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Buffering of Intracellular Calcium in Response to Increased Extracellular Levels in Mortal, Immortal, and Transformed Human Breast Epithelial Cells

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Abstract Extracellular levels of calcium at 1.05 mM or higher induce terminal differentiation and senescence in the mortal (MCF-10M) line of human breast epithelial cells, but does not retard the growth or induce differentiation in the immortal (MCF-10A) and oncogene transformed (MCF-10AneoT) lines. Intracellular levels of calcium and inositol triphosphate were determined in MCF-10M, MCF-10A, and MCF-10AneoT, under conditions of low and high extracellular calcium. We hereby report that increases in extracellular calcium is translated into significant increases in intracellular levels of calcium and inositol triphosphate in MCF-10M, but not in MCF-10A and MCF-10AneoT. This difference in the apparent calcium buffering capacity between the mortal and the immortalized human breast epithelial cells could account for the latter's unperturbed growth potential in high extracellular calcium environment.

Key words: terminal differentiation, senescence, C-Ha-ras oncogene, growth regulation, inositol triphosphate

The growth of human breast epithelial (HBE) cells in culture is strongly regulated by the concentration of calcium in the culture medium [1]. These cells grow and can be subcultured for up to one year if the range of Ca^{2+} concentration in the culture medium is 0.03 to 0.06 mM [1,2]. In this culture condition, the majority of the cells assume a spherical morphology, produce duct-like structures in collagen, display all the ultrastructural features of breast epithelial cells, and maintain their normal diploid karyotype [2–4]. If the calcium concentration in the medium is elevated to 1.05 mM or above, the cells change their morphology to mainly elongated and flattened cells which form tight junctions and domes at confluence [3,5]. This change takes place as early as 5 h after the switch from low to high calcium medium and the cells finally undergo terminal differentiation and stop dividing [2,3]. The calcium induced changes in growth properties has also been reported in other epithelial cells derived from epidermis, bronchus, and esophagus [7–10].

To adequately understand the role of Ca^{2+} in the growth properties of HBE cells, in addition to the mortal cells, we have used a cell line MCF-10A, which was spontaneously immortalized from the mortal human diploid breast epithelial cells [2,5], and its c-Ha-ras oncogene transformed subline (MCF-10AneoT) [6]. Whereas the growth of MCF-10M is negatively regulated by high levels of Ca^{2+} in the medium, MCF-10A and MCF-10AneoT are able to tolerate high levels of calcium [2,5,6]. Since most of the growth control mechanisms influenced by calcium occur in the intracellular milieu and involve other calcium signaling molecules [20], analyses were undertaken to determine whether or not there were differences in the buffering of intracellular calcium levels (Ca_i) as extracellular calcium levels (Ca_o) were changed in the mortal (MCF-10M), immortal (MCF-10A) and transformed (MCF-10AneoT) HBEC lines. Levels of intracellular inositol triphosphate (IP_3), the calcium mobilizing molecule [20], were also determined as a function of the extracellular levels of calcium.

MATERIALS AND METHODS

Cells

The mortal human breast epithelial cells (MCF-10M) were obtained from reduction mam-

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moplasty using methods described elsewhere [11]. The origin and development of MCF-10A has been described [2,5]. This cell line was transformed with the human T-24 mutated c-Ha-ras oncogene to yield the line MCF-10AneoT [6]. The three cell lines were maintained in DMEM/F12 medium containing chelexed 5% equine serum and either 0.04 mM (low calcium medium) or 1.05 mM (high calcium) Ca^{2+} as previously described [3,6].

Intracellular Calcium Measurements

Intracellular calcium levels of attached cells were determined using ACAS-470 (Meridian Instruments, Inc., Okemos, MI) as described [12] with minor modifications. Briefly, the cells were grown in glass coverslips in low calcium medium until 60–80% confluence. The cells were then loaded with 1 μM Indo-1, washed with PBS (2×), and the coverslips were placed in a holder with 1 ml of serum free DMEM/F-12 medium containing 0.04 mM Ca^{2+}. A relative calcium concentration map was obtained after which the medium was replaced with fresh 1 ml of serum free medium containing 1.05 mM Ca^{2+}. After 30 min, another concentration map was obtained. To quantitate intracellular calcium values, the cells (60–80% confluent) were either grown in low calcium medium (day 0) or in high calcium medium (day 1–3) or grown for 3 days in high calcium medium and then returned for 24 h in low calcium medium. The cells were trypsinized, washed with buffer, and loaded with 10 μM Fura-2AM as described by Liao et al. [13], with minor modifications: the buffer contained either 0.04 mM Ca^{2+} (day 0) or 1.05 mM Ca^{2+} (days 1–3). Fluorescence (F) was monitored at 510 nm with SLM 8000-C photon counting spectrophotometer (SLM Instruments Urbana, IL) with excitation wavelength of 340 nm. The Ca_i was calculated as described [14].

