PROSTAGLANDIN $F_2\alpha$ AND INSULIN STIMULATE PHOSPHATE UPTAKE AND (Na^+, K^+)ATPase ACTIVITY IN RESTING MOUSE FIBROBLAST CULTURES

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ABSTRACT The (Na^+, K^+)ATPase transport system in resting 3T3 Swiss mouse fibroblasts is rapidly activated by prostaglandin $F_2\alpha$ and insulin, which initiate DNA synthesis in these cells. Prostaglandin $F_2\alpha$, but not insulin, promotes a rapid increase in P_i uptake which is partially coupled to the Na^+ pump. This rapid activation of both transport systems occurs by a mechanism which does not require fluctuation of cyclic AMP levels or new protein synthesis. A subsequent protein synthesis-dependent increase in P_i uptake is stimulated by insulin and prostaglandin $F_2\alpha$. These results suggest that different types of control of membrane transport occur during growth stimulation.

The stimulation of proliferation of untransformed fibroblasts is associated with rapid changes in surface membrane properties which are subsequently followed by DNA synthesis and cell division (1). When serum is added to resting 3T3 cells, rapid increases in the rates of uridine, P_i, glucose, and Rb^+ uptake are observed, while the intracellular concentration of cyclic AMP (cAMP) is decreased (2-5).

Previous results indicate that the activities of some of these transport systems respond to several independent control mechanisms, rather than to a single regulatory change accomplished by fluctuations in the intracellular levels of cAMP (3). The possibility that the activity of these transport systems can be stimulated selectively by different growth-promoting factors has been suggested (3,6,7).

The present study was designed to extend this concept of control of transport during growth stimulation by using two different growth promoting factors. We report here that prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and insulin, which stimulate DNA synthesis in quiescent 3T3 cells (8), also stimulate

Abbreviations: SQ 20006, 1-ethyl-4-hydrazino-1H-pyrazolo- (3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride; $PGF_2\alpha$, prostaglandin $F_2\alpha$. SFM, serum-free medium.

Rb^+ uptake, used as a measure of the activity of the (Na^+, K^+)ATPase transport system (E.C.3.6.1.3), and P_i uptake. These changes occur independently of changes in the intracellular concentrations of cyclic nucleotides. The activation of P_i uptake by $PGF_2\alpha$ shows a biphasic pattern and is partially dependent upon the activity of the (Na^+, K^+)ATPase transport system.

Materials and Methods: Swiss mouse 3T3 fibroblasts (9) were maintained in Dulbecco's modified Eagle's medium containing 100 units/ml of penicillin, 100 μg/ml streptomycin and supplemented with 10% fetal calf serum. Biotin, vitamin B_{12}, hypoxanthine, glutamic acid and aspartic acid were added at the concentrations described by Holley and Kiernan (10). Subconfluent cultures were grown in 90 mm Nunc Petri dishes at 37°C equilibrated with 10% CO_2 in air and routinely monitored for the absence of mycoplasma contamination.

For transport determinations, the cells were plated in 30 mm dishes with medium supplemented as above but containing 7% fetal calf serum. Cultures were used 3 days after the last medium change. P_i uptake measurement was determined over a 5 min interval as previously described (3). For Rb^+ uptake determinations, the cells were washed twice with serum-free medium and incubated in serum-free medium with experimental additions. At the times indicated, 2.5 μCi of $^{86}RbCl$ was added for 10 min to each culture, at a final concentration of 50 μM RbCl. Then, cells were washed with ice-cold isotonic saline and extracted with 5% trichloroacetic acid at 4°C for 20 min. Radioactivity was measured in 1 ml samples in Aquasol scintillation fluid.

Insulin and N^6-monobutyryl-cAMP were obtained from Sigma and 8-bromo-cGMP from Boehringer. SQ-20006 and prostaglandins were the generous gifts of Dr. M. Chasin, E. R. Squibb & Sons Inc., Princeton, New Jersey, and Dr. John Pike, The Upjohn Company, Kalamazoo, Michigan, respectively. ^{32}Pi and $^{86}RbCl$ were purchased from the Radiochemical Center, Amersham, England.

