The Mitogenic Signaling Pathway but Not the Plasminogen Activator-inducing Pathway of Basic Fibroblast Growth Factor Is Mediated through Protein Kinase C in Fetal Bovine Aortic Endothelial Cells

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Abstract. Basic fibroblast growth factor (bFGF) induces cell proliferation and plasminogen activator (PA) activity in transformed fetal bovine aortic endothelial (FBAE) GM 7373 cells. A similar response is observed after treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In these cells, bFGF and TPA cause activation of protein kinase C (PKC), as demonstrated by the induction of the phosphorylation of an 87-kD PKC substrate in intact cells and by the increase in membrane-associated PKC activity. Activation of PKC by bFGF or TPA is inhibited in cells made PKC-deficient by pretreatment with high concentrations of TPA. The mitogenic activity of bFGF or of TPA is completely inhibited in PKC-deficient cells or in naive cells treated with the PKC inhibitor H-7. However, these cells proliferate in response to serum, epidermal growth factor, and dibutyryl cyclic AMP. Similar results are obtained in normal FBAE AG 7680 cells. These data indicate that activation of PKC is responsible for the mitogenic activity of bFGF in FBAE cells. On the contrary, the PA-inducing activity of bFGF is unaffected by down-regulation of PKC or by treatment with the PKC inhibitor H-7 in both transformed GM 7373 and normal AG 7680 cells. bFGF induces a rapid ^{45}Ca influx in naive and in PKC-deprived GM 7373 cells. In these cells, addition of EGTA to the incubation medium prevents both the ^{45}Ca influx and the increase in PA activity induced by bFGF, without affecting its mitogenic activity. Even though the involvement of PKC in the increase of cell-associated PA activity induced by bFGF can not be completely dismissed, the present results suggest a role of calcium entry in the modulation of the PA-inducing activity of bFGF.

PROTEIN kinase C (PKC)[1] is a calcium and phospholipid-dependent enzyme that is activated by diacylglycerol (Nishizuka, 1984). Several lines of evidence indicate that PKC is involved in mediating the intracellular signaling that triggers various biological processes, including those elicited by certain polypeptide growth factors (Nishizuka, 1986).

Basic fibroblast growth factor (bFGF) is an 18-kD mitogen characterized by its affinity for heparin. In vitro, it induces cell proliferation and a characteristic set of responses, including protease production, depending upon the cell type under investigation. In vivo, bFGF has been shown to induce neovascularization (for a recent review on bFGF see Gospodarowicz et al., 1987). The role exerted by PKC in the transduction signaling mediating bFGF activity is still unclear. bFGF has been shown to activate PKC in Swiss 3T3 and in 3T3-L1 fibroblasts (Blackshear et al., 1985; Tsuda et al., 1985; Kaibuchi et al., 1986). Down-regulation of PKC has been shown to decrease but not to abolish, the ability of bFGF to stimulate cell proliferation (Huang et al., 1988) and to increase ornithine decarboxylase, c-*fos*, c-*myc*, and beta-actin mRNA levels in 3T3 cells (Hovis et al., 1986; Kaibuchi et al., 1986; Stumpo and Blackshear, 1986). On the other hand, PKC activation was not detectable after treatment of Chinese hamster lung CCL39 fibroblasts with bFGF (Magnaldo et al., 1986). Moreover, bFGF has been shown to promote phosphorylation of proteins of 22 and 31 kD and to activate a ribosomal S6 protein kinase in 3T3-L1 fibroblasts made PKC-deficient by long-term treatment with phorbol ester (Blackshear et al., 1985; Pelech et al., 1986).

In cultured bovine capillary endothelial cells, bFGF induces plasminogen activator (PA) and collagenase production, motility, and cell proliferation (Moscatelli et al., 1986; Presta et al., 1986). Preliminary experiments performed in our laboratory had shown that transformed fetal bovine aortic endothelial (FBAE) GM 7373 cells respond to a 24-h treatment with bFGF with a 10–20-fold increase in PA activity and with a 70–80% increase in cell number (see below). The high and rapid response of GM 7373 cells to bFGF prompted

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; db-cAMP, dibutyryl cyclic AMP; FBAE, fetal bovine aortic endothelial; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; PA, plasminogen activator; PKC, protein kinase C.

us to use these cells to evaluate whether bFGF activates PKC and to assess the role of PKC in the transmodulation signaling responsible for the mitogenic activity and for the PA-inducing activity of bFGF.

The results indicate that bFGF induces proliferation of GM 7373 cells via PKC activation. Induction of PA activity by bFGF is instead independent of PKC and it appears to be triggered by calcium uptake from the extracellular environment. Similar results were obtained in an isolate of normal FBAE cells, thus confirming the positive role exerted by PKC in the regulation of cell proliferation in FBAE cells.

