INCREASED OUABAIN-SENSITIVE ^{86}RUBIDIUM^{+} UPTAKE AFTER MITOGENIC STIMULATION OF QUIESCENT CHICKEN EMBRYO FIBROBLASTS WITH PURIFIED MULTIPLICATION-STIMULATING ACTIVITY

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ABSTRACT

Multiplication-stimulating activity (MSA), a protein which stimulates DNA synthesis and growth of chicken embryo fibroblasts, was purified from serum-free medium conditioned by the growth of a rat liver cell line. Purified MSA was shown to rapidly stimulate ouabain-sensitive Na^{+},K^{+}-ATPase activity as measured by both enzyme assay and rate of ^{86}Rubidium uptake. Labeled ouabain binding was also shown to increase after stimulation of quiescent cells by serum or purified MSA. Conditions which interfere with the ability of the cells to accumulate potassium, such as the presence of the specific inhibitor, ouabain; incubation in potassium-free medium; or the presence of the potassium ionophore, valinomycin, were all demonstrated to inhibit the stimulation of DNA synthesis by serum or purified MSA. These results suggest that an early event in the stimulation of DNA synthesis by purified MSA is an activation of membrane Na^{+},K^{+}-ATPase with a resulting accumulation of potassium ions inside the cell.

The mechanism by which serum growth factors stimulate stationary fibroblasts to enter the cell cycle, synthesize DNA, and divide has not yet been defined. However, many investigations strongly indicate that the cell surface plays a crucial role in the regulation of cell proliferation and indeed many parameters of membrane function have been shown to correlate with multiplication rate or with viral transformation. Due to the complexity of serum it has been difficult to determine which of the effects of serum on cells is important for the stimulation of cell multiplication. To probe this question it is necessary to have purified factors which possess growth-stimulating properties. The use of purified factors alleviates the possibility that biochemical events observed after stimulation of cells are due to other substances in serum.

One such purified growth factor is multiplication-stimulating activity (MSA), a polypeptide of about 10,000 mol wt which has been purified from serum-free medium conditioned by the growth of a rat liver cell line (8, 9, 33). This protein has multiplication-stimulating activity for chicken embryo fibroblasts and nonsuppressible insulin-like activity (NSILA) (8). In addition to stimulating DNA synthesis and growth, MSA enhances the transport of glucose and amino acids and is functionally similar to insulin and somatomedin (33).

Several recent investigations have implicated potassium fluxes in the regulation of cell growth (2, 5, 6, 23, 30). Serum has been demonstrated to stimulate ^{86}Rubidium ($^{86}Rb^{+}$) influx in 3T3 cells (24), and increased Na^{+},K^{+}-ATPase activity has been found in virus-transformed cells (13). In or-

der to determine whether this effect of serum on cells is due to a secondary effect of serum, unrelated to the growth response, or is important for the stimulation of cell proliferation, studies with a purified growth factor are needed.

The purpose of this investigation was to determine whether purified MSA would influence the ouabain-sensitive Na^+,K^+-ATPase of stationary chicken embryo fibroblasts. The data indicate that a rapid stimulation of this enzyme activity, resulting in an increase in potassium influx, may be an early regulatory event in the induction of DNA synthesis by purified MSA.

MATERIALS AND METHODS

Reagents

MSA was purified from rat liver cell conditioned medium as described previously (33). Dulbecco's modified Eagle's medium (DME) and calf serum were obtained from Grand Island Biological Co. (Grand Island, N. Y.). [^{3}H]Thymidine (20 Ci/mmol), [^{3}H]ouabain (12 Ci/mmol) and $^{86}Rb^+$ were obtained from New England Nuclear (Boston, Mass.). Valinomycin and ouabain were obtained from Calbiochem (San Diego, Calif.).

