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CALCIUM COMPARTMENTS AND FLUXES ARE AFFECTED BY THE src GENE PRODUCT OF RAT-1 CELLS TRANSFORMED BY TEMPERATURE-SENSITIVE ROUS SARCOMA VIRUS

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SUMMARY: Total cellular calcium content (determined by atomic absorption spectrometry) of Rat-1 cells transformed by temperature-sensitive Rous sarcoma virus decreases with cell density, but is found not significantly different at permissive and at non-permissive temperature. Kinetic analysis of ^{45}Ca efflux from preloaded cells exhibits three separable pools of exchangeable calcium. The ratio of pool size of the fast-exchanging Ca-compartment (bound to cell surface) to pool size of the intermediate Ca-compartment (cytoplasmic) was found to decrease from 2.5 to 1.3 upon shift from non-permissive to permissive temperature. The slowly exchanging Ca-pool (presumably mitochondrial) did not change significantly upon temperature shift. These and further data demonstrate a close correlation between distribution of cellular Ca among different cellular compartments and characteristics of cellular proliferation, both attributable to the function(s) of a single oncogene.

Cellular calcium has been shown to be involved in a multitude of cellular regulation processes (1,2). In particular, evidence has been accumulated that it plays a major role in regulation of mammalian cell proliferation (3-6). Recent investigations on the action of different oncogenes have given rise to the expectation that considerable insights into processes of cell proliferation might be provided using cellular systems transformed by one (or very few) oncogene(s) (7,8). In this respect, many studies have been devoted to cells transformed by the src gene of avian sarcoma virus coding for a 60,000 dalton phosphoprotein ($pp60^{src}$)

ABBREVIATIONS: EGF, epidermal growth factor; ER, endoplasmic reticulum; PDGF, platelet-derived growth factor; $pp60^{src}$, phosphoprotein with 60 kilodalton from Rous sarcoma virus; $p68^{v\text{-}ros}$, protein with 68 kilodalton of avian sarcoma virus UR2.

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which initiates neoplastic transformation and maintains the neoplastic stage in cultured cells (7,9). One of the consequences of expression of this gene product is induction of proliferation of cells maintained in Ca-deficient growth medium (10,11), a condition universally shown to be suitable for proliferation of neoplastically transformed cells, but not of untransformed cells or temperature-sensitively transformed cells at non-permissive temperature (3,10,11). It has been speculated that the transforming gene product pp60^{src} of this virus mimics functions normally elicited by growth factors like EGF or PDGF (12,13). In fact, quite recently these have been shown to raise transiently the intracellular free Ca-concentration (14,15).

For a more detailed understanding of these phenomena, knowledge on cellular Ca-compartments and their changes upon (temperature-sensitive) neoplastic transformation appears to be required. A quantitative, albeit not detailed, characterization of cellular Ca-compartments may be gained by kinetic analysis of radiotracer (^{45}Ca) efflux curves (16).

Using Rat-1 cells transformed by temperature-sensitive Rous sarcoma virus (LA334m), we have, therefore, assayed compartmentation of cell-associated calcium by analysis of ^{45}Ca-efflux kinetics and total calcium by atomic-absorption spectrometry. The present results demonstrate a redistribution of Ca between different cellular compartments upon expression of the (temperature-sensitive) function of Rous sarcoma virus. An abstract on the results of this work has appeared recently (17).

MATERIALS AND METHODS

Cell culture: Fisher rat cells (F 2408, usually referred to as Rat-1) transformed by mutant ts LA 334 m of strain B77 of Rous sarcoma virus (9) were kindly provided by Dr. J. Wyke, London. Stock cultures were maintained in antibiotica-free Dulbecco's modification of Eagle's medium (Flow Laboratories, Bonn) at permissive temperature (36^{o}C). Cells were passaged twice a week at subconfluency. After about 40 passages, cultures were renewed

