Protein kinase C (PKC)- δ / ϵ mediate the PKC/Akt-dependent phosphorylation of extracellular signal-regulated kinases 1 and 2 in MCF-7 cells stimulated by bradykinin

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Abstract

In this paper the signal transduction pathways evoked by bradykinin (BK) in MCF-7 breast cancer cells were investigated. BK activation of the B₂ receptor provoked: (a) the phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2); (b) the translocation from the cytosol to the membrane of the conventional protein kinase C- α (PKC- α) and novel PKC- δ and PKC- ϵ ; (c) the phosphorylation of protein kinase B (PKB/Akt); (d) the proliferation of MCF-7 cells. The BK-induced ERK1/2 phosphorylation was completely blocked by PD98059 (an inhibitor of the mitogen-activated protein kinase kinase (MAPKK or MEK)) and by LY294002 (an inhibitor of phosphoinositide 3-kinase

(PI3K)), and was reduced by GF109203X (an inhibitor of both novel and conventional PKCs); Gö6976, a conventional PKCs inhibitor, did not have any effect. The BK-induced phosphorylation of PKB/Akt was blocked by LY294002 but not by PD98059. Furthermore, LY294002 inhibited the BK-provoked translocation of PKC- δ and PKC- ϵ suggesting that PI3K may be upstream to PKCs. Finally, the proliferative effects of BK were blocked by PD98059, GF109203X and LY294002. These observations demonstrate that BK acts as a proliferative agent in MCF-7 cells activating intracellular pathways involving novel PKC- δ / ϵ , PKB/Akt and ERK1/2.

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Introduction

Bradykinin (BK) belongs to the kallikrein–kinin system and two G-protein-coupled receptors (GPCRs) have been recognised, namely, B₁ and B₂ receptors (Regoli & Barabè 1980, Vavrek & Stewart 1985, Ma *et al.* 1994, el-Dahr *et al.* 1997, Pesquero & Bader 1998). In various cell types the B₂ receptor, as other GPCRs, mediates most of the biological actions of BK via the activation of G_{q/11} protein, the increase of free intracellular calcium concentration ([Ca²⁺]_i) and the activation of various protein kinase C (PKC) isoforms (Enomoto *et al.* 1995, Ankorina-Stark *et al.* 1997, Wiernas *et al.* 1998). It has also been clearly shown that BK treatment of different cell types leads to the activation of the mitogen-activated protein kinase (MAPK) cascade (Jaffa *et al.* 1997, Graness *et al.* 1998, Naraba *et al.* 1998). There are several modes of coupling of the B₂ receptor to the MAPK extracellular signal-regulated kinases 1 and 2 (ERK1/2). For example, in endothelial cells the activation is mediated by Ca²⁺-dependent or -independent, but PKC ϵ -dependent, pathways (Flemming *et al.* 1995, Traub *et al.* 1997). PKC- ϵ is also involved in ERK1/2 activation in fibroblasts, in rat myocytes and in the colon carcinoma cell line SW-480

(Clark & Murray 1995, Clerk *et al.* 1996, Graness *et al.* 1998). In PC-12 pheochromocytoma cells, a Ca²⁺-dependent epidermal growth factor receptor (EGFR) transactivation has been reported to be involved in BK-mediated ERK1/2 activation (Hall 1992); conversely, in A431 epidermoid cells, BK transactivates EGFR and, independently of EGFR, BK activates ERK1/2 through both phosphoinositide 3-kinase (PI3K) and PKC (Graness *et al.* 2000). BK is released by a kallikrein–kinin system, which is also present locally in breast tissue (Hermann *et al.* 1995), where the released kinin could participate in tumourigenesis (Clements & Mukhtar 1977) and angiogenesis (Plendl *et al.* 2000) by increasing vascular blood flow and creating new capillary vessels. We have previously shown that in normal breast cells in primary culture the activation of PKC- δ through the B₂ receptor acts in concert with ERK1/2 and PI3K pathways to induce cell proliferation (Greco *et al.* 2004). Moreover, BK may have a role in breast cancer endorsement and progression since its mitogenic effects are also retained in primary cultured breast tumour cells, due to the operation of novel PKCs and ERK1/2 (Greco *et al.* 2005).

