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Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells

(growth regulation/DNA synthesis/K^+ transport/cyclic nucleotides)

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ABSTRACT **Serum causes a 4-fold increase in $^{86}Rb^+$ (a K^+ tracer) influx in quiescent 3T3 mouse fibroblast cells. It is one of the earliest changes caused by serum, being seen in 2 min and reaching a maximum in 10 min. Removal of serum causes rapid reversal of this effect. Serum acts mainly by increasing the maximum velocity, V_{max}, of entry. Ouabain inhibits entry of $^{86}Rb^+$ (82–90%) both in the presence and absence of serum, but does not alter exit. The rapid increase in cation influx is unaffected by cycloheximide and by changes in cyclic AMP and GMP. Low concentrations of insulin, epidermal growth factor, and prostaglandins (E_1 and $F_{2\alpha}$) produced a smaller (80%) activation of $^{86}Rb^+$ entry. Ouabain, at a level that inhibits cation influx, also prevents the onset of DNA synthesis following serum addition; this is a reversible effect dependent on the concentration of K^+ in the medium. This suggests that cation pumping activity may be required for initiation of DNA synthesis.**

Cultured fibroblasts exist in two states of growth: one of active proliferation and one of reversible arrest in the G1 phase of the cell cycle. Serum stimulates resting cells to reinitiate DNA synthesis and cell division (1). Changes in transport rates for P_i, nucleosides, and glucose (2–5) and in cyclic nucleotide levels (6–8) are, so far, the earliest events detected in this system. Whether or not K^+ uptake is *rapidly* increased by serum is unknown and of importance because the asymmetric distribution of K^+ and Na^+ profoundly affects the regulation of transport of non-electrolytes (9), intracellular osmotic pressure (10), membrane potential (10), glycolytic enzymes (11), macromolecular synthesis (12–14), etc. Furthermore, lectin-stimulated lymphocytes (15) and virus-transformed fibroblasts (16) show increased K^+ transport. Thus, changes in ion pumping activity may be another control point in linking surface and intracellular events.

Here, we report that serum addition rapidly increases the influx rate of $^{86}Rb^+$ into quiescent 3T3 cells. This isotope serves as a K^+ tracer (17). We describe this effect in detail, define its relationship to other early events, and present suggestive evidence that changes in monovalent cation pumping activity may be required for the initiation of DNA synthesis.

MATERIALS AND METHODS

Stock cultures of Swiss mouse 3T3 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified atmosphere of 10% CO_2–90% air at 37°. Cells were subcultured to 30 mm Nunc petri dishes with medium containing 6% serum and used 3–5 days after the last change of medium. After a variable preincubation in 2 ml of fresh medium, with or without 10% fetal calf serum, a solution containing $^{86}Rb^+$ (final concentration 2.5 μCi/ml; 0.1 mM RbCl) was added to the medium. After 10 min (unless otherwise indicated) at 37°, $^{86}Rb^+$ uptake was stopped by sucking off the medium and rapidly washing four times with ice-cold isotonic saline. The cells were extracted at 4° for 20 min with 1.5 ml of 5% trichloroacetic acid and 1 ml of extract was mixed with 10 ml of water to measure Cerenkov radiation. Control cultures were washed immediately after adding isotope. The radioactivity incorporated into cells was not significantly altered by washing at 26° or by using 10 mM Tris·HCl–200 mM choline Cl, pH 7.4 in place of saline. To measure rate of efflux, cells were preloaded in the presence or absence of serum with $^{86}Rb^+$ for 20 min, washed twice with prewarmed medium, and shifted to fresh medium without $^{86}Rb^+$; at different times the $^{86}Rb^+$ remaining in the cells was measured as in the uptake trials.

DNA synthesis was estimated by exposing cells to [^{3}H]thymidine for 24–26 hr and measuring incorporation into trichloroacetic-acid-insoluble material or by radioautography. For incorporation studies, levels of 1 μCi/ml and 3×10^{-6} M were used; for autoradiography the values were 5 μCi/ml and 2×10^{-7} M. Saline-washed cells were assayed for protein (18).

Epidermal growth factor and prostaglandins were the generous gift of Drs. S. Cohn, Vanderbilt, and J. Pike, Upjohn. Isotopically labeled compounds were obtained from The Radiochemical Centre, Amersham.