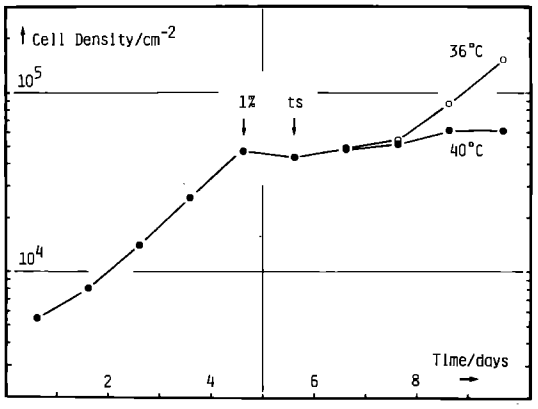

Fig.1. Growth curve of cells used for calcium efflux experiments. Cell cultures were started at 40 °C applying 5% fetal calf serum. After 4.6 days, cells were made quiescent by changing to a medium with 1% fetal calf serum. At 5.6 days, temperature was shifted from non-permissive (40 °C) to permissive (36 °C) temperature (indicated by ts). Throughout the experiment medium renewals were every 48 h.

from frozen stocks. At permissive temperature the cells were fully transformed, whereas at non-permissive temperature (40.5°C) growth characteristics were essentially normal (to be published elsewhere).

Cell preparations used for the present experiments were raised as follows (see Fig.1). Cells from stock culture were seeded at low densities (about $2 \cdot 10^3$ cells/cm^2), and grown at non-permissive temperature in medium containing 5% fetal calf serum. After cell-density had reached a value of 60 to $80 \cdot 10^3$ cells/cm^2, serum concentration was reduced to 1%, immediately arresting the cells in a non-proliferative state in G1 phase of the cell cycle. Usually two days later, experimental plates were shifted to permissive temperature.

Calcium determination by atomic absorption spectrometry: Plates were removed from the incubator and, after removal of the growth medium, dipped five times into ice-cold distilled water. This washing procedure lasted for about 12 sec. Thereafter, the plates were frozen until measurement. After thawing, the cells were removed from the plates in distilled water containing 1 mM $LaCl_3$. The resulting suspension was assayed using an atomic absorption spectrometer Beckman 1272 equipped with an N_2O-acetylene burner. Corrections were made to account for the amount of calcium released from the material of the tissue-culture plate itself.

Calcium efflux: Cells were equilibrated over night in growth medium containing radioactive calcium (0.4 MBq/plate). At the beginning of the efflux experiment, the plates were quickly washed five times (35 sec) in ice-cold medium containing no radioactivity; then, warm medium was added again, periodically replaced, and used for liquid-scintillation counting. All experiments were done in duplicate. At the end of the experiment, cells were scraped off the plates and assayed for remaining radioactivity. Curves of cell-associated radio-activity were constructed by adding up the back-ground-corrected cpm readings starting from the last time point. Data were normalized by dividing each figure by the specific activity of the incubation medium, resulting in mol exchangeable calcium per cell. Data thus obtained were subjected to non-linear regression analysis, yielding pool sizes

and time constants of exchange for the three cellular calcium-exchanging compartments (see below).

Multicompartment model of calcium exchange: Calcium efflux experiments, as described here, usually exhibit curves of the decay of cell-associated radioactivity which can be fitted by three exponential functions with different time constants. We have adopted the following model of cellular calcium compartmentation originally proposed by Borle (16) for a quantitative interpretation of our data. The cell is assumed to consist of three separable calcium-exchanging compartments (S1, S2, S3) in the following arrangement:

$$S3 \underset{k23}{\overset{k32}{\rightleftarrows}} S2 \underset{k02}{\overset{k20}{\rightleftarrows}} O \underset{k10}{\overset{k01}{\rightleftarrows}} S1$$

Pool S1 may be assumed to be membrane-bound Ca, exchanging very rapidly with the growth medium (O); pool S2 comprises the cytoplasmic free calcium pool and cytosolic calcium buffering capacity (most of which probably is ER); compartment S3 is supposed to represent calcium binding to mitochondria (1,2,18-20).

RESULTS

Determination of total cellular calcium by atomic-absorption spectrometry. For both transformed and non-transformed cells we found a pronounced dependence of cellular calcium on cell density (see Fig.2). As cells grow from a density of 10^4 cells/cm^2 to 10^5cells/cm^2 their calcium content decreases roughly by a factor of four. We have not detected any differences in overall calcium content between preparations grown at permissive and non-permissive temperature.