The expression of B₂ receptors is also evident in two breast cancer cell lines, EFM-192A and MCF-7

(Frey *et al.* 1999, Drube & Liebmann 2000). In EFM-192A cells, BK activates ERK1/2 through a PI3K/PKC pathway (Drube & Liebmann 2000). In MCF-7 cells (Frey *et al.* 1999), it is known that BK increases the $[Ca^{2+}]_i$, but no further data about its downstream signalling mechanisms are available. Thus, in this paper we aimed to investigate the intracellular mechanisms activated by BK in MCF-7 cells, paying attention to those pathways previously highlighted in primary normal and cancerous breast cells (i.e. PKCs, PI3K/Akt and ERK1/2 signalling) (Greco *et al.* 2004, 2005); furthermore, the possibility that BK is mitogenic in MCF-7 breast cancer cells was also explored.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotics, glutamine and foetal bovine serum (FBS) were purchased from Celbio (Pero, Milan, Italy). PKCs and ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (DBA, Segrate, Italy) and monoclonal anti-PKB/Akt antibodies from Cell Signalling Technology (Celbio). Gö6976, GF109203X, PKC inhibitors and U73343 were purchased from Calbiochem (Milan, Italy). All other reagents were from Sigma.

Cell culture

Cells from the MCF-7 cell line, derived originally from human breast cancer pleural effusion were propagated in 75 cm² flasks in DMEM containing 10% FBS, 2 mM glutamine and penicillin/streptomycin (100 U/100 mg per ml). Cells were grown at 37 °C in a humidified atmosphere of 95% air:5% CO₂ and were used from passages 8–12. For the experiments, at 75% confluence, the medium was replaced with DMEM without serum and cells were cultured for 48 h.

Immunoblot analysis

Cells in flasks were incubated with agonist and/or inhibitors in DMEM without FBS for the required periods at 37 °C. The stimulation was stopped by transferring the flasks on ice. The cells were extracted with lysis buffer: 50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.25 M sucrose, 10 µg/ml aprotinin and 10 µg/ml leupeptin, and sonicated on ice (3 × 10³ cycles). The mixture was centrifuged for 10 min at 800 g and supernatant was saved and centrifuged at 100 000 g for 1 h; supernatant was taken as the cytosol fraction. The pellet was resuspended in lysis buffer plus 1% TritonX-100 and centrifuged as before; the supernatant was collected as

the membrane fraction. Cellular lysates were used to study the expression of phospho-ERK1/2 or phospho-Akt; cytosols and membrane fractions were collected in order to detect PKC isozymes activation. We evaluated the Na⁺/K⁺-ATPase activity using a coupled enzyme assay method (Norby 1988) to determine the purity of the cell compartment fractions used for PKC immunoblotting. The enrichment factors (enzyme activities of final purified membrane pellet and cytosol compared with those of the initial homogenate) were 54.3 ± 6.2 and not determined (ND) respectively (data not shown). An equal amount of protein was solubilised in sample buffer by boiling for 5 min and subjected to 10% SDS-PAGE followed by electrophoretic transfer on to a polyvinylidene fluoride (PVDF) membrane (Amersham). We used the rabbit antibodies against PKC isozymes and the monoclonal mouse antiserum anti-phosphorylated ERK1/2. Antibody anti-PKC- α was diluted 1:5000, while the other anti-PKC antibodies were diluted 1:1000; the anti-phospho-ERK1/2 and the anti-phospho-Akt antibodies were diluted 1:200 and 1:500 respectively. Membranes were incubated with the appropriate primary antibody and then with peroxidase-conjugated secondary antibodies diluted 1:10 000. As a control, the blots used for active ERK1/2 and PKB/Akt detection were then stripped and re-probed with other antibodies (Promega) which recognise both active and basal forms of the ERK and PKB/Akt enzymes.

