Intracellular ionic changes in normal and transformed human fibroblasts after extracellular Ca^{2+} deprivation

Bonni J. HAZELTON and Joseph T. TUPPER

Department of Biology, Syracuse University, Syracuse, NY 13210, U.S.A.

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The lowering of extracellular Ca^{2+} concentration in the growth medium reversibly blocks normal, but not SV40-transformed, WI38 diploid fibroblasts in the early G_1/G_0 phase of the cell cycle. This growth response is characterized by specific changes in ionic content and transport. Ca^{2+} deprivation (0.03 mM) has little effect on the K^+ content of either normal or transformed cells. Na^+ content, however, is increased nearly 2-fold in the normal cells. This increase is presumably due to a 3-fold increase in unidirectional Na^+ influx in Ca^{2+} -deprived cells. The increased intracellular Na^+ also gives rise to a nearly 3-fold enhancement of the active (ouabain-sensitive) Na^+ efflux. Ca^{2+} deprivation causes only slight increases in Na^+ influx, ouabain-sensitive Na^+ efflux and intracellular Na^+ in the transformed cell. In contrast, the transformed cells lose nearly 60% of their intracellular Ca^{2+} on deprivation, whereas normal WI38 cells lose only 10%. The data suggest that the growth arrest exhibited by the normal cell but not the transformed cell may be related to different membrane-transport and permeability changes in response to Ca^{2+} deprivation.

The Ca^{2+} concentration in the growth medium has a significant effect on the proliferative activity of many normal cells in culture, whereas transformed cells are much more resistant to Ca^{2+} deprivation (e.g. Balk *et al.*, 1973; Boynton *et al.*, 1977; McKeehan & Ham, 1978; Hazelton *et al.*, 1979). Despite the fact that many cell lines exhibit this difference, the physiological basis for growth inhibition in normal cells and its absence in transformed cells has not been determined. A decrease in extracellular Ca^{2+} concentration in the growth medium could affect many aspects of cellular function. Cell-surface-localized Ca^{2+} , either on surface macromolecules or directly associated with the membrane, is decreased on extracellular Ca^{2+} deprivation (Borle, 1968; Tupper *et al.*, 1980a). Such surface Ca^{2+} concentrations are growth- and transformation-sensitive (Tupper & Zorngniotti, 1977; Tupper *et al.*, 1978; Sanui & Rubin, 1978, 1979b; Hazelton & Tupper, 1979) and may relate directly to binding and/or internalization of growth-promoting substances [e.g. insulin (Desai *et al.*, 1978), nerve growth factor (Banerjee *et al.*, 1975)]. The decrease of extracellular Ca^{2+} also alters the transmembrane Ca^{2+} gradient and, on the basis of previous studies, is likely to affect the transmembrane movements of other ions such as Na^+ and K^+ (e.g. Gardos, 1958; Gilbert, 1972; Kolb & Adam,

1976; Smith & Vernon, 1979). In the present study we have evaluated this latter aspect. The effect of Ca^{2+} deprivation on the ionic distributions of the cations Na^+ , K^+ , Ca^{2+} and Mg^{2+} has been monitored. We wished to determine differential responses between the two cell types that could relate to the ability of the transformed cell to escape growth regulation by extracellular Ca^{2+} .

Experimental

WI38 cells and SV40-transformed WI38 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Stock cultures were maintained in Eagle's (1955) basal diploid medium supplemented with 10% (v/v) foetal calf serum and penicillin/streptomycin under humidified 5% CO_2 in air at 37°C. All culture materials were from GIBCO, Grand Island, NY, U.S.A. Stock cultures were passaged at 3–4-day intervals. WI38 cells at passage 20–28 were used for this series of experiments.

The medium used in the Ca^{2+} -deprivation experiments was constructed from Ca^{2+} - and Mg^{2+} -free Eagle's medium supplemented with 10% foetal calf serum pre-treated with the cation-exchange resin Chelex 100 (Bio-Rad, Richmond, CA, U.S.A.), to lower the amount of Ca^{2+} and Mg^{2+} contributed by

the serum. These procedures have been presented in detail elsewhere (Hazelton *et al.*, 1979; Tupper *et al.*, 1980b).

