Endothelin stimulates DNA synthesis in Swiss 3T3 cells

Synergy with polypeptide growth factors

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The vasoactive peptide endothelin is shown to be a potent mitogen for Swiss 3T3 cells. Although endothelin has little effect on DNA synthesis when added alone to cells in serum-free medium, the peptide synergizes very strongly with several other growth factors. A half-maximal response to endothelin is observed at approx. 0.3 nM, with a maximal effect at 3 nM. Over the same concentration range, endothelin stimulates a 2-fold increase in the accumulation of cellular inositol phosphates. Endothelin may prove to be a useful additional agonist for studying the signalling pathways involved in the control of 3T3-cell proliferation.

INTRODUCTION

Endothelin, a 21-amino-acid peptide, was originally isolated from the medium conditioned by pig aortic endothelial cells (Yanagisawa *et al.*, 1988). The peptide is a potent vasoconstrictor through its activity on vascular smooth-muscle cells. Like other vasoconstrictors (e.g. vasopressin, angiotensin II), endothelin has been reported to stimulate the formation of inositol phosphates and Ca^{2+} mobilization in these cells (Van Renterghem *et al.*, 1988). In addition, endothelin has been found to stimulate DNA synthesis in vascular smooth-muscle cells and in rat mesangial cells (Komuro *et al.*, 1988; Nakaki *et al.*, 1989; Simonson *et al.*, 1989).

Swiss 3T3 cells have been used extensively as a model system to study cell growth control and the signalling pathways activated by growth factors. The cells respond to several polypeptide mitogens, including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGF β). In addition, DNA synthesis and proliferation in these cells are also stimulated by the regulatory peptides vasopressin and bombesin (see Rozengurt, 1986). Although the physiological significance of the proliferative effects of neuropeptides remains unknown, there is increasing interest in the possible involvement of this response in several disease states (see Dalsgaard *et al.*, 1989). We report here that endothelin, in synergy with other mitogens, is a potent stimulator of DNA synthesis in Swiss 3T3 cells. The finding of a mitogenic effect of endothelin on this well-characterized cell line should prove helpful in studies both of the control of cell proliferation and of the mechanisms of endothelin action.

MATERIALS AND METHODS

Synthetic endothelin (pig) was from Novabiochem, and other peptides were from Bachem. [*methyl*- ^3H]Thymidine, ^{125}I , *myo*-[2- ^3H]inositol and recombinant

PDGF and FGF were purchased from Amersham International. TGF β was from R&D Systems. EGF and ^{125}I -EGF were prepared as described by Brown & Blakeley (1983). Stock cultures of Swiss mouse 3T3 cells were maintained and passaged as described (Brown & Blakeley, 1983). For experimental use, cells were seeded into 35 mm-diam. culture dishes (Nunc) in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn-calf serum, and used after 7 days' growth, by which time the cells were confluent and quiescent.

For measurement of DNA synthesis, cells were incubated in 2 ml of serum-free Dulbecco's medium containing [^3H]thymidine (1 $\mu\text{Ci/ml}$; 1 μM). Mitogens were added at the concentrations indicated in the legends, and the cells were incubated at 37 °C for 40 h. [^3H]Thymidine incorporation was determined after washing and extracting the cells as described by Brown & Blakeley (1983). For measurement of formation of inositol phosphates, cells were pre-labelled for approx. 72 h by adding *myo*-[^3H]inositol (1 $\mu\text{Ci/ml}$) directly to the growth medium 3–4 days after seeding. The extraction and measurement of the total ^3H -labelled inositol phosphate fraction (inositol mono-, bis-, tris- and tetrakis-phosphate) from mitogen-stimulated cells was performed as described previously (Heslop *et al.*, 1986; Blakeley *et al.*, 1989).

RESULTS

Stimulation of DNA synthesis by endothelin

Endothelin had little effect on DNA synthesis when added alone to quiescent cultures of Swiss 3T3 cells in serum-free medium. However, the peptide interacted synergistically to stimulate [^3H]thymidine incorporation in combination with several polypeptide growth factors (EGF, PDGF, FGF and TGF β). In combination with concentrations of these factors which by themselves produced only a small increase in DNA synthesis, endothelin stimulated responses up to 50% of that induced by 10% serum (Table 1). Endothelin also synergized weakly with the peptide mitogen bombesin,

Abbreviations used: EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGF β , transforming growth factor β ; IGF 1, insulin-like growth factor 1; TPA, 12-*O*-tetradecanoylphorbol acetate.

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Table 1. Synergistic stimulation of DNA synthesis in Swiss 3T3 cells by endothelin and other mitogens

Confluent monolayers of cells were incubated in serum-free Dulbecco's modified Eagle's medium containing [3 H]thymidine and the indicated concentrations of mitogens. After 40 h at 37 °C, the cells were washed and extracted, and incorporated radioactivity was measured. The mean values ($\pm$ S.E.M.) for three separate determinations of [3 H]thymidine incorporation are shown. Similar results were obtained in two additional experiments.