Calcium efflux. The result of a typical experiment comparing cells grown at non-permissive and at permissive temperature is shown in Fig.3. Efflux from cells at non-permissive temperature proceeds relatively fast (90% released after 10 min), whereas efflux of transformed cells is delayed (90% released after 30 min). In order to arrive at a quantitative description of these results, we have interpreted these data in terms of the multi-compartment model presented above. The results are shown in Table 1.

Upon shift to permissive temperature, we find a reduction of the "fast" compartment S1, an increase of pool size S2, and a

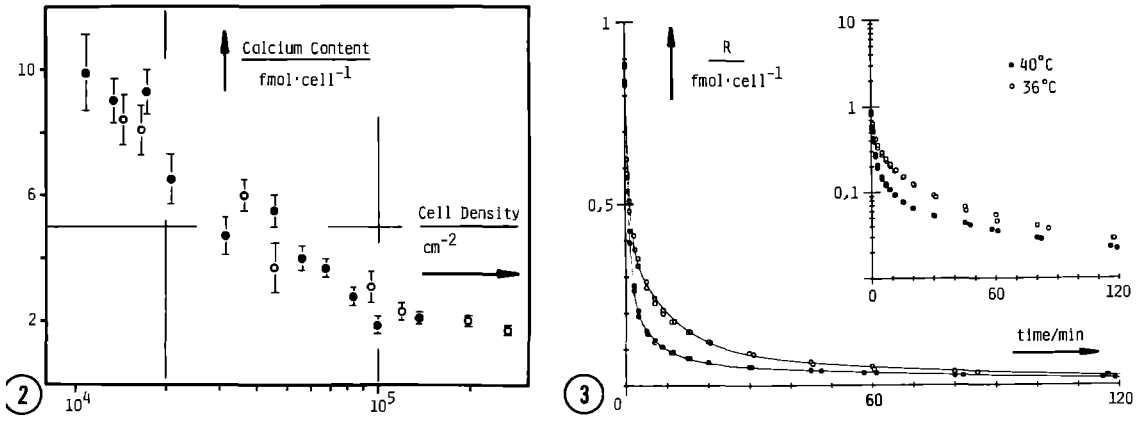

Fig.2. Cellular calcium content determined by atomic absorption as a function of cell density. Closed symbols: Cells grown at non-permissive temperature (40 °C). Open symbols: Cells grown at permissive temperature (36 °C). Error bars indicate standard error of the mean from 2 to 5 independent experiments (i.e. different growth curves).

Fig.3. Calcium efflux versus time. Duplicate experiments are shown. R: Normalized radioactivity associated with the cells (background-corrected cpm-values, divided by specific activity of incubation medium). Closed symbols: non-permissive temperature (40 °C). Open symbols: permissive temperature (36 °C). The inset shows a semilogarithmic plot of the same data.

reduction of the time constant k20 of exchange between pool 2 and the medium. Significant changes of pool size S3 and exchange rates k23 and k32 between pool 2 and pool 3 have not been observed. Notably, total exchangeable calcium, too, does not change significantly upon temperature shift (see Fig.3 and Table 1).

Effects as described here are detectable (though with only marginal significance) as early as 12 to 18 hours after temperature shift. They are accompanied by profound changes in cell morphology and precede the onset of DNA-synthesis, as measured by flow-cytometry (data not shown).

DISCUSSION

The results presented above demonstrate a close correlation between temperature-sensitive expression of the pp60^{src} gene product and the characteristics of cellular compartments for calcium.

Table 1

Pool sizes S1, S2, and S3 in fmol/cell and time constants k10, k20, k23, and k32 in min^{-1} of calcium exchange as obtained from efflux experiments by non-linear regression analysis

	Growth temperature		Differences significant
	40 °C n=21	36 °C n=17	at p = 0.05 (Weir test)
S1	0.593 ± 0.022	0.386 ± 0.025	yes
S2	0.237 ± 0.008	0.310 ± 0.024	yes
S3	0.090 ± 0.012	0.118 ± 0.016	no
k10	1.3*	1.3*	-
k20	0.168 ± 0.009	0.121 ± 0.008	yes
k23	0.013 ± 0.0012	0.015 ± 0.0015	yes
k32	0.034 ± 0.0047	0.040 ± 0.0077	no

40 °C: Control, cells at non-permissive temperature.
36 °C: Cells at permissive temperature; experiments performed for 18 to 72 hours after temperature shift.
n: Number of independent experiments; figures are given ±SEM.
*: This time constant was found to be invariable within experimental error for different preparations. It was, therefore, set constant in order to increase the rigidity of adjustment of parameters by non-linear regression.