Cells for experimental purposes were plated in normal Eagle's medium with 10% foetal calf serum for at least 24 h. The medium was then aspirated and the cells were washed with Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution. The appropriate medium was then added to the plates. An incubation period of 4 days was used before ionic determinations. Cells used for total ionic determinations were washed with 6×3 ml of 200 mM-choline chloride/10 mM-Tris base adjusted to pH 7.4 with HCl at 20°C. For intracellular Ca^{2+} and Mg^{2+} determinations, this wash solution also contained 1 mM-disodium EDTA, which removes primarily surface-bound Ca^{2+} and Mg^{2+} (Borle, 1968; Tupper & Zorngiotti, 1977). Ca^{2+} was measured by incubating the cells in the presence of ^{45}Ca from the time of the change of medium (final specific radioactivity 0.6 Ci/mol of Ca^{2+}). This technique has been described in detail elsewhere (Tupper & Zorngiotti, 1977; Tupper *et al.*, 1978). Cells were washed by the procedures described above, lysed in 1 ml of glass-distilled water, and scraped from the dish with a rubber policeman. A 500 μl portion of the resulting suspension was placed in a counting vial with 10 ml of a toluene-based 2,5-diphenyloxazole/1,4-bis-(5-phenyloxazol-2-yl)benzene radioactivity-counting cocktail with Triton as a solubilizer (Hazelton & Tupper, 1979). From the equilibrium ^{45}Ca distribution and the ^{45}Ca specific radioactivity, the Ca^{2+} content of the cells was determined. The cells used for Mg^{2+} determinations were washed and lysed as above. The Mg^{2+} in the resulting suspension was then determined by atomic-absorption spectrophotometry (Perkin-Elmer 303). Cells to be used for Na^{+} and K^{+} determinations were washed as above, extracted in 1 ml of 15 mM-LiCl, and Na^{+} and K^{+} were determined by flame photometry with Li^{+} as an internal standard.

For unidirectional Na^{+} -efflux measurements, cells were preloaded with ^{22}Na (final specific radioactivity 13 mCi/mol) for 30 min in the 4-day growth medium. They were then washed twice in Ca^{2+} - and Mg^{2+} -free Hanks balanced salts solution, and the appropriate normal or low- Ca^{2+} medium removed from parallel non-radioactive plates was added back. At 5 min intervals for 15 min the medium was removed and replaced with radioisotope-free medium. The ^{22}Na lost plus the ^{22}Na remaining in the cells at the termination of the efflux was monitored in a gamma counter. From plots of the log ($[^{22}\text{Na}]_t/[^{22}\text{Na}]_{t_0}$) (where t and t_0 are the time of sampling and zero time respectively) versus time, the unidirectional efflux rate coefficient was determined. The active (ouabain-sensitive) component of Na^{+} efflux was determined from the difference in ^{22}Na

efflux in the presence or absence of 0.1 mM-ouabain, a concentration at which the $(\text{Na}^{+} + \text{K}^{+})$ -activated ATPase is maximally inhibited in these cells (J. Tupper, unpublished work). Unidirectional Na^{+} influx was determined from the rate of ^{22}Na uptake. The influx of Na^{+} is rapid in the human diploid cells, so sampling was done over a 3 min period at 1 min intervals. Over this period the uptake is linear with time. Ouabain at 0.1 mM was present to prevent pump-mediated extrusion of the radioisotope. ^{22}Na at 6 or 13 mCi/mol, depending on the experiment, was added at zero time. At 1 min intervals the plates were rapidly dipped in four 1-litre 200 mM-choline washes in succession, drained, and ^{22}Na was determined in a gamma counter. Non-specific ^{22}Na retention on the plates was corrected for by subtraction of the non-zero intercepts of the plots of radioactivity accumulated versus time.

Cell counts and modal volumes were obtained by using an electronic cell counter. Protein was determined by a Bio-Rad colorimetric assay (Bradford, 1976), with bovine serum albumin as a standard.