Materials and Methods

Cell Cultures

GM 7373 and AG 7680 cells were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). GM 7373 cells represent an immortalized cell line which was derived from FBAE cells by in vitro transformation with benzo(a)pyrene and correspond to the BFA-lc 1BPT multilayered transformed clone described by Grinspan et al. (1983). AG 7680 cells are an isolate of normal FBAE cells. GM 7373 cells were grown in MEM containing 10% FCS, vitamins, and essential and nonessential amino acids. AG 7680 cells were grown in the same conditions except that FCS concentration was 20%.

Cell Proliferation Assay

The assay was performed on subconfluent cells. To this purpose, GM 7373 or AG 7680 cells were plated in 24-well dishes at 70,000 or 50,000 cells/cm^2, respectively. After 24 h, cultures were washed twice with serum-free medium and incubated in fresh medium containing 0.4% FCS and different concentrations of human recombinant bFGF (obtained from Synergen Inc., Boulder, CO) or of other molecules. 24 h later, cells were washed twice with PBS, trypsinized, and counted by using a Burker chamber. Cultures incubated in fresh medium containing 10% FCS were used as positive controls. For both normal and transformed cells, this treatment resulted in an 80–100% increase of the cell number in respect to cells incubated in 0.4% FCS with no addition.

Plasminogen Activator Assay

GM 7373 or AG 7680 cells were seeded in 96-well dishes at the same density used for the cell proliferation assay. After 24 h, cells were washed twice with serum-free medium and incubated in fresh medium containing 0.4% FCS and different concentrations of bFGF or of other molecules. 24 h later, cell-associated PA activity was measured. To this purpose, cells were washed twice with PBS and were extracted with 100 μl of 0.05% Triton X-100 in 60 mM Tris-HCl, pH 8.5 (T/T buffer). Aliquots of the cell extracts corresponding to 1 μg of protein were incubated in a microtiter plate with 4.2 ng of purified glu-plasminogen (Kabi AB, Stockholm, Sweden) and 42 nmol of the plasmin chromogenic substrate *H*-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide-acetate (American Diagnostica, Greenwich, CT) in 150 μl of T/T buffer. After incubation at 37°C, the plate was read at 405 nm with an automatic microplate reader. Human urokinase (Calbiochem-Behring Corp., La Jolla, CA) was used as a standard.

Down-Regulation of PKC

Long-term treatment with high concentrations of phorbol ester results in down-regulation of PKC in several cell types (Rodriguez-Pena and Rozengurt, 1984; Rozengurt, 1986; Wolfman et al., 1987). Accordingly, subconfluent cultures of GM 7373 or AG 7680 cells were incubated for 16 h in fresh medium containing 0.4% FCS and 3 μg/ml 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). This treatment results in down-regulation of PKC (see Results). In all the experiments, control cultures, herewith referred to as naive cultures, were incubated in the same medium with 0.4% FCS and solvent control. In untreated PKC-deprived cells, cell-associated PA activity was identical to that detectable in untreated naive cultures. Routinely, to evaluate the response of PKC-deprived cells to mitogenic and PA-inducing stimuli, naive and PKC-deficient cells were further incubated for 16 h in fresh medium containing 0.4% FCS and the molecule tested. Then, cell proliferation and cell-associated PA activity were determined as described above. It must be pointed out that no difference in the response of PKC-deprived cells to the mitogens tested was observed when the molecules were added directly to the medium containing TPA, i.e., without medium change, or when the mitogens were administered in fresh medium in the presence or in the absence of 3 μg/ml TPA (results not shown).

PKC Assay

Subconfluent GM 7373 cells were incubated for 16 h in fresh medium containing 0.4% FCS. Then, cells were washed with serum-free medium and incubated for 5 min at 37°C in serum-free medium containing solvent control, 30 ng/ml bFGF, or 10 ng/ml TPA. Then, incubation was terminated by washing the cells twice with ice cold PBS and scraped with a rubber policeman in homogenization buffer which contained 10 mM Hepes buffer (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM EDTA, 0.24 M sucrose, 75 μg/ml PMSF, 110 mU/ml aprotinin, 50 μg/ml leupeptin, and 4 μg/ml pepstatin. Harvested cells were sonicated on ice with six 10-s bursts each preceded by 10-s pause. Homogenates were centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant and the particulate fraction were the cytosolic and membrane fractions, respectively. Membranes were resuspended in homogenization buffer containing 1% NP-40, held at 4°C for 1 h, and centrifuged at 13,000 *g* for 15 min at 4°C to separate detergent-solubilized material. PKC assay on 500-μg aliquots of solubilized membrane fractions was performed as described (Thomas et al., 1987), using 250 μg/ml histone III-S (Sigma Chemical Co., St. Louis, MO) as exogenous substrate.