Cell Culture and Assay for the Stimulation of DNA Synthesis

Primary cultures of chicken embryo fibroblasts were prepared by trypsinization of the body walls of 10–12-day-old embryos. Cells were maintained in DME plus 10% calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For experimentation, secondary cultures were prepared by transferring cells to 35-mm plastic tissue culture dishes at a concentration of 3×10^5 cells per dish in 2 ml of DME containing 0.25% calf serum. Cells prepared in this manner exhibited little if any cell division and entered a resting, quiescent stage in which little DNA synthesis occurred. 3 days after plating, less than 2% of the cells were in S phase at any one time as determined by autoradiography after a 1-h pulse of labeled thymidine (data not shown). At this time, cells were used for experimentation and were stimulated by changing the culture fluid to fresh medium containing serum or MSA and the various test materials. DNA synthesis was determined in stimulated cells by exposure to [^{3}H]thymidine (0.2 μCi/ml) for 1 h during the peak in the rate of DNA synthesis, which occurred at about 12 h (data not shown). At the end of the pulse period, label was removed and the cells were washed twice with cold phosphate-buffered saline (PBS) and twice with cold 10% trichloroacetic acid (TCA). The cells were then fixed for 10 min in ethanol:ether (3:1), air dried, and cells dissolved in 0.5 ml of 0.2 N NaOH. Aliquots were then assayed for acid-insoluble radioactivity by liquid scintillation spectrometry. Serum stimulation of quiescent cells generally resulted in a 10–15 fold increase in thymidine incorporation relative to control cells which were incubated in fresh medium without serum. Duplicate cultures did not vary by more than ± 10%. MSA stimulation resulted in an approximately 6–8 fold increase in thymidine incorporation. During the first 12 h after stimulation no increase in cell number was observed, indicating that most cells were synchronized at a point before S phase.

Rubidium Uptake and Labeled Ouabain Binding

Potassium influx was measured with $^{86}Rb^+$ as a tracer because of its longer half-life and because it has been shown to be taken up by cultured cells in the same way as potassium (13, 35). $^{86}Rb^+$ uptake was determined by directly adding 5μCi $^{86}Rb^+$ to each culture. After 15 min (unless otherwise indicated), uptake was terminated by washing the cells three times with cold PBS and extracting for 1 h with 1 ml of cold 10% TCA. The TCA extract was added to 10 ml of water and the radioactivity was determined by Cerenkov radiation in a liquid scintillation counter.

Labeled ouabain binding was determined by removing the medium and washing the cells once with serum-free medium. One ml of DME containing 2×10^{-7} M [^{3}H]ouabain was added to the cells, and the cultures were incubated for 1 h. At the end of the incubation period, the cultures were washed 3 times with cold 0.15 M NaCl, the cells were dissolved in 1 ml of 1% sodium dodecylsulfate, and aliquots were taken for determination of cell-bound radioactivity. Specific binding was determined by subtracting cell-bound cpm in the presence of excessive unlabeled ouabain (10^{-4} M).

Enzyme Assay

Na^+,K^+-ATPase activity was assayed in crude cell homogenates as previously described by Kimelberg and Mayhew (13) and Kimelberg and Papahadjopoulos (14). Two hr after stimulation, cells to be assayed were washed three times with cold 0.15 M NaCL and frozen at −70°C. Upon thawing, the cells were scraped from the dish with a rubber policeman into 1.5 ml of medium containing 100 mM NaCL, 10 mM KCL, 50 mM Tris acetate, 0.1 mM Na EDTA, and 3 mM $MgCl_2$ at pH 7.2. Enzyme assays were carried out, after brief homogenization in a Dounce homogenizer, by the addition of 5 μmoles of adenosine triphosphate (ATP). Values given are ouabain-sensitive release of $PO_4^=$ from ATP in micromoles per culture in a 1-h incubation.

RESULTS

The effect of mitogenic stimulation by serum or purified MSA on $^{86}Rb^+$ uptake in chicken embryo fibroblasts was determined. As shown in Fig. 1,

