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Roles of Calcium, Serum, Plasma, and Folic Acid in the Control of Proliferation of Normal and Rous Sarcoma Virus-Infected Chicken Fibroblasts

(heat-inactivated plasma/autonomy/wound hormone)

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ABSTRACT **In a culture medium of pH 7.4 and a folic acid concentration of 100 μg/liter that contains 5% heat-inactivated chicken plasma rather than serum, the rate of proliferation of normal chicken fibroblasts is determined by the concentration of calcium. Proliferation, rapid when the calcium concentration is physiological, decreases when the calcium concentration is reduced. At a very low calcium concentration, in this culture medium, normal fibroblasts are maintained without proliferation, whereas those infected with Rous sarcoma virus proliferate rapidly. This proliferative inactivity of normal fibroblasts does not involve contact-inhibition, since the effect is observed at low, as well as higher, culture densities. When a physiological amount of calcium is added to cultures of normal fibroblasts that have been maintained in very low calcium–plasma medium for 3 days, labeled thymidine uptake and protein synthesis are strongly stimulated, and cell division follows. The use of heat-inactivated chicken serum, instead of plasma, in this medium appears to strongly sensitize normal fibroblasts to the mitogenic action of calcium.**

In a plasma-containing culture medium of physiological calcium concentration and a folate concentration of 5 μg/liter, neither normal nor Rous sarcoma virus-infected fibroblasts proliferate to an appreciable extent. The use of serum, however, instead of plasma results in rapid proliferation of both normal and infected cells, as does increase in the folate concentration of the plasma-containing medium to 100 μg/liter.

The fact that while normal fibroblasts are maintained without proliferation in low calcium–plasma medium, Rous sarcoma virus-infected fibroblasts proliferate rapidly, indicates that the effect of calcium is regulatory rather than permissive. These results suggest that the proliferation of normal fibroblasts is initiated by a cellular function involving calcium, and that the autonomous proliferation of the neoplastic fibroblasts results either from increased calcium uptake or from an alteration or a bypass of that function. The results also suggest that serum contains a mitogenic factor(s) not present in plasma, possibly a "wound hormone" for fibroblasts.

A large amount of evidence has appeared that indicates that calcium and the principal hormones of the calcium homeostatic system are major physiological regulators of the proliferation of thymic lymphoblasts and bone-marrow erythroid cells, and perhaps of liver parenchymal cells and peripheral lymphocytes (1). It has also been shown, by S.D.B., that calcium ion concentration *in vitro* controls the proliferation of normal chicken fibroblasts in chicken plasma-containing medium, but does *not* control the proliferation of these cells after they have been infected by the Schmidt–Ruppin strain of Rous sarcoma virus (2, 3). The ability of calcium to regulate normal fibroblast proliferation is obscured in conventional, but less physiological, serum-containing medium (2, 3). This effect is due to the ability of serum to strongly stimulate the proliferation of normal cells.

Since a considerable alteration of a calcium-dependent proliferative control system follows infection of cells by an oncogenic virus, elucidation of the nature of the action of calcium could yield a greater understanding of the loss of proliferative control that is the essence of the neoplastic state. Therefore, in the present study we have located the calcium-sensitive stage of the normal fibroblast's growth-division cycle. Moreover, we will show that the mitogenic properties possessed by serum, but not by plasma, stem from an apparent sensitization of normal cells to the mitogenic action of calcium, as well as from a permissive effect on the proliferation of both normal and infected cells.

MATERIALS AND METHODS

The basic materials and methods used in the experiments reported here were described (2, 3).

Incubation Conditions—Carbon Dioxide. Some of the proliferative effects described previously (2, 3) were of variable magnitude. It was determined that this variability was related to differences in culture medium pH, and that these differences involved, in turn, inadequate control of CO_2 tension in the incubation chambers. For the experiments reported here, the CO_2 content of the humidified air–CO_2 mixture that passed through the incubator chambers was monitored with a Fyrite CO_2 Indicator (Bacharach Instrument Co., Pittsburgh, Pa.) and maintained at 5%. With 5% CO_2 in the incubation atmosphere, the pH of the culture media used in these experiments was 7.4.

Synthetic Medium. The components of the synthetic medium used in these experiments, as modified from those described previously (2, 3), are listed in Table 1.

