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

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INTRODUCTION

The earliest phenotypic change detected in mouse epidermal cells initiated by chemical carcinogens is an altered response to Ca++ in vitro since normal epidermal cells are induced to differentiate in medium with Ca++ > 0.1 mM while neoplastic epidermal cells and keratinocytes transduced with a v-ras" gene are resistant to Ca++. In normal epidermal cells, the elevation of extracellular Ca++ (Ca0) from 0.05 to 1.2 mM causes a biphasic intracellular Ca++ (Ca") 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 Ca,. The transient peak in Ca0 is dependent on a serum component and independent of Ca,, while the sustained plateau is directly dependent on Ca,. The transient response to a serum factor is lost in normal cells after 24 h in 1.2 mM Ca++, 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 Ca response to an increase in Ca0. 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 Ca0. Furthermore, 308 cells remain sensitive to the serum-induced Ca0 transient after 24 h in 1.2 mM Ca++. To determine whether the activating c-ras" mutation in 308 and SP-1 cells was responsible for the altered Ca0 regulation, a v-ras" gene was transduced into normal keratinocytes by a defective retrovirus. This also produces the papilloma phenotype in vivo. Recipient cells were resistant to Ca++-induced terminal differentiation although they did not proliferate in 1.2 mM Ca++. The Ca0 profile in response to 1.2 mM Ca++ was identical in normal and v-ras" transduced keratinocytes, and these cells lost the serum-induced transient Ca0 peak after 24 h. Thus, the activation of the c-ras" gene in 308 or SP-1 cells is probably not solely responsible for the altered Ca0 response in neoplastic cell lines. Sustained physiological elevation of Ca0 may be relevant to the loss of proliferative potential in both normal and v-ras" keratinocytes in 1.2 mM Ca++. In addition, v-ras"-mediated or activated c-ras"-mediated changes in a complementary pathway may contribute to the block in terminal differentiation in neoplastic cells.

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 Ca0 Analysis. Primary BALB/c keratinocytes were isolated as previously described (1). Cells were initiated event in skin carcinogenesis (8), resistance to Ca++-induced differentiation and changes in the regulation of Ca0 could be direct consequences of a ras" gene mutation. Indeed, neoplastic keratinocyte cell lines, which express a mutated c-ras" allele, are Ca++ resistant and have altered intracellular Ca++ responses to a change in Ca0 (9). Furthermore, ras-related proteins Saccharomyces cerevisiae have been directly implicated in the control of Ca++ transport (10).

Biochemical data are consistent with a linkage among ras gene mutations, epidermal neoplasia, and altered Ca++ metabolism. In both normal and neoplastic keratinocytes, an increase in Ca0 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 Ca(45). Keratinocytes transduced with a v-ras" gene by a replication-defective retrovirus also have substantial elevations of these second messengers (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 Ca++-induced differentiation-specific genes such as keratins 1 and 10, filaggrin and loricrin (14). The Ca++-regulated expression of these genes is also inhibited in chemically initiated keratinocytes and v-ras"-transduced epidermal cells (7, 15).

Several studies have indicated that keratinocyte differentiation is associated with a rise in Ca0. Sharpe et al. (16) reported that Ca0 is elevated in human keratinocytes induced to differentiate by Ca++. In mixed colonies of differentiating and basal-like human keratinocytes, the Ca0 is higher in the differentiating cells (16). Maclaurin et al. (17) demonstrated a rise in Ca0 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 Ca0 in normal mouse keratinocytes stimulated by increased Ca0. Furthermore, the Ca0 increase was sustained and graded to the level of Ca0, 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 Ca0 response of normal and neoplastic keratinocytes exposed to changes in Ca0, and to explore the consequences of the expression of a ras oncogene in these responses.

Received 1/17/91; accepted 5/31/91.

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1 This research was supported in part by a grant from the Proctor and Gamble Company.

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4 The abbreviations used are: Ca0, extracellular Ca++; Ca, intracellular Ca++; Fura-2/AM, Fura-2 acetoxymethylester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

5 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 Ca0 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 oncoprotein, primary cultures on coverslips were infected with a replication-defective retroviral vector containing the v-rasα 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-rasα 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 plates with cells on coverslips were monitored for resistance to Ca2+-induced differentiation to ensure that v-rasα 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-rasα 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-rasα 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 Tien 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 (Ditirc Optics, Hudson, MA), and emitted light was collected through a broad-band filter (500 nm; bandpass of 40 nm) (Ditirc 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/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+ 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

Line 308 Keratinocytes

Figure 1. A. Ca, 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 Ca2+ 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 Ca2+ 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. Ca2+ increase in neoplastic cell line 308 is not sustained in 1.2 mM Ca2+ medium. Experimental conditions were as described in A.