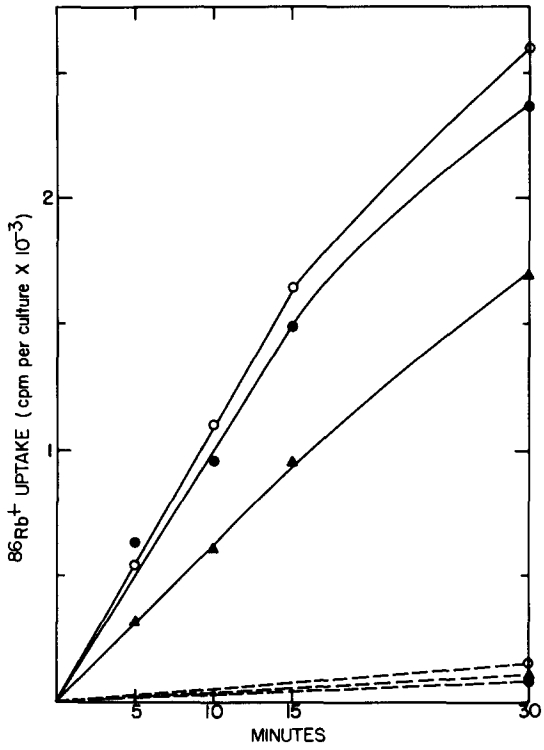

FIGURE 1 Uptake of $^{86}Rb^+$ 2 h after stimulation. Stationary cells were prepared as described in Materials and Methods. 3 days later, cultures were stimulated by changing the culture fluid to fresh medium containing no serum (▲), 2% serum (●), or 1 μg/ml MSA (○) with (---) or without (—) 10^{-5} M ouabain. 2 h later, 5 μCi of $^{86}Rb^+$ were added directly to each culture. Uptake was terminated at the times indicated by washing duplicate cultures three times with cold PBS and extracting for 1 h with 1 ml of cold 10% TCA.

both serum and MSA caused an increase in the rate of uptake of $^{86}Rb^+$ after 2 h of stimulation. It is also evident that both the basal rate and the stimulated rate of $^{86}Rb^+$ uptake in these cells are almost totally inhibited by ouabain, a specific inhibitor of Na^+,K^+-ATPase activity (27, 32). The observed rate of $^{86}Rb^+$ uptake is linear for over 15 min but tends to level off at later times. The plateau level for unstimulated cells is lower than that for stimulated cells (data not shown), indicating that stimulated cells have an increased capacity to accumulate potassium ions.

To insure that the increased rate of $^{86}Rb^+$ uptake seen in stimulated cells was not due to a decreased rate of potassium efflux, the experiment shown in Fig. 2 was conducted. It is evident that stimulation of cells that had been preloaded with $^{86}Rb^+$ did not result in a significant difference in the rate of $^{86}Rb^+$ efflux. Additional experiments in which the efflux was monitored for considerably longer periods of time also showed no significant difference (data not shown).

It is also necessary to point out that the results seen in Fig. 1 are not due to an increase in cell volume after stimulation. Intracellular water space was determined as the intracellular space available to the nonmetabolizable glucose analog, D-[3-O-*methyl*^{3}H]glucose, using the procedure for attached cells described by Kletzien et al. (15). No significant increase in the cell volume of stimulated cells could be detected during the initial 6 h after stimulation (data not shown).

To determine when this increase in cation influx becomes evident after stimulation, a time course of the stimulation of $^{86}Rb^+$ transport was performed and is shown in Fig. 3. Mitogenic stimulation by both serum and MSA causes an enhancement of $^{86}Rb^+$ transport, and this increase is evident as early as 15 min after stimulation and reaches a maximum by 1 h. No increase is seen

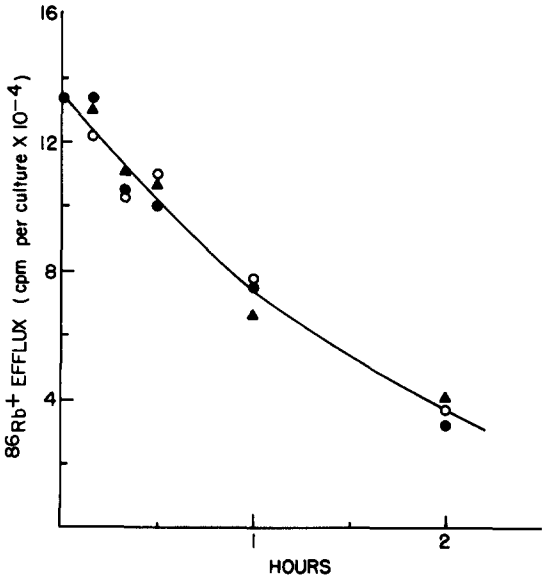

FIGURE 2 $^{86}Rb^+$ efflux. Stationary cells were prepared by growth in low serum-containing medium for 3 days as described in Materials and Methods. 10 μCi $^{86}Rb^+$ were directly added to the culture medium and the cells were incubated for 6 h. Preloaded cells were then washed, and fresh culture fluid was added which contained no serum (▲), 2% serum (●), or 1 μg/ml MSA (○). At the times indicated, duplicate cultures were assayed for the amount of cell-associated radioactivity.

