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# Bradykinin blocks the action of EGF, but not PDGF, on fibroblast division

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Quiescent fibroblasts derived from human fetal lung can be stimulated to reinitiate DNA synthesis by sequential addition of 3 nM IGF-1 and a low concentration (8 pM) of EGF or by continuous exposure to 10% fetal calf serum or 10 ng/ml PDGF. Bradykinin blocks the IGF-1 and EGF-dependent signals without affecting the response to serum or PDGF. It activates protein kinase C and its anti-mitogenic effect is abolished after this kinase has been down-regulated. Bradykinin has no effect on the binding affinity of the EGF receptor whereas phorbol ester induces its 'transmodulation' to low affinity.

Protein kinase C; Epidermal growth factor; Bradykinin; DNA synthesis

## 1. INTRODUCTION

Early intracellular events in the triggering of a growth factor-dependent proliferative program have been well documented. They include activation of tyrosine kinase activities intrinsic to some growth factor receptors [1], activation of phosphatidylinositol breakdown [2] and protein kinase C [3] and fluxes of several ions e.g. $H^+$ [4] and $Ca^{2+}$ [5]. Identification of events specific to one pathway but not another has been less successful. In mouse BALB/c-3T3 cells [6,7] it has been shown that prior exposure to PDGF is required before EGF or a combination of EGF and IGF-1 can commit the cells to DNA synthesis and division. In human diploid fibroblasts, on the other hand, it appears that either PDGF or EGF will suffice to initiate cell division [8], indicating that cellular responses to combinations of growth factors may be specific to the cell type and/or its culture history. In the present work we have observed synergy between EGF and IGF-1 in the human system and examined the action of bradykinin in discriminating between IGF-1/EGF-dependent and PDGF-dependent pathways.

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*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor 1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

## 2. MATERIALS AND METHODS

### 2.1. *Growth factors*

IGF-1 (recombinant) was from Amersham International (England) and EGF from Collaborative Research (USA). PDGF was a gift from Dr C.-H. Heldin.

### 2.2. *Cell culture*

Human fetal lung fibroblasts (Flow Laboratories) were cultured in a 1:1 mixture of DMEM and Ham's F12 nutrient mixture, supplemented with 10% fetal calf serum, 50 $U \cdot ml^{-1}$ penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. Exponentially growing cultures (passages 15–20) were starved in 0.1% serum for 36–48 h to obtain subconfluent, quiescent cells.

### 2.3. *Time-lapse cinematography*

A culture flask containing 5000–6000 starved cells $\cdot cm^{-2}$ and 10 ml of medium (0.1% serum) with appropriate additions was placed in an airstream incubator on an inverted microscope

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equipped with a video camera and a time-lapse video recorder. A field of about 50 well-separated cells was chosen and one picture was recorded every minute. No significant cell death was observed during the experiment. Cell division was scored as the completion of the division furrow between a pair of cells. In experiments where protein kinase C was down-regulated, 150 nM TPA was added to the medium during the starvation period. Growth factor regimes were either 10 ng/ml PDGF ± 100 nM bradykinin or 3 nM IGF-1 given for 4 h, washed out and replaced with 8 pM EGF ± 100 nM bradykinin.

2.4. *Thymidine uptake*

Cells were seeded into 24-well plates and starved to quiescence when still subconfluent. 22 h after the initiation of the treatment with growth factors (as above) the medium was replaced with 1 ml of DMEM containing 2 $\mu$Ci [$^3$H]thymidine (56 Ci·mmol$^{-1}$). After a further 12 h, the cells were rinsed and then fixed in methanol/acetic acid (3:1) for 1 h, washed in distilled water and allowed to dry in air. The wells were coated with a 1:1 dilution of Ilford K2 emulsion in water at 45°C. Excess emulsion was aspirated and the plates dried over silica gel at room temperature for 24 h before storage at 4°C for 3–6 days. Development was by standard photographic techniques.

2.5. *EGF binding assays*

$^{125}$I-EGF was prepared by the iodogen (Pierce) method and purified on a Waters $C_{18}$ SepPak cartridge. Fibroblasts were grown in 50 mm dishes and starved as above. 100 nM bradykinin or 150 nM TPA were added for 2 h at 37°C before rinsing the plates with ice-cold binding buffer (Earle's balanced salts solution buffered with 20 mM Hepes and containing 0.1% bovine serum albumin). 16 pM $^{125}$I-EGF and 0–100 nM unlabeled EGF were added in 2 ml buffer and allowed to equilibrate with the cells for 9 h at 4°C. The cells were washed and then dissolved in 1 M NaOH overnight and the radioactivity counted. Non-specific binding (in the presence of 100 nM unlabeled EGF) was about 5% of total binding. Data were analysed using the LIGAND computer program [9].