Measurement of Intracellular Inositol Triphosphate

The cells at 60–80% confluence were trypsinized and washed in PBS. They were then mixed with 0.2 volume of ice cold 20% perchloric acid and kept on ice for 20 min. Proteins were precipitated by centrifugation at 2,000g for 15 min at 4°C. The supernatants were adjusted to pH 7.5 with 5N KOH and kept ice cold. Precipitated potassium perchlorate was removed by centrifugation. Inositol triphosphate in the supernatants was determined by radioassay using a commercial kit (Amersham, Arlington Heights, IL). Total protein was determined using Biorad protein assay kit (Biorad Lab, Richmond, CA) and the results expressed as pmoles/mg protein.

RESULTS

The relative calcium concentration maps using indo-1 fluorescence dye revealed that the mortal human breast epithelial cells do not effectively buffer their Ca_i against elevated levels of Ca_o, as shown in Figure 1. In five separate determinations, elevation of Ca_o from 0.04 (Fig. 1A) to 1.05 mM (Fig. 1D) resulted in elevated and sustained increases in Ca_i for at least 30 min, as evidenced by Figure 1. The immortal and transformed HBE cells, on the other hand, did not show observable increases in their Ca_i subsequent to the elevation of Ca_o to 1.05 mM, as shown in Figure 1B and 1E and 1C and 1F, respectively.

These Ca_i concentration changes depicted as functions of Ca_o were confirmed and quantitated by fluorescence spectroscopy using fura-2AM. Data from three separate experiments showed that the Ca_i of both mortal and immortal HBE cells growing in medium containing 0.04 mM was maintained at a very low concentration (approximately 20 nM). However, if the extracellular calcium was increased to 1.05 mM, the Ca_i increased 2- to 3-fold in the mortal cells, as depicted in Figures 2 and 3. The immortal and oncogene transformed cell lines on the other hand did not show significant changes in their Ca_i following the elevation of Ca_o from 0.04 to 1.05 mM, as shown in Figure 2. The Ca_i of both MCF-10A and MCF-10AneoT was maintained at around 20 nM regardless of the Ca_o levels. To test whether or not the increased Ca_i in the mortal cells was reversible, cells that had been grown in high calcium medium for 3 days were switched back to low calcium for 24 h. In two separate experiments we showed that Ca_i in MCF-10M cells dropped precipitously subsequent to a switch back to low calcium medium, as shown in Figure 3. The immortal and transformed cells, on the other hand, did not show a significant difference in their Ca_i levels after the switch back to low calcium medium as expected. However, in two out of three separate experiments, the Ca_i of the transformed cells increased following the switch from high to low calcium medium (Fig. 3).

Switching of the mortal cells from low to high calcium medium resulted not only in substantial

