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Serum stimulates the Na^+,K^+ pump in quiescent fibroblasts by increasing Na^+ entry

(transport/membrane ATPase/monensin/growth control/DNA synthesis)

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ABSTRACT Two ionophores (monensin and gramicidin) that carry Na^+ into 3T3 cells markedly enhance the rate of $^{86}Rb^+$ uptake. Ouabain prevents both ionophores from increasing $^{86}Rb^+$ uptake, indicating that the ionophores activate the Na^+,K^+ pump. Measurements of $^{86}Rb^+$ uptake and cell Na^+ and K^+ over a range of monensin concentrations show that the activity of the Na^+,K^+ pump in 3T3 cells is limited by the supply of internal Na^+ and is extremely sensitive to small changes in internal Na^+. Serum rapidly enhances the rate of $^{22}Na^+$ uptake and net Na^+ entry when Na^+ exit is inhibited by ouabain. At 0.3 $\mu g/ml$, monensin increases the rate of net Na^+ entry and activates the Na^+,K^+ pump by the same degree as serum. The stimulation of $^{86}Rb^+$ uptake by serum or the ionophores has an absolute requirement for external Na^+. Thus, serum appears to stimulate the Na^+,K^+ pump in quiescent 3T3 cells by increasing its supply of Na^+.

Fibroblasts in culture undergo arrest in the G_1/G_0 phase of the cell cycle when the medium becomes depleted of growth factors or essential nutrients (1–3). Addition of fresh serum or purified growth factors to quiescent cultures sets in motion a complex array of events which precede DNA synthesis and cell division (4).

One of the more striking changes occurring in the plasma membrane of quiescent fibroblasts soon after the replenishment of growth factors is the activation of the Na^+,K^+ pump which in quiescent 3T3 cells increases about 3-fold within minutes after the addition of fresh serum (5, 6). The purified mitogens, multiplication stimulating activity and fibroblast-derived growth factor, stimulate the Na^+,K^+ pump in quiescent cultures of chicken embryo fibroblasts (7) and 3T3 cells (8), respectively. Ouabain reversibly inhibits the Na^+,K^+ pump in fibroblasts and blocks protein and DNA synthesis, which appear to depend on the level of intracellular K^+ (9). Significantly, fibroblasts arrested in G_0 increase their K^+ content when growth is initiated (5, 6) and cell K^+ and $^{86}Rb^+$ uptake fall when the fibroblasts stop growing at confluency (10). Transformed fibroblasts, which have a characteristically high rate of proliferation, likewise display a higher rate of Na^+,K^+ pumping (11, 12).

This report addresses the mechanism by which serum and growth factors rapidly modulate the Na^+,K^+ pump in fibroblasts. We propose the following model of pump regulation: (*i*) there is a Na^+ channel in the plasma membrane of fibroblasts; (*ii*) growth factors increase Na^+ entry via the channel; (*iii*) the activity of the Na^+,K^+ pump in fibroblasts is limited by the supply of internal Na^+; and (*iv*) growth factors stimulate the pump by activating the Na^+ channel, thereby supplying the pump with more Na^+. Our study of Li^+ transport (13) and the data presented here on Na^+ entry, on the action of ionophores, and on the requirement of external Na^+ for stimulating the pump support the proposed model of regulation.

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MATERIALS AND METHODS

Cell Culture. The Swiss line of fibroblastic mouse 3T3 cells (1, 2) was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described (5). The medium was replaced 3 days after subculture, and assays were usually performed on confluent and quiescent cultures in 33-mm Nunc dishes about 7–14 days later.

Cells Na^+ and K^+. Total cell Na^+ and K^+ were measured with a flame photometer (Corning-EEL, Model 430). Cultures of 3T3 cells (in 90-mm dishes) were washed six times with 0.1 M $MgCl_2$ at 4°C and allowed to drain for a few minutes to improve removal of the final $MgCl_2$ wash. When the dishes were dry, 1.0 ml of 15 mM LiCl containing 1% toluene was added. Toluene helped to lyse the cells and the LiCl served as an internal standard.