Labeling of Cells with $^{32}P_i$

Subconfluent GM 7373 cells were incubated for 16 h in fresh medium containing 0.4% FCS. Then, cultures were washed twice with DME without phosphate and incubated for 1 h in the same medium. The medium was removed and the cells were incubated for 3 h at 37°C with DME phosphate-free containing 50 μCi/ml carrier-free $^{32}P_i$ to label endogenous ATP pool. Then, 100 ng/ml TPA, 100 ng/ml bFGF, or solvent control were added for various times ranging from 15 s to 10 min. The reaction was stopped by removing the medium and rapidly washing the cultures twice with ice-cold 0.15 M NaCl in 20 mM Tris-HCl, pH 7.5. The cells were immediately extracted with 5% TCA at 4°C for 30 min. The acid-soluble materials were removed; the precipitated proteins were washed twice and dissolved with 150 μl of SDS-PAGE reducing sample buffer heated at 100°C. The samples were then transferred to tubes and placed in boiling water bath for 5 min before resolution by SDS-8% PAGE. Then, gels were exposed to Kodak X-Omat AR film for 1–12 h. 87-kD phosphorylation was quantified by soft-laser scanning of the autoradiography of the gel. Similar results were obtained when the 87-kD protein was excised from the gel and counted for radioactivity in a scintillation counter.

^{45}Ca Uptake in GM 7373 Cells

Subconfluent cells in 35-mm dishes were incubated for 16 h in fresh medium containing 0.4% FCS with solvent control or 3 μg/ml TPA. Then, cells were washed with serum-free medium and added with the same medium containing 2 μCi/ml ^{45}Ca (14.5 mCi/mg calcium) and different concentrations of bFGF. After various times, the cells were washed rapidly five times with ice-cold PBS and extracted with 5% TCA at 4°C for 30 min. TCA-soluble material was then collected and counted for radioactivity in a liquid scintillation counter. Radioactivity measured in the TCA-soluble material of cells incubated with ^{45}Ca for 5 s at 4°C represented nonspecifically bound Ca^{2+} and was subtracted from all the values.

Results

Induction of Plasminogen Activator Activity and Cell Proliferation in FBAE Cells

bFGF has been shown to induce cell proliferation and PA activity in bovine capillary endothelial cells (Moscatelli et al., 1986; Presta et al., 1986). On this basis, preliminary experiments were performed on GM 7373 cells to characterize their response to bFGF. When subconfluent cultures of GM 7373 cells were incubated for 24 h with different concentra-

tions of bFGF in the presence of 0.4% FCS, an increase of cell number and cell-associated PA activity was observed (Fig. 1). The PA activity induced by bFGF has been identified as of the urokinase-type PA by SDS-PAGE zymography (results not shown). It is interesting to note that the ED_{50} for the two responses is remarkably different, corresponding to 0.3 ng/ml and 3.0 ng/ml bFGF for the mitogenic activity and the PA-inducing activity, respectively. These data suggest that the PA-inducing activity of bFGF is not related to its mitogenic activity. Indeed, bFGF still stimulates PA activity in GM 7373 cells whose mitogenic response to bFGF is abolished by co-incubation with mitomycin C or by omission of FCS from the assay (results not shown).

To characterize further the capacity of transformed FBAE cells to respond to different stimuli, we evaluated the capacity of TPA, EGF, and dibutyryl cyclic AMP (db-cAMP) to induce cell proliferation and PA activity in GM 7373 cells. As shown in Fig. 1, all the molecules tested exerted a mitogenic response in these cells, but only TPA induced also an increase in PA activity. It must be pointed out that (*a*) both the mitogenic and the PA-inducing responses exerted by TPA were remarkably lower than those exerted by bFGF and (*b*) the PA-inducing activity of TPA was abolished when the molecule was administered at concentrations >1 μg/ml (results not shown), possibly as a consequence of down-regulation of PKC (see below). For all the molecules tested, similar results were obtained on normal FBAE AG 7680 cells (data not shown). These data indicate that among the mitogens tested only bFGF and TPA are able to induce PA activity in FBAE cells and that, in contrast with previous observations on adult bovine capillary and aortic endothelial cells (Gospodarowicz et al., 1978; Doctrow and Folkman, 1987; Morris et al., 1988), both normal and transformed FBAE cells respond to the mitogenic activity of EGF and TPA.

bFGF Induces PKC Activation in FBAE GM7373 Cells

An 87-kD protein has been shown to be a major specific and widespread substrate for PKC (Blackshear et al., 1986; Albert et al., 1987). PKC-dependent phosphorylation of this protein can be observed in experiments performed both on intact cells and on the cytosolic fraction of a variety of cell types (Rodriguez-Pena and Rozengurt, 1986; Blackshear et al., 1986; Mahadevan et al., 1987; Smith and Colburn, 1988), including endothelial cells (Mackie et al., 1986). Preliminary experiments were performed to assess whether this protein was present also in GM 7373 cells. Indeed, when a cytosolic fraction of GM 7373 cells was autophosphorylated in the presence of the PKC activator TPA, the phosphorylation of a band with an apparent molecular mass of 87 kD was observed (data not shown). Moreover, the TPA-dependent phosphorylation of this protein was drastically reduced in the cytosolic fraction of cells preincubated for 16 h with a high concentration (3 μg/ml) of TPA (Maier, J. A. M., unpublished observations), a treatment which causes down-regulation of PKC in several cell types, including endothelial cells (Rodriguez-Pena and Rozengurt, 1984; Rozengurt, 1986; Wolfman et al., 1987; Uratsuji and DiCorleto, 1988). These data therefore indicate that the 87-kD protein represents a major PKC substrate also in GM 7373 cells.