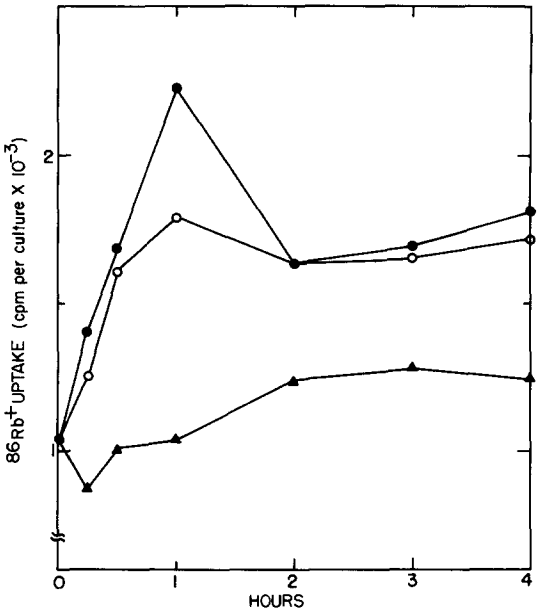

FIGURE 3 Time course of $^{86}Rb^+$ uptake. Stationary cells were prepared by growth in low serum-containing medium for 3 days as described in Materials and Methods. Cells were stimulated by changing the culture fluid to fresh medium containing no serum (▲), 2% serum (●), or 1 μg/ml MSA (○). At the times indicated, 5 μCi $^{86}Rb^+$ were added to duplicate cultures in each set and uptake was measured over a 15-min interval.

when the culture fluid is changed to medium lacking serum or MSA.

Fig. 4 shows the dependence of $^{86}Rb^+$ transport rate at 1 h on the concentration of serum or MSA. Results show that $^{86}Rb^+$ transport increases in linear fashion at low concentrations of serum or MSA. A plateau level is reached in both cases at concentrations which also stimulate maximally DNA synthesis (33, and data not shown).

In view of the above data implicating the involvement of membrane Na^+,K^+-ATPase activity in the stimulation of DNA synthesis by purified MSA, it was of interest to examine the effects of an inhibitor of this enzyme on the incorporation of [^{3}H]thymidine. Ouabain, which has already been shown to inhibit $^{86}Rb^+$ transport (Fig. 1), also totally inhibits the incorporation of [^{3}H]thymidine by fibroblasts stimulated with serum or MSA (Table I). In control experiments (data not shown), this inhibition by ouabain was shown to be completely reversible and is therefore not due to cell killing or cytotoxicity. In addition, this inhibition of incorporation of [^{3}H]thymidine by ouabain is not due to an effect on thymidine transport, an important consideration since Na^+,K^+-ATPase is known to be coupled to several transport processes in addition to cation fluxes (28).

Also shown in Table I is the effect of valinomycin, a potassium ionophore, on DNA synthesis. Valinomycin drastically reduces the levels of [^{3}H]thymidine incorporation, probably by allowing potassium to leak out of the cells. This mode of action for valinomycin inhibition is verified, since, in cells that have been preloaded with $^{86}Rb^+$, the rate of $^{86}Rb^+$ efflux is twice as fast in the presence of valinomycin (data not shown).

