CALCIUM TRANSPORT AND EXCHANGE IN MOUSE 3T3 AND SV40-3T3 CELLS

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ABSTRACT

The kinetics of Ca^{++} uptake have been evaluated in 3T3 and SV40-3T3 mouse cells. The data reveal at least two exchangeable cellular compartments in the 3T3 and SV40-3T3 cell over a 50-min exposure to $^{45}Ca^{++}$. A rapidly exchanging compartment may represent surface-membrane-localized Ca^{++} whereas a more slowly exchanging compartment is presumably intracellular. The transition of the 3T3 cell from exponential growth (at 3 day's incubation) to quiescence (at 7 days) is characterized by a 7.5-fold increase in the size of the fast component. Quiescence of the 3T3 cell is also characterized by a 3.2-fold increase in the unidirectional Ca^{++} influx into the slowly exchanging compartment and a 3.6-fold increase in its size. The increase in size of the slow compartment at quiescence may result from a redistribution of intracellular Ca^{++} to a more readily exchangeable compartment, possibly reflecting a release of previously bound Ca^{++}. In contrast, no significant change in any of these parameters is observed in the proliferatively active SV40-3T3 cells after corresponding periods of incubation, even though these cells attained higher growth densities and underwent postconfluence.

KEY WORDS 3T3 cell · calcium exchange

A substantial amount of experimental evidence indicates that the divalent cations calcium and magnesium are growth-regulating factors in vitro (2, 20, 25). Normal cells in culture require calcium in the medium to maintain proliferative activity while virally, chemically, or spontaneously transformed cells have a greatly decreased calcium requirement (5). This suggests a different mode of calcium metabolism by transformed cells. Extracellular Ca^{++} and Mg^{++} are required for some normal cells to successfully complete the G1 phase of the cell cycle and initiate DNA synthesis (6, 11). These cations are not required for certain transformed cells to progress through G1 phase (9, 18). Despite these differences in divalent cation requirements between normal and transformed cells, virtually nothing is known of the physiological basis for this phenomenon. In an attempt to understand this, we have compared Ca^{++} transport and compartmentalization in the 3T3 cell and its SV40-transformed counterpart.

Calcium has been shown to exist in at least two compartments in HeLa cells (3), pancreas cells (12, 13), heart cells (14), and 3T3 cells (21, 22). In all of these cell types a very rapidly exchangeable compartment exists and it is believed to be surface localized because of its removal by enzymatic (3, 22), chelation (3, 22), or displacement techniques (14), which function primarily, if not exclusively, at the cell surface. The remaining Ca^{++}, which is not removed by these treatments, is considered to be intracellular calcium. It exists in one or more subcellular compartments (4, 17). In a previous study (22), we have determined the

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total Ca^{++} content of 3T3 and SV40-3T3 cells as a function of growth stage and the distribution of Ca^{++} between the cell surface (removable by EGTA and/or trypsin) and the cell interior. Such measurements, however, gave no indication of the subcellular distribution of intracellular Ca^{++} nor of any changes in distribution resulting from changes in proliferation or transformation. In the present study we have undertaken a kinetic analysis of Ca^{++} uptake in the 3T3 and SV40-3T3 cell. This analysis (3) allows for the characterization of two cellular Ca^{++} compartments, their size, rate of exchange, and the unidirectional Ca^{++} flux into them. The data presented indicate clear differences in these compartments as 3T3 cells achieve quiescence. Because the same redistribution is not evident in the proliferating SV40-3T3 cells as they reach confluence and postconfluence, we suggest that Ca^{++} redistribution is related to growth control in the 3T3 cell.

MATERIALS AND METHODS

Mouse BALB/C 3T3 cells (Clone A31, passage 86) were obtained from the American Type Culture Collection (Rockville, Md.). The SV40-transformed counterpart was the gift of Dr. George Poste (Roswell Park Memorial Institute, Roswell Park, N. Y.). Stock lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N. Y.) and penicillin-streptomycin in a humidified, 5% CO_2:air mixture at 37°C. Cells used for experimental purposes were grown in 60-mm plastic culture dishes and were routinely seeded at $\sim 10^5$ cells in 3 ml of medium. To ensure that the cells were at the desired growth stage for the experiments, cell number was monitored. Under the conditions described, 3T3 proliferative activity did not continue beyond the sixth day after plating. Thus, quiescent 3T3 cultures were routinely obtained on the seventh day and exponentially growing cultures on the third day. Identical incubation periods were used to obtain confluent and postconfluent, proliferatively active SV40-3T3 cultures. Exponentially growing populations of 3T3 cells ranged in culture density from 1.0 to 2.2 $\times 10^4$ cells/cm^2 on day 3. SV40-3T3 cells had culture densities of 1.5–6.3 $\times 10^4$ cells/cm^2 on day 3 and both cell types were near confluent or confluent sheets. Quiescent populations of 3T3 cells on day 7 ranged in culture density from 2.1 to 3.1 $\times 10^4$ cells/cm^2 whereas the SV40-3T3 cells ranged from 1.3 to 1.4 $\times 10^5$ cells/cm^2 and were capable of at least one more population doubling. The quiescent 3T3 cells were confluent sheets whereas the SV40-3T3 cells were postconfluent and tightly packed.