On this basis, to evaluate the capacity of bFGF to induce PKC activation in GM 7373 cells, we assessed the effect of bFGF on the phosphorylation of the 87-kD protein in intact cells. bFGF treatment induces an increase in the phosphorylation of several proteins, including an 87-kD protein (Fig. 2 *A*). The pattern of phosphorylation induced by bFGF is identical to that induced by TPA and it is abolished in PKC-deficient cells. Moreover, the kinetic of phosphorylation of the 87-kD protein induced by bFGF is very similar to that induced by TPA, both molecules causing a maximal induction of the 87-kD phosphorylation within 1 min from the beginning of the stimulus (Fig. 2 *B*). A similar kinetic of induction was observed also for the other proteins shown in Fig. 2 *A* whose phosphorylation is stimulated both by bFGF and TPA (data not shown). It is interesting to note that bFGF induces a higher increase of 87-kD phosphorylation (fourfold increase) in respect to TPA (twofold increase). This difference was observed both when the 87-kD phosphorylation was quantified by soft-laser scanning of the autoradiography of the gel (Fig. 2 *B*) or by counting the radioactivity of the band excised from the gel (data not shown). In conclusion, these data suggest that bFGF induces activation of PKC in GM 7373 cells.

Activation of PKC is paralleled by its translocation from the cytosol to the cell membrane (Nishizuka, 1986; Thomas et al., 1987). On this basis, to confirm the capacity of bFGF to activate PKC in FBAE cells, we have measured PKC activity in the solubilized membrane fraction of bFGF or TPA-treated cells. As shown in Fig. 3, both TPA and bFGF induce an increase in membrane PKC activity. As observed for 87-kD phosphorylation, the increase is more pronounced in bFGF-treated cells than in cells stimulated with TPA. No membrane-associated PKC activity was detectable in PKC-deficient cells stimulated with additional TPA or bFGF (Fig. 3). Thus, our data indicate that bFGF induces activation of PKC in transformed FBAE cells.

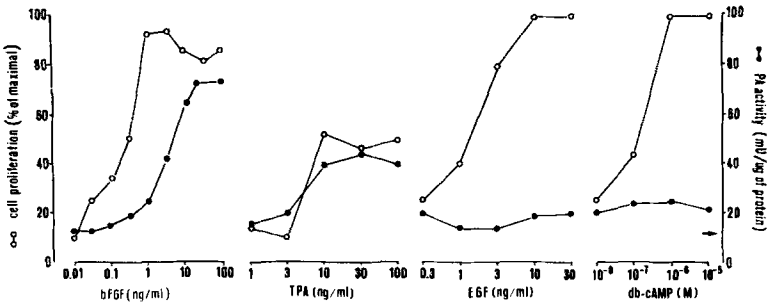

Figure 1. Effect of different mitogens on cell proliferation and cell-associated PA activity in GM 7373 cells. Cells were seeded at 80,000 cells/cm^2. After 24 h, cultures were washed twice with serum-free medium and incubated in fresh medium containing 0.4% FCS and different concentrations of the mitogens tested. Cell number (○) and cell-associated PA activity (●) were evaluated after 24 h of incubation. The arrow indicates the PA activity in control cultures. Cell proliferation is expressed as percent of the proliferation observed in cells grown in 10% FCS.

