Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 115-122

EFFECT OF HEPARIN-BINDING GROWTH FACTORS ON MONOVALENT CATION TRANSPORT IN BALB/C 3T3 CELLS

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Received February 9, 1987

The changes in monovalent cation fluxes induced by class 1 and class 2 heparin-binding growth factors purified from bovine brain were studied in confluent quiescent Balb/C 3T3 cells. Both classes of HBGF's rapidly stimulate the activity of the Na^+-K^+ pump in a concentration-dependent manner that parallels their mitogenic activity. This effect requires the presence of external Na^+ and is abolished by 1 mM amiloride. As with other growth factors, HBGF's increase the activity of the Na^+-K^+ pump by increasing Na^+ influx into the cells via the amiloride-sensitive Na^+-H^+ exchange. These results indicate that rapid activation of cation transport is an early cellular response that follows binding of HBGF's to specific receptors on the surface of target cells.

Heparin affinity chromatography has been used to purify a variety of growth factors from different tissues and species (1). These heparin-binding growth factors (HBGF's) fall into two classes (1,2). Class 1 HBGF's, which have been found in high levels only in neural tissue, are anionic mitogens typified by bovine acidic brain fibroblast growth factor (aFGF). Class 2 HBGF's, which have a wide tissue distribution, are cationic mitogens typified by bovine basic pituitary and brain fibroblast growth factor (bFGF).

HBGF's are potent mitogens for a variety of cell types and both classes stimulate DNA synthesis in target cells at subnanomolar concentrations. Although recent work shows that HBGF's interact with specific receptors on target cells (3,4),

0006-291X/87 $1.50

little is known about specific intracellular events leading to mitogenesis. However, studies on other mitogens, including epidermal growth factor (5), platelet derived growth factor (6), and colony stimulating factor (7), have shown that rapid changes in the fluxes and intracellular concentrations of cations may play a role in the signalling that leads to DNA synthesis and cell proliferation (8). These mitogens increase Na^+ entry through an amiloride sensitive Na^+-H^+ exchanger (9,10). This causes cytoplasmic alkalinization and increases intracellular Na^+ which results in secondary stimulation of the Na^+-K^+ pump (9,11). In addition, the interaction of the mitogens with their specific receptors is associated with an increased concentration of cytosolic Ca^{++} ion (12,13), which appears to mediate many of the subsequent physiological responses.

In this report the effects of HBGF's on ion fluxes in Balb/C 3T3 cells are described. Both aFGF and bFGF increase the activity of the Na^+-K^+ pump and Na^+ entry in confluent quiescent 3T3 cells. These effects are transient, occur within minutes after addition of the mitogens, and exhibit a concentration dependence and a sensitivity to heparin that parallels their mitogenic effects.

METHODS

Growth factor preparation. Bovine brain aFGF and bFGF were purified to homogeneity by ammonium sulfate precipitation, CM-Sephadex C50 ion-exchange, heparin-Sepharose affinity chromatography and Mono-S cation exchange high performance liquid chromatography (HPLC), as described (1). A chemically modified derivative of aFGF was generated by extensive reductive methylation of lysine residues, and the modified derivative separated from residual unmodified aFGF by Mono-S cation exchange HPLC (Harper, J.W., and Lobb, R.R., unpublished observations). This derivative has a reduced ability to stimulate 3T3 cell mitogenesis ($K_{0.5} \simeq 3$ ng/ml), due to a reduced affinity for its cell surface receptor.

Cell culture. Balb/C 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum, 2 mM glutamine, 50 μg/ml gentamicin and 0.5 μg/ml fungizone. Mitogenesis was measured by [^{125}I]-iododeoxyuridine uptake into cellular DNA, and was performed on quiescent confluent 3T3 cells in 96 well plates as described (14). For ion flux assays cells were plated into 35 mm Petri dishes (3×10^4

cells/ml; 3 ml/dish), grown to confluence (2-4 days) and then used 5-7 days later when the cells had become quiescent.