Figure 2. Steady-state Ca2+ 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 Ca2+ averaged from 3 experiments; bars, SE. To determine steady-state Ca2+ cells were loaded with Fura-2 for 2 h in medium with appropriate Ca2+ concentration. †, primary keratinocytes; ‡, line 308.

Figure 3. The early peak in Ca2+ in both normal and neoplastic keratinocytes is dependent on serum. Ca2+ 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.

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

Exposure to 1.2 mM Ca2+ Medium Reduces the Stimulated Ca2+ 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 Ca2+ response to serum within 24 h (Fig. 4A), although the response is intact at 8 h. Since plateau values of Ca2+ are increased at both 8 and 24 value after 30 min to 21 h (Fig. 1). When intermediate times were studied (Fig. 2), Ca2+ 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 Ca2+ in normal cells is as high or higher than the steady-state Ca2+ in 308 cells (Fig. 2).
Normal and v-ras<sup>Ha</sup> Keratinocytes Have Identical Ca<sub>i</sub> Responses to Increased Ca<sub>o</sub>. When the v-ras<sup>Ha</sup> gene is introduced into normal keratinocytes, recipient cells have similarities to 308 and SP-1 cells including resistance to Ca<sup>2+</sup>-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-ras<sup>Ha</sup> mutations (19), v-ras<sup>Ha</sup> keratinocytes provided a model to test whether ras<sup>Ha</sup> gene activation is responsible for altered Ca<sup>2+</sup> regulation in benign neoplastic keratinocytes. When v-ras<sup>Ha</sup>, v-neo', and mock-infected cells in 0.05 mM Ca<sup>2+</sup> medium were switched to 1.2 mM Ca<sup>2+</sup>, the Ca<sub>i</sub> responses were identical (Fig. 5). The three cell types had basal Ca<sub>i</sub> 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-ras<sup>Ha</sup> transduction alone did not reproduce the changes in Ca<sub>i</sub> regulation characteristic of neoplastic keratinocyte cell lines.

To determine whether Ca<sub>i</sub> responses in v-ras<sup>Ha</sup> cells would be altered when smaller changes in Ca<sub>o</sub> were tested, the three groups (control, v-neo', and v-ras<sup>Ha</sup>) were switched from 0.05 to 0.12 mM Ca<sup>2+</sup>, a concentration of Ca<sub>o</sub> known to induce differentiation, suprabasal marker expression, and small increases in Ca<sub>i</sub> in normal cells (24, 46). As shown in Fig. 6, all cells responded similarly, starting at a basal Ca<sub>i</sub> 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 Ca<sup>2+</sup> independent of the v-ras<sup>Ha</sup> gene.

Neoplastic line 308 retained sensitivity to the serum-induced transient Ca<sub>i</sub> peak after 24 h in 1.2 mM Ca<sup>2+</sup>, whereas normal keratinocytes lost this response. Fig. 7 indicates that v-ras<sup>Ha</sup> 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, for either of the virally transduced cells and did not change the plateau value. Thus for all of these parameters of Ca, response, v-

Fig. 7. Expression of the v-ras^{H} gene does not enhance the response of primary keratinocytes to serum after a 24-h exposure to 1.2 mM Ca^{2+}. Normal primary keratinocytes expressing v-ras^{H} (■), v-neo (▲), or mock-infected cells (▲) were maintained in 0.05 mM Ca^{2+} + 8% fetal bovine serum until day 8. After a 22-h exposure to serum-containing medium with 1.2 mM Ca^{2+}, the cells were loaded with Fura-2 for 2 h and rechallenged with serum containing medium with 1.2 mM Ca^{2+}.

DISCUSSION

We have detected aberrations in Ca, metabolism in the benign neoplastic keratinocyte cell lines 308 and SP-1. First, these cells are particularly sensitive to serum-induced Ca, 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 phos-
CALCIUM REGULATION IN EPIDERMAL NEOPLASIA

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 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 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 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 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 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 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 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 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


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