Results and Discussion: Rb^+ uptake is rapidly activated by $PGF_2\alpha$ and insulin as shown in Fig. 1A. Addition of 400 ng/ml of $PGF_2\alpha$ or 200 ng/ml of insulin independently or in combination to quiescent 3T3 cells stimulated Rb^+ uptake 2-fold after 10 min. Addition of 15% serum also produced a rapid and more marked stimulation of Rb^+ uptake, as has also been recently reported (4). Stimulation of Rb^+ uptake 20 min after addition of $PGF_2\alpha$ or serum was not prevented by preincubation of cultures with 10 μg/ml cycloheximide (Table 1).

In contrast, the stimulation of P_i uptake by $PGF_2\alpha$ at 400 ng/ml showed a biphasic activation curve as a function of time, while insulin at 200 ng/ml stimulated only the second component (Fig. 1B). Stimulation of

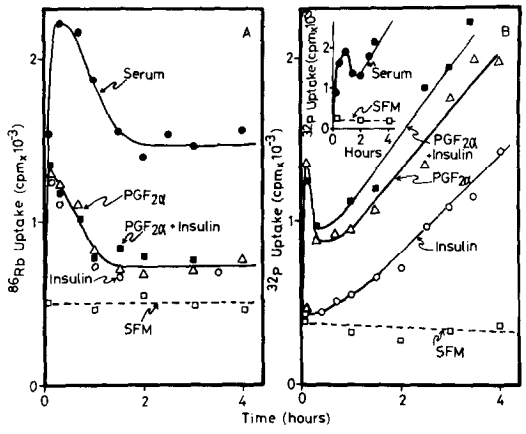

Fig. 1 Kinetics of the changes in Rb^+ uptake (A) and phosphate uptake (B) produced by the addition of $PGF_2\alpha$, insulin and serum to resting 3T3 cells. (Δ) $PGF_2\alpha$ 400 ng/ml, (○) Insulin 200 ng/ml, (■) $PGF_2\alpha$ + Insulin at the same concentration (●) serum 15% and (□) serum-free medium. The uptake of ^{86}Rb and ^{32}P was determined as indicated in Materials and Methods. The insert in B shows the effect of serum on phosphate uptake.

Table 1. Differential effects of cyclic nucleotide-elevating agents and cycloheximide on P_i and Rb^+ uptake stimulated by prostaglandins, insulin and serum.

Additions	$^{32}P_i$ Uptake % Control		^{86}Rb Uptake % Control
	15 min	4 hrs	20 min
Control	100	100	100
$PGF_2\alpha$	330	500	170
Insulin	130	370	170
$PGF_2\alpha$ + Insulin	350	-	160
Serum	820	1200	260
$PGF_2\alpha$ + Cycloheximide	380	260	200
Insulin + Cycloheximide	-	160	-
Serum + Cycloheximide	830	500	260
$PGF_2\alpha$ + SQ 20006	350	-	-
Serum + SQ 20006	830	-	250
8-bromo-cGMP	110	100	87
N^6-monobutyryl cAMP	150	100	100
SQ 20006	100	-	90
8-bromo-cGMP + SQ 20006	90	-	96
PGE_1 200 ng/ml	-	120	-
PGE_1 40 μg/ml	-	470	-

[1]. Cells were preincubated 20 min in serum-free medium with 0.15 mM SQ 20006, 0.1 mM 8-bromo-cGMP, 0.1 mM N^6-monobutyryl cAMP and/or 10 μg/ml cycloheximide where indicated. Then, at zero time, 15% fetal calf serum, 200 ng/ml PGE_1 or 200 ng/ml $PGF_2\alpha$, and/or 200 ng/ml insulin were added to the cells. Transport determinations for $^{32}P_i$ uptake were carried out during the 15-20 min interval after additions at zero time, and ^{86}Rb uptake was determined during the 15-25 min interval after zero time. 100% represents 400 cpm/ml of ^{32}P and 2700 cpm/ml ^{86}Rb.

