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Involvement of protein kinase C in the mitogenic and chemotaxis effects of basic fibroblast growth factor on bovine cerebral cortex capillary endothelial cells

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Basic fibroblast growth factor is increasingly implicated in cellular growth, differentiation, angiogenesis and oncogenesis. In culture, basic fibroblast growth factor greatly improved the growth rate of bovine brain cortex capillary endothelial cells. Down-regulation of protein kinase C by prolonged treatment with phorbol esters prevented the mitogenic effect of basic fibroblast growth factor on capillary endothelial cells. Furthermore, staurosporine, a potent protein kinase inhibitor, showed strong antiproliferative activity against basic fibroblast growth factor-induced endothelial cell growth. Similarly, the chemotaxis effect of basic fibroblast growth factor on capillary endothelial cells was abolished by down-regulation of protein kinase C or by staurosporine treatment. Therefore, it is suggested that protein kinase C could account for part of the angiogenic effect of basic fibroblast growth factor.

Protein kinase C; Fibroblast growth factor; Capillary endothelial cell; Proliferation; Chemotaxis

1. INTRODUCTION

Angiogenesis is a complex process in which capillary blood vessels grow in an ordered sequence of events [1]. Among the substances that are known to act predominantly on capillary proliferation by direct action on vascular endothelial cells are peptidic heparin binding growth factors of the fibroblast growth factor (FGF) family [2]. Although it has been clearly established that acidic and basic FGF participate in the regulation of angiogenesis, both in vitro [2,3] and vin vivo [4,5], their mode of action has not yet been clearly defined. Recent work using Swiss 3T3 fibroblasts, suggested that basic FGF (bFGF) at mitogenic concentrations activated the calcium/phospholipid-dependent protein kinase, protein kinase C (PKC) [6,7].

PKC is a key regulatory enzyme involved in both signal transduction and cellular proliferation of various cell types (for review see [8]). In a recent paper, we demonstrated that the active tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate which activate PKC, greatly improved the growth rate of bovine brain cortex capillary endothelial cells in culture [9] showing that PKC plays a fundamental role in capillary endothelial cell growth. PKC implication in endothelial cell proliferation was further demonstrated by down-regulation experiments and by inhibitory effect of staurosporine, a highly potent inhibitor of PKC [10].

As a step towards understanding bFGF signal transduction in endothelial cells, we have examined the involvement of PKC in the mitogenic and chemotaxis effects of bFGF on bovine cerebral cortex capillary endothelial cells.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Tissue culture reagents were from Boehringer Mannheim (France). Bovine basic fibroblast growth factor (bFGF) was purchased from Amersham (France). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 4α-phorbol-12,13-didecanoate were purchased form Sigma (France). Staurosporine was from Fluka (Germany). All other chemicals were from Prolabo (France).

2.2. *Cell culture*

Capillary endothelial cells were isolated from bovine cerebral cortex, cloned and cultured as described by Gospodarowicz et al. [11]. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 1 ng/ml bovine pituitary FGF (added every other day) until cultures were confluent. Endothelial cell cultures were passaged weekly at a split ratio of 1:4. Capillary endothelial cells were identified as vascular endothelial cells by the presence of factor VIII-related antigen, as previously described [12].

2.3. *Proliferation assays*

Cell proliferation assays were performed in fibronectin-coated 24-well cluster plates (Nunc, Denmark). Cultures to be grown in Dulbecco's modified Eagle medium with 2% foetal calf serum were

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seeded (20 × 10^3 cells/well) and maintained for the length of the experiment without change of the media. 6 h after plating of the cells, different concentrations of the tested compounds were added. Phorbol esters and staurosporine were solubilized in dimethylsulfoxide. bFGF was added every other day. After a 5-day period, triplicate plates were trypsinized and the cell number was determined using a Coulter counter.

2.4. *Chemotaxis assays*

Chemotaxis assays were carried out using a 48-microwell apparatus (Neuroprobe, Cabin John, MD) as described [13]. Test solutions in Dulbecco's modified Eagle medium + 0.2% bovine serum albumin or control media were loaded into each bottom well. A nucleopore membrane with 8 μm pores from Neuroprobe (Cabin John, MD) was appied to the plate. The top plate with upper wells was attached and the apparatus was equilibrated at 37°C for 15 min. The cells used in the assay were detached from the culture flask by trypsin treatment (0.05% trypsin, 0.02% EDTA). After centrifugation (400 × g, 10 min) the cells were resuspended in Dulbecco's modified Eagle medium + 10% foetal calf serum (final concentration: 10^6 cells/ml). Cell suspensions were loaded into each top chamber of the microwell plate (50 × 10^3 cells/well) and the apparatus was incubated at 37°C with 5% CO_2 for 5 h. After disassembling, the non-migrated layer of cells was removed from the membrane using a rubber wiper blade (Neuroprobe). The migrated cells were fixed to the membrane and stained using a M + D Diff quick-staining set (Dade, France). The membrane was rinsed with H_2O and the cells which had migrated were counted under a microscope (250 × magnification). Five fields were counted for each well and 4 replicate wells were run for each experimental protocol. The number of cells per field was converted to cells per mm^2 (0.8 mm^2/field) and averaged. Results were expressed as a percentage of migrating cells per total number of cells originally placed in each well.

