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Stimulation of polyphosphoinositide hydrolysis in Swiss 3T3 cells by recombinant fibroblast growth factors

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Mitogenic concentrations of recombinant acidic or basic fibroblast growth factor (FGF) stimulated the accumulation of [^{3}H]inositol phosphates ([^{3}H]IPs) in Swiss 3T3 cells pre-labelled for 48 h with [^{3}H]inositol. Maximal effects were obtained at 0.3 ng/ml and 3 ng/ml for basic and acidic FGF, respectively. Higher doses of either factor led to a diminished stimulation. FGF also stimulated $^{45}Ca^{2+}$ release from cells pre-labelled with the isotope. However, FGF-stimulated production of [^{3}H]IPs and release of $^{45}Ca^{2+}$ exhibited marked differences when compared with the responses to the peptide mitogen bombesin; the FGF responses were markedly slower and were not inhibited by phorbol esters.

Phosphatidylinositol turnover; Ca^{2+} mobilization; Fibroblast growth factor; Bombesin

1. INTRODUCTION

The acidic and basic forms of FGF are potent stimulators of the proliferation of various mesoderm-derived cell lines (see [1] for review). Although acidic and basic FGF have been demonstrated to bind to the same cellular receptors [2,3], little is known about the signal-transducing pathways activated by receptor occupation. Activation of tyrosine kinase has been observed in some [4,5], but not all [6], studies. Conflicting results have also been obtained in studies of phosphoinositide hydrolysis in response to FGF [7-10]. The recent production of recombinant acidic and basic FGFs has enabled us to investigate the effects of the pure mitogens on the production of inositol phosphates (IPs) in Swiss 3T3 cells and to compare the response with that induced by the potent phosphoinositide-hydrolysing mitogen bombesin [11-13].

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Abbreviations: FGF, fibroblast growth factor; IPs, inositol phosphates; PBS, phosphate-buffered saline; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate

2. MATERIALS AND METHODS

Stock cultures of Swiss 3T3 cells were maintained as described [14]. For experimental use, cells were seeded into 3.5 cm dishes in DMEM supplemented with 10% calf serum and used 7 days later when the cultures were confluent and quiescent. The incorporation of [^{3}H]thymidine was assayed as described [14]. For measurement of inositol phosphates, cells were labelled for the final 48 h of culture by adding *myo*-[^{3}H]inositol (1-2.5 μCi/ml) directly to the growth medium. After careful washing to remove unincorporated label, the cells were incubated in PBS (pH 7.4) containing 10 mM LiCl and mitogens as indicated in the figure legends. At the end of the incubation, [^{3}H]IPs were extracted into 0.5 ml of 15% (w/v) trichloroacetic acid (4°C) and analysed by anion-exchange chromatography [15]. [^{3}H]Inositol mono-, bis- and trisphosphate fractions (IP_1, IP_2 and IP_3 fractions, respectively) were either eluted separately [12], or together as a single fraction (IP_t) [16]. The efflux of $^{45}Ca^{2+}$ from pre-labelled cells was measured as described [12].

Recombinant bovine basic FGF was obtained from Amersham International. Recombinant human acidic FGF [17] was a generous gift from Dr Michael Jaye, Rorer Biotechnology. Both factors were produced in *E. coli* hosts and were purified to apparent homogeneity from bacterial lysates.

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3. RESULTS

3.1. *Stimulation of DNA synthesis by recombinant FGFs*

Basic FGF produced a dose-dependent stimulation of [^{3}H]thymidine incorporation in quiescent cultures of Swiss 3T3 cells. When added alone in serum-free medium, a maximal stimulation approx. 25–40% of that achieved by the addition of 10% calf serum was observed (fig.1 and results not shown). In combination with insulin, the maximal response to basic FGF rose to 90% of the serum response, and the dose for maximal stimulation fell slightly from 0.3 to 0.1 ng/ml (fig.1). Recombinant acidic FGF produced similar results (not shown) although somewhat higher concentrations of the factor (1–10 ng/ml) were required for a maximal response.