2.6. *Two-dimensional gel electrophoresis*

Starved (and in some cases, TPA-treated) cultures in 35 mm dishes were incubated for 4 h at 37°C with 1 ml Hepes-buffered phosphate-free DMEM containing 200 $\mu$Ci [$^{32}$P]orthophosphate. 15 min before the end of this period, they were treated with 100 nM bradykinin or 150 nM TPA. The medium was removed and the cells washed rapidly with ice-cold phosphate-buffered saline. They were then dislodged by scraping into 200 $\mu$l ice-cold lysis buffer (20 mM Hepes, 50 mM benzamidine, 10 mM EDTA, 100 mM NaF, 5 mM 2-mercaptoethanol, 10 $\mu$M sodium orthovanadate, 1% Triton X-100, pH 7.4), vortexed vigorously and centrifuged. Sample preparation and electrophoresis were essentially as described [10]. 30 $\mu$g of protein were loaded onto each iso-electric focusing gel (1.6% pH 4–6, 0.4% pH 3–10 LKB-Ampholines). Electrophoresis in the second dimension was on 10% acrylamide gels in the presence of SDS.

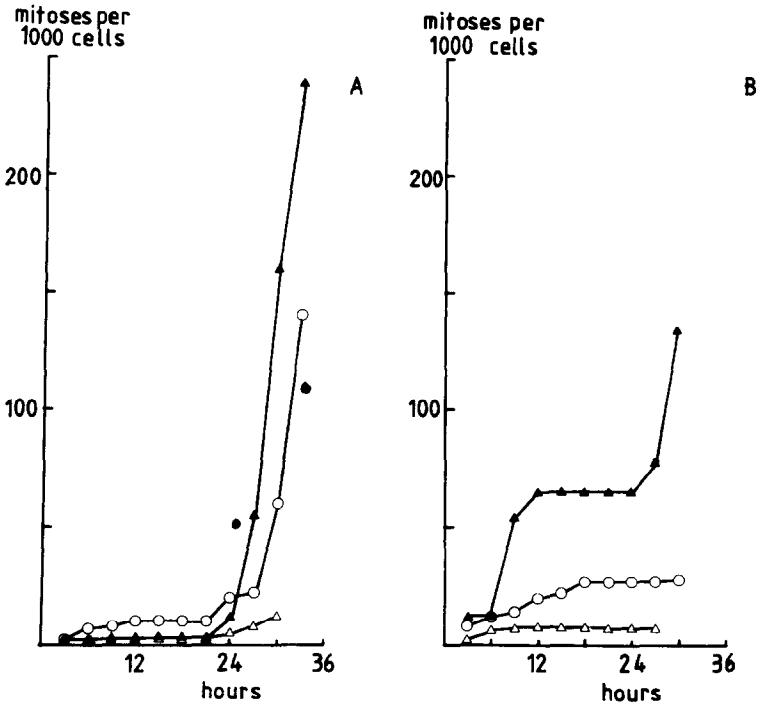


Fig.1. Time-lapse cinematography. Video recording was started at the beginning of the treatment regime. (A) Effects of serum and PDGF: (△—△) 0.1% serum; (▲—▲) 10% serum; (○—○) PDGF; (●) PDGF + bradykinin. (B) Effects of IGF-1, EGF and bradykinin: (○—○) IGF-1; (▲—▲) IGF-1 + EGF; (△—△) IGF-1 + EGF + bradykinin.

## 3. RESULTS AND DISCUSSION

PDGF or 10% serum both induced a mitotic response in quiescent cells after a lag period of about 28 h (fig.1A) and further addition of bradykinin was without effect. The sequential combination of IGF-1 and 8 pM EGF caused a small wave of mitosis after 8 h and a second larger wave after 24–26 h (fig.1B). IGF-1 alone had only marginal effect. Bradykinin abolished both waves of mitosis. The early response to IGF-1/EGF needs further work to assess its provenance but distinguishes this cellular response from that to PDGF or serum.

We confirmed the action of bradykinin on cell division by assaying DNA synthesis. 10% serum caused a 10-fold increase in nuclear labeling by [$^3$H]thymidine (fig.2). The IGF-1/EGF combination, but neither factor given alone, elicited a strong response, which was abolished by bradykinin.