The absence of potassium from the medium also is inhibitory to DNA synthesis (Table I), again suggesting the importance of potassium influx for progression into S phase to occur. These three experiments with the inhibitors ouabain and valinomycin and potassium-free medium all demonstrate an inhibitory effect on the stimulation of DNA synthesis by serum or MSA. All of these procedures have the same effect on cells, namely the deprivation of potassium. These results, coupled to the rubidium uptake data presented earlier, suggest that the intracellular accumulation of potassium after stimulation by serum or purified MSA is a necessary early event leading to DNA

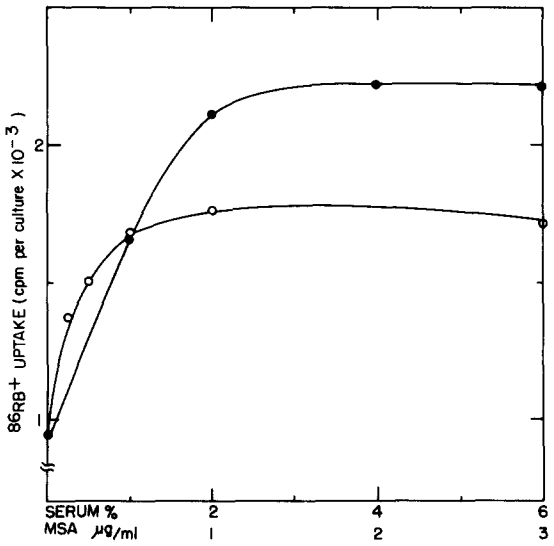

FIGURE 4 Dose-response curve. Stationary cells were prepared by growth in low serum-containing medium for 3 days as described in Materials and Methods. Cells were stimulated by changing the culture fluid to fresh medium containing various concentrations of serum (●) or purified MSA (○). $^{86}Rb^+$ transport was measured 2 h after stimulation during a 15-min interval after the addition of 5 μCi $^{86}Rb^+$ to duplicate samples.

TABLE I

*Effect of Ouabain, Valinomycin and the Absence of K^+ on Serum- and MSA-Induced DNA Synthesis**

Inhibitor	[³H]Thymidine incorporation			Inhibition	
	Control w/o Serum	10% Serum‡	MSA 1 μg/ml	Serum	MSA
	cpm per culture			%	
None	690	9,040	3,560	0	0
Ouabain					
(10^{-5} M)	170	610	390	94	89
(10^{-4} M)	90	95	110	99	97
Valinomycin					
(0.1 μg/ml)	500	2,240	1,620	75	54
(1.0 μg/ml)	440	1,260	1,580	86	56
Potassium-free medium	55	280	350	97	90

* Stationary cultures of chicken embryo fibroblasts were prepared as described in Methods and Materials. After 3 days, cells were stimulated by changing the culture fluid to fresh medium containing the indicated additions. At 12 h, [³H]thymidine incorporation was determined. Values shown are the averages of duplicate cultures. Duplicates did not vary by more than 10%.

‡ Calf serum used in the potassium-free medium was extensively dialyzed against 0.15 M NaCl to remove potassium. This serum was equally as active as undialysed serum in stimulating DNA synthesis in complete medium.

synthesis. It appears likely that serum growth factors might act by initially stimulating ion fluxes by activating membrane Na^+,K^+-ATPase activity. The increased $^{86}Rb^+$ transport and the apparent requirement for a functional Na^+,K^+-ATPase encourage such speculation.

To verify this data, direct attempts to measure increased Na^+,K^+-ATPase activity in stimulated cells were carried out. The Na^+,K^+-ATPase activities shown in Table II were measured in cell homogenates and were calculated as μmol of inorganic phosphate released from ATP per culture in a 60-min incubation time. The amounts shown are those sensitive to 10^{-3} M ouabain and are the means of triplicate samples. The differences in enzyme activity between control and stimulated cells were found to be significant by statistical analysis at the 95% confidence level. Thus, it is possible to demonstrate an increased ouabain-sensitive Na^+,K^+-ATPase activity after stimulation by MSA or serum.

The results of labeled [³H]ouabain binding experiments are also shown in Table II. Specific ouabain binding was always greater in cultures stimulated with serum or MSA; however, the differences were statistically significant only at the 80% confidence level. These data indicate that the activation of Na^+,K^+-ATPase activity by serum or MSA might involve an uncovering of sodium pump sites in the membrane. However, this result does not rule out other possibilities such as an increase in the affinity of the enzyme for ouabain.