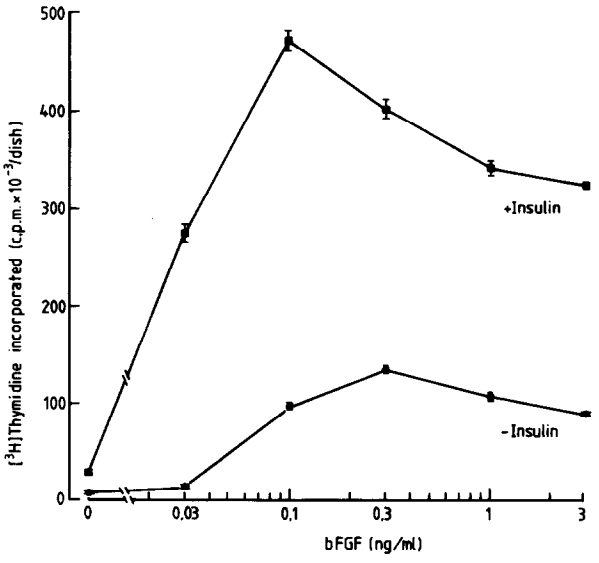

Fig.1. Stimulation of DNA synthesis by recombinant FGF. Confluent monolayers of cells were incubated for 40 h at 37°C in 2 ml of serum-free DMEM containing [^{3}H]thymidine (1 μCi/ml) and the indicated concentration of basic FGF in the presence (■) or absence (●) of insulin (1 μg/ml). Cultures were washed, extracted, and incorporated radioactivity was measured. Each point represents the mean value (±SE) for three determinations of [^{3}H]thymidine incorporation on separate dishes of cells. Error bars not visible fall within the symbol. [^{3}H]Thymidine incorporation in response to the addition of 10% (v/v) calf serum was 541 × 10^3 cpm/dish.

3.2. *Production of inositol phosphates in response to FGF*

The time course for the accumulation of ^{3}H-labelled IP_1, IP_2 and IP_3 fractions in quiescent Swiss 3T3 cells stimulated by basic FGF is shown in fig.2. A gradual accumulation of all three fractions was observed, although it was evident that the rise in the IP_3 fraction preceded the rise in the IP_2 fraction which preceded the rise in the IP_1 fraction. Similar results were obtained in several experiments using either basic or acidic FGF with no significant differences between the time courses of the responses. In contrast, the mitogenic peptide bombesin [18], which has previously been shown to stimulate polyphosphoinositide hydrolysis in these cells [11–13], generated a markedly biphasic response which was most evident in the IP_3 and IP_2 fractions (fig.2).

The stimulation of the production of [^{3}H]IPs in response to FGF was dose-dependent. Maximal responses were seen with concentrations of 0.3 and 3 ng/ml for basic or acidic FGF, respectively (fig.3). These doses are similar to those required for maximal stimulation of DNA synthesis by the factors (fig.1 and results not shown). An intriguing feature of the effects of both basic and acidic FGF is the pronounced reduction in the response at higher doses of agonist. This phenomenon, which was very reproducible, was most marked for the FGF-stimulated formation of IPs (fig.3) but was also seen in DNA synthesis assays (fig.1). The cause of this effect is not known but it is not simply due to FGF-induced cell detachment, since parallel FGF-treated cultures showed no significant decrease in cell numbers.

Although the stimulation of phosphoinositide hydrolysis in Swiss 3T3 cells by bombesin or vasopressin is inhibited by phorbol esters [12], the response to platelet-derived growth factor (PDGF) was reported to be unaffected by these agents [19]. We have therefore investigated the effect of TPA on the FGF-stimulated production of [^{3}H]IPs. The response to FGF, like the response to PDGF, was not inhibited; indeed FGF-stimulated formation of [^{3}H]IPs was reproducibly potentiated by TPA (table 1 and results not shown). In contrast, the responses to bombesin and vasopressin were markedly inhibited (table 1).

3.3. *$^{45}Ca^{2+}$ efflux from cells stimulated by FGF*

FGF stimulated the release of $^{45}Ca^{2+}$ from prelabelled Swiss 3T3 cells (fig.4). However, it was