Measurements of $^{86}Rb^+$ and $^{22}Na^+$ influx. Confluent quiescent 3T3 cells in 35 mm dishes were washed three times at 37°C with a balanced salt solution (BSS) (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 20 mM Hepes buffered to pH 7.4 with Tris base) and then equilibrated for 10 min at 37°C in BSS containing 10 mM glucose and 1 mg/ml bovine serum albumin. This medium was aspirated and replaced with 1 ml of BSS containing either the K^+ analog $^{86}Rb^+$, (2-3 μCi/ml; 2-12 mCi/mg) or $^{22}Na^+$, (3 μCi/ml; 300 mCi/mg). After different time intervals the influx of ions was terminated by rapidly washing the cells six times with ice-cold 100 mM $MgCl_2$ (buffered with 10 mM Hepes-Tris, pH 7.4). In experiments performed at lower Na^+ concentrations NaCl was replaced with choline chloride to maintain osmolarity. Cell associated radioactivity was extracted and counted as described (15). The ouabain-sensitive $^{86}Rb^+$ influx was used as an indicator of the activity of the Na^+-K^+ pump and was calculated as the difference in the initial rates of $^{86}Rb^+$ influx in the absence and presence of 2 mM ouabain, added simultaneously with the isotope. To determine the influx of $^{22}Na^+$ the concentration of Na^+ in the incubation medium was reduced to 30 mM to increase the specific activity of the isotope and 2 mM ouabain was present in the buffers to prevent backflux of the tracer through the Na^+-K^+ pump. All flux assays were performed in triplicate and the protein content of each sample was determined in duplicate by the method of Bradford (16). Initial rates of $^{86}Rb^+$ or $^{22}Na^+$ influx (nmol/mg protein/min), were calculated from the cell associated radioactivity (cpm/mg protein) divided by the specific activity of the isotope in the flux medium (cpm/nmol) and the time interval.

Determination of intracellular Na^+. After treatment in the presence of 2 mM ouabain, the cultures were rapidly washed six times with ice-cold $MgCl_2$ washing solution. One milliliter of 0.1 N HNO_3 was added to each dish and the total cell Na^+ content was measured by atomic absorption spectrometry.

RESULTS AND DISCUSSION

Effect of HBGF's on ouabain-sensitive $^{86}Rb^+$ influx. The ouabain-sensitive (OS) $^{86}Rb^+$ influx, i.e., the influx of $^{86}Rb^+$ mediated through the Na^+-K^+ pump, was determined from the uptake of the radioactive tracer in the absence and presence of ouabain. The basal OS $^{86}Rb^+$ influx of confluent quiescent 3T3 cells was $\simeq$ 12 nmol/mg protein/min, and the ouabain-insensitive $^{86}Rb^+$ influx was $\simeq$ 10 nmol/mg protein/min. These values are in agreement with those reported for other cell lines (15). Figure 1 shows that aFGF (1 ng/ml) induced a rapid and transient increase in the OS $^{86}Rb^+$ influx, which was detected within minutes after mitogen addition. Figure 1 also shows that the OS $^{86}Rb^+$ influx was stimulated even when the mitogen was added simultaneously with the isotope, that the maximum effect was

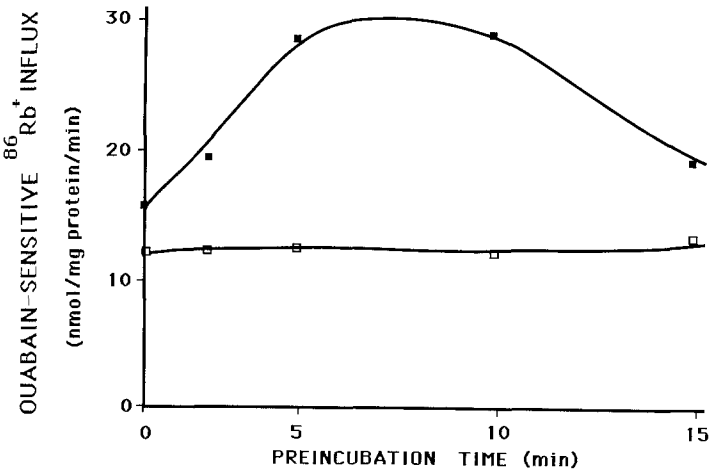

Figure 1. Time course of the effect of aFGF on ouabain-sensitive $^{86}Rb^+$ influx into 3T3 cells. The cells were preincubated for different time intervals in the presence (■) and absence (□) of 1 ng/ml of aFGF. The medium contained (mM) 130 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 20 Hepes buffered to pH 7.4 with Tris base. At the end of the preincubation time the influx was started by adding the tracer $^{86}Rb^+$ (≃ 2μCi/ml) to the medium, with and without ouabain (2 mM). The incubation time was 5 minutes.

observed when the cells were preincubated with mitogen for 5-10 minutes, and that the effect of the mitogen was diminished when the preincubation time was extended to 15 minutes. The ouabain-insensitive influx of $^{86}Rb^+$ was not changed by the presence of aFGF.

The stimulation of the OS $^{86}Rb^+$ influx was dependent upon the aFGF concentration (Fig. 2A). Moreover, this concentration dependence parallelled the mitogenic effect of the growth factor as determined by DNA synthesis (Fig. 2B). Thus, both the Na^+-K^+ pump and the synthesis of DNA were half maximally stimulated ($K_{0.5}$) at ≃ 0.3 ng/ml and approached saturation at ≃ 0.8 ng/ml. bFGF also stimulated OS $^{86}Rb^+$ influx in a concentration-dependent manner (Fig. 2C) that parallelled DNA synthesis (not shown; see ref. 1). These results suggest that the stimulation of both the activity of the Na^+-K^+ pump and the synthesis of DNA induced by HBGF's is triggered by common cellular events elicited by the interaction of the growth factors with their specific receptors.