Calcium-free synthetic medium was prepared by mixture of the following components, in appropriate proportions: glass-distilled water; 10× salt solution, including lactate and pyruvate; 50× amino acids in 1 N HCl; 100× glutamine, asparagine·H_2O and tryptophan; 1000× vitamins; 1000× Ampicillin; 100 mM $MgSO_4 \cdot 7H_2O$; 1 N NaOH (NaOH was added to neutralize the HCl that was used to dissolve the amino acids. The amount of NaCl in the 10× salt solution was reduced so that the NaCl content of the finished medium was that given in Table 1). After mixture, the completed,

TABLE 1. *Composition of synthetic medium, mg/liter*

NaCl 6800	Cystine 24	Tyrosine 36
$NaHCO_3$ 2200	Glutamic acid 10	Valine 46
KCl 445	Glycine 17	Thiamine·HCl 0.10
$NaH_2PO_4 \cdot H_2O$ 101	Histidine 31	Pyridoxal·HCl 0.05
$CaCl_2$ 0–160	Hydroxyproline 10	Nicotinamide 0.10
$MgSO_4 \cdot 7H_2O$ 168	Isoleucine 52	Riboflavin 0.01
Glucose 4000	Leucine 52	*d*-Biotin 0.01
Lactic acid 100	Lysine 47	Calcium pantothenate 0.15
Sodium pyruvate 6	Methionine 15	
Glutamine 60	Phenylalanine 32	Choline chloride 1
Asparagine 10	Proline 30	Folic acid 0.005*
Arginine 70	Serine 12	Inositol 2
Alanine 30	Threonine 48	Ampicillin 100
Aspartic acid 10	Tryptophan 10	

* In medium for primary and secondary cultures, folic acid was used at 5 μg/liter. In experiments, folate was used at concentrations of 5–100 μg/liter. All experimental media also contained hypoxanthine and xanthine, each at 10 μM.

calcium-free synthetic medium was thoroughly bubbled with 5% CO_2 in air.

Calcium was added to culture media from a 100 mM $CaCl_2$ stock. Folic acid in excess of 5 μg/liter was added, by dilution, from a 100 mg/liter sodium folate stock. Hypoxanthine and xanthine were included in media used for experiments at 10 μM each, a physiological concentration. They were added from a 10 mM stock in 0.1 N NaOH.

Preparation, Growth, and Passage of Cultures; Infection with Schmidt–Ruppin Rous Sarcoma Virus. Primary and secondary cultures were grown, and secondary cultures were infected with Rous sarcoma virus, in synthetic medium containing 1.44 mM calcium, with 10% heat-inactivated commercial chicken serum. Primary cultures were prepared, from the pectoral muscles of 8-week-old COFAL-negative cockerels, by the method given previously (2, 3). No supplementary folic acid was used for infection of secondary cultures with sarcoma virus.

Secondary cultures of normal and sarcoma virus-infected fibroblasts were passaged to yield the tertiary cultures of normal and infected cells used for experiments. Cells for tertiary cultures were seeded into 35-mm plastic tissue culture dishes. The seeding medium was 91% calcium-free synthetic medium–4% 2.5 mM EGTA in calcium-free synthetic medium–5% heat-inactivated plasma (total calcium, 2.5 mM). On the day after seeding, tertiary cultures were changed to test media. Test media were changed on day 2 and on each day thereafter.

Components and Construction of Experimental Media. Heat-inactivated plasma and heat-inactivated serum were prepared as described (2, 3).

Test media for the experiments represented in Figs. 1–4 were made with very low calcium–plasma medium ($[Ca^{2+}] \rightarrow 0$), or very low calcium–serum medium ($[Ca^{2+}] \rightarrow 0$) as a base, and addition of appropriate amounts of calcium or folate. Very low calcium–plasma medium was composed of 90% calcium-free synthetic medium (containing hypoxanthine and xanthine at 10 μM), 5% 2.5 mM EGTA in calcium-free synthetic medium, and 5% heat-inactivated plasma. Since the total calcium concentration of cockerel plasma was 2.5 mM (10 mg %), the concentration of available calcium in the very low calcium–plasma medium should approach zero. Although the serum and plasma used in the experiment represented in Fig. 4 were prepared from the same lot of pooled cockerel blood, the serum contained calcium at a total concentration of 9.5 mg %, rather than 10 mg % as did the plasma. Accordingly, very low calcium–serum medium ($[Ca^{2+}] \rightarrow 0$) for this experiment was composed of 90% calcium-free synthetic medium, 4.75% 2.5 mM EGTA in calcium-free synthetic medium, and 5% heat-inactivated serum.