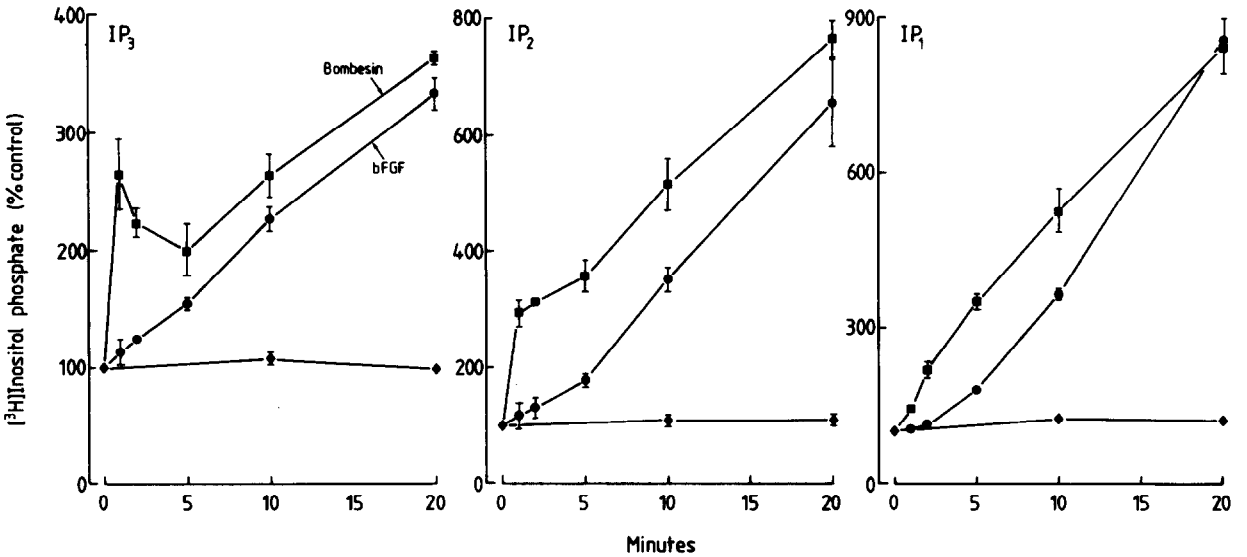

Fig.2. Time courses of FGF- and bombesin-stimulated production of inositol phosphates in Swiss 3T3 cells. Cells were stimulated at 37°C for the indicated times (in the presence of 10 mM LiCl) by 0.3 ng/ml recombinant basic FGF (●), 3 nM bombesin (■), or no addition (◆). After extraction of the cells, [^{3}H]IP fractions were separated and measured. The combined results from two experiments are shown. Each point represents the mean value (±SE) for three determinations from separate dishes of cells.

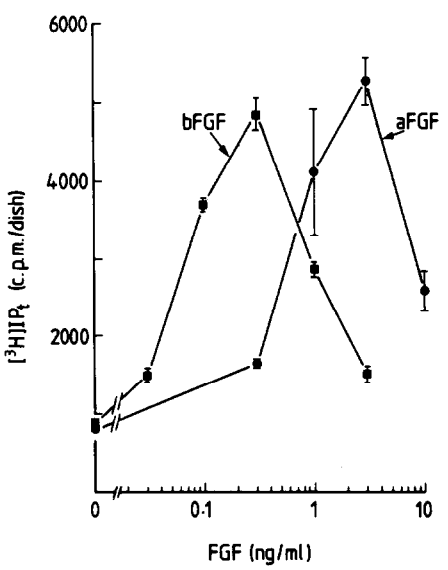

Fig.3. Dose response for FGF-stimulated accumulation of inositol phosphates. Cells were stimulated at 37°C for 30 min by recombinant acidic (●) or basic (■) FGF in the presence of 10 mM LiCl. After extraction of the cells, the [^{3}H]IP$_t$ fraction was separated and measured. Each point represents the mean value (±SE) of three or four determinations from separate dishes of cells.

observed that FGF-stimulated $^{45}Ca^{2+}$ release was considerably slower than that induced by bombesin even when both mitogens were tested at concentrations that caused maximal stimulation of phosphoinositide hydrolysis and DNA synthesis (fig.4). When submaximal doses of bombesin were used, the peak release of $^{45}Ca^{2+}$ still occurred during the first 2-min interval after agonist addition ([12] and results not shown). Although it is tempting to

Table 1

Effects of TPA on the production of [^{3}H]IPs in response to mitogens

Addition	[^{3}H]IP$_t$ (cpm/dish)	
	−TPA	+TPA
None	202 ± 27	200 ± 18
Basic FGF (0.3 ng/ml)	1028 ± 124	1433 ± 21
PDGF (10 ng/ml)	3842 ± 342	4270 ± 210
Bombesin (3 nM)	2962 ± 192	1817 ± 84
Vasopressin (100 nM)	830 ± 40	339 ± 23