Ion Fluxes. $^{22}Na^+$ uptake was assayed after equilibrating 90-mm cultures in growth medium containing choline Cl instead of NaCl. The replacement of NaCl with choline Cl reduced the concentration of Na^+ in the medium to 50 mM and was done to increase the specific activity of the isotope. Ouabain was present at 1 mM to prevent Na^+ from exiting via the Na^+,K^+ pump. After a 5-min incubation at 37°C in a humidified atmosphere of 10% CO_2/90% air in 2 ml of medium, 0.100 ml of $^{22}Na^+$ containing about 3×10^6 cpm (Amersham/Searle, 0.2 mCi/μg of Na^+) was added. Uptake was stopped 3 min later, by placing the dishes on ice and washing each rapidly six times with 10 ml of 0.1 M $MgCl_2$. After removal of the last $MgCl_2$ wash, 1.0 ml of 5% trichloroacetic acid was added to extract radioactivity. $^{22}Na^+$ was counted in a gamma counter optimized for $^{22}Na^+$ (Intertechnique, CG 2000). Uptake is expressed in μmol of Na per mg of cell protein.

The uptake of $^{86}Rb^+$ was assayed as described (5), except that 100 mM $MgCl_2$ at 4°C, instead of 150 mM NaCl, was used to wash the cells for the purpose of removing external isotope. The use of $MgCl_2$ instead of NaCl improved the retention of internal K^+ and Li^+. The cells were equilibrated in the assay medium for 5 min prior to initiating a 10-min uptake with the addition of 50 μl of $^{86}Rb^+$ containing about 2×10^6 cpm (Amersham/Searle, 5 mCi/mg of Rb^+). Whenever ouabain was present, the concentration was 1 mM. DNA synthesis and total cell protein were assayed as described (5).

RESULTS

Serum Enhances Na^+ Entry in Quiescent 3T3 Cells. Fig. 1 shows that the addition of fresh serum increases markedly the rate of $^{22}Na^+$ uptake and net Na^+ entry in cells arrested in G_1/G_0. $^{22}Na^+$ does not appear to enter fibroblasts by cotransport with amino acids because ouabain, which inhibits amino acid uptake, increases $^{22}Na^+$ uptake. Moreover, serum stimulates $^{22}Na^+$ entry in a medium devoid of amino acids.

Stimulation of the Na^+,K^+ Pump by Monensin. Monensin is a carboxylic acid ionophore that forms an uncharged complex with Na^+ and protons and catalyzes the electroneutral exchange

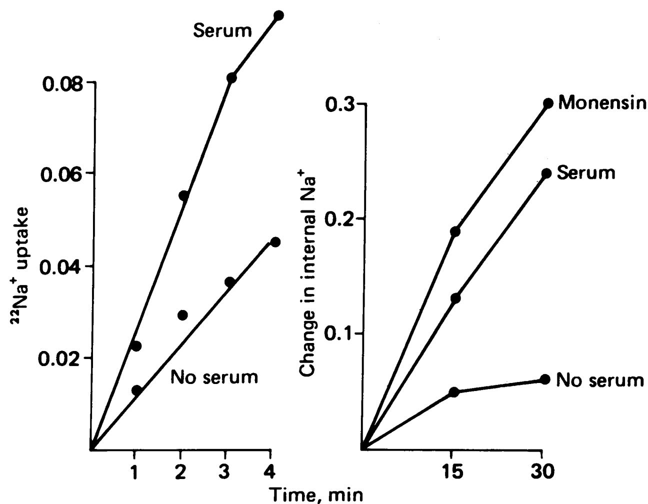

FIG. 1. Effect of serum and monensin on $^{22}Na^+$ uptake and net Na^+ entry in quiescent 3T3 cells. (*Left*) Quiescent cultures of Swiss 3T3 cells grown in 90-mm dishes were washed twice with growth medium in which the NaCl was replaced by equimolar choline Cl. Then, the cultures received 2 ml of the same medium containing 1 mM ouabain in the absence or presence of 10% dialyzed fetal bovine serum. The cultures were incubated for 5 min at 37°C and the uptake was initiated by adding $^{22}Na^+$. All other experimental details were as described in *Materials and Methods*. (*Right*) Quiescent cultures of 3T3 cells in 90-mm dishes were washed once with growth medium and incubated with 5 ml of medium containing 1 mM ouabain either in the absence or presence of 10% fetal bovine serum or 0.4 μg of monensin per ml. After 15 or 30 min of incubation, the cultures were washed and total cell Na^+ was measured. The Na^+ content of parallel cultures not exposed to ouabain was also measured. The value (0.22 μmol/mg of protein) was substracted from the Na^+ concentrations measured in the presence of ouabain, so the values plotted represent net increase of total cell Na^+ (μmol/mg of protein).