Proteins were detected using enhanced chemiluminescence (ECL; Amersham). The intensity of the bands was quantified by scanning densitometry using the NIH Image 1.62 software (NIH, Bethesda, MD, USA).

Proliferation assay by cell count

MCF-7 cells were seeded at 2.5×10^4 cells/well on 24-well plates in DMEM growth medium with 10% FBS and incubated overnight at 37 °C in a humidified environment containing 5% CO₂ to allow the adherence. The medium was changed to FBS-free growth medium for 48 h to induce quiescence. Agonists and inhibitors were diluted in FBS-free growth medium, and cells were counted in a Burkert cell-chamber (Sigma) 24 h after treatment.

Statistics

Experimental points represent the mean $\pm$ S.D. of three replicates measured on three cell cultures. Statistical analysis was carried out using Student's *t*-test for unpaired samples and ANOVA with Bonferroni–Dunn's test. Significance levels were chosen as $P < 0.05$ for Student's *t*-test and $P < 0.005$ for Bonferroni–Dunn's test.

Results

BK provokes the phosphorylation of ERK1/2

Serum-starved MCF-7 cells were incubated for various periods (1, 5, 10, 20, 30, 60 and 180 min) with 0.1 µM BK;

Figure 1 Activation of ERK1/2 by BK. MCF-7 cells were treated with 0.1 μM BK for increasing incubation times (A) or with increasing concentrations of BK for 5 min (B). Cell lysates were subjected to SDS-PAGE and probed with anti-phospho-ERK1/2, and then stripped and re-probed with anti-ERK1/2. Representative autoradiographs are shown and results are expressed as percentage ratio over cells at time 0 min (A) or cells incubated in medium only (B) (control). Different letters indicate statistical differences found using Bonferroni–Dunn’s test.

using the anti-phospho-ERK antibody, two bands of relative molecular mass (M_r) 42 000 and 44 000 were detected in cell lysates, corresponding to Tyr²⁰⁴-phosphorylated ERK1 and 2 respectively. Phosphorylation was maximal at 5.0 min and returned to unstimulated levels at 60 min (ANOVA, $P < 0.0001$) (Fig. 1A). Thus, in order to study the dose-dependent phosphorylation of ERK1/2, cells were stimulated for 5 min using increasing concentrations of BK (0.001, 0.01, 0.1, 1.0 μM), and Fig. 1B shows that the maximum effect was obtained with 0.1 μM BK (ANOVA, $P < 0.0001$).

PD98059, a specific MEK inhibitor (Alessi *et al.* 1995), completely inhibited ERK1/2 phosphorylation at 10 μM (ANOVA, $P < 0.0005$) (data not shown).

The B₂ receptor is responsible for BK-mediated ERK1/2 activation

The receptor involved in BK effects was characterised using Hyp3-BK and Lys-BK, B₂ and B₁ receptor inhibitors respectively. We stimulated cells with and without 0.1 μM BK for 5.0 min after a 45 min treatment with increasing concentrations of B₂ (0.01, 0.1, 1.0 and 10 μM) or B₁ (1.0, 10 and 100 μM) inhibitors. Hyp3-BK reduced the BK-mediated ERK1/2 phosphorylation dose dependently (ANOVA, $P < 0.0001$) (Fig. 2A), while Lys-BK did not have any effect (ANOVA, $P > 0.005$) (Fig. 2B), demonstrating that B₂ was responsible for the BK effects.