To monitor the kinetics of Ca^{++} uptake, cells were pulsed for from 30 s to 50 min with $^{45}Ca^{++}$ (final sp act of 0.6 Ci $^{45}Ca^{++}$/mol Ca^{++}). $^{45}Ca^{++}$ was added directly into the culture medium in which the cells were originally plated. The $[Ca^{++}]_0$ ranged from 1.9 to 2.2 $\times 10^{-3}$M. At the appropriate times, plates were washed six times with 5 ml of 200 mM choline chloride, 10 mM Trizma-base, adjusted to pH 7.4 with HCl. The wash procedure was completed within ~1 min. Cells were extracted in 1 ml of glass-distilled water and thoroughly scraped from the dish with a rubber policeman. The suspension was drawn into an automatic pipette four times to ensure homogeneity. A 500-μl aliquot was counted in a liquid scintillation counter in 10 ml of 2,5-diphenyloxazole (PPO)-1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)-based counting solution containing Triton X as a solubilizer. A portion of the remaining suspension was used to determine protein by the Lowry method. Details for measurement of specific activity, verification of the wash procedures, correction for nonspecific retention of $^{45}Ca^{++}$ by the culture dishes, and measurement of total cell Ca^{++} levels have been presented (21, 22).

The $^{45}Ca^{++}$ uptake data are fit by a three-compartment model (suggested to be extracellular, cell surface, intracellular) as described by Borle (3).

RESULTS AND DISCUSSION

Ca^{++} uptake in an exponentially growing and a quiescent population of 3T3 cells is illustrated in Fig. 1*A*. The slopes of the uptake were calculated at successive intervals and plotted semilogarithmically (Fig. 1*B* and *C*). The graphical analysis reveals at least two Ca^{++} compartments, a fast and a slow phase, the kinetics of which differ substantially between the two growth conditions. The parameters of the two compartments were analyzed graphically and are summarized for the two cell types in Table I. This method of analysis assumes that the compartments are in parallel. If the compartments are in series, however, the size of the slow compartment may be slightly overestimated (3, 10), but the error is not serious if the fast and slow rate constants differ by a factor of 10 or more. Examination of the fast and slow rate constants for individual experiments indicates a minimal 10-fold difference and maximal 33-fold difference.

In 3T3 cells the size of the rapidly exchanging Ca^{++} compartment increases 7.5-fold ($P < 0.001$) at quiescence. This observation is in good agreement with our previous studies which demonstrated that the fraction of Ca^{++} removable from 3T3 cells by the Ca^{++} chelator EGTA increased sevenfold at quiescence (22). A twofold increase in Ca^{++} removable by trypsin treatment has also been observed in growing versus quiescent 3T3 cells (23). It is likely that this rapidly exchanging compartment represents surface-localized Ca^{++}