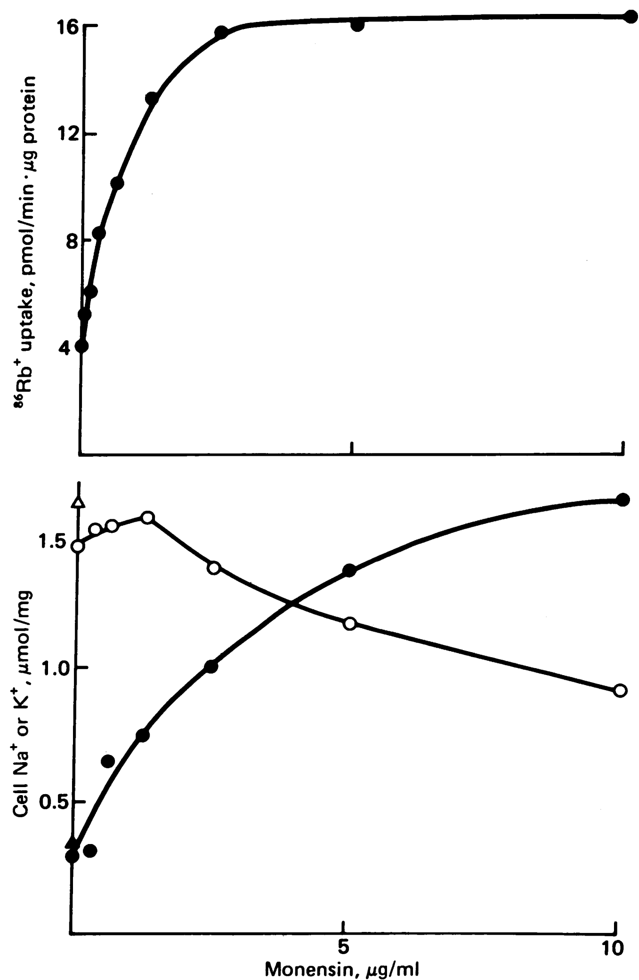

FIG. 2. Effect of different concentrations of monensin on $^{86}Rb^+$ uptake and on total cell Na^+ and K^+. (*Upper*) Quiescent cultures of Swiss 3T3 cells grown in 30-mm dishes (70 μg of protein/dish) were incubated in the presence of different concentrations of monensin. After 5 min, $^{86}Rb^+$ was added to the dishes and the uptake was terminated 10 min later. All other experimental conditions were as described in *Materials and Methods*. (*Lower*) Measurements of total cell Na^+ and K^+ were carried out as described in the legend to Fig. 1 and in *Materials and Methods*. The cultures were exposed to different concentrations of ionophore for 15 min. The triangles show total cell Na^+ (▲) or K^+ (△) 15 min after adding 10% serum in growth medium. ●, Na^+; ○, K^+.

of the two ions across membranes (14). Monensin binds K^+ but with a lower affinity than protons or Na^+. At 0.3 μg/ml, monensin stimulates the Na^+,K^+ pump about 2- to 3-fold (Fig. 2), which is similar to the stimulation by serum (5). Significantly, this level of monensin produces about the same increase in the rate of net Na^+ entry as does serum (Fig. 1). Monensin maximally stimulates the rate of $^{86}Rb^+$ uptake, by 4- to 6-fold (Fig. 2). Maximal stimulation requires about 3 μg of ionophore per ml, but higher concentrations are not inhibitory (Fig. 2). Ouabain prevents monensin from increasing $^{86}Rb^+$ uptake, indicating that the ionophore activates $^{86}Rb^+$ entry via the Na^+,K^+ pump (Fig. 4).