RESULTS

Addition of serum for 20 min to quiescent 3T3 cells stimulates $^{86}Rb^+$ uptake up to 4-fold whether or not K^+ is present in the medium during exposure to serum or during $^{86}Rb^+$ uptake. The serum effect saturates at a concentration of 10% serum. With growing cells, the stimulation by serum is only 20–40%. Kinetic studies (Fig. 1A, insert) reveal that the rate of $^{86}Rb^+$ transport increases within 2 min after serum addition and reaches a maximum by 10 min (Fig. 1). An activation by serum of $^{86}Rb^+$ influx within 2 min was also seen at a lower Rb^+ concentration (0.1 mM rather than 0.5 mM). This increase in rate is reversible within 15 min on removal of serum (Fig. 1B). Re-addition of serum to these cultures elicits once more a rapid increase in rate of uptake of $^{86}Rb^+$. Clearly, the rate of $^{86}Rb^+$ transport is rapidly altered by some sort of switch mechanism upon the addition to or removal of serum from quiescent cells.

Does serum increase the rate of influx or reduce the rate of efflux of $^{86}Rb^+$? Fig. 2A shows a 3.5-fold increase in rate of $^{86}Rb^+$ influx after serum addition. By contrast, the rate of efflux of isotope from cells pre-loaded with $^{86}Rb^+$ is not appreciably affected by serum (Fig. 2B).

That the increase in rate of $^{86}Rb^+$ influx produced by serum is mediated by the Na^+ pump is shown by the

Abbreviation: PG, prostaglandin.

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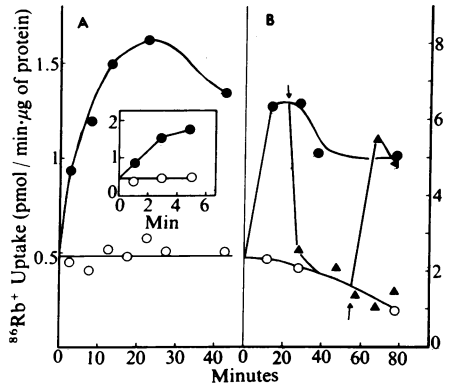

FIG. 1. (A) Kinetics of stimulation by serum of Rb^+ uptake in quiescent 3T3 cells. The cultures were washed twice with KCl-free medium and in the presence (closed symbols) or absence (open symbols) of fetal calf serum. Uptake of $^{86}Rb^+$ (0.5 mM final concentration, 5 μCi per dish) measured over a 5 min interval and the time points are plotted for the middle of the 5 min pulse. Thus, for the 7.5 min time point, cells were pulsed with $^{86}Rb^+$ between 5 and 10 min after the change of media. *Insert:* Kinetics of stimulation at early time intervals. Transport rates were determined in cultures shifted to serum-free or serum-containing medium and exposed to isotope from 0 to 2 min, 2 to 4 min, or 4 min to 6 min. (B) Effect of removal and re-addition of serum on Rb^+ uptake by 3T3 cells. Cultures were incubated in medium with (●) or without (○) 10% serum in the presence of 5 mM K^+. The "5 min rate" of Rb^+ uptake was measured as in (A), at different times. At 22 min (arrow pointing down), the medium containing serum was removed from some of the dishes which were then washed three times and incubated with warmed, serum-free medium. The rate of uptake of Rb^+ was measured at different times thereafter. At 55 min (arrow pointing up), serum was added back to some of these dishes and transport rates were determined afterwards.

