

CALCIUM CONTENT AND DISTRIBUTION AS A FUNCTION OF GROWTH AND TRANSFORMATION IN THE MOUSE 3T3 CELL

JOSEPH T. TUPPER and FLAVIA ZORNIOTTI

From the Department of Biology, Syracuse University, Syracuse, New York 13210

ABSTRACT

Total Ca content and that fraction of Ca sensitive to removal by the chelator ethylene glycol-bis(β -aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA) have been investigated in the mouse 3T3 cell as a function of growth stage, transformation with SV40 virus, and serum levels of the media. Cells were allowed to grow through several doublings in media containing ^{45}Ca . The cellular content of ^{45}Ca was used to assess total cell Ca. That fraction of ^{45}Ca removed by EGTA was presumed to represent primarily surface-localized Ca. The data are expressed on a per cell volume basis to compensate for size differences as a function of growth stage and transformation. During exponential growth phase, the 3T3 cell contains 525 pmol Ca/ μl cell volume. Of this, approx. 457 pmol/ μl is not removable by EGTA and, presumably, is cytoplasmically located. This value is in close agreement with previous studies on the HeLa cell (470 pmol Ca/ μl cell water after removal of the surface Ca). The low level of EGTA-removable Ca present in the 3T3 cell during early exponential growth (68 pmol Ca/ μl cell volume) increases progressively with increasing cell density, and upon quiescence it is sevenfold greater. In contrast, SV40-transformed 3T3 cells growing exponentially possess total levels of Ca which are approximately two-thirds the levels of the normal 3T3 cell. However, their EGTA-sensitive Ca is not significantly different from that of exponentially growing, normal 3T3 cells. As the transformed cells continue to grow at high density, their total Ca and their sensitivity to EGTA do not change, in contrast to the normal 3T3 cell. Thus, an increase in Ca associated with the cell surface appears to be correlated with growth inhibition. This has been investigated further by regulating growth of the normal and transformed cell with alterations in the serum levels of the media. In 4% calf serum the normal cell is stopped from continued proliferation. Growth stoppage under these conditions is characterized by a nearly fourfold increase in EGTA-removable Ca, similar to the increase observed upon quiescence in depleted 10% serum. Similar treatment of the transformed cell does not reduce its growth rate, nor does it significantly alter Ca distribution. However, at 0.5% medium serum levels, the SV40 3T3 growth rate is substantially reduced and, under these conditions, EGTA-removable Ca increases twofold.

KEY WORDS calcium · mouse 3T3 cells · transformation · calcium transport

Calcium ion is required for continued cellular proliferation of a wide variety of cell types (for re-

views, see references 19 and 20). This requirement differs in certain normal vs. virally transformed cells carried in *in vitro* culture. Both SV40-transformed mouse 3T3 cells and Rous sarcoma virus-infected chicken fibroblasts require lower levels of extracellular Ca for continued proliferation as compared to their nontransformed counterparts (1, 9). It has also been demonstrated that elevation of extracellular Ca alone, under certain conditions, can induce quiescent mouse 3T3 cells to enter a proliferative state and initiate DNA synthesis (8, 11). Calcium and serum act synergistically in this regard, suggesting that serum stimulation of growth may be mediated, in part, through changes in cellular Ca content or localization (11). Both Ca and serum have effects on intracellular cyclic nucleotide levels, the latter having been suggested as primary factors in growth regulation (for reviews, see references 19 and 21). Furthermore, it has been suggested that divalent cations such as Ca and Mg mutually regulate growth and that the effects of Ca on cellular proliferation may reflect an interaction of Ca with Mg-binding sites (16). It has also been observed that, as with Ca, Mg is required for entrance into DNA synthesis and that the amount of Mg required is related to the medium serum levels (13).

These observations strongly suggest that divalent cations have a primary role in growth regulation and that this role involves a synergism between Ca and/or Mg and serum. The differential requirements of certain normal vs. transformed cells for Ca (1, 9) as well as serum (2, 12) suggest that an alteration in the synergism between serum and divalent cations may be involved in the loss of growth regulation *in vitro* and *in vivo*. To assess this possibility, we have investigated Ca content and distribution between the cell surface and the intracellular environment in the normal and SV40-transformed mouse 3T3 cell, using a Ca chelator as a marker for surface Ca. We have also manipulated the growth of both cell types by alteration of the serum levels of the media and have evaluated the effect of this treatment on the various Ca parameters. The data indicate that the normal vs. transformed 3T3 cells differ as regards total Ca content and that the distribution of this Ca changes as normal cells enter a quiescent state. Furthermore, we observe that serum is able to modulate certain facets of cellular Ca.

MATERIALS AND METHODS

Mouse BALB/c 3T3 cells (clone A31) were obtained

from the American Type Culture Collection (Rockville, Md.). The SV40-transformed counterpart was the generous gift of Dr. George Poste (Roswell Park Memorial Institute, Roswell Park, N. Y.). Stock lines were grown in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N. Y.) and gentamicin (50 $\mu\text{g}/\text{ml}$) in a 5% CO_2 -air mixture at 37°C. During the course of the experiments reported here the normal 3T3 cell, when plated at 4.8×10^3 cells/cm² in 3 ml of medium, routinely became quiescent at $1.4\text{--}1.8 \times 10^4$ cells/cm² on a 21-cm² growth surface. Cells to be used for experimental purposes were generally grown in 60-mm plastic dishes.

