Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 32-40

EARLY AND LATE MITOGENIC EVENTS INDUCED BY FGF ON BOVINE EPITHELIAL LENS CELLS ARE NOT TRIGGERED BY HYDROLYSIS OF POLYPHOSPHOINOSITIDES

Michel Moenner°, Isabelle Magnaldo*, Gilles L'Allemain*, Denis Barritault°, and Jacques Pouysségur*

° Université Paris XII, Laboratoire de biotechnologie des cellules eucaryotes, Avenue du général de gaulle, 94010, Créteil, France
* Centre de Biochimie-CNRS, Université de Nice, Parc Valrose, 06034 Nice, France

Received May 25, 1987

Basic or acidic forms of FGF, a potent mitogen for Bovine Epithelial Lens cells caused a rapid and transient rise in cytoplasmic Ca^{2+} followed by an increase in intracellular pH of 0.4 units. When cells were labeled at equilibrium with [^{3}H]-inositol, no significant breakdown of polyphosphoinositides (in the presence of 20 mM LiCl) could be detected in response to 10-100 ng/ml of FGF. Similarly, fetal calf serum efficiently reinitiated DNA synthesis in these cells with little stimulation of polyphosphoinositide hydrolysis. In contrast, prostaglandin $F2_\alpha$ and angiotensin II, two weak mitogens for BEL cells, were found potent agonists of polyphosphoinositide breakdown. These results strongly indicate that the mitogenic action of FGF is not coupled to phospholipase C activation, a conclusion consistent with the fact that the FGF-induced $[Ca^{2+}]_i$ rise is strictly dependent upon external Ca^{2+}.

The response of cells to mitogens via their receptors is mediated by a variety of successive intracellular events leading to DNA synthesis and cellular division. Among the intracellular responses induced by growth factors action, a common set of early events such as Na^+/H^+ antiporter stimulation leading to cytoplasmic alkalinization, and mobilization of intracellular Ca^{2+} has been described.

However, recent reports have pointed out differences in respective potentialities of growth factors to induce breakdown of polyphosphoinositides. While many of the growth-promoting agents were reported as agonists of the inositol lipid hydrolysis (1),

Abbreviations: bFGF, basic Fibroblast Growth Factor; FCS, Fetal Calf Serum; Quin-2/AM, quin-2-tetra-(acetoxymethyl) ester; HBS, Hepes-Buffered Saline; BSA, Bovine Serum Albumin; EGTA, Ethylene Glycol bis (B-aminoethyl) -1- piperazine ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; BEL cells, bovine epithelial lens cells; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$.

0006-291X/87 $1.50

several data indicated that EGF failed to stimulate this pathway in some cells (2-4) but not in others (5,6).

Apparently, these contradictory reports seem to be largely explained by differences in the number of EGF receptors (6) and differences in basal activity of phospholipase C. A similar controversy emerged concerning the mitogenic signal transduction of FGF. This growth factor has been reported to act *via* inositol lipid breakdown and C-kinase activation in 3T3 cells (7) and independently of these pathways in hamster fibroblasts (8). In view of these different behaviours, and for a better understanding of the relative importance of the mechanisms supposed to be involved in the mitogenic response, we decided to further investigate the initial mitogenic events triggered by FGF.

FGF stimulates a broad variety of mammalian cells and has been shown to be present in numerous tissues (9). Since our early interest has been the study of the role of retina in lens growth and differentiation, leading to the discovery and purification of the Eye-Derived Growth Factor I and II (retinal form of basic and acidic FGF)(10), we naturally studied the effects of EDGF/FGF on phosphatidylinositol turnover, calcium and pH variation on cultured Bovine Epithelial Lens cells (BEL). These cells in which we have characterized bFGF receptors (11) are highly responsive to EDGF/FGF. Here we show that FGF induces in these cells early and late mitogenic events *via* a signaling pathway independent of inositol lipid breakdown. This finding reinforces our conclusion based on hamster fibroblast studies (8).