Cell Counts. Test media were aspirated from dishes and 1 ml of calcium- and magnesium-free balanced salt solution, with 0.10% Difco trypsin and 0.01% Dow Corning Antifoam-F, was added. After 1 hr in the incubator, two drops of calf serum and two drops of 20-times physiological concentra-

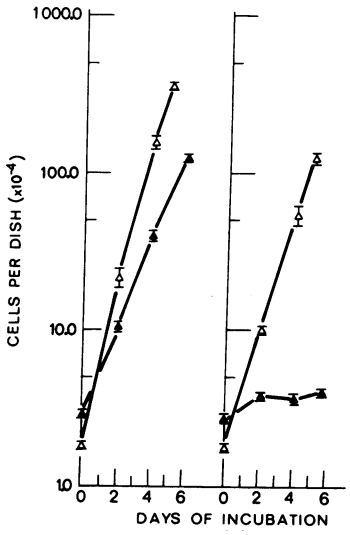


FIG. 1. Proliferation of normal (▲——▲) and Rous sarcoma virus-infected (△——△) chicken fibroblasts in plasma-containing medium at physiological (1.4 mM) (*left*) and very low ($[Ca^{2+}] \rightarrow 0$) (*right*) calcium concentrations. Medium contained 0.1 mg/liter of folic acid, 10 μM hypoxanthine, and 10 μM xanthine.

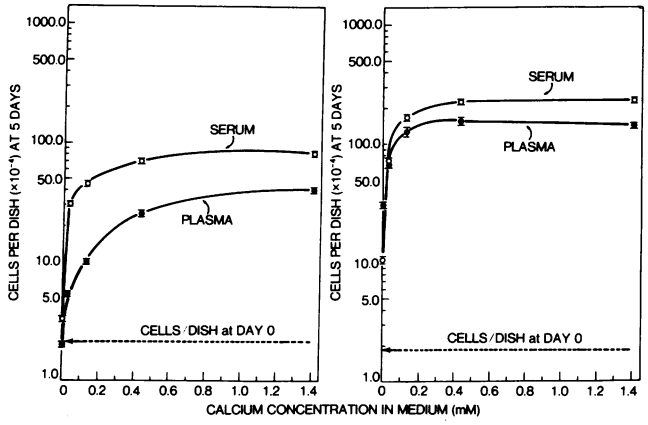
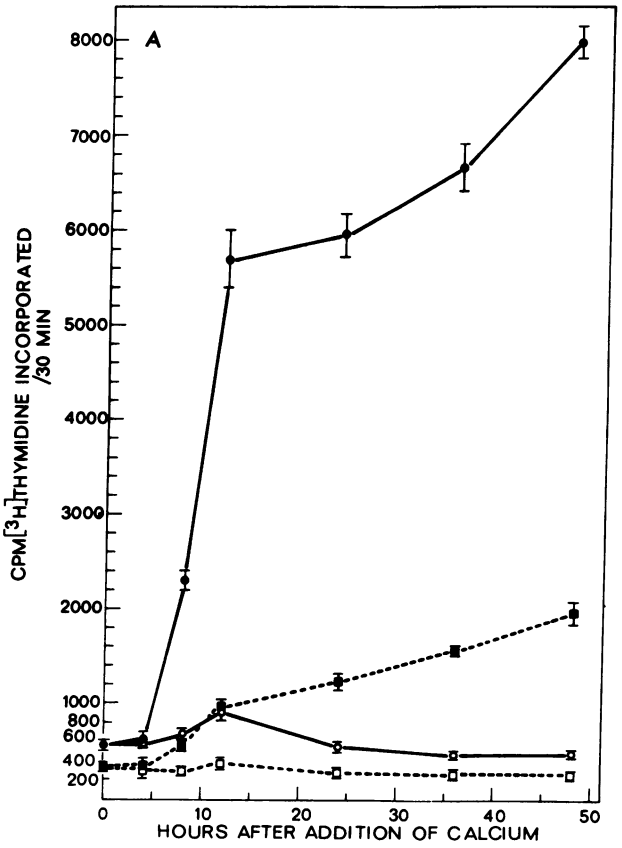


FIG. 2. Effect of calcium concentration on the proliferation of normal (*left*) and Rous sarcoma virus-infected (*right*) fibroblasts in plasma- and serum-containing media (see Fig. 1.). Heat-inactivated serum and heat-inactivated plasma were prepared from the same lot of pooled cockerel blood.