Role of phospholipase C-β (PLC-β) in the effects of BK on MCF-7 cells

Frey *et al.* (1999) have demonstrated that BK increases $[Ca^{2+}]_i$, suggesting the involvement of the phospholipid pathway. To confirm the involvement of PLC-β in the ERK1/2 phosphorylation, cells were incubated for 45 min with 0.1, 1.0 and 10 μM U73122, a PLC-β inhibitor, and then stimulated with 0.1 μM BK for 5.0 min. As shown in Fig. 3, U73122 completely inhibited the phosphorylation of ERK1/2 induced by BK (ANOVA, $P < 0.0001$). The addition of 10 μM U73343 (an inactive analogue of U73122), used as negative control, for 45 min before 0.1 μM BK stimulation did not reduced ERK1/2 phosphorylation (Fig. 3).

BK-mediated PKC isozyme cytosol-to-membrane translocations

The PLC activity triggers the translocation from cytosol to membrane and thus the activation of PKC isozymes, either conventional (PKC-α, -β), or novel (PKC-δ, -ε, -η and -θ), and such activation may be a mitogenic signal for breast cells (Lafon *et al.* 1995, Greco *et al.* 2003, 2004, 2005).

Incubation of cells with 0.1 μM BK provoked the translocation of PKC-α, -δ and -ε only, with maximal activation occurring at 5 min (Fig. 4A); all the other isoforms expressed by the MCF-7 cells and reported previously (Muscella *et al.* 2003) did not show any cytosol-to-membrane translocation (Fig. 4A). The role of PKC isozymes in ERK1/2 activation was studied by incubating cells for 45 min with or without 0.1 and 10 μM Gδ6976 (a PKC-α inhibitor), or 0.1 and 1.0 μM GF109203X (an inhibitor of both conventional and novel PKCs), before treatment with 0.1 μM BK for 5 min. GF109203X completely inhibited the ERK1/2 phosphorylation induced by BK, while Gδ6976 did not,

Figure 2 ERK1/2 activation by BK is B_2 dependent. MCF-7 cells were treated for 45 min with increasing concentrations of Hyp3-BK (B_2 inhibitor) (A) or Lys (des-Arg-Leu)-BK (B_1 inhibitor) (B) before adding 0.1 μ M BK. Cell lysates were subjected to SDS-PAGE, probed with anti-phospho-ERK1/2, and then stripped and re-probed with anti-ERK1/2; results are expressed as the percentage ratio over cells incubated in medium only (C, control). Different letters indicate statistical differences found using Bonferroni–Dunn’s test.

indicating a role for the novel PKC- δ / ϵ isozymes only (Student’s *t*-test, $P < 0.005$; compared with control, cells incubated with BK only) (Fig. 4B).

BK provokes the phosphorylation of PKB/Akt

A time-course study was conducted to determine the incubation times required for BK to stimulate phosphorylation of PKB/Akt. MCF-7 cells were stimulated

Figure 3 PLC is involved in BK-dependent ERK1/2 activation. MCF-7 cells were treated for 45 min with increasing concentrations of U73122 or 10 μ M U73343, before stimulation with 0.1 μ M BK for 5 min. Cell lysates were subjected to SDS-PAGE, probed with anti-phospho-ERK1/2, and then stripped and re-probed with anti-ERK1/2; results are expressed as the percentage ratio over cells incubated in medium only (C, control). Different letters indicate statistical differences found using Bonferroni–Dunn’s test.

with 0.1 μ M BK, the concentration able to maximally activate ERK1/2, for periods of 5–220 min. The resolved proteins were immunoblotted with monoclonal anti-phospho-Akt antibody which recognises PKB/Akt phosphorylated at Ser⁴⁷³. As shown in Fig. 5A, treatment of MCF-7 cells with BK stimulated PKB/Akt phosphorylation maximally at 60 min and then declined gradually (ANOVA, $P < 0.0001$) to basal level. Cells were then stimulated for 60 min using increasing concentrations of BK (0.01, 0.1 and 1.0 μ M) and Fig. 5B shows that 0.1 μ M BK was the concentration able to give the highest activation of the enzyme (ANOVA, $P < 0.0001$).