MATERIALS AND METHODS

Materials- Acidic and basic FGF were purified to homogeneity from bovine brain by acetic acid extraction followed by affinity chromatography on heparin-Sepharose as described (10).Prostaglandin $F_{2\alpha}$ and fatty acid free bovine serum albumin were purchased from Sigma and quin-2/AM was from Calbiochem. Fetal calf serum was from Seromed. [Methyl-^{3}H] -thymidine, [7-^{14}C] benzoic acid and myo-[2-^{3}H(n)]-inositol were from NEN, France. Angiotensin II was a gift from Dr Vincent (Université de Nice).

Cell culture- Bovine Epithelial Lens cells (12) were maintained in Dulbecco's modified Eagle's medium (H21 from Gibco) supplemented with 10% fetal calf serum, penicillin (50 unit/ml) and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells were used between the 9th and 20th passages (doubling time of 4 to 5 days). Before experiments, cell monolayers were arrested in G0/G1 by incubation for 24 hours in serum-free DMEM.

Assay for phosphoinositides turnover- Confluent BEL cells in 35 mm dishes were labeled with [^{3}H]-inositol (2 µCi/ml) for 5 days in a DMEM free of inositol and containing 10% FCS. Confluent cells were then made quiescent in the same medium without FCS for 24 hours. Before experiments, cells were treated 20 min with 20 mM LiCl in the labeled culture medium and were then stimulated with the indicated agents. Incubations were stopped by quickly removing the medium, and adding 0.5 ml of 10% (w/v) $HClO_4$. Separation

of free inositol, and inositol phosphates was carried out essentially as previously described (14).

Calcium mobilization- Measurements of cytoplasmic free Ca^{2+} concentrations were realized as described by Moolenaar et al.(15). Monolayers of quiescent BEL cells on 3x1 cm rectangular glass coverslips were loaded for 40 min at 37°C in HBS containing 50 μM of quin-2/AM. Cells were then washed twice by incubation for 10 min in HBS. Quin-2 loaded monolayers were then inserted vertically into a thermostated cuvette in a Perking-Elmer 3000 spectrofluorometer. Fluorescence was continuously recorded at an excitation wavelength of 339 nm and emission wavelength of 492 nm. Values of intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) were calculated from fluorescence signals according to Tsien et al. (16).

Cytoplasmic pH measurements- Confluent cultures in 35 mm dishes were made quiescent and incubated for 15 min at 37°C with a Hepes-buffered saline solution (HBS) consisting of 130mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM glucose and 20 mM Hepes/NaOH, pH=7.4. 1 μCi/ml of [^{14}C]benzoic acid was then added 15 min before either fetal calf serum, FGF or bovine serum albumin. Variations of intracellular pH were determined as reported (13).

Mitogenesis assay- Reinitiation of DNA synthesis was measured on confluent BEL cells in 24 well-plates after a 24 hours incubation in DMEM 0% FCS. Stimulations were realized by incubating the quiescent cells monolayers with the mitogens. After 20 hours, 2μCi/ml of [methyl-^{3}H]-thymidine was added and the incubation with growth factors was further allowed for 28 hours. Cells monolayers were then fixed with 5% trichloroacetic acid for 20 min at 4°C and consecutively washed. Cells were finally extracted with 0.1N NaOH and taken for liquid scintillation counting.

RESULTS

FGF does not induce the turnover of inositol phospholipids

We analyzed the capacity of FGF and fetal calf serum to stimulate the hydrolysis of polyphosphoinositides. We first chose these two agents because they are the best growth promoting-agents for BEL cells. After prelabelling at equilibrium with [^{3}H]inositol and in the presence of 20 mM LiCl to amplify the detection of phospholipase C activation, neither FGF nor 10% FCS was capable to significantly induce the production of inositol phosphates in quiescent BEL cells. Fig.1 shows that even after a 60 min incubation time with LiCl and a maximal mitogenic concentration of bFGF (10 to 100 ng/ml), no increase in inositol phosphates was detected. The absence of effect with FCS was somewhat surprising since the same batch of serum used in quiescent hamster fibroblasts and under the same assay conditions was found very potent to induce the breakdown of polyphosphoinositides (14).