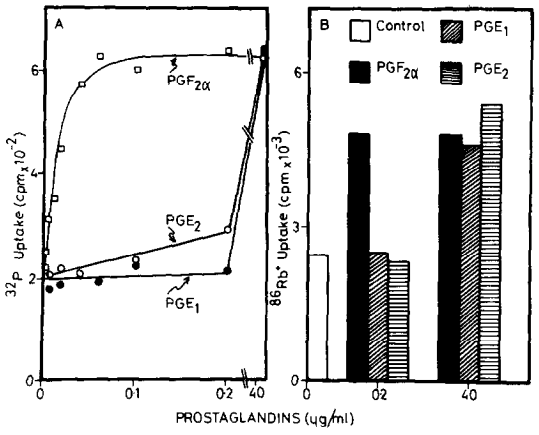

Fig. 2 Effect of different prostaglandins on the early increase in phosphate uptake (A) and ^{86}Rb uptake (B) in quiescent 3T3 cells. Cells were washed 4 times with serum free medium (phosphate-free in A) and incubated for 15 min with addition of (□) $PGF_2\alpha$ (○) PGE_2 or (●) PGE_1. Then the cells were pulsed for 5 min with $^{32}P_i$ as indicated in Materials and Methods. For ^{86}Rb uptake determinations the cells were incubated 15 min with the additions (symbols as in the figure) and then labelled for 10 min.

P_i uptake by 15% serum showed a similar type of biphasic activation to that observed in the presence of $PGF_2\alpha$ (Insert, Fig. 1B).

The increase in P_i uptake observed during the 5 to 10 min interval after $PGF_2\alpha$ or serum addition was not affected by preincubation of cells with 10 μg/ml cycloheximide, while the component of P_i uptake stimulation observed 4 hr after addition of $PGF_2\alpha$, insulin or serum was completely blocked (Table 1). When $PGF_2\alpha$ and insulin were added together, no synergistic effect upon either Rb^+ or P_i uptake was observed (Fig. 1), in contrast with their synergistic effects on growth (8). $PGF_2\alpha$ was more effective on a concentration basis compared with other prostaglandins in activating both P_i and Rb^+ uptake. Addition of $PGF_2\alpha$ in a concentration range of 5 ng/ml to 40 μg/ml stimulated the early phase of P_i uptake, reaching a maximum increase of 3-fold over controls at 100 ng/ml, while at the same concentrations PGE_2 or PGE_1 showed no effect (Fig. 2A). However, at 40 μg/ml, PGE_1 and PGE_2 produced the same effect as 200 ng/ml $PGF_2\alpha$, activating both the early phase of P_i uptake (Fig. 2A) and the

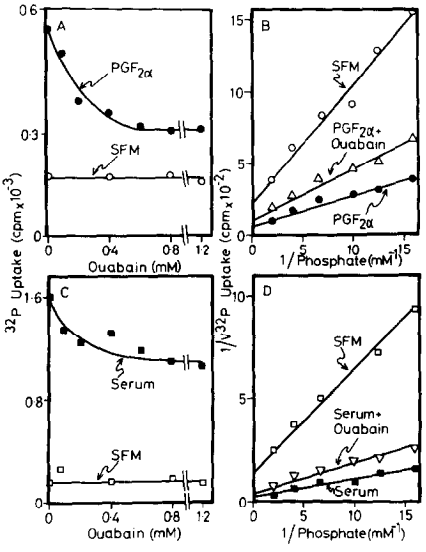

Fig. 3 Effect of ouabain on stimulation of P_i uptake by $PGF_{2\alpha}$ (A) and serum (C). Cultures were washed 3 times with serum-free medium minus phosphate and preincubated at different concentrations of ouabain for 20 min. Then $PGF_{2\alpha}$ at 400 ng/ml (●) or 15% serum (■) were added for 15 min before determination of uptake rates. Double reciprocal plots of uptake as a function of P_i concentration, and the effect of ouabain, after addition of $PGF_{2\alpha}$ (B) or serum (D). Cell were preincubated with 1.2 mM ouabain as above where indicated. $PGF_{2\alpha}$ (400 ng/ml) in the presence (Δ) or absence (●) of ouabain, or 15% serum in the presence (▽) or absence (■) of ouabain were added for 15 min before determination of uptake rates for $^{32}P_i$ (50 μCi μmol^{-1}, 5 μCi ml^{-1}). (○,□) represent uptake in serum-free medium (SFM).

second phase (Table 1). Similarly, 40 μg/ml of PGE_1 or PGE_2 could produce the activation of Rb^+ uptake at 20 min which was observed with 200 ng/ml $PGF_{2\alpha}$ (Fig. 2B).