A variety of procedures were evaluated for the assay of ⁴⁵Ca content and distribution as a function of growth and transformation (see Results). The following procedures were found to prove both most reproducible and efficient.

To monitor total cell Ca and surface localized Ca (as defined as that fraction of Ca removed by the chelator (ethylene glycol-bis[β -aminoethyl ether]*N,N,N',N'*-tetraacetate [EGTA])), cells were grown in ⁴⁵Ca over various time intervals. Uptake experiments indicated that within 24 h the bulk of cellular ⁴⁵Ca had exchanged with ⁴⁵Ca, thus verifying the use of isotope detection as a monitor of total cell Ca (see Results).

To monitor calcium as a function of growth and transformation, the cells were plated at 1×10^3 cells per 60-mm plate in 3 ml of MEM plus 10% calf serum. At this time, enough ⁴⁵Ca was added to yield approx. 2 μCi ⁴⁵Ca/ml (final specific activity). The isotope was added in a 100- μl aliquot of filter-sterilized, serum-free MEM. For experiments in which the level of medium serum was to be changed to modulate growth of the cells, different plating procedures were followed. Reduction of serum levels would halt or slow continued proliferation under certain conditions. Thus, it was necessary to adjust the initial plating densities to assure that enough cells would be present to allow assay of ⁴⁵Ca content. For plates in which cell growth would be slowed, initial plating densities were elevated such that at the time of ⁴⁵Ca assay, the density of the cells on the plate was comparable to that of the growing cells. For these experiments, all cells were initially plated in MEM plus 10% calf serum. Approx. 24 h later, this medium was removed from the plates and replaced with medium of equivalent or lowered serum levels (see Results). At this time, ⁴⁵Ca was added to all plates as described above.

To determine total or EGTA-removable Ca, the following procedure was utilized. The growth medium was removed, and the plate was washed with 5 ml of 200 mM choline chloride, 1 mM Tris-OH, pH 7.4 with HCl or this solution plus 1 mM disodium-EGTA. The 5 ml was added by directing the stream of fluid against the side of the dish, using an automated pipetting device. The plate was washed with a second 5-ml aliquot of the appropriate solution, and this was discarded. A third 5-ml aliquot was added, and the cells were removed from the dish

with a rubber policeman and placed on a glass fiber filter in a multi-well vacuum system. The wash solution was removed under vacuum, and the cells were washed a fourth time. The rubber policeman was rinsed between successive plates with EGTA. The filters were then assayed for ^{45}Ca content (see below). The procedure requires high ^{45}Ca specific activities due to the relatively low Ca content of the cells and the low number of cells present during early growth phases. This, coupled with the fact that Ca binds rather tenaciously to the culture dishes, makes care in the washing procedures a critical aspect of the assay.

The filters were counted in 5 ml of a 2,5-diphenyloxazole (PPO), 1,4-bis[2(5-phenyloxazolyl)]benzene (PO-POP) based counting solution containing Triton X as a solubilizer. The filters were shaken vigorously in the counting vial and counted minimally 24 h after the assay. Blank filters were run in triplicate, as were all samples, and washed the same way as the experimental dishes. Blank filters were prepared from cell-free dishes containing growth medium and ^{45}Ca from the experiment in question. With the specific activities used in these experiments, these filters, washed in the absence of EGTA, contained approx. 120 cpm vs. approx. 80 cpm in the presence of EGTA. The filters containing cells were at the least twice these background levels during early exponential growth phase and at the least fourfold these levels beyond the 2nd day of growth (see Figs. 4 and 5).

The specific activity of the medium (cpm ^{45}Ca /mol ^{40}Ca) was monitored on each day that an assay was conducted. 50- μl aliquots of the growth medium were initially counted in vials containing filters on which isotope-free cells were washed. Subsequent experiments indicated that the presence of the filter and the cells did not alter counting efficiency. The levels of ^{40}Ca in the medium were determined by means of an automatic fluorometric titration of the Ca-calcein complex with EGTA (7) using a Corning calcium analyzer (Corning Glass Works, Science Products Div., Corning, N. Y.). The system, with modification, was capable of a resolution of 2 nmol of Ca. Over the course of the experiments, medium Ca levels ranged from 2.1 to 2.9 mM.

The data are expressed on a per cell volume basis to compensate for differences in size between the normal and SV40-transformed 3T3 cell and for changes in size as a function of the density of cells in the culture. For each experiment and on each day of ^{45}Ca assay, replicate plates of the appropriate cell line were treated with trypsin-EDTA and sized and counted in MEM on a Coulter electronic cell counter (Coulter Electronics Inc., Hialeah, Fla.). It should be noted that expression of the data on a per cell basis, although misleading due to changes in cell size as a result of growth and transformation, does not alter any of the major conclusions drawn in this study since the changes in Ca content and distribution do not parallel changes in cell volume, but rather they are a function of growth and transformation.

RESULTS

An Evaluation of the Calcium

Assay Procedure

Detailed observations on Ca content and distribution in *in vitro* cell lines is, at present, limited to the work of Borle with HeLa cells (4, 5, 6). The work on the HeLa cell was not directed to an analysis of cell Ca as related to growth nor, by necessity, as related to a normal vs. transformed cell. In an effort to evaluate such relationships, we have chosen to use the content and distribution of ^{45}Ca as a monitor of ^{40}Ca in the intact cell. Borle (4) and Borle and Briggs (7) have successfully used the method of fluorometric titration to determine ^{40}Ca content in the HeLa cell (4, 7). At present, our system is not capable of the resolution required for such an analysis with the 3T3 cell.