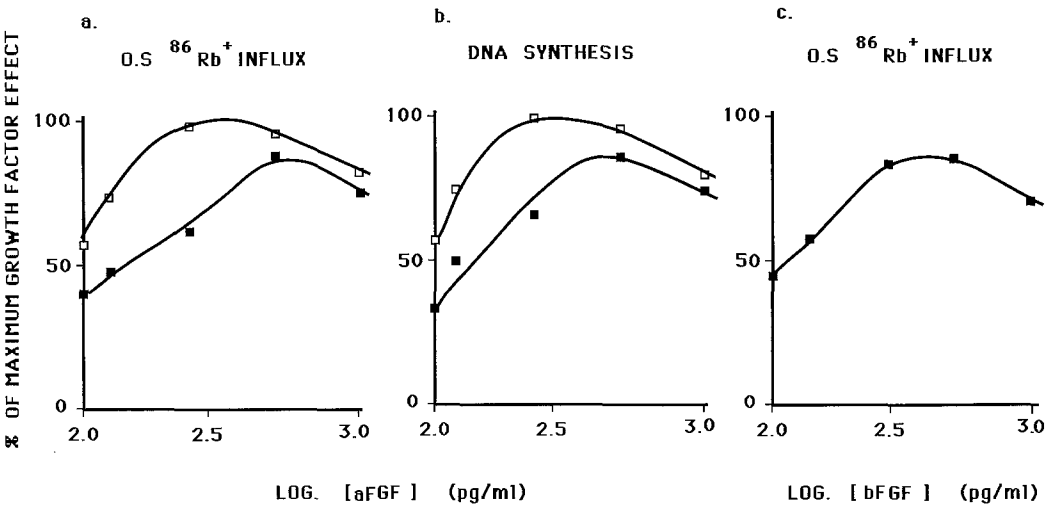

Figure 2. Concentration dependence of the stimulation by aFGF of A) ouabain-sensitive $^{86}Rb^{+}$ influx, and B) DNA synthesis in 3T3 cells, and by bFGF of ouabain-sensitive $^{86}Rb^{+}$ influx. Before starting the influx assay, the cells were preincubated with aFGF for 2.5 min in the presence (□) and absence (■) of heparin (10 μ/ml), or preincubated with bFGF for 5 min. The incubation time was 5 min. Other experimental conditions were the same as those in Fig. 1. Results are expressed as a % of the maximum effect of either HBGF. In the absence of heparin the OS $^{86}Rb^{+}$ uptake at saturating concentrations of both mitogens was 30 nmol/mg protein/min, and in the absence of mitogen 12 nmol/mg protein/min.

This conclusion is further supported by experiments with aFGF chemically modified by reductive methylation. The modified mitogen has a 10-fold decreased mitogenic activity as compared with unmodified aFGF and exhibits a similar reduction in its ability to stimulate the Na^{+}-K^{+} pump ($K_{0.5}$≃3 ng/ml) in 3T3 cells (not shown).

Since heparin enhances the mitogenic effect of aFGF, but not bFGF, towards Balb/C 3T3 cells (1), its effect on the stimulation of the Na^{+}-K^{+} pump by HBGF's was investigated. Both the stimulation of the Na^{+}-K^{+} pump (Fig. 2A) and the stimulation of DNA synthesis (Fig 2B) by aFGF were enhanced by the presence of heparin (10 μg/ml) in the incubation medium. In contrast, the stimulation of the pump by bFGF was not affected by heparin (not shown). The nature of the apparent enhancement of aFGF activity by heparin is unclear, but may be due in part to the ability of heparin to stabilize aFGF to inactivation, either during the time

course of the assays, or during storage (3,17,18). Our results show that the stimulation of OS $^{86}Rb^+$ influx by aFGF, an assay which takes 10-15 min, is enhanced by added heparin in a manner which parallels the enhancement of the mitogenic activity of aFGF in an assay which takes 48 h. Thus, inhibition by heparin of aFGF inactivation over the time course of mitogenesis assays cannot be the sole reason for heparin enhancement.