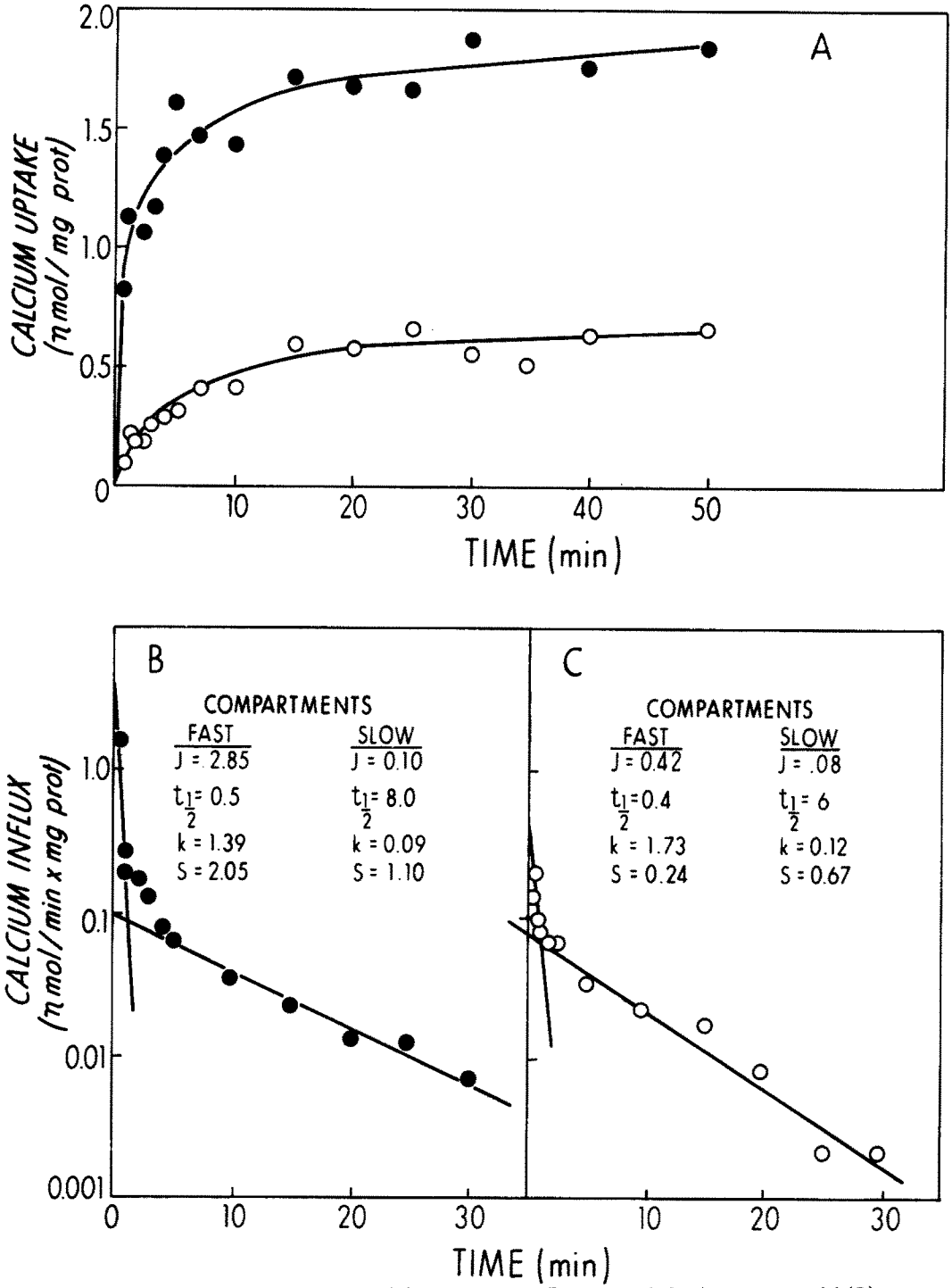

FIGURE 1 Calcium uptake by 3T3 cells. (*A*) The kinetics of ^{45}Ca accumulation in an exponential (○) or quiescent (●) 3T3 cell population. Cells were plated at 4.8×10^3 cells/cm^2 and on day 3 and day 7 were assayed for ^{45}Ca uptake as described in Materials and Methods. (*B* and *C*) The change in rate of ^{45}Ca accumulation (nmol/mg·min) was determined over intervals from the uptake curves of *A*, and these data are plotted semilogarithmically against time of exposure to ^{45}Ca. From the intercepts and slopes of the kinetic functions, the size (S, nmol/mg protein), half-time of exchange ($t_{1/2}$, min), rate constant (k, 1/min), and influx (J, nmol/min·mg protein) of each compartment were determined. These are listed for the (*B*) quiescent and (*C*) exponential cells. The data are typical of three such experiments summarized in Table I.

TABLE I

*The Parameters of Ca^{++} Uptake Associated with Growth and Transformation of the 3T3 Cell**

	Exponential 3T3	Quiescent 3T3	*P* value	Low density SV40-3T3	High density SV40-3T3
Slow compartment					
J (nmol/mg protein × min)	0.04 ± 0.02	0.13 ± 0.02	<.05	0.12 ± 0.03	0.11 ± 0.01
$t_{1/2}$ (min)	8.4 ± 1.2	9.2 ± 0.66	NS	9.5 ± 2.0	12.9 ± 3.4
k (min^{-1})	0.09 ± 0.01	0.08 ± 0.01	NS	0.08 ± 0.02	0.06 ± 0.01
S (nmol/mg protein)	0.48 ± 0.18	1.72 ± 0.43	<.05	1.47 ± 0.31	2.15 ± 0.78
Fast compartment					
J (nmol/mg protein × min)	0.26 ± 0.06	1.9 ± 0.49	<.05	0.90 ± 0.23	0.97 ± 0.36
$t_{1/2}$ (min)	0.65 ± 0.11	0.67 ± 0.17	NS	0.63 ± 0.03	0.70 ± 0.06
k (min^{-1})	1.16 ± 0.21	1.16 ± 0.23	NS	1.24 ± 0.17	1.01 ± 0.08
S (nmol/mg protein)	0.22 ± 0.03	1.66 ± 0.27	<.001	0.73 ± 0.19	0.93 ± 0.28
Total calcium (nmol/mg protein)	5.27 ± 1.6	5.14 ± 0.24	NS	2.03 ± 0.42	3.03 ± 0.88
Relatively inexchangeable Ca^{++} (nmol/mg protein)	4.57 ± 1.7	1.32 ± 1.0	NS	None	None