As the pool size S3 of the slow compartment does not change significantly upon transformation of cells by shift to the permissive temperature, the major effect of transformation is a striking redistribution of surface-bound calcium S1 to the intracellular compartment S2, yielding S1/S2 = 1.25 at 36°C, whereas S1/S2 = 2.50 at 40°C.

The observed decrease by a factor of 1.5 of surface-bound Ca upon expression of transformation might be due to the following effects: (A) a decrease of cellular surface area at fixed surface-charge density, (B) a decrease of the affinity to calcium and/or the density of negative surface charges at fixed cellular surface area. As cellular volume determined by uptake of ^{14}C-o-methyl-

glucose (data not shown) does not decrease upon transformation, possibility (A) does not appear to be supported by our (preliminary) data. A more detailed analysis of this question by characterization of the morphology of the cell surface using scanning electron microscopy is under work in our laboratory. Concerning the alternative (B), efflux experiments performed at different external Ca-concentrations (to be published elsewhere) do not exhibit a significant change of affinity for binding of Ca to the extracellular compartment S1. Thus, the present data indicate that the decrease of S1 upon transformation is due to a decrease of the (negative) cell-surface charge density. This interpretation of our present data is of considerable interest, as a modulation of cell-surface charge density can be expected to strongly affect the passive fluxes of all ions across the plasma membrane. In fact, transformation of 3T3 mouse cells by the DNA-virus SV40 results in a decrease of cell-surface charge density by a factor of two, as derived from the dependence of passive K^+-fluxes on extracellular Ca^{2+}-concentration (21,22). As the gangliosides appear to represent a major fraction of cellular surface charge (23), it is of interest that a general effect of cellular transformation was shown to be an alteration of ganglioside metabolism (24). These data, taken together, raise the interesting possibility that cellular transformation generally is associated with a decrease of cell-surface charge density.

Interpretation of the characteristics of the second Ca-compartment S2 is complicated by the fact that it comprises the free cytoplasmic Ca^{2+} pool as well as Ca bound to intracellular sites (most probably to the endoplasmic reticulum). The pool size of the free cytoplasmic Ca^{2+} can be neglected due to its very low concentration of about 10^{-7} M (14,15). Thus, our results indicate that the intracellular sites of pool 2 (presumably the

ER) bind more Ca upon transformation. This effect may be the consequence of (i) an increased binding affinity of sites of pool 2 for calcium (at fixed or even lowered free cytoplasmic Ca^{2+}-concentration), or (ii) an increased free cytoplasmic Ca^{2+} concentration (at fixed binding affinity of pool 2). In order to arrive at a definite conclusion, levels of free cytoplasmic Ca^{2+} should be known. Work with this aim is in progress in our laboratory, using Quin 2 as a probe for free cytoplasmic Ca^{2+}. In this context, it appears of considerable interest that recent work (25) demonstrated a significant stimulatory effect of the active $pp60^{src}$ gene product on phosphorylation of phosphatidylinositol. Similar results were obtained for the $p68^{v\text{-}ros}$ gene product of avian sarcoma virus UR2 (26). Interestingly, polypeptide growth factors like EGF and PDGF have been shown recently to raise transiently intracellular free Ca^{2+}-concentration (14,15), and concurrently affect inositol lipid metabolism (27). According to recent results, break-down of polyphosphoinositides results in formation of inositol triphosphate, which in turn acts in different cellular systems by releasing Ca from internal stores, viz. the endoplasmic reticulum (28-30). Clearly, further work is needed to differentiate hereby between short-term effects mediated by humoral effectors (12,13,15,28-30) from long-term effects due to neoplastically activated oncogenes (10,11,13,25,26, this work).

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