To validate this finding it was crucial to demonstrate that BEL cells can indeed respond to some well known agonists of phosphoinositide metabolism. Three agents, AlF_4^-, angiotensin II and $PGF_{2\alpha}$ were able to increase respectively 1.6-, 2.5- and 5-fold the basal level of inositol phosphates after 60 min of incubation in the presence of Li^+. During this period of stimulation, intracellular free-[^{3}H]-inositol declined proportionally

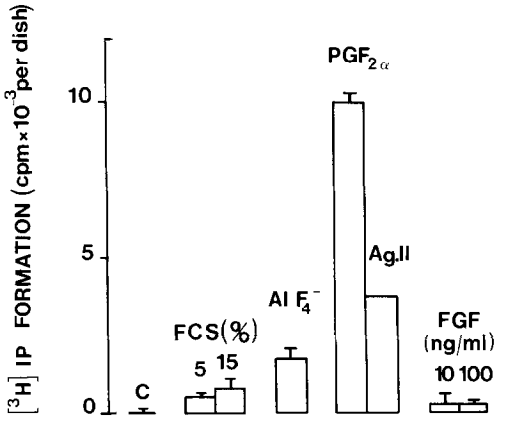


Figure 1. Effect of bFGF and various agents on the inositol phosphates release (IP_1,IP_2,IP_3) on BEL cells. G0-arrested BEL cells were incubated for 60 min in the presence of 20 mM LiCl with bFGF (10 ng/ml and 100 ng/ml) or different other effectors. FCS was tested at concentrations of 5% and 15%. $PGF_{2\alpha}$ was used at the concentration of 500 ng/ml and angiotensin II (Ag II) at 4.10^{-7}M. AlF_4^- was a mixture of $AlCl_3$ (10 μM) and NaF (5 mM). (C) represents the control experiment without any effector and after 60 min of incubation. Values are the average of duplicate dishes.

to the amount of inositol phosphates accumulated in response to the three agonists (not shown). As expected, free $[^3H]$-inositol remained constant during maximal FGF stimulation.

FGF stimulates a rise in cytoplasmic Ca^{2+}

A first set of experiments using cells loaded with quin-2 is presented in Fig.2a. The fluorescence signal from resting cells indicated a basal cytoplasmic Ca^{2+} concentration of 130-160 nM. This value was found consistent with other reports on different cell types (17,18). Stimulation of BEL cells with bFGF in these conditions induced a rapid (20-30 sec) increase in $[Ca^{2+}]_i$ up to 350 nM and then a slow decrease back to the basal level (over 8 min). When external Ca^{2+} was reduced to 1 μM in the presence of 1 mM EGTA, addition of bFGF failed to stimulate the rise in $[Ca^{2+}]_i$ (Fig.2b). Readdition of external Ca^{2+} (1 mM) to the same cells restored rapidly the increased level of cytoplasmic Ca^{2+} in the presence of bFGF. Same results were obtained with acidic FGF (not shown).

FGF stimulates cytoplasmic alkalinization

Fig.3 shows that bFGF induced an alkalinization of the cytoplasm with a maxima reached after 15 min of incubation. This variation of the pH_i was still detectable after 30 min and paralleled the effect obtained after cellular stimulation with FCS. Both bFGF (10

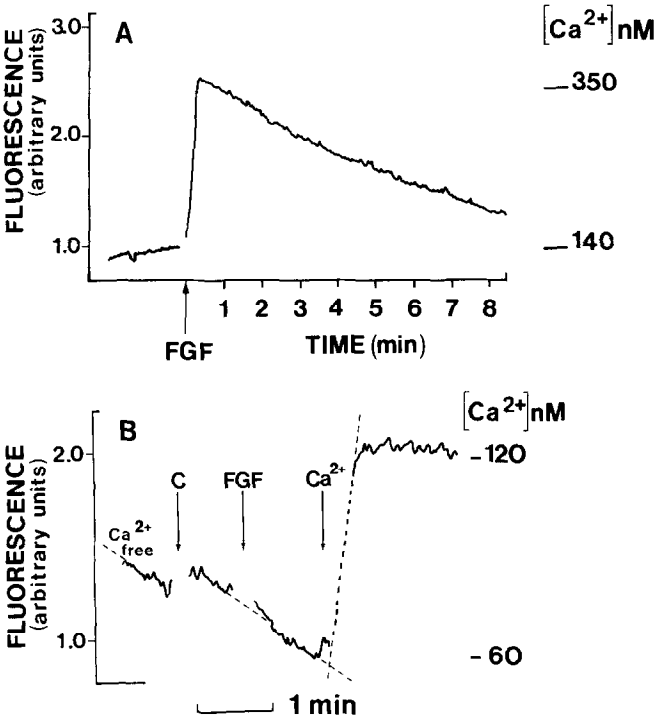
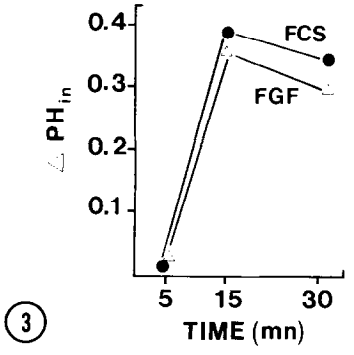