Both the activation of P_i and Rb^+ uptake produced by the different factors was not affected by conditions shown to affect the intracellular levels of cyclic nucleotides. Addition of N^6-monobutyryl cAMP or 8-bromo-cGMP, separately, or in combination with SQ 20006 (an inhibitor of the cyclic 3'5' nucleotide phosphodiesterase (11)), to resting cells did not produce an increase in P_i or Rb^+ uptake. The rapid activation of the Na^+ pump and P_i uptake stimulated by $PGF_{2\alpha}$ or serum was not prevented by preincubation of the cells with SQ 20006 (Table 1A) suggesting that a drop in cAMP does not mediate this response. Furthermore, the addition of either 200 ng/ml insulin or 40 μg/ml PGE_1 stimulated the rapid increase

Table 2. Effect of ouabain on Rb^+ uptake stimulated by $PGF_2\alpha$, and serum

Additions	% Uptake Rate control
Control	100
$PGF_2\alpha$	190
$PGF_2\alpha$ + Ouabain 0.8 mM	50
Serum	390
Serum + Ouabain 0.8 mM	80

Resting cultures of 3T3 cells were preincubated with ouabain in serum-free medium for 20 min, and then exposed to 400 ng/ml of $PGF_2\alpha$ or 15% Fetal calf serum for 15 min. The uptake of ^{86}Rb was measured as described in Fig. 2. 100% represents 500 cpm/ml.

in the rate of Rb^+ uptake (Fig. 2B) and the second phase of P_i uptake activation (Table 1), yet these compounds have opposite effects on intracellular levels of cAMP (5).

The rapid stimulation of P_i uptake by $PGF_2\alpha$ and serum is due to an increase in the maximal velocity (Fig. 3). No change in the apparent Michaelis constant (Km) was observed and the Km of about 0.4 mM obtained was similar to values recently reported (12,13).

The early increase in P_i uptake stimulated by $PGF_2\alpha$ or serum is partially coupled to the (Na^+, K^+)ATPase transport system. Preincubation of cells with 1.2 mM ouabain for 20 min prior to the addition of $PGF_2\alpha$ or serum resulted in 50% and 30% inhibition of P_i uptake respectively (Fig. 3A,C). The coupled activity of P_i uptake and the Na^+ pump is only partial, since 0.8 mM ouabain was sufficient to reduce serum or $PGF_2\alpha$-stimulated Rb^+ uptake to the control level (Table 2). Complete inhibition of Rb^+ uptake by ouabain cannot be achieved in this medium due to decreased sensitivity of rodent cells to ouabain (14).

$PGF_2\alpha$ and insulin have recently been shown to stimulate DNA synthesis in resting Swiss 3T3 cells (3). The present results indicate that $PGF_2\alpha$ and insulin also rapidly increase the rate of P_i uptake and the activity of the (Na^+, K^+)ATPase transport system independ-

ently of fluctuations in the intracellular levels of cyclic nucleotides. Interestingly, concentrations of $PGF_2\alpha$ causing stimulation of the (Na^+, K^+)ATPase transport system and P_i uptake correlate with those which stimulate DNA synthesis in quiescent Swiss 3T3 cells (8). This correlation exists for the whole cell population. However, we do not know whether it is also valid for individual cells since only 14% of the cells have initiated DNA synthesis after 26 hr of $PGF_2\alpha$ stimulation (8).

The mechanisms of activation of the Na^+ pump and P_i uptake by $PGF_2\alpha$, insulin or serum are not understood. A plausible explanation for the effect of these hormones and serum on Rb^+ uptake and the early phase of P_i uptake could be a direct interaction with the cell membrane, since we observed no dependence on protein synthesis or cyclic nucleotides for this stimulation. Also, these results indicate that the second phase of stimulation of P_i uptake by $PGF_2\alpha$, insulin or serum requires an intracellular response involving protein synthesis, but not mediated by cyclic nucleotides.

We provide evidence that $PGF_2\alpha$ and insulin both stimulate the Na^+ pump in resting 3T3 cells but only $PGF_2\alpha$ can produce the early phase of P_i uptake activation, despite the partial coupling between the activity of these two uptake systems. This supports previous views that the interaction of hormones with the cell surface involves multiple mechanisms controlling transport and growth (3,7). A final question unresolved is whether these early transport changes triggered by serum or hormones are necessary for stimulation of cell proliferation in resting 3T3 cultures (7).

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