Results

The growth of WI38 and SV40-transformed WI38 cells in normal and low- Ca^{2+} medium is presented in Fig. 1. Proliferation of the WI38 cell is

Fig. 1. Growth of WI38 cells and SV40-transformed WI38 cells in control and low- Ca^{2+} medium

Cells were plated and incubated in normal basal Eagle's medium for 24 h. Day 0 represents the day when the cells were changed to medium containing 2 mM- Ca^{2+} (●) or 0.03 mM- Ca^{2+} (○) prepared as described in the text. Cell number was monitored at the times indicated, each point representing the mean of three determinations.

inhibited within 48 h after Ca²⁺ deprivation, whereas SV40-transformed WI38 cells are only slightly affected by the low-Ca²⁺ medium. These data are in agreement with previous observations on normal and transformed human diploid fibroblasts under similar ionic conditions (Boynton *et al.*, 1977; McKeehan & Ham, 1978). Evidence has accumulated which indicates that conditions that affect the growth rate of cells also result in changes in ion transport and intracellular ionic content. Therefore, cells incubated for 4 days in control and low-Ca²⁺ medium were analysed for cellular contents of Na⁺, K⁺, Ca²⁺ and Mg²⁺. The data are summarized in Table 1. Mg²⁺ and K⁺ contents in both WI38 and SV40-transformed WI38 cells grown in Ca²⁺-deficient medium are not significantly different from values measured in the control cells. Decreasing the Ca²⁺ in the growth medium does, however, result in a 72% increase in intracellular Na⁺ concentration in the WI38 cells ($P < 0.01$). A 28% increase in Na⁺ ($P < 0.05$) is observed in the SV40-transformed

WI38 cells. A differential response to Ca²⁺ deprivation is also seen between the two cell lines when Ca²⁺ content is examined. After 4 days in 0.03 mM-Ca²⁺ medium, normal cells lost 10% of their intracellular Ca²⁺ as compared with cells in 2.0 mM-Ca²⁺. In contrast, the SV40-transformed WI38 cells exhibit a 57% loss ($P < 0.01$) in intracellular Ca²⁺ content in Ca²⁺-deficient media.

If it is assumed that the ionic activity coefficients for Na⁺ are similar in the extracellular medium and the cytoplasm, then the steady-state 1.7-fold increase in Na⁺ in the normal cell after Ca²⁺ deprivation could be due to an enhanced inward Na⁺ leak, a decrease in active outward Na⁺-pump activity, or a combination of both. We have investigated the mechanism of this change by evaluating the kinetics of ²²Na influx and efflux in the two cell types. The total and ouabain-sensitive ²²Na effluxes were evaluated after 4 days of Ca²⁺ deprivation and are presented in Table 2. Total unidirectional Na⁺ efflux is increased 1.8-fold in

Table 1. Ionic contents in control and Ca²⁺-deprived WI38 and SV40-transformed WI38 cells

WI38 and SV40-transformed WI38 cells were plated at 10⁴ cells/cm² in normal basal Eagle's medium. After 24 h the medium was changed to Ca²⁺ control medium (2.0 mM) or 0.03 mM-Ca²⁺ medium. On day 4 the cells were analysed for intracellular ionic contents by the procedures described in the text. The results presented are means $\pm$ S.E.M. for a minimum of 14 individual determinations. The P values in parentheses were determined by Student's t test and indicate the degree of significance of the change in ionic content between control and Ca²⁺-deprived cells; N.S., no significant change ($P > 0.05$).

	Intracellular Ca ²⁺ (nmol/mg of protein)	Intracellular Mg ²⁺ (nmol/mg of protein)	Na ⁺ concn. (mM)	K ⁺ concn. (mM)
WI38 cells				
Control Ca ²⁺	4.8 $\pm$ 0.5 (N.S.)	62 $\pm$ 4 (N.S.)	28 $\pm$ 3 (0.01)	123 $\pm$ 4 (N.S.)
Ca ²⁺ -deprived	4.4 $\pm$ 0.6	61 $\pm$ 5	48 $\pm$ 7	133 $\pm$ 9
SV40-transformed WI38 cells				
Control Ca ²⁺	3.6 $\pm$ 0.2 (<0.01)	59 $\pm$ 5 (N.S.)	25 $\pm$ 3 (<0.05)	145 $\pm$ 8 (N.S.)
Ca ²⁺ -deprived	1.6 $\pm$ 0.1	58 $\pm$ 4	32 $\pm$ 2	159 $\pm$ 8