3. RESULTS AND DISCUSSION

3.1. *Involvement of PKC in the mitogenic effect of basic fibroblast growth factor on bovine cerebral cortex capillary endothelial cells*

When tested at concentrations ranging from 0.1 to 5 ng/ml, bFGF greatly improved the proliferation of endothelial cells (fig.1). The concentration of bFGF required to obtain optimal cell proliferation was observed to be 1 ng/ml. This result is in general agreement with other studies performed on vascular endothelial cells from different origins [11,14]. To investigate the exact role of PKC in bFGF-induced capillary endothelial cell growth, cells were exposed to 200 nM TPA for 72 h. It is now well known that prolonged treatment with phorbol esters leads to down-regulation of intracellular PKC [9]. Fig.1 shows that this preincubation strongly affects the mitogenicity of bFGF. 4α-phorbol-12,13-dideca-noate, known to be inactive for PKC, is without effect of bFGF mitogenicity on endothelial cells. In a recent paper, we have shown that staurosporine, a microbial alkaloid and actually the most potent inhibitor of protein kinase [10], inhibits TPA-induced bovine cerebral cortex capillary endothelial cell proliferation [9]. Therefore, to further demonstrate the fundamental role of PKC in bFGF-induced endothelial cell growth, the effect of staurosporine was investigated. Incubation of endothelial cells with increasing concentrations of staurosporine in the presence of either bFGF (1 ng/ml)

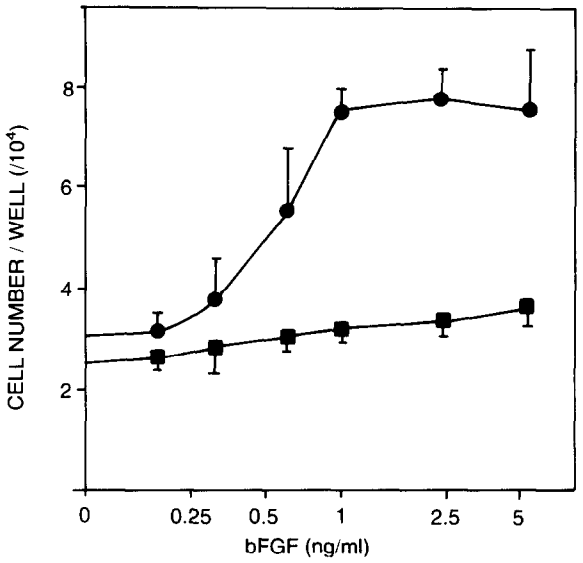

Fig.1. Effect of bFGF on the growth of capillary endothelial cells, effect of TPA preincubation. Confluent endothelial cells in 75 cm^2 culture flasks were incubated for 72 h in the absence (●) or presence (■) of 200 nM TPA. After 3 washes in Dulbecco's modified Eagle medium, cells were trypsinized, seeded into 24-well cluster plates (20 × 10^3 cells/well) and grown in Dulbecco's modified Eagle medium suppemented with 2% foetal calf serum and increasing concentrations of bFGF. After 5 days in culture, triplicate wells were trypsinized and cells were counted. Data are expressed as mean cell density ± SEM.

or TPA (10 nM) results in strong inhibition of cellular growth (fig.2). Half-maximal inhibitory doses (IC_{50}) were respectively 0.7 and 1.3 nM indicating in both cases that the antiproliferative activity of staurosporine against bFGF or TPA mitogenic activity occurs in a range of concentrations which corresponds to its known inhibitory action of PKC [10].

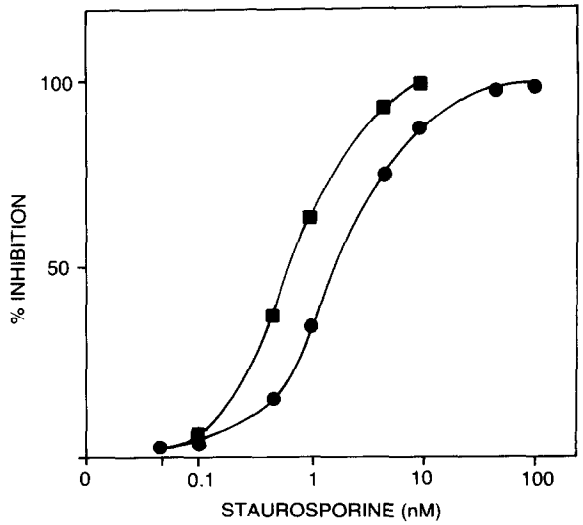

Fig.2. Effect of staurosporine on bovine cerebral cortex capillary endothelial cell growth. Cells were seeded at 20 × 10^3 cells/well and incubated with increasing concentrations of staurosporine in Dulbecco's modified Eagle medium supplemented with 2% foetal calf serum and bFGF (1 ng/ml) (■) or TPA (10 nM) (●). Cell numbers were determined after 5 days in culture. Data are expressed as the mean of 3 experiments.