The use of ^{45}Ca to monitor total cell Ca assumes that the isotope is in total equilibrium with cell Ca. Since the cells are grown in the presence of ^{45}Ca , this assumption is valid under conditions in which several population doublings have occurred, i.e., the cellular Ca of newly formed cells must come from the medium. After three cell doublings, a minimum of 87.5% of the total cell Ca must be in equilibrium with the ^{45}Ca of the medium. This example represents the extreme case which assumes that the Ca of the initial cell population does not exchange with the isotope. This is, of course, not the case. Fig. 1 illustrates the uptake of ^{45}Ca by the SV40 3T3 cell as a function of time. Beyond approx. 7 h after addition of ^{45}Ca , the slope of the cpm of ^{45}Ca retained by the cells nearly parallels the increase in cell number, indicating that the Ca of newly forming cells is in equilibrium with the ^{45}Ca of the growth media. If the uptake of ^{45}Ca is expressed as a function of the total volume of the cells on the plate, it is seen that this value equilibrates sometime between 7 and 15 h. Our earliest assays of ^{45}Ca content are done at 24 h after addition of the isotope. Thus, it would appear that, from this point on, the content of the isotope is a measure of the total cell Ca. Preliminary experiments in which atomic absorption spectroscopy was used to determine ^{40}Ca content of the cells are consistent with the isotopic measurements. In the quiescent 3T3 cells used in this study, ^{40}Ca was determined to be 1.153 ± 78 pmol/ μl cell volume ($N = 6$, SEM). The ^{45}Ca equilibrium method yields, 1.103 ± 110 pmol/ μl

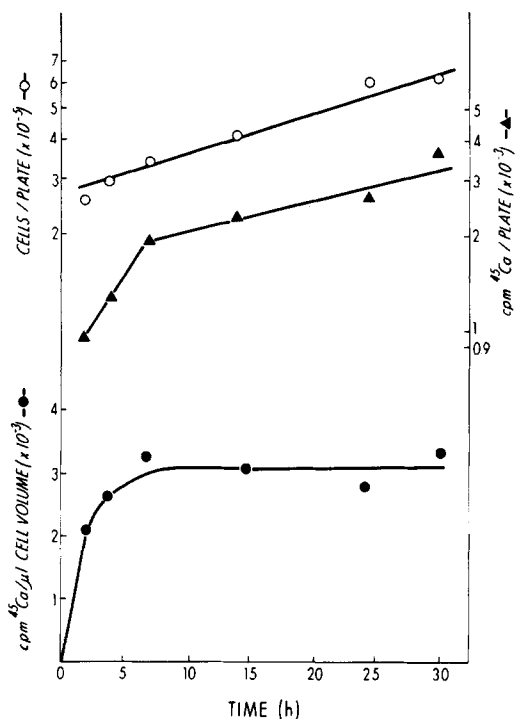


FIGURE 1 Calcium uptake and equilibration in the SV40 3T3 cell. 1 day after plating, an aliquot of ^{45}Ca was added to a series of cultures, and the cellular content of the isotope was monitored as described in Materials and Methods at the times indicated. Cell number and average volume were determined in parallel plates. All points are the mean of duplicate samples.

cell volume ($N = 12$, Fig. 3). It should be noted that the data of Fig. 1 are not meant to analyze the kinetics of Ca uptake nor its compartmentation. A series of experiments¹ aimed at determining these parameters in the 3T3 cell indicate that Ca is taken up into at least two definable kinetic compartments, a very rapidly exchanging compartment presumably representing the surface Ca and a much more slowly exchanging compartment which is presumably bounded by the surface membrane. This is consistent with similar studies on the HeLa cell (5, 6).

Borle has presented evidence (4) that successive washes with trypsin, EDTA, or trypsin-EDTA in combination effectively remove the same amount of Ca from the cell and that this Ca is predominantly surface localized. Our observations are

¹ Hazelton, B., and J. T. Tupper. Manuscript in preparation.

consistent with this conclusion. As shown in Fig. 2, by the fourth wash the cpm of ^{45}Ca in the filtrate is not significantly different from background levels. Successive washes beyond this point do not reduce the amount of ^{45}Ca associated with cells trapped on the filter. This is true for cells washed in the presence or absence of EGTA, thus substantiating that complete removal of EGTA-sensitive Ca is achieved by the fourth wash. In preliminary experiments, cells were washed with trypsin-EDTA vs. EGTA, and no difference was observed in the results. These data are summarized in Table I. Furthermore, we would note that comparable studies carried out on normal and transformed 3T3 cells, using trypsin rather than EGTA