Addition	[3 H]Thymidine incorporated (c.p.m./dish)	
	No endothelin	+ endothelin (3 nM)
Expt. 1		
None	3612 $\pm$ 84	6483 $\pm$ 297
Insulin (1 μ g/ml)	8700 $\pm$ 200	19556 $\pm$ 1270
EGF (30 ng/ml)	18156 $\pm$ 263	129918 $\pm$ 6990
TGF β (0.3 ng/ml)	24479 $\pm$ 437	77732 $\pm$ 6323
PDGF (1 ng/ml)	14722 $\pm$ 1590	102188 $\pm$ 3439
Basic FGF (0.03 ng/ml)	8353 $\pm$ 474	99526 $\pm$ 4313
Bombesin (0.3 nM)	10260 $\pm$ 2355	25432 $\pm$ 1310
TPA (2 nM)	10427 $\pm$ 143	83105 $\pm$ 2207
Newborn-calf serum (10 %)	245038 $\pm$ 7883	Not done
Expt. 2		
None	6021 $\pm$ 1012	8088 $\pm$ 135
Vasopressin (0.1 nM)	7333 $\pm$ 169	102563 $\pm$ 858

but more strongly with vasopressin and with the tumour promotor 12-*O*-tetradecanoylphorbol acetate (TPA) (Table 1).

The dependence of [3 H]thymidine incorporation on the concentration of endothelin in the presence or absence of a fixed concentration of EGF is shown in Fig. 1(a). A

half-maximal response was obtained at an endothelin concentration of approx. 0.3 nM, with a maximal stimulation at approx. 3 nM. The pronounced synergy between EGF and endothelin was also seen in the converse experiment, where cells were exposed to increasing concentrations of EGF in the absence or presence of 3 nM-endothelin (Fig. 1b). Marked synergy between endothelin and EGF was also evident when the proportion of labelled nuclei was measured by autoradiography. For example, only $2.5 \pm 0.3\%$ and $1.2 \pm 0.2\%$ of cells respectively were labelled after treatment with EGF (20 ng/ml) or endothelin (3 nM) alone, whereas the proportion rose to $25.5 \pm 0.7\%$ when the two factors were added together. Values for unstimulated and serum-stimulated cultures were $< 1\%$ and $> 95\%$ respectively.

In contrast with the marked synergistic interactions described above, endothelin showed a limited ability to potentiate the mitogenic effect of insulin (Table 1). Similarly, limited synergy was seen when insulin-like growth factor 1 (IGF 1) was tested in the concentration range 1–30 ng/ml (results not shown). This is an interesting result, since, under the same conditions, bombesin and vasopressin synergize very effectively with either insulin or IGF1 (see Rozengurt, 1986).

Endothelin stimulates production of inositol phosphates in Swiss 3T3 cells

Endothelin has been reported to stimulate phosphoinositide hydrolysis in vascular smooth-muscle cells (Resink *et al.*, 1988; Van Renterghem *et al.*, 1988). We have therefore investigated the effect of endothelin on the formation of inositol phosphates in Swiss 3T3 cells. In the presence of LiCl, the peptide stimulated a 2-fold increase in the accumulation of inositol phosphates, with a maximal response at approx. 3 nM-endothelin (Fig. 2). In five separate experiments, the maximal accumulation of inositol phosphates in response to this concentration of endothelin was $209 \pm 13\%$ of that in unstimulated cultures. Thus, although highly reproducible, the endothelin-mediated effect was smaller than that usually seen

Fig. 1. Synergistic stimulation of DNA synthesis by endothelin and EGF

[3 H]Thymidine incorporation was measured after a 40 h incubation in medium containing increasing concentrations of either endothelin (a) or EGF (b), in the presence (●) or absence (○) of 20 ng of EGF/ml (a) or 3 nM-endothelin (b). Mean values ($\pm$ S.E.M.) for three determinations on separate dishes of cells are shown. Error bars not visible lie within the symbol. [3 H]Thymidine incorporation in response to the addition of 10% newborn-calf serum to the cultures was 185×10^3 and 147×10^3 c.p.m./dish in experiments (a) and (b) respectively.

Fig. 2. Stimulation of phosphoinositide hydrolysis in Swiss 3T3 cells by endothelin

Total [³H]inositol phosphates formation was measured after a 30 min incubation with the indicated concentrations of endothelin in the presence of LiCl (10 mM). Mean values ($\pm$ S.E.M.) for three separate determinations are shown. Very similar results were obtained in two additional experiments.