MCF-7 cells were also pre-treated for 45 min with 10 and 30 μ M PD98059 and/or 0.1 μ M BK for 60 min; as shown in Fig. 5C, PD98059 did not have any effect on the BK-provoked PKB/Akt phosphorylation (Student’s *t*-test, $P > 0.05$; compared with control, cells incubated with BK only).

Effect of LY294002 on ERK1/2 and PKB/Akt phosphorylation induced by BK

We further examined the effect of the PI3K inhibitor LY294002 on BK-induced PKB/Akt activation. LY294002 is a specific inhibitor of PI3K at low micromolar concentrations, but has no inhibitory effect on a number of tyrosine kinases at 50 mM concentration (Vlahos *et al.* 1994).

Pre-treatment of MCF-7 cells with 5 and 15 μ M LY294002 attenuated the BK-stimulated phosphorylation

Figure 4 Involvement of PKC isozymes in BK-dependent mitogenic effects. (A) Representative autoradiographs of PKC isozyme translocations from cytosol (C) to membrane (M) provoked by 0.1 μM BK at the indicated periods. (B) MCF-7 cells were treated with and without 0.1 and 10 μM Gö6976 or 0.1 and 1.0 μM GF109203X (GF) for 45 min and then stimulated for 5 min with 0.1 μM BK. Cell lysates were subjected to SDS-PAGE, probed with anti-phospho-ERK1/2 and representative autoradiographs of ERK1/2 phosphorylation are shown. Result are expressed as the percentage ratio over cells incubated in medium only (C, control). Asterisks indicate statistical significance ($P < 0.005$) for Student's *t*-test compared with control, i.e. cells treated with BK only. * $P < 0.01$; ** $P < 0.001$.

of PKB/Akt in a concentration-dependent manner (Student's *t*-test, $P < 0.05$; as compared with the cells stimulated by agonist alone), showing a complete inhibition at 15 μM LY294002 (Fig. 6A). Moreover, LY294002 concentration-dependently inhibited the phosphorylation of ERK1/2 stimulated by BK (Student's *t*-test, $P < 0.05$; as compared with the cells stimulated by agonist alone) (Fig. 6B).

Effect of PKCs inhibition on PKB/Akt phosphorylation induced by BK

Cells were pre-treated with 10 μM Gö6976 or 1.0 μM GF109203X for 45 min and then stimulated or not with

0.1 μM BK for 60 min; as shown in Fig. 7A, only GF109203X inhibited the BK-dependent PKB/Akt phosphorylation, with Gö6976 showing no effects (Student's *t*-test, $P < 0.005$; as compared with the cells stimulated by BK alone) (Fig. 7A).

We also investigated the effect of PI3K inhibition on PKC-δ and -ε cytosol-to-membrane translocation obtained in cells stimulated with 0.1 μM BK for 5 min. The pre-treatment of MCF-7 cells for 45 min with 15 μM LY294002 completely inhibited the BK-induced translocation of both PKCs (Fig. 7B).

Proliferative effects of BK in the MCF-7 cell line

Increasing concentrations of BK (0.001, 0.01, 0.1, 1.0 μM) were used to stimulate serum-starved MCF-7 cells for 24 h and cell proliferation was quantified by cell count. BK increased cell proliferation in a dose-dependent manner with maximum effect at 0.1 μM (ANOVA, $P < 0.0001$) (Fig. 8A).

The MEK inhibitor PD98059 (0.01, 0.1, 1.0, 10 and 20 μM) was administered for 45 min before stimulating the cells with 0.1 μM BK for 24 h. PD98059 provoked a significant dose-dependent decrease in the BK-induced proliferation of MCF-7 cells (ANOVA, $P < 0.0005$). A threshold decrease was observed at 0.01 μM PD98059, with a maximum at 10 μM (Fig. 8B).

The role of PKC-δ and -ε isozymes in the BK mitogenic pathway was studied by incubating the cells for 45 min with or without GF109203X (0.01, 1.0 and 10 μM) before treatment with 0.1 μM BK for 24 h. A dose of 1.0 μM GF109203X completely inhibited the proliferation induced by BK, thus indicating a role for the novel PKC isozymes (ANOVA, $P < 0.0005$) (Fig. 8C).