Figure 2. Changes in free intracellular Ca^{2+} in BEL cells following addition of bFGF. A) bFGF (15 ng/ml) was added on cells loaded with quin 2 and incubated in HBS containing 1mM $CaCl_2$. B) Quin-2 fluorescence signal after cellular incubation for 10 min in the same medium as above, plus 2 mM EGTA (Ca^{2+}-free). Successive following injections were carried through : (C) Control injection of HBS-2mM EGTA, bFGF (26 ng/ml) and finally 2 mM $CaCl_2$ (1mM final excess of external Ca^{2+}) in the continuous presence of bFGF. The results are presented as the relative change in quin-2 emission intensity.

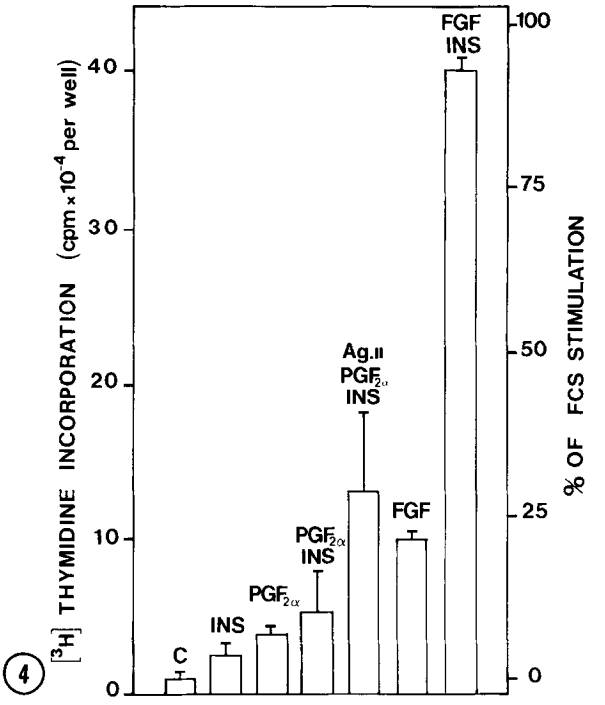
ng/ml) and fetal calf serum (10 %), were used at amounts that ensured maximal mitogenic response on these target cells.

Stimulation of DNA synthesis on quiescent BEL cells.

Having characterized two agonists of inositol lipid breakdown ($PGF_{2\alpha}$ and angiotensin II) and two non-agonists (FGF and FCS) for BEL cells, it was of interest to compare their relative potency to trigger DNA synthesis. Growth-arrested BEL cells were stimulated with maximal concentration of each agent alone or in combination with insulin. Fig.4 shows that bFGF stimulated by itself the incorporation of [^{3}H]-thymidine on these cells. This stimulation was strongly potentiated (3-4 fold) by insulin, giving a similar degree of stimulation to that obtained with 10 % FCS (a 35-fold increase above control). In contrast, the two agonists of inositol lipid breakdown are weak mitogens. The concentration of $PGF_{2\alpha}$ required to give optimal results in the mitogenic assay was 500 ng/ml (not shown). At that



③



④

Figure 3. Effect of bFGF on the cytoplasmic pH in quiescent BEL cells as a function of incubation time. G0-arrested cells in 35 mm dishes were equilibrated for 30 min in HBS at 37°c. 1µCi/ml [^{14}C]benzoic acid was then added to the medium 15 min before the addition of (▲) bFGF 10 ng/ml, (●) FCS 10%. Wells were then rapidly washed with ice cold buffer PBS and the radioactivity counted after cellular extraction with 0.1 M NaoH. Points represent mean values from duplicate dishes.