The Relationship between Internal Na^+ and Na^+,K^+ Pump Activity. The effect of monensin on the Na^+ and K^+ content of 3T3 cells may be seen in Fig. 2. Monensin strikingly increases internal Na^+, confirming the conclusion that the ionophore increases the supply of Na^+ to the pump. At low concentrations of monensin a small, but significant, increase in internal K^+ occurs. This small increase in cell K^+ is nearly the same as that observed after a 15-min incubation of quiescent 3T3 cells in fresh serum (Fig. 2). The maximal increase in total cell K^+ (about 50%) occurs about 4 hr after serum replenishment (ref. 6; unpublished results). Higher levels of monensin or longer incubation times (60 min) cause a net exit of K^+ from the cells, which may result from the binding of K^+ by monensin at the inner side of the plasma membrane at which the ratio of K^+ to Na^+ is high.

By combining the measurements of $^{86}Rb^+$ uptake with those of cell Na^+ and K^+ over a range of monensin concentrations (Fig. 2), we obtained a quantitative estimate of the dependence of the Na^+,K^+ pump on internal Na^+. Fig. 3 shows the rate of $^{86}Rb^+$ uptake as a function of cell Na^+ and as a function of cell Na^+/K^+. Clearly, the activity of the pump in quiescent fibroblasts is highly sensitive to small changes in total cell Na^+. In the absence of ouabain, serum slightly increases total cell Na^+.

Na^+ Requirements for Activating the Na^+,K^+ Pump. Fig. 4 shows that the stimulation of $^{86}Rb^+$ uptake by serum or monensin requires external Na^+. At 30 mM Na^+ the stimulation of the pump by serum falls to 50% of the control value (Fig. 5). Because lowering internal Na^+ will at some point diminish pump activity, it is particularly significant that the basal rate of $^{86}Rb^+$ uptake was not markedly altered at low levels of external Na^+ (Fig. 5). The requirement of external Na^+ for the rapid activation of the Na^+,K^+ pump in quiescent cells supports the view that the effect of serum on the pump is mediated by an increase in its supply of Na^+.

The sigmoidal relationship between $^{86}Rb^+$ uptake and external Na^+ (Fig. 5) shows that at low Na^+ levels serum cannot

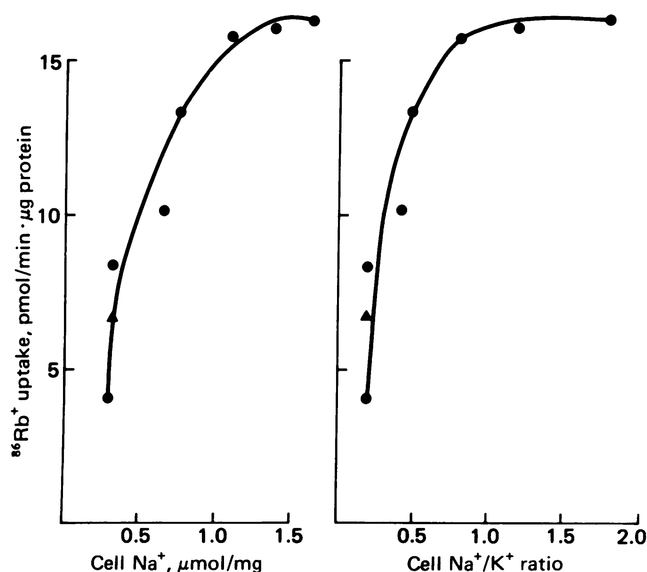

FIG. 3. The activity of the Na^+,K^+ pump in 3T3 cells as a function of total cell Na^+ (*Upper*) and the ratio of cell Na^+ to K^+ (*Lower*).

increase the supply of Na^+ to the pump. If Na^+ enters 3T3 cells by a Na^+ channel as we have suggested (13), then the rate of Na^+ entry would be severely affected by reducing its transmembrane gradient.