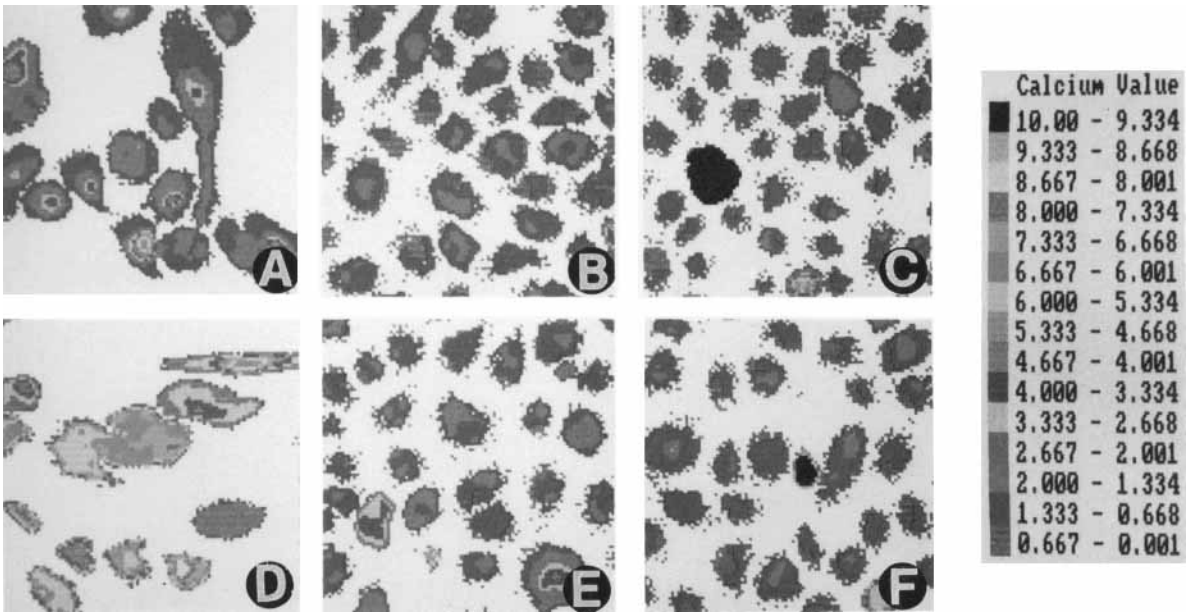

Fig. 1. Relative levels of Ca_i in HBEC expressed as a function of Ca_o. Levels of Ca_i in MCF-10M, MCF-10A, and MCF-10AneoT in medium containing 0.04 mM Ca^{2+} are shown in **A, B, and C,** whereas Ca_i levels 30 min after the switch to medium containing 1.05 mM Ca^{2+} are shown in **D, E, and F,** respectively.

and significant increase in Ca_i, but also in significant increases in inositol triphosphate, as depicted in Figure 4. The sustained increases in intracellular IP_3 closely matched Ca_i increases over the three-day period subsequent to the switch to high calcium medium. The intracellular levels of IP_3 in the immortal and transformed HBE cells, on the other hand, did not change significantly, but remained more or less at the basal level (Fig. 4).

DISCUSSION

The data reported herein show that there is a marked difference in the manner in which mortal HBE cells maintain Ca_i compared with their immortal counterparts when Ca_o is increased. It is well established that nearly all cell types maintain their Ca_i at a very low concentration compared with Ca_o [for review, see 15]. To do this, cells have evolved elaborate calcium buffering

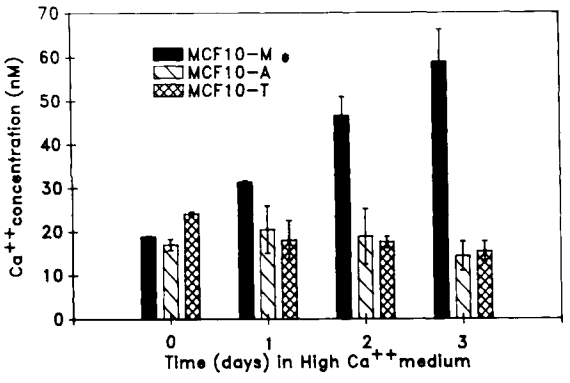

Fig. 2. Quantitation of Ca_i in MCF-10M, MCF10A, and MCF-10AneoT. The cells were grown in medium containing 0.04 mM calcium and then switched to medium containing 1.05 mM Ca^{2+} for the specified periods. The cells were trypsinized, washed, and loaded with fura-2A for Ca_i as described in Materials and Methods (*$P < 0.001$).

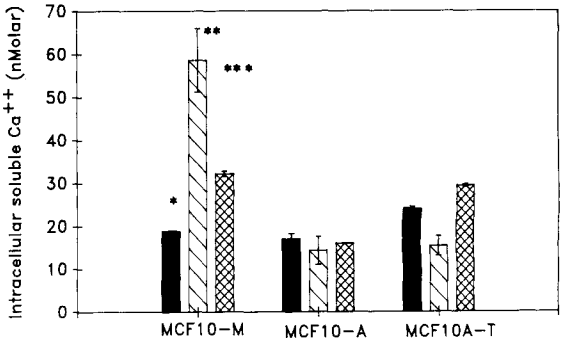

Fig. 3. Reversibility of Ca_i increases in MCF-10M, MCF-10A, and MCF-10AneoT in response to low and high Ca_o. The cells were grown in medium containing 0.04 mM Ca^{2+} (solid bars) or 1.05 mM Ca^{2+} (left hatched bars) for 3 days before harvesting. Part of the cells that had been grown in medium containing 1.05 mM Ca^{2+} for 3 days were replated in 0.04 mM Ca^{2+} medium for 24 h, trypsinized, washed, and Ca_i was determined (cross-hatched bars) (* vs. **$P < 0.001$; ** vs. ***$P < 0.001$).