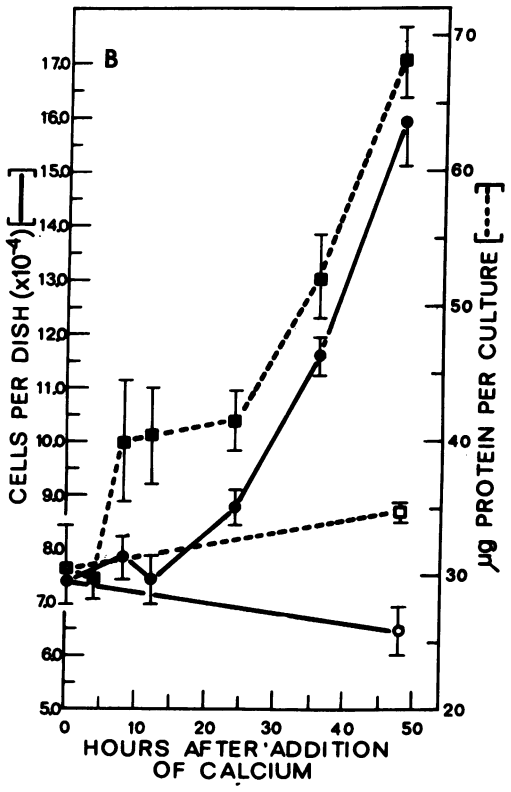


FIG. 3A, B. Initiation of fibroblast proliferation by calcium. Cultures of normal fibroblasts were maintained for 3 days in very low calcium–plasma medium ($[Ca^{2+}] \rightarrow 0$). At "zero" time, enough sterile 100 mM $CaCl_2$ solution was added to some of the dishes to give a final calcium concentration of 1.5 mM (*closed symbols*). *A: Solid lines;* hot acid-soluble cpm; *dashed lines,* cold acid-soluble cpm.

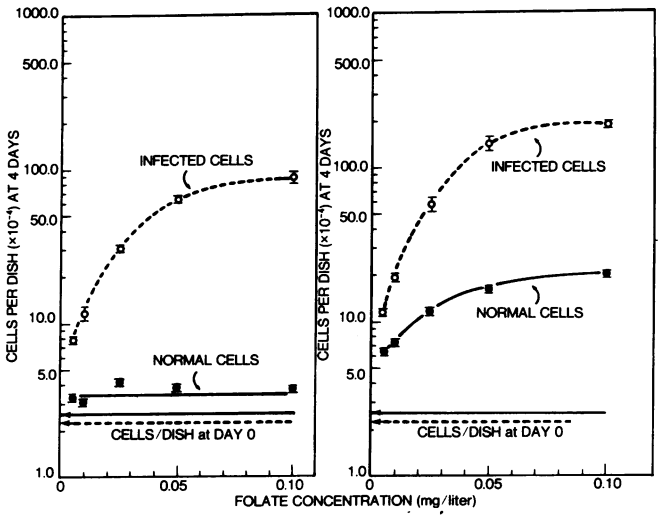


FIG. 4. Effect of folic acid concentration on the proliferation of normal and Rous sarcoma virus-infected fibroblasts in plasma-containing medium at physiological (1.2 mM) (*right*), and very low ($[Ca^{2+}] \rightarrow 0$) (*left*) calcium concentrations. Medium contained 10 μM hypoxanthine and 10 μM xanthine.

tion calcium and magnesium solution were added. Contents of the dishes were triturated 21 times with a disposable Pasteur pipette, then transferred to a tube containing 8.9 ml of Isoton diluting fluid (Coulter Electronics Inc., Hialeah, Fla.). The diluted cell suspensions were counted with a Coulter model B Electronic Cell Counter with a 100-μm aperture. All experimental points reported (cell counts, thymidine incorporation, and protein per culture) represent the means ± SEM of determinations on four culture dishes.