Finally, the role of the PI3K/Akt pathway was investigated using LY294002. Cells were pre-treated with or without LY294002 (5, 15 and 50 μM) for 45 min and then stimulated for 24 h with 0.1 μM BK. A dose of 15 μM LY294002 completely inhibited the proliferation induced by BK (ANOVA, $P < 0.0001$) (Fig. 8D).

Discussion

The proliferation of breast epithelial cells is a key underlying event involved in breast diseases including cancer. The proliferative response of breast epithelial cells may be regulated by autocrine and paracrine stimuli. The effects of BK, to which breast epithelial cells may be exposed for either short or prolonged durations, have been explored using primary cultured normal and cancerous human breast epithelial cells (Greco *et al.* 2004, 2005). It was found that in both normal and cancerous cells BK has consistent mitogenic effects exerted through the ERK1/2 pathway. Such effects were also indicated by others in

Figure 5 Akt phosphorylation stimulated by BK. MCF-7 cells were stimulated with BK (0.1 μ M) for increasing incubation times (A) or with increasing BK concentrations for 60 min (B). The resolved proteins were immunoblotted with anti-phospho-Akt, and after stripping with anti-total Akt. Results are expressed as the percentage ratio over cells incubated at time 0 or in medium only. Different letters indicate statistical differences found using Bonferroni–Dunn’s test. (C) Cells were pre-treated for 45 min with 10 and 30 μ M PD98059 and then stimulated or not with 0.1 μ M BK for 60 min. The resolved proteins were immunoblotted with anti-phospho-Akt.

EFM-192A, a breast cancer cell line (Drube & Liebmann 2000). In another well characterised breast cancer cell model, MCF-7, information regarding the role of BK on cell proliferation has so far not been published; however, previous studies have shown that the MCF-7 cells were

indeed a target of BK, in as much as it evoked a substantial intracellular free Ca^{2+} increase (Frey *et al.* 1999). In this study, we demonstrated that BK induces activation of ERK1/2 via the B_2 receptor. The intracellular routes by which this activation occurred were studied and involved

Figure 6 Role of PI3K on Akt and ERK1/2 phosphorylation induced by BK. MCF-7 cells were pre-treated for 45 min with 5 and 15 μM LY294002 (LY) and then stimulated with 0.1 μM BK for 60 or 5 min, for Akt or ERK1/2 activation respectively. Cells were also treated for 45 min with 5 and 15 μM LY294002 only. The resolved proteins were immunoblotted with anti-phospho-Akt, and after stripping with anti-total Akt (A) or with anti-phospho-ERK1/2 and then stripped and re-probed with anti-ERK1/2 (B). Results are expressed as the percentage ratio over cells incubated in medium only. Asterisks indicate statistical significance for Student's *t*-test compared with the cells stimulated by agonist alone. **P*<0.01; ***P*<0.001.

the operation of novel PKCs and PI3K/Akt pathways, ending in the stimulation of cell proliferation. Drube and Liebmann (2000), have also shown the involvement of the PI3K pathway in the activation of the MAPK cascade, but here we show for the first time in breast cancer cells, that novel PKC isozymes can act as a link between the B₂ receptor and Akt phosphorylation.

Figure 7 Role of the novel PKCs in the PI3K/Akt pathway. (A) Serum-starved cells were pre-treated with 10 μM G6976 or 1.0 μM GF109203X for 45 min and then stimulated or not with 0.1 μM BK for 60 min. Asterisks indicate statistical significance for Student's *t*-test compared with the cells stimulated by agonist alone. (B) LY294002 (15 μM) was also administered for 45 min before 0.1 μM BK for 5 min; the resolved membrane (M) and cytosolic (C) protein fractions were then probed with antiserum anti-PKC-δ or -ε.