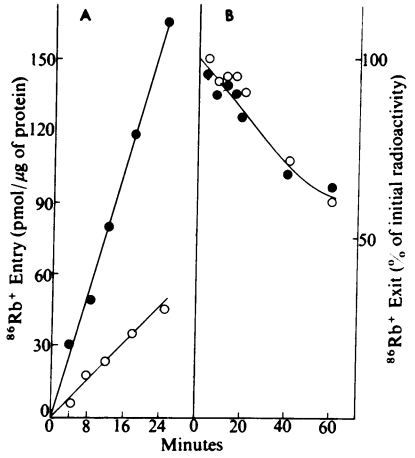

FIG. 2. Serum stimulates Rb^+ influx (Panel A) but does not stimulate Rb^+ efflux (Panel B). (A) The usual growth medium of quiescent 3T3 cells was replaced by serum-free medium (open symbols) or by medium containing 10% fetal calf serum (closed symbols). After 20 min at 37° the cultures were exposed to $^{86}Rb^+$ for different times as indicated. (B) Quiescent 3T3 cells, exposed to serum-free medium (open symbols) or to medium containing 10% fetal calf serum (closed symbols) were loaded with $^{86}Rb^+$ for 20 min in the presence of the usual concentration of K^+ (5 mM). Thereafter, the cells were rapidly washed twice with prewarmed serum-containing or serum-free medium, and incubation was continued to allow efflux of the accumulated radio isotope to occur. The radioactivity still present in the cells at each time interval is expressed as % of that present at the end of the 20 min labeling period. This corresponded to 6120 cpm per dish (4.4 nmol of $^{86}Rb^+$) for cells in serum-free medium and 20,230 cpm per dish (14.7 nmol of $^{86}Rb^+$) for cells in serum-containing medium.

marked sensitivity of both stimulated and basal uptake to the specific inhibitor ouabain; rate of influx decreases by 82–90% (Fig. 3B). When the concentration of K^+ in the medium is reduced 10-fold, there is a striking increase in sensitivity to ouabain of both the stimulated and basal rates (Fig. 3A). This would be expected if the changes in $^{86}Rb^+$ influx are largely mediated by fluctuations in the activity of the Na^+ pump (19)†. In contrast to the influx data, $^{86}Rb^+$ efflux with or without serum was not inhibited by 1 mM ouabain (data not shown). A small ouabain-insensitive fraction of uptake is also increased by serum (Fig. 3).

The rapid stimulation of rate of $^{86}Rb^+$ influx is due to an increase in maximum velocity V_{max} with no change in apparent Michaelis constant K_m (Fig. 4). The K_m, 1.4 mM, agrees with reported values for HeLa cells (20). The rapidity of serum stimulation and the fact that 15 μg/ml of cycloheximide has no effect argues against *de novo* synthesis of transporter molecules.

In addition to serum, insulin and epidermal growth factor stimulate $^{86}Rb^+$ uptake in a concentration-dependent manner (Fig. 5A and B), although to a lesser extent. Epidermal growth factor acts at the same low concentration needed for specific binding to surface receptors in fibroblasts (21, 22).

We also examined the possible role of cyclic nucleotides. The drop in cAMP caused by serum (6–8) is unlikely to mediate the activation of $^{86}Rb^+$ uptake because the following cAMP-elevating agents fail to prevent this stimulation: theophylline (1–2 mM), 1-ethyl-4-hydrazino-1-H-pyrazolo-(3,4-6)-pyridine-5-carboxylic acid, ethyl ester·HCl (SQ20006) (23) (0.2–0.5 mM), isobutyl methylxanthine (0.5 mM), prostaglandin E_1 (PGE_1) (0.25–25 μg/ml), N^6-monobutyryl cAMP (0.5 mM), 8-bromo-cAMP (0.5 mM), and combinations of PGE_1 (25 μg/ml) with theophylline (1.2 mM) or SQ20006 (0.25 mM). Furthermore, in the absence of serum, PGE_1 elevates cAMP levels (3, 7, 24) and yet it *stimulates* rather than inhibits the uptake of $^{86}Rb^+$ (Fig. 5C). Another reason for thinking that cAMP does not mediate this uptake

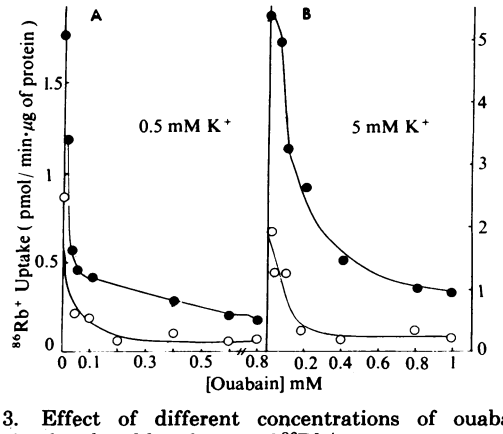

FIG. 3. Effect of different concentrations of ouabain on serum-stimulated and basal rates of $^{86}Rb^+$ transport at two different levels of K^+. Quiescent cultures of 3T3 cells were washed twice with KCl-free medium and exposed to medium containing 0.5 or 5 mM K^+ in the absence (open circles) or in the presence (closed circles) of 10% fetal calf serum and different concentrations of ouabain freshly prepared. After 20 min incubation the cultures were labeled with $^{86}Rb^+$ for 10 min. Uptake was determined as described in *Materials and Methods*.