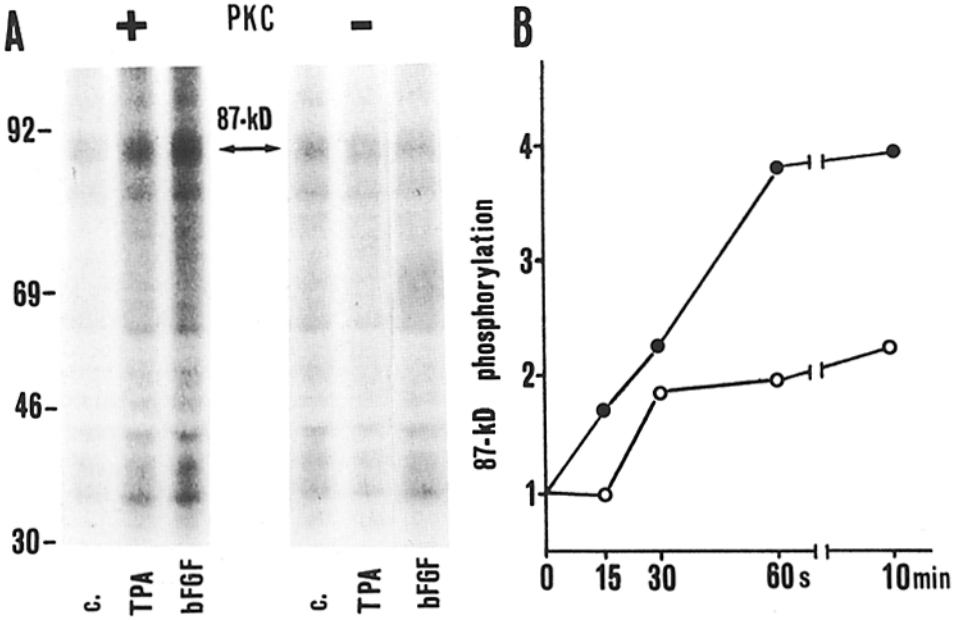

Figure 2. 87-kD phosphorylation in intact GM 7373 cells. (*A*) Naive cultures (+ PKC) and cultures made PKC-deficient by 16 h incubation with 3 μg/ml TPA (− PKC) were incubated for 3 h with 50 μCi/ml $^{32}P_i$. Then, cells were treated for 5 min with solvent control (*c.*), 100 ng/ml TPA, or 100 ng/ml bFGF. Cells were extracted and all the TCA-precipitable material of each sample was run on SDS 8% polyacrylamide gel. Similar results were obtained in three independent experiments. (*B*) Naive cultures were incubated for 3 h with 50 μCi/ml $^{32}P_i$. Then, cells were treated for the times indicated with 100 ng/ml TPA (○) or with 100 ng/ml bFGF (●). Cells were extracted and all the TCA-precipitable material of each sample was run as in *A*. 87-kD phosphorylation was quantified by soft-laser scanning of the autoradiography of the gel. Values are shown as fold increase in respect to cultures treated with solvent control. Each point represents a single determination. Similar results were obtained in three independent experiments.

The Mitogenic Activity of bFGF Depends on PKC Activity

Long-term treatment with high concentrations of TPA results in down-regulation of PKC in several cell types, including endothelial cells (Rodriguez-Pena and Rozengurt, 1984; Rozengurt, 1986; Wolfman et al., 1987; Uratsuji and DiCorleto, 1988). Accordingly, we have observed that 16-h treatment with 3 μg/ml TPA down-regulates PKC in GM 7373 cells (see above). Therefore, to assess the role of PKC in mediating the mitogenic and the PA-inducing activity of bFGF in transformed FBAE cells, we have measured the effect of bFGF on cell proliferation and PA activity in PKC-deficient cells. As shown in Fig. 4, down-regulation of PKC completely abolishes the mitogenic activity of bFGF but it does not affect its PA-inducing activity. As expected, instead, down-regulation of PKC completely abolishes both the increase in PA activity and the increase in cell number induced by TPA. However, PKC-deficient cells fully respond to the proliferative stimulus exerted by serum, EGF, or db-cAMP, thus indicating that the lack of mitogenic response of PKC-deficient cells to bFGF and TPA is specific for these molecules. These data therefore suggest that the mitogenic activity of bFGF, but not its PA-inducing activity, is mediated by PKC.

1-(5-isoquinolinesulfonyl)-2-methylpeperazine dihydrochloride (H-7) is an inhibitor of PKC and it has been shown to inhibit several PKC-dependent responses when administered to cultured cells (Kawamoto and Hidaka, 1984; Inagaki et al., 1985; Hidaka and Hagiwara, 1987). Moreover, H-7 inhibits the PKC-dependent 87-kD phosphorylation induced by TPA in the cytosolic fraction of GM 7373 cells (Maier, J. A. M., unpublished observations). On this basis, to confirm the role of PKC in mediating the mitogenic activity of bFGF, we have measured cell proliferation of GM 7373 cells

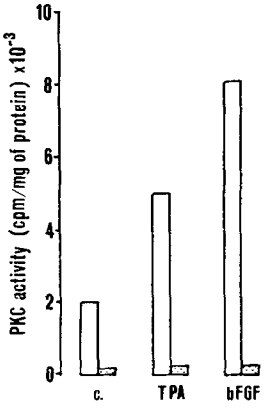

Figure 3. PKC activity in GM 7373 cell membranes. Naive cultures (□) and PKC-deficient cultures (▨) were incubated for 5 min with solvent control, 10 ng/ml TPA, or 30 ng/ml bFGF. Then, detergent-solubilized membrane fractions were prepared and assayed for PKC activity as described in Materials and Methods. Each value is the mean of two determinations in triplicate.

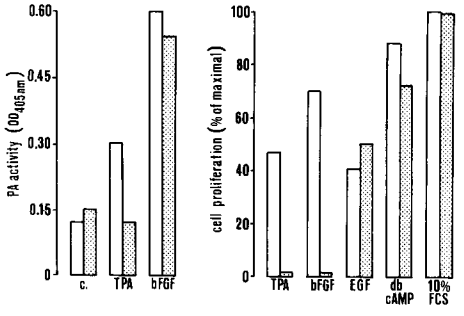

Figure 4. Effect of PKC deprivation on cell proliferation and PA activity in GM 7373 cells. Naive cultures (□) and PKC-deficient cultures (▨) were incubated in fresh medium containing 10% FCS or 0.4% FCS added with solvent control (*c.*), 10 ng/ml TPA, 30 ng/ml bFGF, 30 ng/ml EGF, or 10^{-5} M db-cAMP. After 16 h, cell number and cell-associated PA activity were determined. Cell proliferation is expressed as percent of the proliferation observed in naive cells grown in 10% FCS.