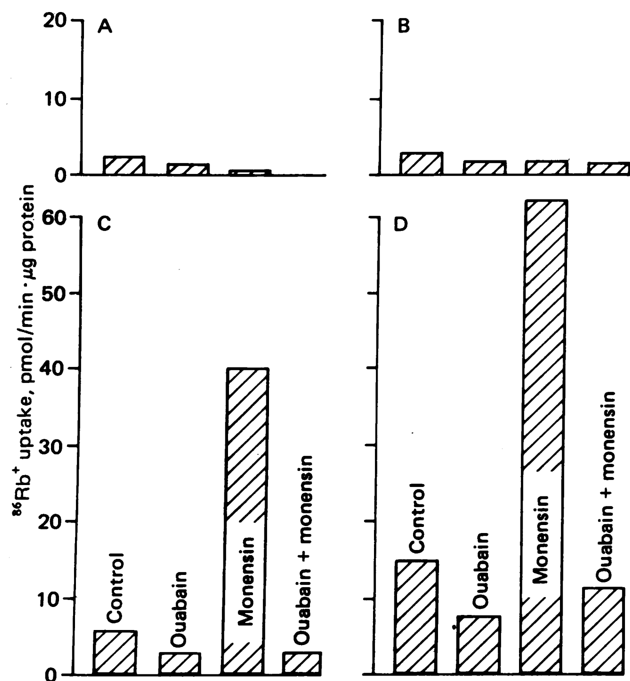

FIG. 4. Ouabain sensitivity and Na^+ dependence of the stimulation of the Na^+,K^+ pump by monensin. In order to obtain a medium completely devoid of Na^+ the following buffer solution was used: 100 mM choline Cl, 25 mM $MgCl_2$, 5 mM KCl, and 20 mM Tris/Hepes, pH 7.2. The cultures were washed three times with the above mentioned buffer and incubated with 2 ml of the same buffer containing various additions as noted on the graph. After 5 min, $^{86}Rb^+$ uptake was measured. The concentrations of monensin and ouabain were 5 μg/ml and 1 mM respectively. Na^+-free medium: no serum (*A*) and 10% serum (*B*). Medium containing 100 mM Na^+: no serum (*C*) and 10% serum (*D*). Control, growth medium.

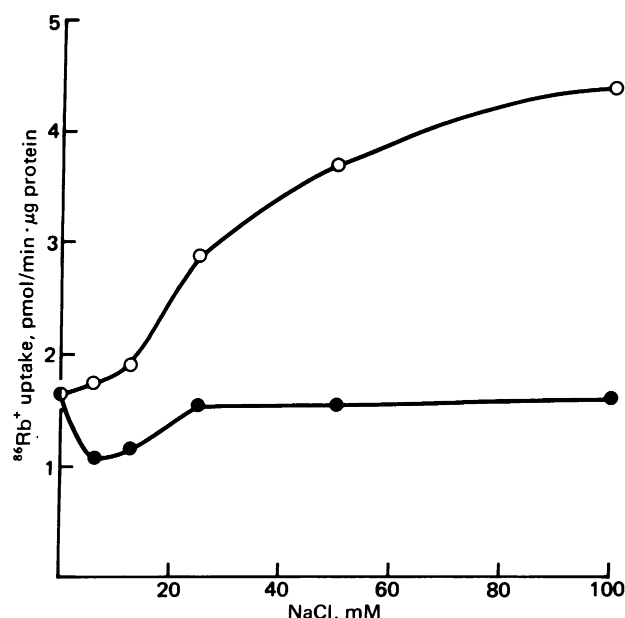

FIG. 5. Dependence of $^{86}Rb^+$ uptake on Na^+ concentration in the absence (●) and presence (○) of 10% serum. Quiescent cultures of 3T3 cells grown in 30-mm dishes were washed three times and incubated in 2 ml of a buffer solution whose composition was as described in the legend to Fig. 4. NaCl was replaced by equimolar choline Cl. All other experimental conditions were as described in *Materials and Methods*.