As in other cell lines, the activity of the Na^+-K^+ pump in 3T3 cell membranes is highly sensitive to changes in the internal Na^+ content of the cells (11). Treatment of Balb/C 3T3 cells with aFGF increased the internal Na^+ concentration of these cells. After 10 minutes incubation in the presence of 2 mM ouabain, the intracellular Na^+ concentration in the absence of mitogen was 450 ± 15 nmol/mg protein (n=6). In the presence of 0.8 ng/ml of aFGF the intracellular Na^+ concentration increased to 580 ± 10 nmol/mg protein (n=6). Moreover, Figure 3 shows that aFGF did not stimulate the OS $^{86}Rb^+$ influx into 3T3 cells when the influx was measured in Na^+-free medium (choline substitution). These results suggest that HBGF's stimulate the Na^+-K^+ pump by increasing cell Na^+. Other studies (19) show that Na^+ entry into 3T3 cells occurs, at least in part, through the amiloride-sensitive exchange of external Na^+ for internal H^+ (Na^+-H^+ exchange). Consistent with this suggestion, Figure 3 also shows that aFGF failed to stimulate the OS $^{86}Rb^+$ influx when 1 mM amiloride was present in the incubation medium.

Effect of HBGF's on unidirectional $^{22}Na^+$ influx. Figure 4 shows that aFGF rapidly increased the unidirectional $^{22}Na^+$ influx into confluent, quiescent 3T3 cells. The presence of 1 mM amiloride in the incubation medium completely abolished the stimulation of the unidirectional $^{22}Na^+$ influx produced by the growth factor. These results indicate that HBGF's, like a variety of other growth factors (8), stimulate Na^+ influx through the amiloride-sensitive Na^+-H^+ exchanger.

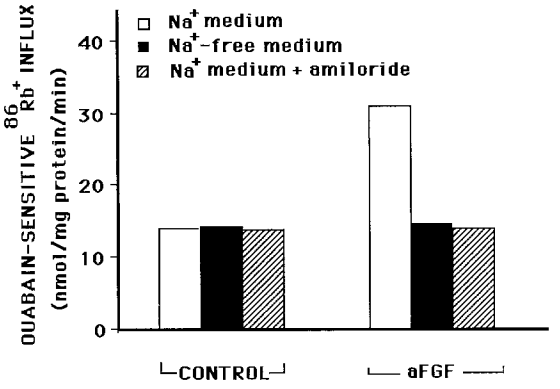

Figure 3. Effects of amiloride, and absence of external Na^+, on the stimulation by aFGF of the OS $^{86}Rb^+$ influx into 3T3 cells. The ouabain-sensitive $^{86}Rb^+$ influx was determined in the presence and absence of 1 ng/ml aFGF in Na^+ medium (white), Na^+ medium with 1 mM amiloride (dark) and Na^+-free medium (choline substitution, gray).

Activation of the Na^+-H^+ exchanger has been proposed to be triggered by different cellular events such as early cytosolic acidification and activation of tyrosine-specific kinases or protein-kinase C which can be activated by diacylglycerol released during agonist-induced breakdown of phosphoinositides (20-22). The mechanism by which HBGF's stimulate amiloride-sensitive Na^+ influx remains to be determined. However, a link

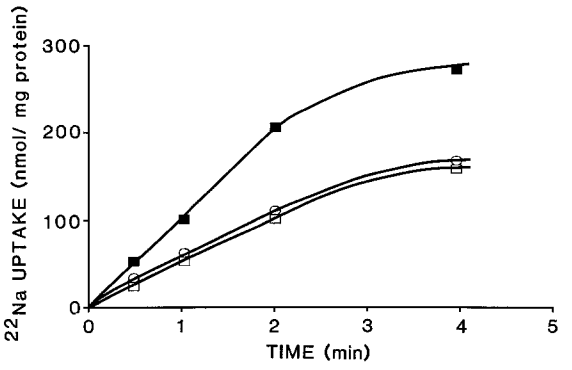

Figure 4. Effect of aFGF on the unidirectional $^{22}Na^+$ influx into 3T3 cells. The medium contained (mM) 30 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 2 ouabain, and 20 Hepes buffered to pH 7.4 with Tris base. The influx was started by adding ≈ 3 μCi/ml of $^{22}Na^+$ and stopped after different time intervals. (□) Control; (■) aFGF (0.8 ng/ml); (○) aFGF (0.8 ng/ml) and amiloride (1 mM). Amiloride and aFGF were added simultaneously with the isotope.

between bFGF proliferation and diacylglycerol activation of protein kinase C has already been suggested (23).

The present report shows that HBGF's, like other mitogens, affect monovalent cation transport across cell membranes. These results further support the concept that, upon binding of growth factors to their specific cell surface receptors, early changes in the ionic composition of the cell are linked to the metabolic events that lead to cell proliferation.

ACKNOWLEDGMENTS

We thank Sandra Vaters for excellent technical assistance. This work was supported by Grant NS23367-01 from the National Institute of Neurological and Communicative Disorders and Stroke (JAH), and by Grant HL 36373-01 from the National Heart, Lung, and Blood Institute (RRL),

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