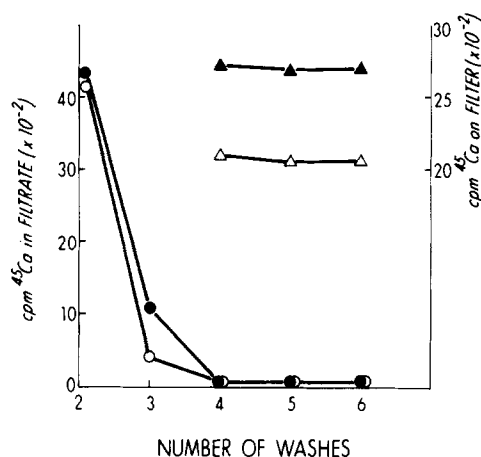


FIGURE 2 The effect of washing on cellular and surface-localized ^{45}Ca in the SV40 3T3 cell. Cells were grown in ^{45}Ca for 3 days and at the time of assay were at a density of 1.3×10^5 cells/cm². At this time, the growth medium was removed from the plates, and the cells were washed with 5 ml of 200 mM choline chloride, 1 mM Tris-Cl with or without 1 mM disodium-EGTA (wash no. 1, not shown). A second 5-ml aliquot of the appropriate solution was added, and the cells were removed from the plate, in this solution, onto glass fiber filters, and the wash was removed under vacuum (wash no. 2). Subsequent 5-ml washes were carried out on the filters. The filtrate was trapped in tubes within the filter apparatus. Aliquots of the filtrate were assayed for ^{45}Ca content during successive washes by removal of the tubes and replacing them with clean tubes. Likewise, different filters were retrieved after the fourth, fifth, or sixth wash, as indicated. The points are means of samples done in triplicate. The cpm of ^{45}Ca in the filtrate beyond the third wash are equivalent to background levels. Circles refer to the filtrate, triangles to the filter. Open symbols represent washes in the presence of EGTA.

TABLE I
Effect of Trypsin-EDTA and EGTA on Removal of Surface Calcium from the 3T3 Cell

⁴⁵ Ca on filter (cpm)	Wash procedure		
	Control	trypsin-EDTA	EGTA
	498 ± 12	241 ± 52	263 ± 30

Cells were plated at 1×10^5 cells/60 mm plate in 3 ml of MEM plus 10% calf serum. 2 days later, ⁴⁵Ca was added to each plate, and the cells were assayed on day 4. ⁴⁵Ca content of the filters was determined as described in Materials and Methods. EDTA and EGTA were present at 1 mM concentrations and trypsin at 0.25% (wt/vol). In the case of the trypsin-EDTA procedure, the first two washes were discarded, and the cells were allowed to remain in trypsin-EDTA until they had visibly lifted from the plate (6 min). They were then transferred to the filters. The data are the mean ± SE of triplicate samples.

to remove surface Ca, have produced results that are in qualitative agreement with our studies (see Discussion). We believe that it is important to point out that washing of cells in the absence of EGTA, in all likelihood, removes some fraction of surface localized Ca. Thus, the difference in Ca content observed after washing in the Ca chelator may represent an underestimate of the total amount of surface Ca. This in turn leads to the conclusion that the changes in EGTA-removable Ca associated with growth and/or transformation may, in fact, be larger than observed. We have also seen that the wash procedures in the absence of EGTA do not eliminate differences in Ca transport properties. Transport studies¹ indicate a four-fold difference in the half-time of exchange of the fast Ca compartment in normal vs. transformed 3T3 cells. Washing procedures used for these studies were similar to those of the present study.

Calcium Content and Distribution in the 3T3 vs. the SV40 3T3 Cell

Fig. 3 summarizes our observations comparing the two cell lines during exponential growth phase. At cell culture densities ranging from 4 to 8×10^3 cells/cm², the cells are preconfluent. In four separate experiments in which ⁴⁵Ca was assayed, the 3T3 cell was $2,718 \pm 65 \mu\text{m}^3$ (SE, $N = 9$) in volume at these culture densities. In comparison, and within these same experiments, the SV40 3T3 cell possessed a vol of $1,702 \pm 29 \mu\text{m}^3$ (SE, $N = 6$). On a per volume basis, the normal 3T3 cell has a total Ca content of approx. 525 pmol/μl of cell volume, and this is significantly higher ($P <$

0.001) than that observed for the transformed cell (approx. 350 pmol/μl). This difference holds for the Ca not removable by EGTA and presumably located intracellularly. The normal cell has 2.2-fold greater intracellular Ca than the transformed

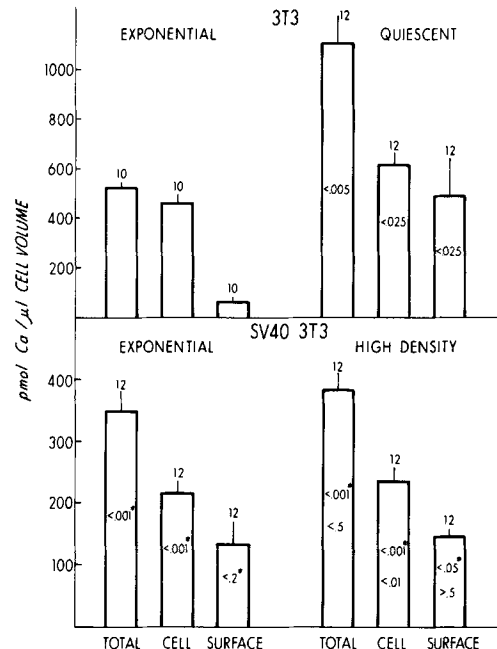


FIGURE 3 Calcium content and distribution as a function of growth stage and transformation in the 3T3 and SV40 3T3 cell. Total Ca refers to calcium content after washing in the absence of EGTA. Cell Ca represents Ca remaining after washing with EGTA. Surface Ca refers to the difference between total vs. cell Ca. Ca content in pmol/μl cell volume was determined from the specific activity of the medium (cpm/mol Ca) and the total microliters of cells per plate done on the day of assay for both cell lines in duplicate for each experiment. Exponential cells ranged in culture density from 4 to 8×10^3 cells/cm². Quiescent 3T3 cells had saturation densities of 1.2 – 1.8×10^4 cells/cm². High density SV40 3T3 cells were 1.8 – 2.4×10^5 cells/cm². The data are expressed as mean ± SE. The number above each error bar is the number of separate plates assayed. The data represent cultures from four different cell platings for each condition. Probabilities were determined by analysis of variance and the *t* test. The probabilities without an asterisk refer to the level of significance when the comparison is made within the same cell type at a different growth condition, e.g., the total Ca in exponential 3T3 cells is significantly different from that in quiescent 3T3 cells at a <0.005 level. The probabilities marked by an asterisk refer to comparisons between normal vs. SV40 3T3 cells in exponential growth or at quiescence vs. high culture density.

cell ($P < 0.001$). In contrast, there is no significant difference ($P < 0.2$) in EGTA-removable Ca between the two cell lines during exponential growth.