TABLE II

*Na^+,K^+-ATPase Activity, and Ouabain Binding after Stimulation by Serum or MSA**

System	Na^+,K^+-ATPase activity ouabain-sensitive release of PO_4	Specific [³H]ouabain binding
	μmol/60 min/culture	*cpm/culture*
Control (no serum)	0.076 ± .006	550 ± 90
10% serum	0.112 ± .008	740 ± 95
MSA (*1 μg/ml*)	0.090 ± .003	675 ± 120

* Stationary cultures were prepared as described in Materials and Methods. After 3 days, cells were stimulated by changing the culture fluid to fresh medium containing no or 10% serum or 1 μg/ml MSA. 2 h after stimulation, triplicate cultures were assayed for enzyme activity and quadruplicate cultures were assayed for ouabain binding. Data are given ± one standard deviation.

DISCUSSION

The data reported in this communication clearly demonstrate that purified MSA from rat liver cell-conditioned medium stimulates potassium transport after addition to stationary chicken embryo fibroblasts. This increased potassium transport is due to an activation of Na^+,K^+-ATPase activity as indicated by direct measurements of enzyme activity and by the marked sensitivity to the specific inhibitor, ouabain. Other techniques which drastically reduce the capacity of the cells to accumulate potassium such as potassium-free conditions or the presence of the potassium ionophore, valinomycin, also inhibit DNA synthesis.

Increased specific ouabain binding was also demonstrated after stimulation by MSA. Ouabain

binding has been used to estimate the number of sodium pumps present on the cell surface (10); however, it is difficult to eliminate the possibility that increased binding is due to changes in the affinity of the enzyme for the inhibitor (2). Most important was the direct demonstration of an increased ouabain-sensitive Na^+,K^+-ATPase activity after stimulation of stationary cells. These results suggest that serum and purified MSA may exert their mitogenic effect by stimulating membrane Na^+,K^+-ATPase activity. This may involve a direct interaction of the growth factor with the enzyme on the cell surface or it might be a result of secondary interactions with other membrane components involved in growth control. It is not yet possible to speculate as to whether the cells respond to (*a*) an increase in the rate of potassium influx *per se*, or (*b*) an increased intracellular potassium concentration, or (*c*) a change in transmembrane potential. Investigations are currently in progress to examine these possibilities.

Cation fluxes have previously been implicated in the regulation of cell proliferation. Rates of DNA synthesis have been shown to vary in proportion to the external potassium ion concentration (16, 21) and potassium uptake increases in lymphocytes upon stimulation with mitogens (2, 22). Other workers have correlated internal potassium ion concentrations with proliferative rate in mouse lymphoblasts (5, 6), and increased rates of potassium uptake have been demonstrated in virus-transformed 3T3 and BHK cells (13). It has also been suggested that the electrical transmembrane potential is involved in contact inhibition of cell division (4). Cellular growth has been found to be directly related to the amount of sodium pumping activity in mouse lymphoblasts (30), and serum has been shown to stimulate ouabain-sensitive $^{86}Rb^+$ influx in 3T3 cells (24) although no changes in enzyme activity of cell homogenates were detected.

Ouabain is known to prevent the stimulation of lymphocytes by phytohemagglutinin (PHA) (22, 23) and it inhibits the multiplication of Ehrlich ascites cells (19), canine kidney cells (1), BHK cells (20), and mouse lymphoblasts (5).

It is interesting to note that other investigators have demonstrated that insulin, a known growth-promoting protein, stimulates (Na^+,K^+)-Mg^{++}-ATPase in rat uterus (17), diaphragm (12) and liver (18). Insulin also stimulates Na^+,K^+-ATPase activity in rat (3) and frog (10) muscle.

Membrane Na^+,K^+-ATPase is responsible for the intracellular accumulation of potassium ions and the maintenance of membrane potential. However, in some systems this enzyme activity is coupled to the transport of sugars and amino acids (28). A vast literature exists which demonstrates that glucose transport rates increase rapidly after the addition of growth-promoting substances to cultured cells (25, 26, 29, 33, 34), and it has been suggested that changes in nutrient transport rates are critical to the regulation of cell growth (7, 11). It is conceivable that the stimulation of cell multiplication by purified growth factors involves the direct activation of membrane Na^+,K^+-ATPase with secondary enhancement of nutrient transport rates. Such an interaction is currently under active investigation.

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