Effects of Other Ionophores on $^{86}Rb^+$ Uptake. Gramicidin forms a positively charged complex with cations and catalyzes an electrically impelled or electrogenic movement of the ions through membranes. Gramicidin has about equal affinity for Na^+ and K^+ (14). This ionophore more than triples the rate of $^{86}Rb^+$ uptake in 3T3 cells (Fig. 6). At the high ratio of Na^+ to K^+ in growth medium (30:1), ouabain prevents gramicidin from increasing $^{86}Rb^+$ uptake, indicating that under these conditions gramicidin, like monensin, produces a true activation of the Na^+,K^+ pump. At low levels of external Na^+, $^{86}Rb^+$ will enter 3T3 cells via gramicidin, and this movement is insensitive

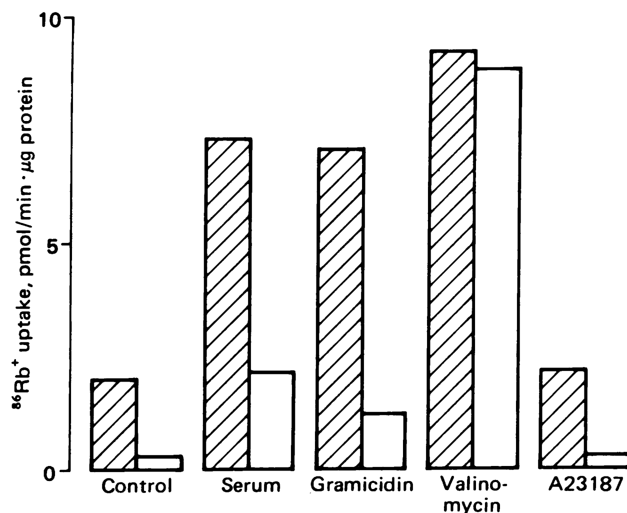

FIG. 6. Effects of gramicidin, valinomycin, and A23187 on $^{86}Rb^+$ uptake. Quiescent cultures of 3T3 cells grown in 30-mm dishes were incubated in growth medium alone (control) or in medium containing 10% fetal bovine serum or different ionophores. The incubations were carried out in the absence (▨) or presence (□) of 1 mM ouabain. The concentrations of the ionophores were 1.2, 10, and 1.0 μg/ml for gramicidin, valinomycin, and A23187, respectively.

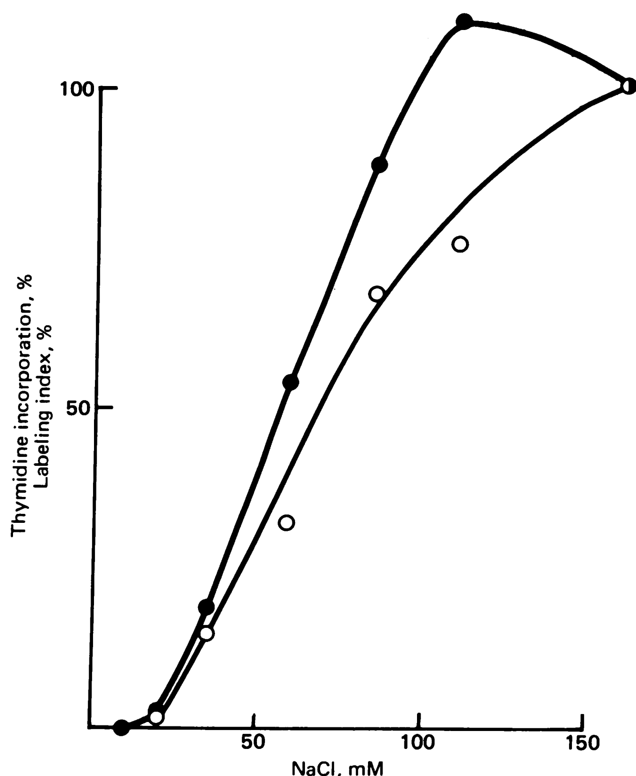

FIG. 7. The stimulation of DNA synthesis caused by serum depends on the concentration of Na^+ in the medium. Quiescent cultures of 3T3 cells were washed five times with growth medium in which the NaCl and $NaHCO_3$ were replaced by 20 mM Tris/Hepes buffer (pH 7.2) plus 150 mM choline Cl. The cultures were incubated in the presence of 10% fetal bovine serum (extensively dialyzed against 0.14 M choline Cl), and different concentrations of Na^+ for 40 hr. The ionic strength was maintained by appropriate changes in choline Cl concentration. The incubations were carried out at 37°C in humidified air (CO_2 was not supplied). DNA synthesis was determined either by incorporation into trichloroacetic acid-insoluble material (●) or by radioautography (○) as described (5). The curves represent percentages of the values obtained at 150 mM Na^+. At this Na^+ concentration there was 3.2×10^5 cpm of [^{3}H]thymidine incorporated into acid-insoluble material and there was 41% labeled nuclei.

to ouabain. Nor is $^{86}Rb^+$ entry via the K^+-specific ionophore, valinomycin, inhibited by ouabain (Fig. 6). Finally, the divalent cation ionophore, A23187, fails to significantly alter $^{86}Rb^+$ uptake (Fig. 6), suggesting that an increase in internal Ca^{2+} and perhaps Mg^{2+} does not stimulate the pump.