Table 2. Components of unidirectional Na⁺ efflux in control and Ca²⁺-deprived WI38 and SV40-transformed WI38 cells

Cells were plated as in Table 1. On day 4 they were pre-loaded with ²²Na as described in the text and the unidirectional efflux rate coefficient (k_0 , min⁻¹) was determined in the presence or absence of 0.1 mM-ouabain. The unidirectional Na⁺ efflux was then calculated from the relationship J_0 (mol/litre per min) = $k_0 \cdot C_i$, where C_i is the internal Na⁺ concentration in mmol/litre of cell volume, as determined by flame photometry. The data are the means for duplicate samples from two separate experiments.

	Efflux (mmol/litre per min)			
	WI38 cells		SV40-transformed WI38 cells	
	Control	Ca ²⁺ -deprived	Control	Ca ²⁺ -deprived
Total Na ⁺ efflux	5.0	9.1	3.8	4.6
Ouabain-sensitive Na ⁺ efflux	0.8	2.4	1.0	1.3

normal Ca^{2+} -deprived cells. The active ouabain-sensitive fraction of the efflux increases nearly 3-fold. The SV40-transformed WI38 cells exhibit a smaller increase in total unidirectional Na^+ efflux, but there is no change in the active component of Na^+ efflux. Thus the data indicate that a decrease in active Na^+ extrusion is not the mechanism by which the large increase in intracellular Na^+ occurs in the WI38 cell. The uptake of ^{22}Na was monitored to evaluate the effect of Ca^{2+} deprivation on the unidirectional Na^+ influx, which is presumed to represent the passive-inward Na^+ leak. Fig. 2 illustrates that Ca^{2+} deprivation results in a 3-fold enhancement of unidirectional Na^+ influx in normal WI38 cells. Only a slight increase is observed in the transformed cell, which is consistent with the substantially smaller increase observed in intracellular Na^+ . It should be noted that a portion of the ^{22}Na influx could represent Na^+/Na^+ exchange and not a leak pathway. However, such fluxes would not contribute to the net increase in intracellular Na^+ observed in the Ca^{2+} -deprived cells. The magnitude of the Na^+ efflux in the normal cell when expressed per gram of protein ($30\mu\text{mol}/\text{min}$ per g) is very similar to values for ^{22}Na efflux in another study on

the human fibroblast ($42\mu\text{mol}/\text{min}$ per g; Villereal, 1980) with medium in which choline was substituted for extracellular Na^+ . This also indicates that Na^+/Na^+ exchange does not represent a substantial portion of the unidirectional Na^+ efflux. The data also demonstrate that both the normal and transformed Ca^{2+} -deprived cells have achieved a new steady-state Na^+ distribution, since the total unidirectional Na^+ influx and efflux are similar in either cell (Table 2 and Fig. 2).

Discussion

The present data are in agreement with previous studies evaluating the effects of Ca^{2+} deprivation on the Na^+ and K^+ contents of mammalian cells. Lowering of Ca^{2+} concentration to the $10\mu\text{M}$ range causes substantial increases in intracellular Na^+ , with much smaller concomitant losses of intracellular K^+ (e.g. Morrill *et al.*, 1964; Morrill & Robbins, 1967; Moscatelli *et al.*, 1979), or with little change in intracellular K^+ concentrations (Smith & Vernon, 1979; the present work). The data from the current study suggest that the increase in intracellular Na^+ in the WI38 cell occurs as a result of increased Na^+ permeability and not as a result of altered active Na^+ extrusion. The resistance of the tumour cells to the Ca^{2+} deprivation, as regards an increased Na^+ leak, is consistent with a previous study. Smith & Vernon (1979) did not detect a significant increase in the Na^+ permeability coefficient in Ehrlich ascites-tumour cells as a result of Ca^{2+} deprivation. The current data also suggest that the increase in Na^+ observed represents an increase in free intracellular Na^+ and not enhanced Na^+ binding. The $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}$ is stimulated by intracellular Na^+ (Garay & Garrahan, 1973). The stimulation of the ouabain-sensitive component of the Na^+ efflux observed in the present study is consistent with activation of the $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}$ by increased cytoplasmic activity of Na^+ in Ca^{2+} -deprived cells. It is worth noting that this elevated ATPase may change the intracellular ATP/ADP ratio, which in turn could be related to the inability to proliferate in low Ca^{2+} concentrations.

