-ras²⁶ keratinocytes mimic the normal cell response. In this experi-
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\(^{Hd}\) keratinocytes were similar to normal cells and different from neoplastic line 308.

The normal profile of Ca\(_i\) response to v-ras\(^{Hd}\) 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\(^{Hd}\) 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\(^{Hd}\) 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 triphosphate (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 phos-
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 changes in Ca²⁺-sensitive cells.

The mutated ras<sup>G12D</sup> allele present in 308 and SP-1 cells is believed to be central in their neoplastic phenotype. ras gene mutations can alter Ca²⁺ metabolism in 3T3 cells. For example, the ras oncogene blunts the Ca²⁺ transient seen after exposure of 3T3 cells to serum or platelet-derived growth factor (36, 37). Expression of the ras oncogene also stimulates phosphatidylinositol turnover in 3T3 cells, generating inositol phosphates (38–40). However, our studies with v-ras<sup>Ha</sup> keratinocytes suggest that the ras oncogene is not responsible for the changes in Ca²⁺ responses of neoplastic cell lines 308 and SP-1. v-ras<sup>Ha</sup> keratinocytes do not differ from normal cells in the magnitude of the serum-induced transient, the sustained plateau phase of the Ca²⁺ response, or the loss of serum responsiveness after 24 h in 1.2 mM Ca²⁺. Yet these cells are neoplastic and resistant to Ca²⁺-induced differentiation. v-ras<sup>Ha</sup> 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 Ca²⁺ regulation in neoplastic keratinocyte cell lines.

There are biological differences between v-ras keratinocytes and 308 or SP-1 cells. For example v-ras<sup>Ha</sup> keratinocytes do not terminally differentiate in 1.2 mM Ca²⁺, 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 Ca²⁺, 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 Ca²⁺ correlates to the loss of proliferation in normal epidermal cells (41, 42), and this is not changed by ras<sup>G12D</sup> oncogene alone. Therefore, the development of cell lines such as 308 or SP-1 through a high-Ca²⁺ selection protocol (19) would require an alteration in Ca²⁺ regulation in addition to the ras gene mutation. This combination would provide both resistance to Ca²⁺-induced terminal differentiation and sustained proliferative capability to Ca²⁺ > 0.1 mM.

We conclude that the major alteration in Ca²⁺ 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 Ca²⁺ during 1.2 mM Ca²⁺ 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<sup>Ha</sup> keratinocytes with regard to the regulation of Ca²⁺ are not associated with malignant progression. All three neoplastic cell types studied were benign. The expression of the v-fos oncogene in v-ras<sup>Ha</sup> 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 Ca²⁺ metabolism can distinguish benign from malignant keratinocytes.

ACKNOWLEDGMENTS

We thank Margaret Taylor for assistance with the manuscript.

REFERENCES


4211
CALCIUM REGULATION IN EPIDERMAL NEOPLASIA


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


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/51/16/4206

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.