Thymidine Incorporation. Cells were exposed to 5.0 μCi of [^{3}H]thymidine (20 Ci/mmol) per ml of medium for 30 min. The monolayers were then washed twice with normal saline. Cultures were trypsinized and triturated as above, but with the addition of 10 mM unlabeled thymidine to the trypsinizing solution. After trypsinization and trituration, contents of the dishes (1.1 ml) were transferred to 15-ml conical centrifuge tubes containing 1.1 ml of cold 14% $HClO_4$ (perchloric acid), and the tubes were shaken to mix their contents. The centrifuge tubes containing the extraction mixtures were held at 4°.

The tubes were centrifuged, and the supernatants were transferred to scintillation vials containing 10 ml of Omnifluor (New England Nuclear)–dioxane cocktail and counted in a Beckman LS 255 scintillation counter. These counts were from cold $HClO_4$-soluble material, and were considered to reflect thymidine pool sizes.

The precipitate was washed three times with cold 7% $HClO_4$, then held at 80° for 30 min in 1 ml of 7% $HClO_4$. The tubes were spun at 2000 rpm in an International PRJ centrifuge and the supernatants were transferred to scintillation vials containing 10 ml of Omnifluor–dioxane cocktail for counting. These hot $HClO_4$-soluble counts were considered to represent incorporation of thymidine into DNA.

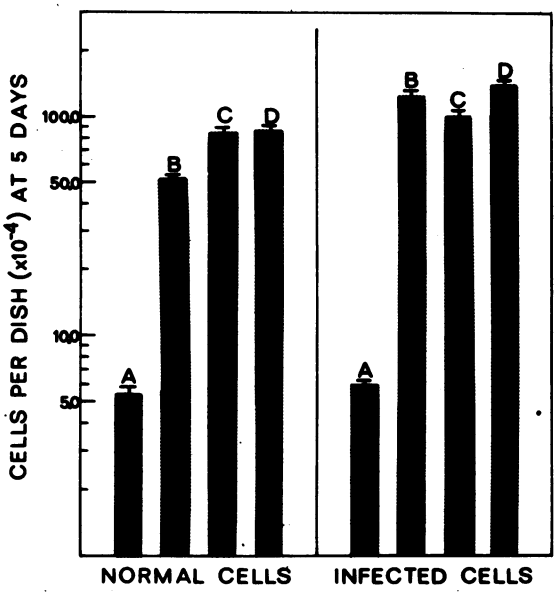


FIG. 5. Stimulation of fibroblast proliferation by chicken serum, but not chicken plasma, or by folic acid in a culture medium of physiological calcium concentration (1.4 mM). Media contained hypoxanthine and xanthine, each at 10 μM. Heat-inactivated serum and heat-inactivated plasma were prepared from the same lot of pooled cockerel blood. The zero-time values for culture dishes of normal and infected cells were, respectively, $2.8 \pm 0.1 \times 10^4$ and $3.0 \pm 0.1 \times 10^4$ cells per dish. *A*, plasma–5 μg/liter folate; *B*, plasma–100 μg/liter folate; *C*, serum, 5 μg/liter folate; *D*, serum, 100 μg/liter folate.

Determination of Protein per Culture. Test media were aspirated and monolayers were washed twice with normal saline. 1.0 ml of 1 N NaOH was added, and the dishes were incubated for 1 hr at 41°. Contents of the dishes were triturated and then transferred to 15-ml conical centrifuge tubes, which were held at room temperature. Protein was measured according to the method of Lowry *et al.* (4), with bovine-albumin fraction V as a standard.

DISCUSSION OF RESULTS

Normal chicken fibroblasts did not multiply in a medium containing chicken plasma and a very low calcium concentration ($[Ca^{2+}] \rightarrow 0$), but cells infected with the Rous sarcoma virus proliferated rapidly under these conditions (Fig. 1). More detailed examination of the calcium dependence of normal cell proliferation showed that maximum proliferation occurred when the extracellular calcium concentration was about 0.5 mM (Fig. 2). However, the proliferative activity of infected cells, which was already high at very low calcium concentrations, reached its maximum when the extracellular calcium concentration was only about 0.1 mM (Fig. 2). Therefore, infection with Rous virus appears either to increase calcium uptake by the cells or to functionally alter or largely bypass a normal, calcium-dependent system for the control of proliferation.