The specific binding of 16 pM $^{125}$I-EGF to bradykinin-treated cells was not significantly different from control (96%) whereas binding to TPA-treated cultures was markedly reduced (60% of control, mean of three experiments). Computer analysis revealed the binding curves to have both high and low affinity phases (dissociation constants of 40–100 pM and 8–12 nM, respectively) for the control and bradykinin-treated cells but that only low affinity sites remained after TPA treatment. We conclude that TPA causes the well-documented transmodulation of the EGF receptor to low affinity [11] but that bradykinin under our conditions does not. This rules out a reduction by bradykinin of the occupancy of the EGF receptor (in the presence of the low concentration of EGF) as the basis of its anti-mitogenic effect.

This effect was found to be dependent on protein kinase C activity. In cells whose kinase levels had been down-regulated by exposure to 150 nM TPA over 48–60 h bradykinin increased the number of cells entering mitosis and shortened the time required to reach maximal mitotic activity (fig.3). Whether this results from a loss of a normal damping action of protein kinase C is unclear. TPA was still mitogenic under these conditions, suggesting the persistence of some protein kinase C activity, but insufficient to mediate any inhibitory response to bradykinin.

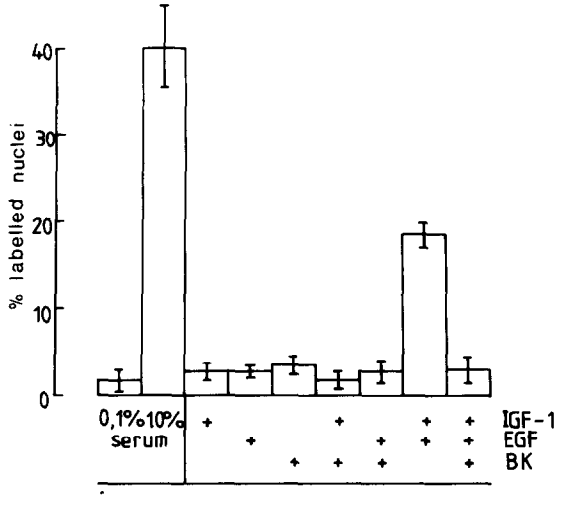


Fig.2. [$^3$H]Thymidine uptake and its response to growth factors. The results represent the means (± SE) of quadruplicate estimates of the percentage of cell nuclei labeled with [$^3$H]thymidine.

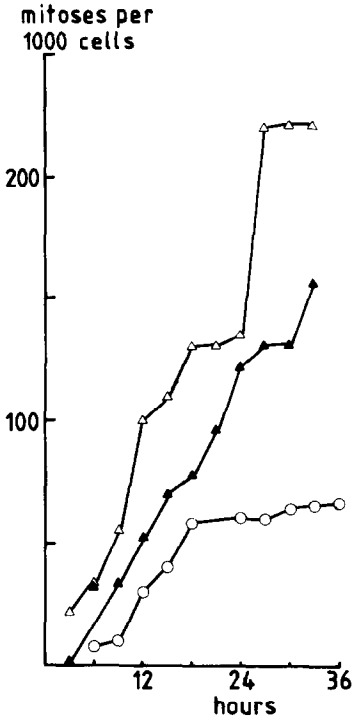


Fig.3. Time-lapse cinematography after down-regulation of protein kinase C. (o—o) TPA; (▲—▲) IGF-1 + EGF; (Δ—Δ) IG-1 + EGF + bradykinin.