† On repeated trials, we have been unable to detect changes in NaK ATPase activity of cell homogenates, to correlate with the rapid increase in ouabain-sensitive $^{86}Rb^+$ uptake after serum addition.

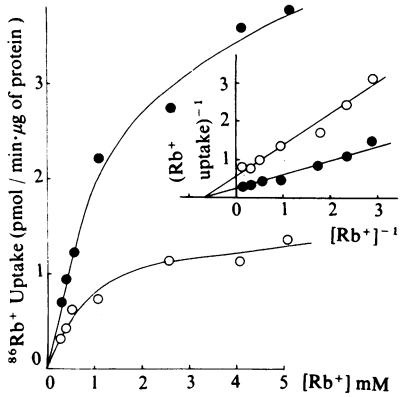

FIG. 4. Rate of influx of $^{86}Rb^+$ as a function of Rb concentration, in the presence or absence of serum. The usual medium of quiescent 3T3 cells was replaced by serum-free (open circles) medium or by medium containing 10% dialyzed serum (closed circles). Twenty minutes later the cultures were rapidly washed twice with pre-warmed KCl-free medium and then incubated with medium containing different concentrations of Rb^+ labeled with $^{86}Rb^+$ (5 μCi/ml), for 10 min, during which uptake was linear with time. Again, serum was present (●) or absent (○). Each point represents the average of two determinations.

effect is that $PGF_{2\alpha}$ also increases $^{86}Rb^+$ entry (Fig. 5C) but it changes cAMP levels very little (7, 24). Exposure of quiescent cultures to 8-bromo-cyclic guanylic acid (10^{-7}–10^{-3} M) for 15–60 min in serum-free medium has no effect. The serum stimulation of $^{86}Rb^+$ influx proceeds unaffected in phosphate-free medium.

Ouabain, which inhibits growth of several cell lines (14, 25), prevents the initiation of DNA synthesis in serum-stimulated 3T3 cells (Fig. 6), as indicated by radioautography (Fig. 6A, and B), thymidine incorporation (Fig. 6C and D), and by measuring total DNA (data not shown). This ouabain effect is reversible (not shown), is obtained at different serum levels (Fig. 6A), and is produced in the concentration

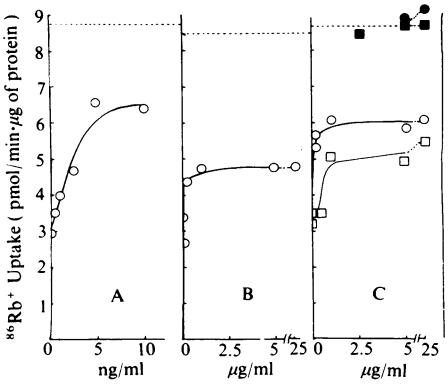

FIG. 5. (A) Effect of different levels of epidermal growth factor on the rate of ^{86}Rb influx in quiescent 3T3 cells. The peptide was diluted in medium containing 50 μg/ml of crystalline bovine albumin and added to the cultures for 20 min. Then $^{86}Rb^+$ uptake was measured as under *Materials and Methods*. (B) Effect of different levels of bovine insulin (26.4 IU/mg) on $^{86}Rb^+$ uptake. The hormone was dissolved at 5 mg/ml in 6 mM HCl and then diluted in nutrient medium. Other experimental details were as in A. (C) Effect of different levels of prostaglandins E_1 (squares) and $F_{2\alpha}$ (circles) in the absence (open symbols) or in the presence (closed symbols) of 10% dialyzed fetal calf serum. Prostaglandins were dissolved in ethanol (10 mg/ml); a fixed amount of ethanol (0.25%) was present in all the determinations. Other experimental details were as described in A. The broken lines represent the level of uptake stimulated by a 20 min exposure to serum in each experiment.