in the presence of H-7 and bFGF. H-7 completely inhibits the mitogenic activity of bFGF, as well as that exerted by TPA, but not that exerted by serum (Fig. 5 *A*). The inhibition of the mitogenic activity of bFGF by H-7 was dose-dependent (Fig. 5 *B*) with an ID_{50} equal to 6 ± 2 μM (n = 5), identical to the K_i value of this compound for PKC (Hidaka et al., 1984). However, as observed in PKC-deficient cells, cells treated with H-7 still respond to bFGF in terms of PA activity (Fig. 5 *B*), further suggesting that the PA-inducing activity of bFGF in GM 7373 cells is independent from PKC.

bFGF and PKC Activation in Normal FBAE AG 7680 Cells

In contrast with our findings on transformed FBAE GM 7373 cells, PKC activation exerted by phorbol ester has been shown to inhibit or to unaffect cell proliferation in adult bovine capillary or aortic endothelial cells (Doctrow and Folkman, 1987; Morris et al., 1988). To rule out the possibility that the positive modulation of cell proliferation exerted by PKC in GM 7373 cells depends upon their transformed status, we evaluated the role of PKC in the transduction of bFGF activity in normal FBAE AG 7680 cells. AG 7680 cells respond to TPA and bFGF with an increase in cell number and cell-associated PA activity (see above). AG 7680 cells preincubated for 16 h with 3 μg/ml TPA are still able to respond to bFGF with an increase in PA activity, but not with an increase in cell proliferation. TPA-pretreatment, instead, completely abolishes the mitogenic and the PA-inducing activity of additional TPA also in AG 7680 cells, without affecting the mitogenic activity of EGF and FCS (data not shown). Thus, these data indicate that PKC activation positively modulates the mitogenic but not the PA-inducing activity of bFGF also in normal FBAE cells and suggest that PKC might exert a different modulation of cell proliferation in fetal in respect to adult bovine aortic endothelial cells.

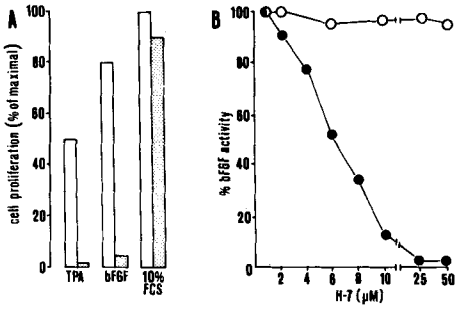

Figure 5. Effect of the PKC inhibitor H-7 mitogenic and PA-inducing activity of bFGF. (*A*) GM 7373 cells were incubated in fresh medium containing 10% FCS or 0.4% FCS added with 30 ng/ml bFGF or 10 ng/ml TPA (□). Half of the cultures received also 10 μM H-7 (▨). After 24 h, cell number was determined. Cell proliferation is expressed as percent of the proliferation observed in cells grown in 10% FCS with no addition. (*B*) GM 7373 cells were incubated in fresh medium containing 0.4% FCS and 30 ng/ml bFGF in the presence of increasing concentrations of H-7. After 24 h, cell number (●) and cell-associated PA activity (○) were determined. Values are expressed as percent of the activity exerted by 30 ng/ml bFGF in the absence of H-7. No modification of cell number and cell-associated PA activity was induced by H-7 in control untreated cultures.

bFGF Induces Ca^{2+} Influx in FBAE Cells

In fibroblasts and in epithelial lens cells, bFGF has been shown to cause an increase in the cytoplasmic free Ca^{2+} that is strictly dependent upon the presence of calcium in the external medium (Magnaldo et al., 1986; Moenner et al., 1987). Therefore, we investigated whether bFGF was able to elicit Ca^{2+} influx also in GM 7373 cells. To this purpose, we measured the uptake of ^{45}Ca in GM 7373 cells treated with bFGF. The ionophore A23187 was used as a positive control. As shown in Fig. 6 *A*, 100 ng/ml bFGF induces a rapid increase of Ca^{2+} uptake, comparable to that exerted by A23187. The increase of Ca^{2+} uptake was dose dependent, with a half-maximal stimulation at 3 ng/ml bFGF (Fig. 6 *B*). Similar results were obtained also in PKC-deprived cells (data not shown). The uptake was completely abolished by 5-min preincubation with 1 mM EGTA (results not shown). These data therefore indicate that bFGF is able to induce a Ca^{2+} influx in naive and PKC-deprived GM 7373 cells. Ca^{2+} influx was observed also in normal FBAE AG 7680 cells treated with 100 ng/ml bFGF (results not shown).