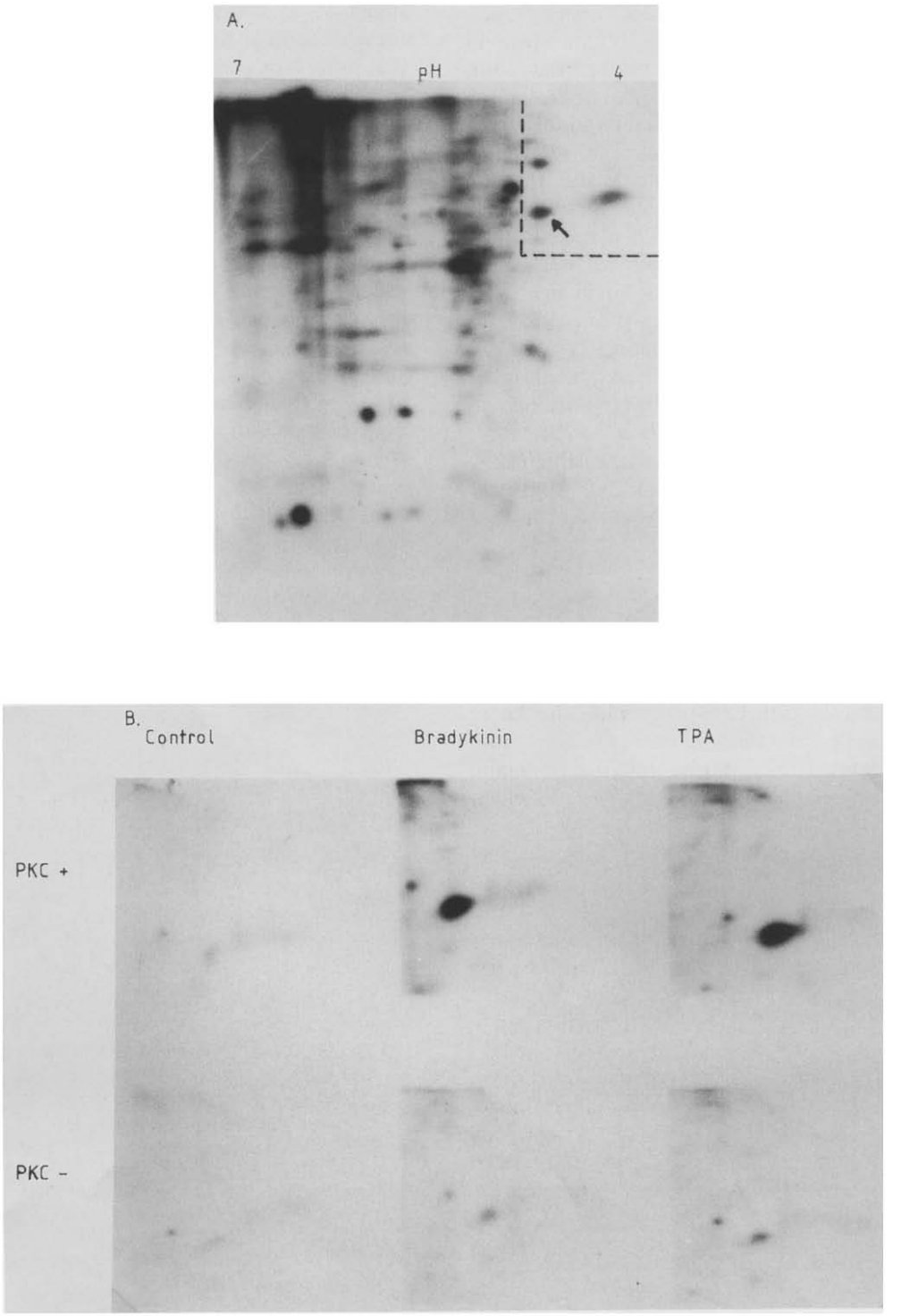


Fig.4. Two-dimensional gel electrophoresis of $^{32}P$-labeled cells. (A) Autoradiogram from a control sample. The position of the 87 kDa spot is indicated by an arrow and the corner cut out for part B outlined. (B) The 87 kDa region of gels from experimental samples.

We assayed the protein kinase C activity more directly by comparing the cellular protein phosphorylation patterns produced by bradykinin or TPA. An acidic phosphoprotein of approximate molecular mass 87 kDa can be readily resolved by two-dimensional electrophoresis and is believed to be a substrate for protein kinase C in vivo [12,13]. In its stimulation of '87K' phosphorylation in control, but not in chronically TPA-treated cells, bradykinin was indistinguishable from TPA (fig.4). It is clear that it must activate at least some subclasses of protein kinase C.

Our results on the IGF-1/EGF synergy are not peculiar to lung fibroblasts and our first observations of this phenomenon were during studies on human embryonic corneal fibroblasts [14]. This particular mitogenic pathway can be distinguished from that employed by serum or PDGF by its different timing pattern and its sensitivity to bradykinin.

The reported effects of activators of protein kinase C on EGF-induced DNA synthesis have been contradictory. In mouse 3T3 cells, TPA and EGF act synergistically [15] but in human fibroblasts TPA antagonizes the action of EGF [16]. Our results are consistent with the latter report.

In the light of recent observations of many subclasses of protein kinase C [17], some of which have been demonstrated to show differing activities toward the EGF receptor [18], we suggest that bradykinin activates species of this enzyme which can phosphorylate the 87 kDa protein and interfere with EGF-, but not PDGF-stimulated mitosis, but cannot cause transmodulation of the EGF receptor. Whether they can phosphorylate the EGF receptor is still an open question since there are a number of candidate substrate sites on that molecule, in addition to the Thr-654 residue which has been implicated in transmodulation [19]. Other possibilities are that these protein kinase species act at other loci specific to the EGF pathway, an avenue we are currently pursuing, or that intracellular release of inositol trisphosphate [2] may be responsible for the constellation of effects reported here.

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