Fig. 3. Inhibition of ¹²⁵I-EGF binding by endothelin

Confluent monolayers of control (○) or protein kinase C-depleted (●) cells were incubated in binding medium containing ¹²⁵I-EGF (0.5 ng/ml) and the indicated concentration of endothelin for 45 min at 37 °C. The cells were washed, dissolved in 0.5 M-NaOH, and cell-associated radioactivity was measured as described by Brown & Blakeley (1983). The values shown are the means for duplicate determinations of the percentage inhibition of ¹²⁵I-EGF binding. The protein kinase C-depleted cells were prepared by adding TPA (300 nM) to the growth medium for 48 h before the experiment. TPA (10 nM) inhibited ¹²⁵I-EGF binding by 82% and 9% in control and pretreated cultures respectively.

in response to vasopressin, and considerably smaller than the response to bombesin, which causes up to 15-fold increases in the accumulation of inositol phosphates (see Blakeley *et al.*, 1989; Brown *et al.*, 1989).

Inhibition of ¹²⁵I-EGF binding by endothelin

In these and other cell types, the binding of ¹²⁵I-EGF to its cellular receptors is inhibited by agonists which activate protein kinase C. The mechanism involves a decrease in receptor affinity after phosphorylation of its cytoplasmic domain by the kinase (see Schlessinger, 1986). Endothelin inhibited ¹²⁵I-EGF binding to Swiss 3T3 cells over a similar dose-response range as was found to stimulate phosphoinositide hydrolysis and DNA synthesis (Fig. 3). In four separate experiments, the maximal inhibition observed varied between 34% and 50%. Down-modulation of protein kinase C by prolonged pretreatment with TPA (see Stabel *et al.*, 1987) decreased, but did not eliminate, the inhibitory effect of endothelin on ¹²⁵I-EGF binding (Fig. 3), suggesting that both protein kinase C-dependent and -independent pathways may be involved. The possibility that multiple pathways regulate EGF-receptor phosphorylation and affinity state has been suggested previously (Davis & Czech, 1987; Corps & Brown, 1988).

DISCUSSION

Although discovered as a potent vasoconstrictor, endothelin has been found to stimulate DNA synthesis in vascular smooth-muscle cells, and it has been speculated that this effect might be related to the development of atherosclerosis (Komuro *et al.*, 1988). Simonson *et al.* (1989) found that endothelin also stimulates DNA synthesis in mesangial cells, a perivascular smooth-muscle-like cell from the kidney glomerulus. However, those authors noted that endothelin did not affect [³H]thymidine incorporation by quiescent Swiss 3T3 cells. Their failure to observe an effect of endothelin on 3T3 cells was probably due to the fact that endothelin was not tested in combination with other growth factors or mitogens. In the present study, we have observed a marked stimulation of [³H]thymidine incorporation in Swiss 3T3 cells by endothelin only in the presence of synergizing co-mitogens. During preparation of this manuscript, Takuwa *et al.* (1989) reported that endothelin induced a small stimulation of DNA synthesis in Swiss 3T3 cells and that this response was potentiated by IGF1. Our results support this finding and extend it by showing a more marked synergy with other growth factors.

There is conflicting evidence on whether endothelin stimulates phosphoinositide hydrolysis in vascular smooth-muscle cells and whether inositol polyphosphates mediate the rise in intracellular Ca²⁺ concentration stimulated by the peptide (Yanagisawa *et al.*, 1988; Resink *et al.*, 1988; Van Renterghem *et al.*, 1988; Hirata *et al.*, 1988). In Swiss 3T3 cells, Takuwa *et al.* (1989) reported that endothelin stimulated the production of inositol phosphates and diacylglycerol, and also enhanced phosphorylation of an 80 kDa acidic protein which is regarded as a marker substrate for activation of protein kinase C. In the present study, we observed a reproducible, but relatively small, stimulation of the accumulation of inositol phosphates in response to endothelin.

Activation of the inositol lipid signalling pathway has

been implicated in the mechanism of action of several mitogens (see Whitman & Cantley, 1988). Although endothelin shares with vasopressin and bombesin the ability to stimulate phosphoinositide hydrolysis in Swiss 3T3 cells, we have observed a number of differences between the mitogenic effects of the peptides. Firstly, vasopressin and bombesin synergize strongly with insulin to stimulate DNA synthesis (see Rozengurt, 1986). In contrast, in the present experiments, the synergy between endothelin and insulin was much weaker than that observed between endothelin and other factors such as EGF or FGF. Secondly, endothelin synergizes strongly with TPA, whereas vasopressin does not synergize with this agent (Dicker & Rozengurt, 1980). Finally, endothelin can synergize with bombesin or vasopressin to stimulate DNA synthesis. Taken together, these results suggest that the signalling pathways activated by these three mitogenic peptides may not be identical.

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