Figure 4. Initiation of DNA synthesis on serum-deprived BEL cells in response to different stimuli. Cells were incubated with the following effectors: C, control (no addition); insulin (Ins), 10 µg/ml; PGF$_{2\alpha}$, 500ng/ml; bFGF, 5 ng/ml. For the combination experiments: Ins/PGF$_{2\alpha}$, Ins/PGF$_{2\alpha}$/AgII and Ins/bFGF, results were obtained with identical respective concentration of these agents. Angiotensin II (Ag II) was used to the final concentration of 400 ng/ml. Values were obtained by counting the thymidine incorporation in cells after 48 hours of incubation with the different agonists. Results were expressed as total cpm incorporated (left) or as the percentage (right) of the values obtained with 10% FCS.

concentration, the individual effect of PGF$_{2\alpha}$ on DNA synthesis was small but significant (3- to 4-fold increase). Association of PGF$_{2\alpha}$ with insulin alone or with a combination of insulin and angiotensin II induced a further increase in DNA synthesis. Nevertheless it is interesting to note that for this cell system, the best mitogenic responses are triggered by growth promoting agents not coupled to polyphosphoinositide hydrolysis.

DISCUSSION

Our results indicated that reinitiation of DNA synthesis on quiescent BEL cells after stimulation by FGF is associated with activation of intracellular early ionic events such

as an increase in pH_i and $[Ca^{2+}]_i$, as described for other cells and growth factors (13,15,17,18). These results were expected since cytoplasmic Ca^{2+} has been previously proposed as a key signal of growth factor action (17), and since the increase of pH_i has also been demonstrated to play a permissive role for G0/G1 progression and commitment to DNA synthesis (19,20).

The question we addressed in this paper was to know whether early and late mitogenic events elicited by FGF are transduced through the inositol lipid signaling pathway in BEL cells. Surprisingly, FGF was shown unable to stimulate phosphatidylinositol hydrolysis in this cell system. This finding, which contrasts with a report in Swiss 3T3 cells (7), confirms and extends our previous studies on Chinese hamster lung cells (CCL39) (8).

Indirect experiments measuring variations of cytoplasmic Ca^{2+} after cellular stimulation with FGF led to the same conclusion. Indeed, we showed that the observed rise in intracellular Ca^{2+} on BEL cells was dependent upon the presence of external Ca^{2+}, as it has already been described for EGF (15,18). The demonstration that FGF failed to induce mobilization of Ca^{2+} from intracellular stores maybe correlated to the lack of production of IP_3, since it is now well established that these two events are closely linked (21,22).

However, we demonstrated the ability of BEL cells to activate polyphosphoinositides breakdown since proper agonists like $PGF_{2\alpha}$, angiotensin II and the combination of the chemical agents $AlCl_3$ and NaF (presumably acting as AlF_4^-(23)) acted positively on this pathway. The mitogenic effect of $PGF_{2\alpha}$ and its potentiality to activate polyphosphoinositide hydrolysis has already been described on resting Swiss 3T3 cells (24) while angiotensin II has been shown to be potent on adrenal cortex (25).

In our report, results were only related to the basic form of the FGF, but identical data were obtained with acidic FGF. This probably reflected a similar intracellular pathway of stimulation for both growth factors and correlated with the recent data (26) on the existence of common(s) receptor(s) for acidic and basic FGF.

In conclusion, our results indicated that the polyphosphoinositide cycle was not involved in the mitogenic pathway of FGF. Taking in consideration that the mitogenic events we described in this work for FGF are identical to those reported for EGF, an interesting point is to determine if the general properties of both the FGF and EGF

receptors maybe identical, and particularly if the binding of FGF on its cellular receptor is influenced consecutively to the activation of the protein kinase C, through the indirect mechanism of the transmodulation. This also raised the hypothesis that a tyrosine kinase activity might well be associated to the receptor of FGF. Recently, a report on such an activity has been described for the bovine Brain-derived Growth Factor (27), a polypeptide related to the acidic FGF.