Figs. 4 and 5 illustrate the changes in Ca content and distribution observed as a function of growth in single experiments on the 3T3 and SV40 3T3 cell, as monitored on a daily basis. Four such experiments are summarized in Fig. 3. Quiescence of the 3T3 cell is defined as the absence of continued increases in cell number. Parallel experiments indicated that incorporation of [^3H]thymidine into acid-precipitable material was less than 10% of

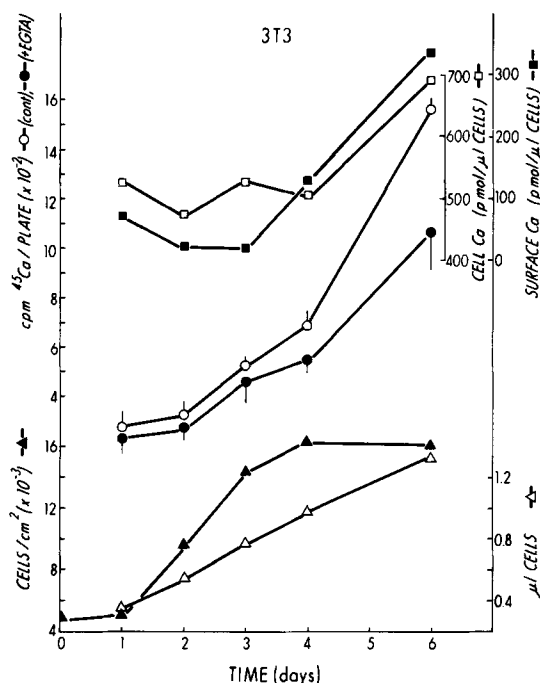


FIGURE 4 Calcium content and distribution as a function of exponential growth vs. quiescence in the 3T3 cell. Cells were plated on day zero at 4.8×10^3 cells/cm². At this time, a total of 6 μCi ^{45}Ca was added to each plate. 1 day later and at subsequent intervals, the cells were assayed for total and EGTA-removable Ca as described in Materials and Methods. All ^{45}Ca assays were run in triplicate for each point, and the cpm $^{45}\text{Ca}/\text{plate}$ is given as mean \pm SE. Specific activity of the growth medium (cpm $^{45}\text{Ca}/\text{mol Ca}$) was monitored on each day of assay, and the cell and surface Ca were computed on the basis of this value and the cpm/plate under the different washing conditions. Cell number and volume were determined in duplicate on each day of assay from parallel cell cultures. Data from four such experiments comparing exponential growth phase vs. quiescence are summarized in Fig. 3.

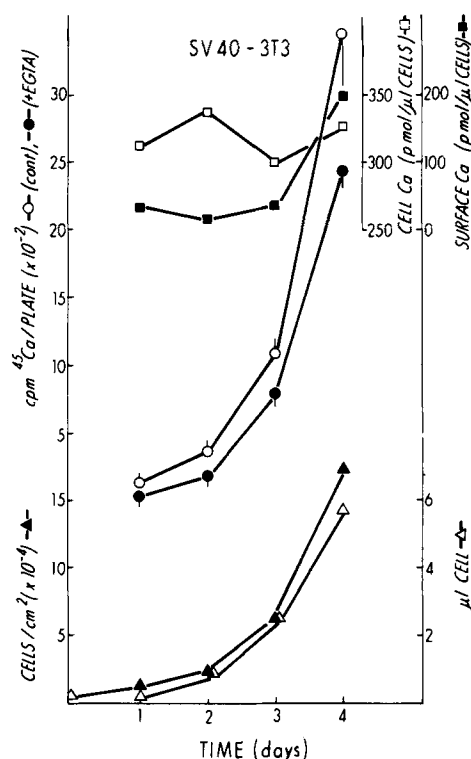


FIGURE 5 Calcium content and distribution as a function of exponential growth at low density vs. high culture density in the SV40 3T3 cell. The details for this experiment are equivalent to those in Fig. 4. The two experiments were run in parallel. Data from four such experiments are summarized in Fig. 3.

that observed in equivalent cultures given fresh medium and pulsed with [^3H]thymidine 12 h after refeeding. At quiescence the 3T3 cell had achieved densities of $1.2\text{--}1.8 \times 10^4$ cells/cm². In four experiments the mean volume of the cells at quiescence was $3,749 \pm 269 \mu\text{m}^3$ (SE, $N = 6$). As seen in Figs. 3 and 4, the absence of continued proliferation is accompanied by substantial changes in Ca distribution. As compared to the exponentially growing 3T3 cell, total Ca increases by greater than twofold ($P < 0.005$). However, this increase is primarily due to a 7.5-fold increase in Ca removed by EGTA (65–485 pmol/ μl ; $P < 0.025$).