Cells were stimulated at 37°C for 30 min (in the presnece of 10 mM LiCl) by the indicated concentrations of mitogens in the presence or absence of TPA (30 nM). After extraction of the cells into cold trichloroacetic acid, the [^{3}H]IP$_t$ fraction was measured. Each point represents a mean value (±SE) for three determinations from separate dishes of cells

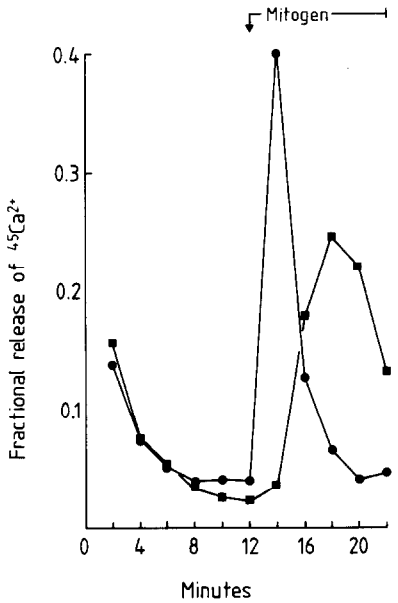

Fig.4. Different time courses for FGF- and bombesin-mediated calcium release. Confluent monolayers of cells were pre-labelled with $^{45}Ca^{2+}$ by an overnight incubation with the isotope (4.4 μCi/ml). The cells were washed and $^{45}Ca^{2+}$ efflux was measured during successive 2-min intervals into PBS (pH 7.4) containing acidic FGF (3 ng/ml; ■) or bombesin (10 nM; ●). The amount of $^{45}Ca^{2+}$ leaving the cells during each 2-min interval is expressed as a fraction of that present in the cells at the beginning of the interval. In unstimulated cells, the fractional release after minute 12 was <0.04 at all time points.

speculate that the slower release of $^{45}Ca^{2+}$ in response to FGF (fig.4) reflects the slower stimulation of PIP_2 hydrolysis in response to this factor (fig.2), a more detailed comparison of the production of 1,4,5-IP_3 and the rise in intracellular [Ca^{2+}] will be required to test this possibility.

4. DISCUSSION

Both basic and acidic recombinant FGFs stimulate the formation of [^{3}H]IPs in quiescent Swiss 3T3 cells pre-labelled with [^{3}H]inositol. Basic FGF was found to be 5–10-fold more potent than acidic FGF although the maximal response to both factors was of a similar size. Using highly purified natural factors, Bohlen et al. [20] found that basic FGF was substantially more potent than acidic FGF in stimulating DNA synthesis in several cell lines. It has been suggested that this reflects the apparent higher affinity of basic FGF over acidic FGF for their shared cellular receptors [1].

Previous studies on the stimulation of phosphoinositide hydrolysis by FGF have generated conflicting results. At concentrations up to 100 ng/ml, FGF failed to stimulate inositol phosphates production in hamster fibroblasts [8] or bovine capillary endothelial cells [10]. In contrast, FGF stimulated the production of diacylglycerol and the incorporation of $^{32}P_i$ into phosphatidylinositol in Swiss 3T3 cells [7,9] although in these studies the formation of IPs was not investigated directly. The reasons why different cell lines should show qualitatively different responses is not clear. Similar conflicting results have been observed in studies using another polypeptide mitogen, epidermal growth factor (EGF). Thus, although EGF stimulates the formation of IPs in A431 cells [21,22], it does not stimulate a significant response in 3T3 cells [23] or in hamster lung fibroblasts [24]. The reasons for this difference are not known but it is probable that the very high number of EGF receptors on A431 cells is involved since it was recently shown that EGF stimulates IPs formation in other carcinoma cell lines which over-express the EGF receptor [22]. However, Swiss 3T3 cells are reported to possess only 6×10^4 FGF receptors per cell [3] which is only 2–3-fold more than the values reported for endothelial cells and human fibroblasts [25].

The time course of FGF-stimulated IPs formation is markedly different from that induced by bombesin. This finding, together with the differential sensitivity to phorbol esters, raises the possibility that fundamentally different mechanisms are responsible for coupling FGF receptors and bombesin receptors to a phosphoinositidase. It is not yet unequivocally established whether FGF receptors, like the receptors for EGF, PDGF and several other polypeptide growth factors, possess integral tyrosine kinase activity. However, in this context, it is interesting that the time course of the FGF-stimulated accumulation of IPs is very similar to that observed for the response to PDGF in 3T3 cells [26] and to EGF in A431 cells [22].

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