The PA-inducing Activity of bFGF Depends on Ca^{2+} Influx

PKC-deficient and H-7–treated FBAE cells still respond to bFGF with an increase of PA activity (see above), suggesting that the PA-inducing activity of bFGF does not depend on PKC. Since calcium is an important intracellular signal (Rasmussen, 1986) and since bFGF induces Ca^{2+} influx with a dose-response similar to that observed for the induction of PA activity, we investigated the role of external calcium in mediating the PA-inducing activity of bFGF. As a preliminary experiment, we assessed whether a Ca^{2+} influx was sufficient to elicit an increase of PA activity in GM 7373 cells. Indeed, 30 ng/ml of the calcium ionophore A23187 induces a threefold increase in PA activity. The effect is dose-dependent with a half-maximal stimulation at 10 ng/ml. No significant effect is instead exerted by A23187 on GM 7373 cell proliferation (results not shown). These data therefore

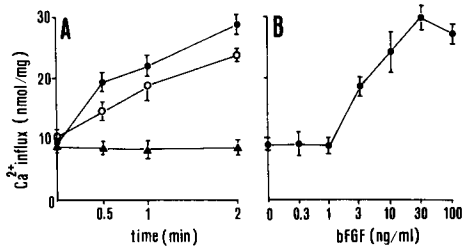

Figure 6. Ca^{2+} influx in GM 7373 cells. (*A*) 35-mm dish cultures were incubated in fresh medium containing 0.4% FCS for 16 h. Then, cells were incubated in serum-free medium containing 2 μCi/ml ^{45}Ca and no additions (▲), 100 ng//ml bFGF (●), or 200 ng/ml A23187 (○). At the times indicated, cells were washed and intracellular ^{45}Ca was counted by liquid scintillation spectrometry as described in Materials and Methods. Values are the mean ± SEM of three determinations. (*B*) GM 7373 cells were incubated for 2 min in serum-free medium containing 2 μCi/ml ^{45}Ca and different amounts of bFGF. Intracellular ^{45}Ca was evaluated as in *A*. Values are the mean ± SEM of three determinations. Similar results were obtained in three independent experiments.

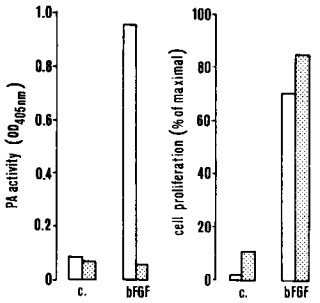

Figure 7. Effect of EGTA on mitogenic and PA-inducing activity of bFGF in GM 7373 cells. Subconfluent cultures were incubated in fresh medium with 0.4% FCS containing no additions (*c.*) or 100 ng/ml bFGF, in the absence (□) or in the presence (▨) of 1 mM EGTA. After 24 h, cell number and cell-associated PA activity were determined. Cell proliferation is expressed as percent of the proliferation observed in cells grown in 10% FCS with no additions.

suggest that calcium uptake might be an event sufficient to stimulate PA activity in GM 7373 cells.

To evaluate whether calcium uptake was responsible for the PA-inducing activity of bFGF, GM 7373 cells were treated with bFGF in the presence of 1 mM EGTA. As shown in Fig. 7, PA-inducing activity of bFGF was completely inhibited in these experimental conditions. GM 7373 cells, however, still responded to bFGF with an increase in cell number comparable to that observed in cells maintained in the regular medium, indicating that removal of free-calcium from the medium is not harmful for these cells in our experimental conditions. Inhibition of the PA-inducing activity of bFGF was observed also in PKC-deprived cells treated with bFGF in the presence of 1 mM EGTA or in naive cells stimulated with bFGF in a calcium-free medium (data not shown).

Discussion

Here we have shown that bFGF induces activation of PKC in transformed FBAE cells. This has been demonstrated by the capacity of bFGF to increase the phosphorylation of a major specific 87-kD PKC substrate in intact GM 7373 cells and to increase membrane-bound calcium, phospholipid-dependent PKC activity. Similar results were obtained in parallel experiments where GM 7373 cells were treated with the phorbol ester TPA. Interestingly, the increase of 87-kD phosphorylation and of membrane-bound PKC activity induced by bFGF is higher than that induced by TPA. It is tempting to relate these findings with the higher mitogenic potency of bFGF in respect to TPA in FBAE cells. PKC is known to exist in different forms derived from distinct genes. These forms differ in terms of enzymological characteristics, tissue expression, and intracellular localization (Nishizuka, 1988). Recently, the presence of different forms of PKC in endothelial cells has been proposed (Uratsuji and DiCorleto, 1988). It is then possible that in FBAE cells bFGF and TPA may activate different PKCs capable of eliciting a different mitogenic response. Further experiments are necessary to assess this hypothesis.

The capacity of bFGF to induce PKC activation, evaluated as 87-kD phosphorylation, has been already reported for Swiss 3T3 and 3T3-L1 fibroblasts (Tsuda et al., 1985; Blackshear et al., 1985; Kaibuchi et al., 1986). On the contrary, PKC activation, evaluated as down-regulated of EGF receptors, was not detectable in CCL39 hamster fibroblasts treated with bFGF (Magnaldo et al., 1986). It is therefore possible to hypothesize that the demonstration of the capacity of bFGF to activate PKC might depend on the cell type under investigation and/or on the assay used.