The intracellular concentrations of Ca^{2+} and Mg^{2+} observed in the WI38 cell are quite similar to values reported for 3T3 cells (Rubin *et al.*, 1978; Hazelton & Tupper, 1979) and chick-embryo fibroblasts (Sanui & Rubin, 1977). The retention of intracellular Ca^{2+} by the normal WI38 cell after extracellular Ca^{2+} deprivation is also consistent with previous observations on the chick-embryo fibroblast (Moscatelli *et al.*, 1979). However, a Ca^{2+} loss after Ca^{2+} deprivation has been observed in the 3T3 cell (Rubin *et al.*, 1978). The large loss of Ca^{2+} by the SV40-transformed WI38 cell is of particular

Fig. 2. Na^+ uptake after Ca^{2+} deprivation of WI38 and SV40-transformed WI38 cells

Cells were plated as in Table 1. On day 4 they were assayed for ^{22}Na uptake as described in the text. The symbols represent WI38 cells in 2.0 mM-Ca^{2+} (Δ) or 0.03 mM-Ca^{2+} ($\blacktriangle$) and SV40-transformed WI38 cells in 2.0 mM-Ca^{2+} ($\circ$) or 0.03 mM-Ca^{2+} ($\bullet$). The data are means $\pm$ S.D. for duplicate samples.

interest as regards the continued growth of these cells in low-Ca²⁺ medium. There is no direct evidence that these two phenomena are related. However, it is conceivable that this loss of Ca²⁺ represents a mechanism for maintenance of physiological intracellular Ca²⁺ concentrations. It has been indicated that Na⁺ induces Ca²⁺ loss from isolated mitochondria (Carafoli & Crompton, 1978). If this phenomenon occurs *in situ*, then the increased Na⁺ concentration induced by Ca²⁺ deprivation could cause a release of mitochondrial Ca²⁺. Concomitant elevation of cytoplasmic Ca²⁺ could be growth-inhibitory (e.g. Marcum *et al.*, 1978). A mechanism for extrusion of Ca²⁺ at the plasma-membrane level would serve to remove cytoplasmic Ca²⁺ and maintain physiological concentrations of intracellular Ca²⁺. At present we have not determined the mechanism of Ca²⁺ loss in the transformed cell, e.g. through a passive leak or an active extrusion mechanism, owing to the absence of a specific inhibitor of the Ca²⁺ pump and the lack of a measure of actual intracellular Ca²⁺ activity. This is no direct evidence that the total Ca²⁺ concentrations measured in either cell type are proportional to cytoplasmic Ca²⁺ ionic activity (e.g. Hazelton & Tupper, 1979). Attempts to induce a Ca²⁺ loss from either the normal or SV40-transformed cell by increasing intracellular Na⁺ with ouabain have proved unsuccessful, as also seen in the 3T3 cell (Sanui & Rubin, 1979a). Thus, simple shifts in intracellular Na⁺ concentrations are not sufficient to mimic the responses of these cells to Ca²⁺ deprivation. This also supports previous observations, which indicate that changes in intracellular Na⁺ or K⁺ concentrations associated with growth or transformation are not likely, in themselves, to be regulatory mechanisms of cell proliferation (see, e.g. Ledbetter & Lubin, 1977; Tupper & Zografos, 1978; Moscatelli *et al.*, 1979).

The present study indicates that extracellular Ca²⁺ deprivation does not inhibit the growth of the human fibroblast by a simple loss of intracellular Ca²⁺. In the absence of information on actual cytoplasmic Ca²⁺ activity, it is not possible at this time to determine alterations in this parameter that could well be mediated by manipulation of Ca²⁺ externally. It is clear, however, that in this cell line there exist specific differences in the response of a transformed counterpart to Ca²⁺ deprivation, particularly as regards transmembrane movements of Na⁺ and Ca²⁺. Further studies may ascertain whether these differences relate directly to growth regulation.

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