* ^{45}Ca uptake was determined as described in Materials and Methods, and the uptake data were plotted as in Fig. 1 *B* and *C*. From these plots, the various parameters were determined. The data are summarized above and represent the mean ± SEM for a minimum of three separate experiments for each growth condition. The probability values presented are derived from comparisons made between exponential and quiescent 3T3 cells. Probability values for SV40-3T3 cells were omitted because there are no significant changes. The level of significance was determined with the Student's *t* test.

and that the increase demonstrated kinetically represents the absolute increase in EGTA-removable Ca^{++} observed previously. Equally striking is the 3.6-fold increase ($P < 0.05$) in the size of the more slowly exchanging Ca^{++} compartment in the 3T3 cell at quiescence. This is particularly interesting because the absolute amount of cell Ca^{++} undergoes no significant change at quiescence (22, see also Table I). This increase represents either a release of bound Ca^{++} or the movement of Ca^{++} into the slowly exchangeable compartment from a large and even more slowly exchanging Ca^{++} pool. It is also important to note that the apparent single slow compartment may represent several slowly exchanging compartments which cannot be distinguished by the present technique (e.g., see reference 13). It is impossible to distinguish between these alternatives at this time.

Quiescence of the 3T3 cell is also characterized by a 3.2-fold increase in the unidirectional Ca^{++} influx of the slow compartment. This flux presumably represents a transmembrane movement of Ca^{++}. The augmented influx may simply reflect the change in Ca^{++} pool size because both are increased to the same extent and there is no significant change in the rate constant.

Exponential 3T3 cells are characterized as having 13.8 ± 3.1% of their total Ca^{++} exchangeable over the 50-min incubation, and this percentage increases significantly ($P < 0.001$) to 65.2 ± 6.3% at quiescence (Table I). No significant change in the size of the rapidly exchanging compartment occurs in the SV40-3T3 cell as a result of achieving postconfluence. This is consistent with the absence of any change in EGTA-removable Ca^{++} under similar conditions (22). The size of the slow compartment in the transformed cell also shows no modification as a function of culture density, and the absolute values for these parameters lie intermediate to growing versus quiescent 3T3 cells. The total Ca^{++} present in SV40-3T3 cells, as in the 3T3 cells, does not change upon reaching postconfluence. However, in contrast to the 3T3 cells, in both low density and postconfluent SV40-3T3 cultures virtually all of the Ca^{++} is exchangeable over the 50-min incubation period (Table I). These differences represent very basic differences in Ca^{++} metabolism in the normal and transformed 3T3 cell which may be directly related to their different growth characteristics.

Inherent in the mechanism of divalent cation regulation of growth is the concept that intracellular activity of an ion such as Ca^{++} is tightly regulated and undergoes transient changes involved in activation or repression of various cellular functions (2, 25). Our present data suggest that growth arrest of the 3T3 cell is characterized by enhanced surface Ca^{++} and a large increase in

intracellular exchangeable Ca^{++}. If the increased exchangeable Ca^{++} observed upon quiescence of the normal cell is in equilibrium with the cytosolic Ca^{++}, then an increase in Ca^{++} activity is implied. One mechanism by which elevated Ca^{++} activity could block cells in the G_0, G_1 phase of the cell cycle relates to the effect of this ion on microtubule assembly. In association with Ca^{++}-activated modulator protein, micromolar amounts of Ca^{++} can inhibit or reverse microtubule assembly (15). The requirement of an intact microtubule array for G_1 progression is shown by the fact that microtubule-disrupting agents prevent serum-stimulated neuroblastoma cells (1) and chick embryo fibroblasts (16), and lectin-stimulated lymphocytes (8), from entering S phase. The elevated surface Ca^{++} associated with quiescence may reflect increased cell adhesiveness (24) and reduced motility of quiescent cells (7). It is likely to be, in part, a result of enhanced levels of Ca^{++}-binding surface macromolecules such as heparin sulfate, which is known to increase in quiescent cells (e.g., reference 23).

The morphological basis for the increase in Ca^{++} exchange is unknown. It may represent the release of bound Ca^{++} or the movement of Ca^{++} from one intracellular compartment to another compartment which is more readily exchangeable over the time interval monitored. For example, it has been observed that microsomes isolated from quiescent 3T3 cells have enhanced Ca^{++} uptake capability as compared to those isolated from growing populations of 3T3 cells (19). The *in situ* significance of this is unclear, but such observations are consistent with changes in intracellular Ca^{++} distribution and exchange associated with changes in proliferative activity.

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