TPA induces cell proliferation in naive but not in PKC-deprived GM 7373 cells, thus indicating a positive role of PKC activation in the regulation of cell proliferation in these cells. Accordingly, activation of PKC appears to be responsible for the mitogenic activity of bFGF. Indeed, PKC-deprived cells do not respond to bFGF in terms of cell proliferation. Moreover, the mitogenic activity of bFGF is inhibited when the cells are incubated in the presence of the PKC inhibitor H-7. The ID_{50} of H-7 was 6 μM, equal to the K_i value of this compound for PKC (Hidaka et al., 1984). Thus, the possibility that the inhibitory effect exerted by H-7 on the mitogenic activity of bFGF might depend upon an inhibition of protein kinase(s) different from PKC (Hidaka et al., 1984) appears to be unlikely. PKC-deprived or H-7-treated cells were still able to respond to different mitogens, e.g., EGF, db-cAMP, or serum, indicating that the lack of mitogenic response to bFGF and TPA was indeed due to the down-regulation or inhibition of PKC activity and not to an impairment of the capacity of these cells to proliferate. Similar results were obtained with normal FBAE AG 7680 cells, thus indicating that these findings are not due to the transformed status of GM 7373 cells.

The positive role of PKC activation in the regulation of cell proliferation of FBAE cells is in contrast with previous reports on the lack of effect or on the inhibitory effect of phorbol ester on proliferation in adult bovine endothelial cells (Doctrow and Folkman, 1987; Morris et al., 1988). Here, we have shown that both normal and transformed FBAE cells respond to TPA administration with an increase in cell number and that this increase is prevented in PKC down-regulated cells. In parallel experiments we have confirmed the inhibitory effect of TPA on proliferation of adult bovine aortic endothelial cells (data not shown). It is therefore possible to hypothesize that the control exerted by PKC activation on endothelial cell proliferation might change during ontogenetic development. This appears to be of particular relevance in experiments aimed to characterize the role exerted by PKC in endothelial cells during angiogenesis, especially when in vivo assays on chicken embryo chorioallantoic membrane are used. Recently, an apparent discrepancy between the capacity of phorbol esters to induce angiogenesis in the chorioallantoic membrane assay and the lack of mitogenic activity of these molecules in cultured adult bovine capillary endothelial cells has been reported (Morris et al., 1988). On the basis of our results, the different degree of ontogenetic development between the endothelial cells of the two biological assays might account for this discrepancy.

bFGF can still cause an increase of PA activity in GM 7373 cells treated with the PKC inhibitor H-7 or in cells rendered PKC-deficient by preincubation with high concentrations of TPA. Several criteria indicate that PKC was effectively down-regulated in TPA-pretreated cells. In these cells: (*a*) no PKC-dependent phosphorylation of the 87-kD substrate was observed in the cytosolic fraction or in intact cells treated with bFGF or additional TPA; (*b*) no increase in membrane-associated PKC activity was observed after treatment with bFGF or additional TPA; (*c*) no stimulation of cell proliferation was elicited by bFGF or additional TPA, as well as by

the active synthetic diacylglycerol 1,2-dioctanoyl-sn-glycerol (data not shown). In agreement with these observations in transformed GM 7373 cells, bFGF retains its PA-inducing activity in normal AG 7680 cells pretreated with high concentrations of TPA or incubated with the growth factor in the presence of H-7 (data not shown). Thus, even though the existence of down-regulation-resistant PKC(s) has been proposed (Kariya and Takai, 1987), our results strongly suggest that the PA-inducing activity of bFGF, at variance with its mitogenic activity, is triggered by an H-7 resistant, PKC-independent pathway in FBAE cells.

Several lines of evidence indicate that extracellular calcium is involved in the increase of PA activity induced by bFGF in FBAE cells: (*a*) the calcium ionophore A23187 induces an increase of PA activity; (*b*) bFGF induces a ^{45}Ca influx similar to that exerted by A23187 in naive and PKC-deficient cells; (*c*) the concentration of bFGF necessary to elicit a half-maximal increase of Ca^{2+} uptake is identical to that necessary to induce a half-maximal increase of PA activity; (*d*) 1 mM EGTA completely inhibits the ^{45}Ca influx and the increase of PA activity induced by bFGF in naive and PKC-deprived cells. However, it must be pointed out that the increase of PA activity which follows A23187 administration is remarkably lower than that observed in bFGF-treated cells, suggesting that Ca^{2+} influx might be only part of the intracellular mechanism triggering the PA-inducing activity of bFGF. Similar conclusions have been drawn for the ornithine decarboxylase-inducing activity of bFGF in PKC-deprived fibroblasts (Hovis et al., 1986).

In conclusion, our results indicate that bFGF induces at least two distinct intracellular signaling pathways in FBAE cells: a PKC-dependent pathway responsible of its mitogenic activity and a calcium-dependent, PKC-independent pathway responsible for its PA-inducing activity.

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