ACKNOWLEDGEMENTS

We wish to thank J.Courty for providing purified FGF, Y.Courtois and O.Lagente for opening their laboratory and supplying with BEL cells. This work was supported by the Association de la Recherche sur le Cancer, la Ligue Nationale contre le Cancer, Naturalia et Biologia, the Ministère de l'Education Nationale and the Ministère de la Recherche et de la Technologie.

BIBLIOGRAPHY

1) Berridge ,M.J., and Irvine ,R.F. (1984) Nature 312, 315-321.
2) L'Allemain, G. and Pouysségur, J (1986) FEBS Lett. 197, 344-348.
3) Besterman, H.M., Watson, S.P. and Cuatrecasas, P. (1986) J. Biol. Chem. 261, 723-727.
4) Taylor ,D., Uhing ,R.J., Blackmore ,P.F., Prpic ,V., and Exton ,J.H. (1985) J. Biol. Chem. 260, 2011-2014.
5) Hepler ,J.R., Nakahata ,N., Lovenberg ,T.W., DiGuiseppi ,J., Herman ,B., Earp ,H.S., and Harden ,T.K. (1987) J.Biol.Chem. 262,2951-2956.
6) Wahl ,M.I., Sweatt ,J.D. and Carpenter ,G. (1987) Biochem. Biophys. Res. Commun. 142, 688-695.
7) Tsuda, T., Kaibuchi, K., Kawahara, Y., Fukuzaki, H., and Takai, Y. (1985) FEBS Lett. 191, 205-210.
8) Magnaldo, I., L'Allemain, G., Chambard, J.C., Moenner, M., Barritault, D., and Pouysségur, J. (1986) J.Biol.Chem. 261, 16916-16922.
9) Gospodarowicz, D., Neufeld, G., and Schweigerer, L., (1986) Cell Differenciation, 19, 1-17.
10) Courty, J., Loret, C., Moenner, M., Chevallier, B., Lagente, O., Courtois, Y., and Barritault, D., (1985) Biochimie 67, 265-269.
11) Moenner, M., Chevallier, B., Badet, J., and Barritault, D., (1986) Proc.Natl.Acad.Sci. U.S.A. 83, 5024-5028.
12) Barritault, D., Arruti, C., and Courtois, Y., (1981) Differentiation 18, 29-42.
13) L'Allemain, G., Paris, S., and Pouysségur, J., (1984) J.Biol.Chem. 259, 5807-5815.
14) L'Allemain, G., Paris, S., Magnaldo, I., and Pouysségur, J (1986) J.Cell.Physiol. 129, 167-174.
15) Moolenaar, W.H., Aerts, R.J., Tertoolen, L.G., and de Laat,S.W., (1986) J.Biol.Chem. 261, 279-284.
16) Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell.Biol. 94, 325-334.
17) Moolenaar, W.H., Tertoolen, L.G. and de Laat, S.W. (1984) J. Biol. Chem. 259, 8066-8069.
18) Hesketh ,T.R., Moore, J.P, Morris, J.D., Taylor, M.V., Royers, J., Smith, G.A. and Metcalfe, J.C. (1985) Nature, 315, 481-484.
19) Pouysségur, J., Sardet, C., Franchi, A., L'Allemain, G., and Paris, S. (1984) Proc.Natl.Acad.Sci. U.S.A. 81, 4833-4837.

20) Pouysségur, J., Franchi,A., L'Allemain, G. and Paris,S. (1985) FEBS Lett. 190, 115-119.
21) Streb, H., Irvine, R., Berridge, M., and schultz, I. (1983) Nature 306, 67-69.
22) Burgess, G., Godfrey, R., Mc Kinney, J., Berridge, M., Irvine, R., and Putney, J. (1984) Nature, 309, 63-66.
23) Guillon, G., Mouillac, B., and Balestre, M.N. (1986) FEBS Lett. 204, 183-188.
24) Macphee, C.H., Drummond, A.H., Otto, A.M. and Jimenez de Asua, L. (1984) J. Cell. Physiol. 119, 35-40.
25) Farese, R.V., Larson, R.E., and Davis,J.S. (1984) Endocrinology, 114, 302-304.
26) Neufeld, G., and Gospodarowicz, D. (1986) J.Biol.Chem. 261, 5631-5637.
27) Huang, S.S., and Huang, J.S. (1986) J.Biol.Chem. 261, 9568-9571.