Na^+ Requirement for DNA Synthesis. The stimulation of DNA synthesis by serum in quiescent 3T3 cells depends on the concentration of Na^+ in the growth medium. Reducing external Na^+ below 100 mM inhibits DNA synthesis until at 20 mM it is completely blocked (Fig. 7). The Na requirement was the same when different solutes (choline Cl, sucrose) were used as osmotic replacements for NaCl, and Na^+ replenishment almost completely restores DNA synthesis even after 3 days in 10 mM Na^+. Because K^+ accumulation is obligatorily linked to Na^+ extrusion, the Na^+ dependence of cell growth probably reflects, at least in part, the Na^+ requirement of the ouabain-sensitive Na^+,K^+ pump ATPase.

DISCUSSION

We propose that serum accelerates the Na^+,K^+ pump by enhancing Na^+ entry and availability to the Na^+ transport site of the pump, which is on the cytoplasmic side of the lipid bilayer (15). The following lines of evidence support the model. (*i*) Serum and purified growth factors (unpublished results) more than double the rate of $^{22}Na^+$ entry and the accumulation of Na^+ in the cell when the exit of this ion is prevented by ouabain. Furthermore, we conclude from our study of Li^+ transport in fibroblastic cells (13) that serum and purified growth factors activate an amiloride-sensitive Na^+ channel in the plasma membrane. (*ii*) We found that the Na^+,K^+ pump in Swiss 3T3 cells is extremely sensitive to fluctuations in internal Na^+. The pump activity in intact cells, as measured by the rate of $^{86}Rb^+$ influx, increases severalfold when certain ionophores are used to raise internal Na^+. Two ionophores that catalyze Na^+ entry, gramicidin and monensin, markedly accelerate the pump, whereas the divalent cation ionophore, A23187, had little if any significant effect on $^{86}Rb^+$ uptake. The acceleration of $^{86}Rb^+$ entry by monensin has an absolute requirement for external Na^+ and does not occur when ouabain is present to block the pump. The quantitative relationship between pump activity and internal cations indicates that even small increases in internal Na^+ markedly enhance pump activity. (*iii*) The stimulation of the pump by serum requires external Na^+. Lowering the Na^+ concentrations of the medium below 60 mM markedly reduces the effect of serum, until at 10 mM serum has no stimulatory effect at all on the pump. All these results are consistent with the proposal that Na^+ entry plays a critical role in the stimulation of the Na^+ pump. However, the elevation in Na^+,K^+ pump activity that serum produces in quiescent 3T3 cells may or may not result solely from an increase in Na^+ availability. An allosteric activation of the pump by serum and growth factors may contribute to the activation that occurs when the Na^+ supply to the pump is increased because, when Na^+ is made freely available by monensin, $^{86}Rb^+$ uptake is still stimulated by serum (Fig. 3). Furthermore, because the distribution of Na^+ within the cell might be heterogeneous (16), the model only requires an increase in Na^+ in a cellular "pool" that is freely accessible to the Na^+ pump rather than in total cell Na^+.

The present demonstration that the Na^+,K^+ pump in intact 3T3 cells is highly sensitive to internal Na^+ is similar to the dependence on internal Na^+ displayed by the Na^+,K^+ pump in erythrocyte ghosts (17). In addition, an increased passive movement of Na^+ into erythrocytes from patients with certain hereditary anemias may account for the abnormally high activity of the Na^+,K^+ pump in these cells (18–20). Our findings demonstrate the relevance of Na^+ supply to the hormonal modulation of the pump in homogeneous cultures of fibroblastic cells. Obviously, the rapid control by serum of the pump in fibroblasts (5) contrasts mechanistically with the synthesis of more pump molecules by HeLa cells after a prolonged exposure (24 hr) to high internal Na^+ (21, 22).