At equivalent times after plating the SV40 3T3 cells were evaluated and compared to their exponential counterparts as well as to the quiescent 3T3 cell (Figs. 3 and 5). At the time at which the normal cell had ceased proliferation, the SV40 3T3 cells were in continued exponential growth and had achieved culture densities ranging from

1.8×10^4 to 2.4×10^4 cells/cm². Their average volume was $1,521 \pm 86 \mu\text{m}^3$ (SE, $N = 6$) over these density ranges. One of four experiments in which total and EGTA-removable Ca were monitored as a function of growth of the SV40 3T3 cell is presented in Fig. 5. In this particular experiment, an increase in EGTA-removable Ca was observed only on day 4. However, in evaluating all four experiments, no significant rise in EGTA-removable Ca was observed ($P > 0.5$, Fig. 3). Also, as is summarized in Fig. 3, there is no substantial difference in their Ca content as a result of achieving the high culture densities, when compared to the same cells in exponential growth at preconfluence. In contrast, when compared to quiescent 3T3 cells, they maintain 2.6-fold less cell Ca ($P < 0.001$) and 3.3-fold less surface Ca ($P < 0.05$).

We believe that these observations are particularly pertinent in two regards. First, they substantiate the procedures used in the ⁴⁵Ca assay since the method detects substantial changes in the normal 3T3 cell whereas similar changes, over a four-fold increase in culture density, are not observed in the transformed cell. It is unlikely that an artifact associated with the washing of the cells during exponential growth and giving rise to the differences observed in the two cell lines at this growth stage would not in turn be carried over to later growth stages. Secondly, the data suggest that the exponential state of growth in the 3T3 cell is associated with low amounts of surface Ca whereas quiescence is paralleled by a substantial increase in this Ca. Transformation with SV40 virus lowers gross intracellular levels of Ca. However, it does not substantially alter the EGTA-removable Ca on a cell volume basis but, rather, it appears to maintain it at a level associated with continued proliferation in the normal cell.

Calcium Content and Distribution in the 3T3 vs. SV40 3T3 Cell as a Function of Medium Serum Levels

Our observations to this point suggest a role for Ca removable by EGTA, and presumably surface localized, in growth inhibition. Furthermore, they indicate that the transformed cell maintains lower total Ca levels as compared to the normal cell. It is known that the proliferation of the 3T3 cell can be regulated by the level of serum in the growth medium and that the requirement for serum is substantially lower for the transformed 3T3 cell as

compared to the normal counterpart (10, 11). These observations provide a basis for further evaluation of the interrelationship between Ca and cell proliferation. Factors such as serum, which can modulate growth, should in turn modulate Ca, if these phenomena are truly related. On the basis of previous studies (2, 12), we have established conditions for our cells in which continued proliferation is halted or substantially slowed by serum deprivation. As illustrated in Fig. 6, reduction of serum levels from 10% to 4% halts continued cell division of the normal 3T3 cell approx. 1 day after serum deprivation. A similar reduction in serum has little effect on the SV40 3T3 cell, and levels of 0.5% serum are required to substantially reduce continued proliferation. These observations are consistent with previous studies, (e.g., reference 2). Calcium content and distribution have been evaluated under these conditions.

All cells were plated in MEM plus 10% calf serum. 1 Day later, the medium was removed and replaced with fresh MEM containing 10% or 4%

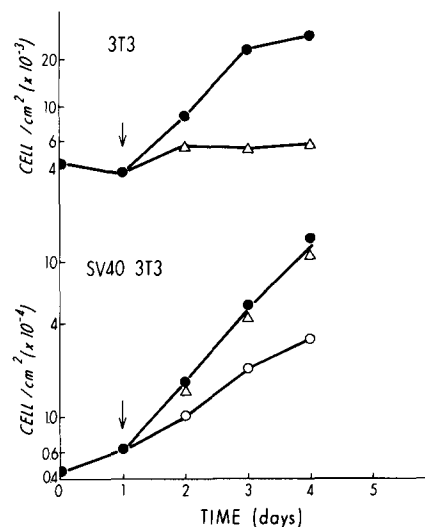


FIGURE 6 Growth of 3T3 and SV40 3T3 cells at various serum levels. Cells were plated at the densities indicated in 3 ml of medium and allowed to grow 1 day. The medium containing 10% serum was then changed (arrow) and 3 ml of medium containing 10% (●—●), 4% (△—△), or 0.5% (○—○) serum was added. In experiments in which ⁴⁵Ca content was determined after serum deprivation, the isotope was added at the time of the medium change. The cells were then assayed, as described in Materials and Methods, 24 or 48 h after addition of the isotope.

serum (3T3) or 10%, 4%, or 0.5% serum (SV40 3T3). Within each experiment, original plating densities were known, and the effect of serum deprivation on growth was confirmed on the day of the assay by determining cell numbers and average volumes on parallel plates. Assay of Ca concentration in the medium of differing serum levels was done using fluorometric titration. Medium with 10% serum contained approx. 2.2 mM Ca. This was reduced approx. 2% and 4% by 4% and 0.5% serum, respectively. The data from four separate experiments on each cell type are summarized in Fig. 7. Reduction of serum levels to 4% increases EGTA-insensitive Ca levels in the 3T3 cell 1.5-fold ($P < 0.005$). Paralleling this is a 3.9-fold increase in EGTA-removable Ca ($P < 0.01$). Reduction of serum levels to 4% with the SV40 3T3 cell has little effect on cell proliferation (Fig. 6). Similarly, it has little effect on EGTA-sensitive or -insensitive Ca levels ($P > 0.5$ for both cases). However, SV40 3T3 cell proliferation is significantly reduced at 0.5% serum levels, and this is accompanied by a twofold increase in the Ca removed by EGTA ($P < 0.05$).