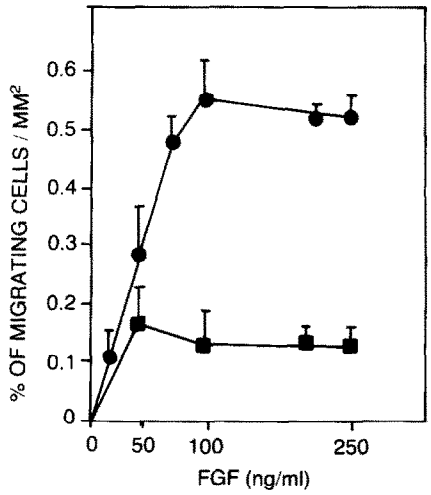

Fig.3. Effect of TPA preincubation on the chemotactic effect of bFGF on bovine cerebral cortex capillary endothelial cells. Increasing concentrations of bFGF were analysed for their ability to promote endothelial cell migration in the Boyden chamber (see section 2 for experimental details). Cells were preincubated for 48 h in the absence (●) or presence (■) of TPA (200 nM). Results are expressed as percent of migrating cells per total number of cells added in each well (50×10^3 cells/well). Data are reported as mean ± SEM cell number for 3 replicate chemotaxis assays.

3.2. *PKC involvement in the chemotactic response of bovine cerebral cortex capillary endothelial cells to basic fibroblast growth factor*

When capillary endothelial cells were placed in the upper compartment of a modified Boyden chamber, we observed that increased migration occurred with as little bFGF as 20 ng/ml in the lower compartment (fig.3). The optimal concentration of bFGF for maximum chemotaxis was 100 ng/ml. In this model, bFGF was 5–10-fold more potent than other well known chemotactic agents like prostaglandin-E_1 or fibrinogen (not shown). Furthermore, capillary endothelial cells proved to be more sensitive to bFGF than bovine aortic endothelial cells or human umbilical vein endothelial cells [14]. To determine the true contribution of PKC in the chemotactic effect of bFGF, endothelial cells were exposed to 200 nM TPA for 48 h and tested for their ability to migrate in response to bFGF. Under these conditions, bFGF showed slight migratory activity but maximal migration was 5-fold lower than in controls, indicating that loss of intracellular PKC leads to strong inhibition of the chemotactic effect of bFGF. Similarly, staurosporine inhibited the migration of endothelial cells in a concentration-dependent manner (IC_{50} = 8 nM) (fig.4).

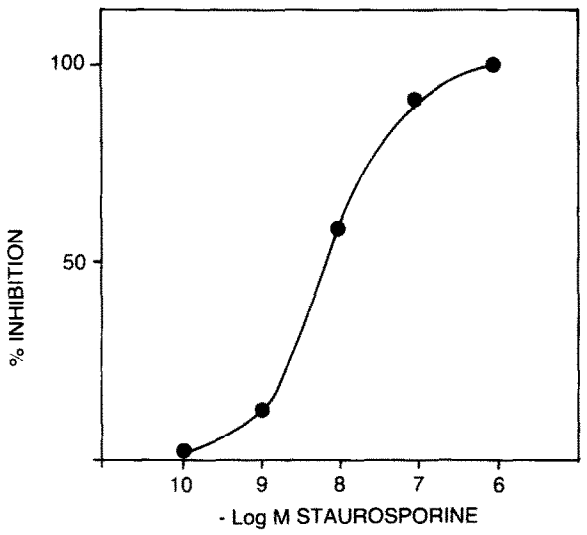

Fig.4. Effect of staurosporine on the chemotaxis response of bovine brain cortex capillary endothelial cells to bFGF. Chemotaxis was assayed in the modified Boyden chamber (see section 2 for details). Increasing concentrations of staurosporine were incubated with 250 ng/ml bFGF. Results are reported as mean of 3 replicate chemotaxis assays.

Therfore, although the precise role played by bFGF in angiogenesis is not easily predicted by in vitro experiments, it is likely that its effect on capillary growth is mediated in part through activation of PKC. This is further supported by previous work showing that application of phorbol esters to microvessels mimics some of the events involved in angiogenesis in vitro and in vivo [15,16]. Hence, newly developed inhibitors of this enzyme could prove to be potent antitumoral agents.

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