The use of serum instead of plasma obscured the proliferative differences between normal and Rous virus-infected fibroblasts. The experimental curves shown in Fig. 2 suggest that this result was due to a capacity of serum to strongly sensitize normal cells to the action of calcium. Thus, normal cells did not proliferate in very low calcium medium containing serum ($[Ca^{2+}] \rightarrow 0$), but elevation of the calcium concentration, only slightly, to 25 μM increased their proliferative activity to its half-maximal value (Fig. 2). In contrast to its striking effect on normal cells, the substitution of serum for plasma did not substantially alter the response of infected cells to variation of the calcium concentration between 0.025 and 1.4 mM (Fig. 2).

When a physiological amount of calcium was added to cultures of normal fibroblasts that had been maintained, without proliferation, in very low calcium–plasma medium ($[Ca^{2+}] \rightarrow 0$), labeled thymidine uptake and protein synthesis were strongly stimulated, and cell division followed (Fig. 3). Between 4 and 8 hr after restoration of a physiological extracellular calcium concentration (1.5 mM), there was a striking increase in the rate of incorporation of [^{3}H]thymidine into hot $HClO_4$-soluble material (DNA), without an equivalent increment in the cellular content of cold acid-soluble thymidine (the thymidine pool) (Fig. 3*A*). Coincident with the initiation of DNA synthesis, protein synthesis began, and sometime between 8 and 20 hr later, the cells began to divide (Fig. 3*B*). These observations suggest that calcium controls the proliferation of normal fibroblasts by initiating some critical process in the phase of the cell cycle (G1) prior to DNA synthesis that determines the operations of the necessarily large complex of reactions leading to mitosis and cytokinesis.

Once calcium has activated the growth of normal fibroblasts, the subsequent level of proliferative activity is determined by the availability of folic acid (Fig. 4). Folate concentrations as high as 0.1 mg/liter did not stimulate proliferatively inactive normal fibroblasts in very low calcium–plasma medium (Fig. 4), but did stimulate the proliferation of cells that had been "activated" by culture in 1.2 mM calcium (Fig. 4). Rous sarcoma virus-infected cells, on the other hand, are proliferatively active in very low calcium–plasma medium, and folate was consequently able to stimulate their proliferation in the presence or absence of calcium (Fig. 4). Therefore, manipulation of the folate concentration can greatly magnify the calcium-dependent difference between the proliferative activities of normal and infected cells.

Serum has a second property that plasma lacks, in addition to its apparent ability to sensitize normal cells to calcium: In a plasma-containing culture medium of physiological calcium concentration and folate concentration 5 μg/liter, neither normal nor Rous virus-infected fibroblasts proliferate to an appreciable extent. The use of serum, however, instead of plasma, results in rapid proliferation of both normal and infected cells, as does an increase in the folate concentration of the plasma-containing medium to 0.1 mg/liter (Fig. 5).

In summary, the fact that while normal fibroblasts are maintained without any proliferation in low calcium–plasma medium, Rous virus-infected fibroblasts proliferate rapidly, indicates that the effect of calcium is regulatory rather than permissive. These results suggest that the proliferation of normal fibroblasts is initiated by a cellular function involving calcium. The autonomous proliferation of the neoplastic fibroblasts appears, on the other hand, to result either from an increased capacity for calcium uptake or an alteration or bypass of the normal calcium-dependent proliferation-control system.

The best demonstration of the difference between the proliferative properties of normal and infected cells is obtained

with a "very low calcium" medium containing chicken plasma and 0.1 mg of folic acid per liter. The universal practice of using serum, instead of plasma, obscures this difference, since serum contains a factor(s) that first appears to greatly sensitize normal cells to calcium and then, like folate, promotes the subsequent multiplication of the activated cells. In the animal, this serum factor(s) would appear only locally and transiently in a blood-clot in an injured tissue, where it might act as a "wound hormone" that could initiate, or at least promote, cell proliferation (2, 3).

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