We show here that in MCF-7 cells a functional B₂ BK receptor enables ERK1/2 phosphorylation. The expression of BK receptors has been proved in a small cell lung carcinoma cell line H-69, in a breast cancer cell line EFM-192A, in a colon carcinoma cell line SW-480 (Drube & Liebmann 2000) and in the androgen-independent prostate cancer cells PC3 (Barki-Harrington & Daaka 2001). Most of the biological actions of the B₂ receptor are mediated via Gq/11 protein leading to an increase in [Ca²⁺]_i and PKC activation in different cell types (Enomoto *et al.* 1995, Ankori-Stark *et al.* 1997,

Figure 8 BK as a mitogen for breast cancer cells. (A) Serum-starved MCF-7 cells were incubated with increasing concentrations of BK for 24 h. Cell proliferation was measured by cell count using a Burkler cell chamber and results were compared with cells incubated in medium only. Serum-starved MCF-7 cells were treated with increasing concentrations of PD98059 (B), GF109203X (C) or LY294002 (LY; D), before treatment for 24 h with 0.1 μ M BK. Cell proliferation was measured by cell count using a Burkler cell chamber and results were compared with controls (letter 'C' in panels B–D), i.e. cells incubated in medium only. Different letters indicate statistical differences found using Bonferroni–Dunn's test.

Wiernas *et al.* 1998). The involvement of PLC in the mitogenic effect of BK in MCF-7 cells has been proved using U73122 which totally inhibited this effect (Fig. 3). As a result of PLC activation, B_2 exerted the cytosol-to-membrane translocation of the conventional PKC- α and the novel PKC- δ and - ϵ (Fig. 4); the latter were clearly linked to the ERK1/2 pathway since their inhibition, but not the inhibition of PKC- α obtained by Gö6976 (Fig. 4), blocked the phosphorylation of ERK1/2. The involvement of novel PKCs in the ERK1/2 activation by BK has also been shown previously in normal and cancerous breast cells in primary culture (Greco *et al.* 2004, 2005). This transduction mechanism seems therefore to be a hallmark in the way in which ERK1/2 is linked to BK signalling in the human breast, MCF-7 cells included. Indeed, MAPK may be coupled to the B_2 receptor in several ways. In B_2 -transfected COS-7 cells both the activation of the PKC pathway and the EGFR transactivation comes about before the activation of MAPK (Adomeit *et al.* 1999). In PC-12 cells, EGFR transactivation (Zwick *et al.* 1997) as well as activation of a Pyk2/Src pathway (Dikic *et al.* 1996) are involved in BK-mediated MAPK activation. Translocation of PKC isozymes to the

membrane are mitogenic signals for the cells (Lafon *et al.* 1995, Greco *et al.* 2003, 2004) and can result in the phosphorylation of ERK1/2. In A431 cells, B_2 induces MAPK activation through a pathway involving both PI3K and PKC, and resulting in the inhibition of EGFR activity (Graness *et al.* 1998).

PI3K activity has, from the start, been linked with many aspects of cell transformation processes, including increased cell growth, proliferation and survival, adhesion, metastasis and angiogenesis (Roymans & Slegers 2001). Disturbances in the PI3K pathway block MAPK activation (Hawes *et al.* 1996, Kranenburg *et al.* 1997, Wennstrom & Downward 1999) suggesting a role for PI3K in MAPK function. The PI3K/Akt pathway in MCF-7 cells appears to be involved in ERK1/2 activation since LY294002 provoked a complete inhibition of the BK-induced phosphorylation of ERK1/2 (Fig. 6B). Several lines of evidence have indicated that the serine/threonine Akt (also known as protein kinase B, PKB) mediates many of the downstream events controlled by PI3K (Crowder & Freeman 1998, Riera *et al.* 2003). PKB/Akt is activated by phospholipid binding, by PDK1 phosphorylation at Thr308 (Alessi *et al.* 1996) and by