Growth inhibition by serum deprivation is expected to mimic entrance into quiescence since it is generally accepted that quiescence is the result of a reduction in necessary serum factors required for continued growth. Thus, the changes in Ca associated with quiescence would be expected to occur upon serum deprivation if, in fact, they are related to growth inhibition. Although the absolute magnitude of the change in Ca sensitive to chelation by EGTA is not equivalent (3.9- vs. 7.5-fold), the results are consistent with the prediction. Serum deprivation of the 3T3 cell also gives rise to a 1.5-fold increase in cell Ca, and this is quite comparable to the 1.4-fold increase observed as the cells become quiescent under normal growth conditions. The correlation between elevated surface Ca and growth inhibition is further substantiated by the observations on the SV40 3T3 cell. Only serum levels which significantly retard continued proliferation give rise to a change in Ca sensitive to removal by EGTA.

DISCUSSION

Borle (4, 5, 6) has investigated the content and distribution of Ca in the HeLa cell as well as the kinetics of Ca transport. With a fluorometric analysis for ^{45}Ca , it has been determined that the level of cell Ca in the HeLa cell (after removal of the surface coat) is 470 pmol/ μl of cell water. De-

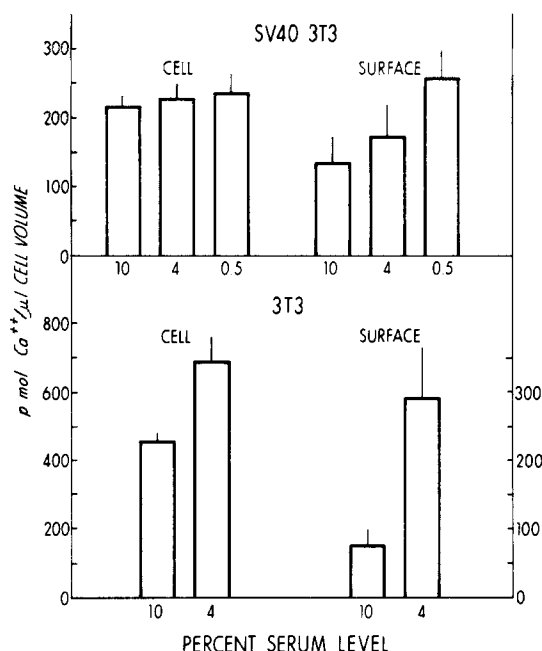


FIGURE 7 Effect of serum on surface membrane-bound calcium and intracellular calcium in the 3T3 cell and the SV40 3T3 cell. Surface values represent the amount of Ca removed by washing with EGTA, as described in Materials and Methods. Cell values represent the amount of Ca insensitive to removal by EGTA. Data are expressed as mol Ca/ μl cell volume, based on ^{45}Ca content of cells and medium specific activity (cpm ^{45}Ca /mol Ca) determined on the day of assay. Cell number and average cell volume were determined in duplicate on the day of assay for each level of serum and in each experiment. The data represent the mean \pm SE for four separate cell populations for each cell type in which the total number of samples assayed under each condition was minimally nine. By analysis of variance and the *t* test, the differences in the various experimental values obtained were significant at the following levels: SV40 3T3 cells; cell Ca in 10%, 4%, and 0.5% serum, no significant difference. Surface Ca in 10% vs. 0.5% serum, $P < 0.05$. 3T3 cells; cell Ca in 10% vs. 4% serum, $P < 0.005$. Surface Ca in 10% vs. 4% serum, $P < 0.01$.

pending on the cell type and growth stage in question, our present values for cell Ca range from approx. 200–600 pmol/ μl of cell volume. Thus, it would appear that the two different methods used for analysis of cell Ca are in reasonably good agreement. This, however, does not appear to be the case as regards EGTA-removable Ca. In the HeLa cell, treatment with EDTA or trypsin removes 4.57 nmol/ μl cell water. In the 3T3 cell, the maximum Ca removed is approx. 0.5 nmol/ μl . One obvious possibility is the difference in cell

type. However, other factors bear consideration. The studies on the HeLa cell were conducted after preincubation in serum-free balanced salt solution. In our studies, serum deprivation enhances the Ca removed by EGTA. Thus, the difference observed may, in part, be due to the absence of serum. Also, the analysis of Ca in the HeLa cell was carried out after washing of the cells while still in a monolayer condition. Our cells are removed from the monolayer, and this procedure may reduce the absolute amount of Ca associated with the cell surface. We would note that ongoing studies with human diploid fibroblasts indicate that exponential growth stages of both the normal and transformed lines are also characterized by low levels of EGTA-removable Ca. This suggests that this property is not necessarily unique to the 3T3 cell. Furthermore, the transformed diploid fibroblast cell has a volume considerably greater than that of the normal cell, in contrast to the 3T3 cell. However, it maintains levels of Ca substantially below that of the normal cell, as observed with the 3T3 cell. Langer and Frank (14) have evaluated the amount of surface-localized Ca in cultured heart cells using lanthanum displacement of ^{45}Ca . They also observe a low level of surface-localized Ca. Of the total Ca in the cells, 15% is displaced by lanthanum.