A selective alteration of internal ions with ionophores may provide insight into the relationships among the various metabolic changes that occur after serum or growth factors are added to quiescent fibroblasts. Ca^{2+} has been suggested to have a role in stimulating aerobic glycolysis (23), which in 3T3 cells seems to result from the activation of phosphofructokinase (24). Monensin also stimulates glycolysis in quiescent 3T3 cells (unpublished) but the mechanism is not yet known. Internal K appears to be required for initiation of the proliferative response in quiescent 3T3 cells (5, 6). So far, attempts to stimulate DNA synthesis with monensin have failed, but this is not surprising in light of the appreciable loss of K^+ from cells exposed to the ionophore, which has some affinity for K^+. Nevertheless, the removal of external Na^+ blocks the proliferative response of 3T3 cells to serum, and the increase in Na^+ entry caused by serum appears to explain the stimulation of the Na^+,K^+ pump and increase in cell K^+. Thus, the hypothesis that an increase in the Na^+ permeability of 3T3 cells sets in motion an array of ion redistributions and metabolic events leading to rapid growth remains an attractive possibility.

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1. Todaro, G. & Green, H. (1963) *J. Cell Biol.* **17,** 299–313.
2. Todaro, G. J., Matsuija, Y., Bloom, S., Robbins, A. & Green, H. (1967) in *Growth Regulating Substances for Animal Cells in Culture,* eds. Defendi, V. & Stoker, M. (Wistar Institute, Philadelphia), pp. 87–98.
3. Holley, R. S. (1975) *Nature (London)* **258,** 487–490.
4. Rozengurt, E. (1978) in *Surface Properties of Normal and Neoplastic Cells,* ed. Hynes, R. (J. Wiley & Sons, Sussex, England), in press.
5. Rozengurt, E. & Heppel, L. A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4492–4495.
6. Tupper, J. T., Zorgniotti, F. & Mills, B. (1977) *J. Cell. Physiol.* **91,** 429–440.
7. Smith, G. (1977) *J. Cell Biol.* **73,** 761–767.
8. Bourne, H. R. & Rozengurt, E. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4555–4559.
9. Ledbetter, M. L. S. & Lubin, M. (1977) *Exp. Cell Res.* **105,** 223–236.
10. Brown, K. D. (1977) *J. Supramol. Struct.,* Suppl., **1,** 150.
11. Banerjee, S. P. & Bosmann, H. B. (1976) *Exp. Cell Res.* **100,** 153–158.
12. Tupper, J. T. (1977) *J. Cell. Physiol.* **93,** 303–308.
13. Smith, J. B. & Rozengurt, E. (1978) *J. Cell. Physiol.,* in press.
14. Christensen, H. N. (1975) *Biological Transport* (W. A. Benjamin, Reading, MA), pp. 310–314.
15. Baker, P. F. (1972) *Metab. Pathways* **6,** 243–268.
16. Lev, A. A. & Armstrong, W. McD. (1975) *Curr. Top. Membr. Transp.* **6,** 59–123.
17. Garay, R. P. & Garrahan, P. J. (1973) *J. Physiol.* **231,** 297–325.
18. Bertles, J. F. (1957) *J. Clin. Invest.* **36,** 816–824.
19. Zarkowsky, H. S., Oski, F. A., Sha'afi, R., Shohet, S. B. & Nathan, D. G. (1968) *N. Engl. J. Med.* **278,** 573–581.
20. Wiley, J. S. (1969) *Nature (London)* **221,** 1222–1224.
21. Cook, J. S., Vaughan, G. L., Proctor, W. R. & Brake, E. J. (1975) *J. Cell. Physiol.* **86,** 59–70.
22. Boardman, L., Huett, M., Lamb, J. F., Newton, J. P. & Polson, J. M. (1974) *J. Physiol.* **241,** 771–794.
23. Diamond, I., Legg, A., Schneider, J. A. & Rozengurt, E. (1978) *J. Biol. Chem.* **253,** 866–871.
24. Schneider, J. A., Diamond, I. & Rozengurt, E. (1978) *J. Biol. Chem.* **253,** 872–877.