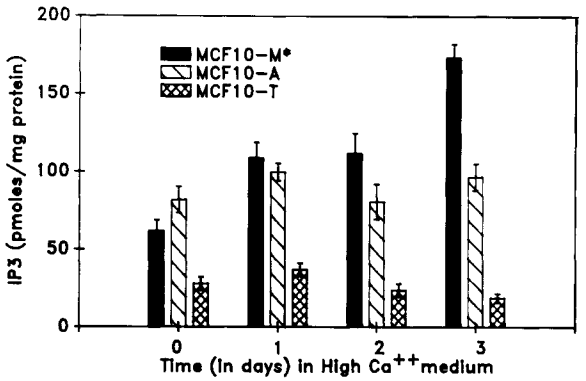

Fig. 4. Quantitation of intracellular IP_3 in MCF-10M, MCF-10A, and MCF-10AneoT. The cells were grown in medium containing 0.04 mM Ca^{2+} and then switched to medium containing 1.05 mM Ca^{2+} for the specified periods. The cells were then trypsinized, washed with PBS, and IP_3 was determined as described in the text (*$P < 0.001$).

systems. These include high affinity calcium binding proteins such as calmodulin [16] and Ca-ATPase pump, which actively pumps calcium out from the cells against a calcium concentration gradient [17]. Opposed to these calcium buffering and export systems are the calcium influx pathways which increase intracellular calcium such as the putative calcium channels [15,18], Na^+/Ca^{2+} exchanger which drives Ca^{2+} into the cells as Na^+ is driven out [19] and second messenger molecules such as IP_3, which mobilize calcium from its intracellular stores [20].

Any of these calcium buffering or influx pathways may be modified in either the mortal or immortal human breast epithelial cells to account for the differential calcium buffering capacity in these cells. Calcium efflux studies (data not shown) indicated no difference in the rate at which ^{45}Ca is extruded from MCF-10M, MCF-10A, and MCF-10AneoT cells subsequent to increasing extracellular calcium. Without ruling out the possible modification of the calcium efflux pathways, our present data tend to favor the calcium influx pathways as the ones crucial for the significant increases in Ca_i in the mortal cells. The fact that Ca_i increases in mortal cells is closely correlated to increases in IP_3, raises the possibility that increases in extracellular calcium activate phospholipase C, resulting in the hydrolysis of phosphoinositol 4,5 bisphosphate phosphate (PIP2) to diacylglycerol and IP_3 [20–22]. The increased IP_3 may, in turn, activate the so called second messenger operated Ca^{2+} channels resulting in calcium influx from the external milieu [23]. It has been shown that the bombesin induced mobilization of IP_3 and calcium in the human breast cancer cell line MCF-7 partly depends on the extracellular calcium since the calcium increases could be inhibited by the calcium channel blocker Ni^{2+} [24].

These data show that the ability of calcium to influence in a negative way the growth of normal human breast and other cells of epithelial origin may be solely due to their inability to buffer Ca_i against increases in Ca_o. The calcium mediated induction of terminal differentiation of MCF-10M requires sustained increases in Ca_i. The increased Ca_i may then activate a series of enzymes which have been implicated in terminal differentiation such as protein kinase C [21]. The increased intracellular calcium may also activate Ca^{2+}-Mg^{2+}-dependent endonuclease, the enzyme system responsible for the fragmentation of cellular DNA leading to programmed cell death [25–27]. Studies directed at various aspects of calcium involvement in terminal differentiation and senescence in HBE cells including programmed cell death are currently being carried out in our laboratory. A detailed knowledge of these mechanisms will lead to a better understanding of growth regulation of human breast epithelial cells both in vitro and in vivo.

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