To our knowledge, and at the time of this writing, no other published studies exist dealing with Ca content and distribution in *in vitro* cultured lines and the comparison of these parameters as a result of growth stage and viral transformation. However, we have received a personal communication from Dr. M. Del Rosso regarding similar studies on normal and transformed 3T3 cells.² By means of ^{45}Ca labeled cells treated with trypsin to remove the surface coat, they have also observed low levels of trypsin-sensitive Ca in exponentially growing 3T3 cells. Upon quiescence, this level of Ca increases twofold when expressed on a milligram protein basis. Surface Ca in Py-3T3 and SV40 3T3 cells is similar to that of exponentially growing normal 3T3 cells. These observations are consistent with the present study. We have also been informed by Dr. A. Boynton (National Research Council, Ottawa, Canada) that he observed lower levels of total Ca in SV40 3T3 cells as

² Vannucchi, S., M. Del Rosso, C. Cella, and V. Chiarugi. 1977. Institute of General Pathology, University of Florence, Florence, Italy. Manuscript submitted for publication.

compared to normal 3T3 cells on a per milligram protein basis in both sparse and dense cultures. These observations are also in agreement with the present study.

Substantial interest has arisen as regards the interrelationship between divalent cations, serum, and growth. Dulbecco and Elkington (11) have demonstrated a synergism between medium Ca levels and serum with the 3T3 cell. Elevation of medium Ca upon serum deprivation will enhance final cell saturation densities. Furthermore, elevated Ca alone will stimulate quiescent cells to enter DNA synthesis, as also observed by Boynton and Whitfield (8). A similar synergism between Mg and serum has been observed by Kamine and Rubin (13) using the chicken embryo fibroblast. The mechanism by which elevated Ca alone induces DNA synthesis in quiescent cells is not known. An elevation of extracellular Ca might be expected to increase cell surface levels of Ca (4). However, the present data suggest that elevated surface Ca is associated with cessation of growth and not growth induction. Since intracellular Ca activity in the 3T3 cell is likely to be very low as compared to extracellular Ca activity, it is possible that elevation of medium Ca levels leads to enhanced passive Ca influx. A resultant elevation of intracellular Ca may be related to the induction of growth, as also suggested by Dulbecco and Elkington (11). Recent experiments in our laboratory indicate that serum-induced proliferation of quiescent 3T3 cells is characterized by an increased Ca uptake by the cells and a concomitant increase in intracellular Ca. A parallel loss of surface Ca also occurs.³ Changes in serum levels, whether through manipulation of the medium or exhaustion by the cells, may also give rise to changes in extracellular and intracellular Ca activity and intracellular Ca distribution. The present estimates of cell Ca, using ^{45}Ca content, represent gross Ca levels. They do not reflect Ca activity and cytoplasmic distribution nor any changes induced in these parameters as a result of transformation and/or growth state. Future kinetic analysis of ^{45}Ca compartmentation will yield some information in this regard. Ca is also known to alter both cAMP and cGMP metabolism (e.g., references 18, 19, and 21). Thus, the gross differences in Ca levels observed in the normal vs. transformed 3T3 cell and the changes in Ca distribution associated

³ Tupper, J. T., M. Del Rosso, B. Hazelton, and F. Zorngiotti. Manuscript submitted for publication.

with the quiescent state may be related to changes in cAMP and cGMP levels, both of which have been suggested as regulators of cell proliferation. Furthermore, the mobility of macromolecules in the surface membrane could alter membrane-localized enzyme activity. Ca has been shown to alter the mobility of surface macromolecules (15).

Quiescent 3T3 cells may be induced to proliferate by means of addition of fresh serum (17). The present data would suggest that a drop in EGTA-removable Ca would parallel entry of the cells into a proliferative state. Our recent experiments indicate this to be true. Within 0.5 h after addition of serum to quiescent cells EGTA-removable Ca is reduced to levels found in growing cells and distinct changes in Ca content and transport occur.³ Preliminary observations of Bissell and Rubin (3) suggest that this is also true in quiescent cultures of chicken fibroblasts. When stimulated to proliferate with fresh serum, these cells undergo an immediate twofold increase in the rate of loss of ⁴⁵Ca. The cells were preloaded with ⁴⁵Ca. Thus, the data do not distinguish the loss of Ca from the cell and/or the surface. However, the result is consistent with a loss of surface-localized ⁴⁵Ca as a result of serum stimulation.

Surface membrane properties are likely to be most meaningful if expressed on a surface area basis. However, realistic estimates of the functional surface area of monolayer cell cultures and changes in this parameter as a function of growth and transformation are plagued with technical difficulties. When comparisons between different growth stages or different cell types are being made, consideration must be given to inherent differences in cell morphology. Protein content and cell volume have generally been used as indices of these differences. However, these parameters need not directly reflect cell surface area. Thus, the changes in Ca distribution observed in the present study could indeed reflect changes in cell surface area. It is unlikely, however, that the differences in intracellular Ca observed between the normal and transformed cell result from differences in cell surface area properties. It is also possible that the changes in surface Ca reflect alterations in other surface components which bind Ca. For example, cell coat polysaccharides are ligands of Ca and they undergo changes as a function of cell growth (e.g., reference 10). Thus, changes in membrane-localized divalent cations may be a consequence of growth and transformation rather than positive or negative regulators of

these processes. Their specific role remains to be evaluated.

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