phosphorylation within the carboxy-terminus at Ser⁴⁷³. While in primary cultured normal and cancerous breast cells it was shown that the activity of PI3K is required to bring about the effects of BK on ERK1/2 (Greco *et al.* 2004, 2005), no data were provided regarding the involvement of the PI3K downstream kinase PKB/Akt. We showed here that indeed BK phosphorylates PKB/Akt in MCF-7 cells both time and dose dependently, and that this effect was not reduced by PD98059; indicating that ERK1/2 are not required for the PKB/Akt activation mediated by BK (Fig. 5). Conversely, when the PI3K pathway is inhibited by LY294002, the BK-dependent phosphorylation of PKB/Akt is obviously inhibited, but also the phosphorylation of ERK1/2 appeared blunted, suggesting that the activation of PI3K/Akt may be upstream to ERK1/2 activation (Fig. 6). Novel PKCs could be a link connecting PI3K to ERK1/2 since GF109203X completely blocked the phosphorylation of PKB/Akt (Fig. 7A). In fact, the phosphorylation of Akt was maximal at 60 min (Fig. 5), whereas ERK1/2 appeared to be maximally phosphorylated at 5 min (Fig. 1). Nevertheless, after 5 min the phosphorylation of Akt was about 1.7-fold the basal unstimulated level; we hypothesise that this activity is sufficient to activate the MAPK cascade.

In epithelial breast cells, BK provokes a PKC-dependent activation of the ERK1/2 signalling cascade leading to stimulation of cell proliferation (Greco *et al.* 2004, 2005); data shown here also indicate that the BK-provoked PKC, PKB/Akt and ERK1/2 activation are a mitogenic signal for MCF-7 cells. Of these pathways, PKB/Akt appears responsible for the activation of novel PKCs, while ERK1/2 are ultimately responsible for cell proliferation and depend upon both novel PKCs and PI3K activities. These data are in accordance with substantial evidence about the involvement of PI3K in BK-provoked cell proliferation, as shown in different human cancer cell lines – such as the EFM-192A (breast), the EFE-184 (endometrium) and the T-24 (bladder) cell lines (Drube & Liebmann 2000).

Here we showed for the first time that by inhibiting PI3K/Akt activity the BK-dependent PKC- δ and - ϵ translocations are prevented (Fig. 7B), indicating a PI3K/Akt-dependent regulation of PKC. As discussed above, the PKC-dependent regulation of Akt is well known, but so is a PI3K/Akt-dependent modulation of PKC activity. In fact, several authors (Zhang *et al.* 1995, Akimoto *et al.* 1996, Moriya *et al.* 1996), suggested that PI3K activity through the generation of 3'-phosphorylated lipids can act as a second messenger for the regulation of most PKC isozymes. In addition, Akt appears to associate to PKC- δ , through the PH domain and Akt was shown to phosphorylate PKC- δ *in vitro* (Konishi *et al.* 1996). Thus a cross-regulation of PI3K/Akt and PKCs in the regulation of the MAPK cascade mediated by BK might be an interesting hypothesis, although it requires further investigation.

In conclusion, we have demonstrated that BK brings about a signalling pathway through the B₂ receptor involving a PI3K/Akt-dependent activation of ERK1/2 mediated by PKC- δ / ϵ isozymes. These BK effects are of physiological importance since they lead to the stimulation of cell proliferation. Kinins also increase vascular permeability, thus facilitating tumour metastasis (Robert & Gulick 1989, Marceau 1995) and, after infiltration into normal adjacent tissues, tumour cells can chemotactically attract inflammatory cells (Traub *et al.* 1997) thereby regulating angiogenesis (Dlaminini *et al.* 1999). Accordingly, it would be reasonable to conclude that BK might have a significant role in breast cancer progression. Finally, the intracellular signalling and the mitogenic effects exerted by BK in the MCF-7 cell line are similar overall to those found in primary cultured breast epithelial cells, suggesting that MCF-7 may be a useful cell model in which to continue the studies on BK pathophysiology in the human breast.

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