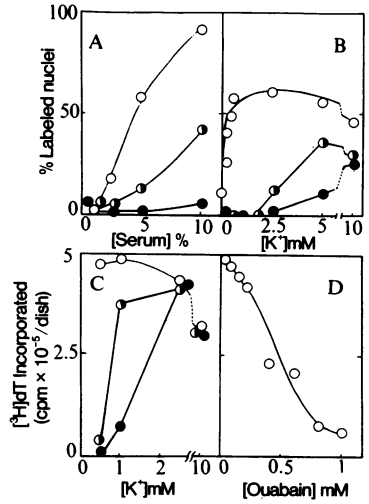

FIG. 6. The stimulation of DNA synthesis caused by serum can be inhibited by ouabain. (A) Cultures of quiescent 3T3 cells were exposed to different concentrations of serum in the presence of zero (○) 0.4 mM (◑) or 0.8 mM (●) ouabain. Incorporation of [^{3}H]thymidine was revealed by radioautography, scoring 1000 cells and averaging two determinations for each point. (B) A similar experiment, except that cells were washed twice with K-free medium and then incubated in the presence of 10% dialyzed serum and different concentrations of K^+ with either zero (○), or 0.25 mM (◑) or 0.5 mM (●) ouabain. In parts (C) and (D) the incorporation of [^{3}H]thymidine was measured by scintillation counting. After labeling, the cultures were washed with isotonic saline, extracted with 5% trichloroacetic acid three times for 15 min, washed two times with ethanol, air dried, dissolved in 0.1 N NaOH, and counted in acidified Triton X-100 –toluene. (C) The incorporation of [^{3}H]thymidine was measured as a function of K^+ concentration in the medium without (○) and with 0.1 mM (◑) or 0.2 mM (●) ouabain. (D) Incorporation of [^{3}H]thymidine in cultures exposed to different concentrations of ouabain in regular medium.

range that inhibits cation influx (Fig. 6D). This inhibition of DNA synthesis depends dramatically on K^+ concentration; the large inhibition of thymidine incorporation obtained at 0.5 mM K^+ is completely reversed by raising the level of K^+ by 10-fold. This indicates that the effect of ouabain is mediated by inhibition of the sodium pump.

DISCUSSION

The present results show that serum added to quiescent fibroblasts rapidly increases the rate of $^{86}Rb^+$ influx but has only a slight effect on the rate of cation efflux. This activation involves the Na^+ pump as indicated by the ouabain sensitivity of the fluxes. Ouabain inhibits entry of $^{86}Rb^+$ by 82–90% without altering the rate of efflux. Thus, only entry appears to be mediated by the Na^+ pump. The absence of ouabain-sensitive $^{86}Rb^+$ efflux argues against the existence of a pump-mediated $^{86}Rb^+$ $-K^+$ exchange (26, 27)‡. Furthermore, the ouabain inhibition of basal and serum-stimulated fluxes is specific, as shown by the marked dependence of the effect on the K^+ concentration of the medium (10, 19). All of these findings support the notion that serum stimulates the Na^+-pump-mediated uptake of K^+.

‡ The intracellular concentration of K^+ was also determined by flame photometry after exposing quiescent 3T3 cells to medium with or without 10% serum for 40 min and 60 min. The values (μmol of K^+/mg of protein) were 0.65 and 0.74 in the presence of serum; in its absence 0.51 and 0.48. The initial concentration was 0.59 μmol/mg of protein. Quintuplicate assays were performed.

The rapidity of serum stimulation (activation begins in seconds) and its ready reversal suggest that fluctuations in pump activity may reflect the interaction of serum factors with the cell surface. Indeed, low concentrations of pure substances like prostaglandins (E_1 and $F_{2\alpha}$), insulin, and epidermal growth factor are also able to stimulate the uptake of $^{86}Rb^+$. The peptide hormones are known to interact with specific surface receptors in fibroblasts (21, 22, 28). Clearly, there are rapid, cycloheximide-insensitive, mechanisms for regulation of active transport which do not depend on *de novo* synthesis of transporter molecules.

This early increase in ion pump activity raises important questions about its relation to other early changes and to late events such as the initiation of DNA synthesis. Our data suggest that the activation of Rb^+ influx is largely independent of changes in cyclic nucleotides and of increased fluxes of inorganic phosphate. Since ouabain has been found to depress the rate of entry of inorganic phosphate (11, 29) it is, in fact, conceivable that increased entry of phosphate is partly mediated by increased activity of the Na^+ pump. Our data also indicate that ouabain prevents the onset of DNA synthesis after serum addition to quiescent 3T3 cells; the drug is less efficient when DNA synthesis is under way (unpublished results). Although the mechanism of this inhibition remains to be established, the data do suggest that a continuous pumping of ions is required for the initiation of DNA synthesis.

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