Bradykinin stimulates cell proliferation through an extracellular-regulated kinase 1 and 2-dependent mechanism in breast cancer cells in primary culture

S Greco, M G Elia, A Muscella, S Romano, C Storelli
and S Marsigliante

Laboratory of Cellular Physiology, Department of Biological and Environmental Sciences and Technologies, Ecotekne, Università di Lecce, Monteroni,
Via Provinciale per Monteroni, 73100 Lecce, Italy

(Requests for offprints should be addressed to S Marsigliante; Email: santo.marsigliante@unile.it)

Abstract

We have previously reported that bradykinin (BK) represents an influential mitogenic agent in normal breast glandular tissue. We here investigated the mitogenic effects and the signalling pathways of BK in primary cultured human epithelial breast cells obtained from a tumour and from the histologically proven non-malignant tissue adjacent to the tumour. BK provoked cell proliferation, increase in cytosolic calcium, activation of protein kinase C (PKC)- α , - β , - δ , - ϵ and - η and phosphorylation of the extracellular-regulated kinases 1 and 2 (ERK1/2). The following compounds blocked the proliferative effects

of BK: Hyp3-BK, a B₂ receptor subtype inhibitor; U73122, a phospholipase C- β inhibitor; GF109203X, a protein kinase C (PKC) inhibitor; and PD98059, a mitogen-activated protein kinase kinase inhibitor. Gö6976, a Ca²⁺-dependent PKC inhibitor, did not have any effect. In conclusion, the mitogenic effects of BK are retained in peritumour and tumour cells; hence, it is likely that BK has an important role in cancer endorsement and progression.

Journal of Endocrinology (2005) **186**, 291–301

Introduction

Bradykinin (BK) is a kinin liberated from kininogens by the enzymatic action of kallikreins and participates in a wide range of physiological effects such as organ perfusion, systemic blood pressure, sodium and water homeostasis, regulation and maturation of growth factors and inflammation (Chen *et al.* 1988, Yu *et al.* 1998). BK exerts its action via two known receptors, namely, B₁ and B₂ receptors (Regoli & Barabé 1980, Vavrek & Stewart 1985, Ma *et al.* 1994, el-Dahr *et al.* 1997, Pesquero & Bader 1998). The kallikrein–kinin system is implicated in tumourigenesis through the actions of kinins (Robert & Gulick 1989, Maeda *et al.* 1999), since released kinins increase vascular blood flow and promote the supply of nutrients and oxygen to the tumour. This may be important in the processes of tumourigenesis and angiogenesis (Clements 1997, Plendl *et al.* 2000). BK is present in tumours of the stomach, pituitary, uterus and breast (Koshikawa *et al.* 1992, Jones *et al.* 1992, Clements & Mukhtar 1977, Hermann *et al.* 1995, Rehbock *et al.* 1995). Breast cancer is the most common cancer in women worldwide and continues to be a major health problem (Harris *et al.* 1992, Henderson 1993, Henson & Tarone 1994). The evolution of breast cancer and the relationships of genetic predispos-

ing factors with somatic changes are very complicated. Genetic and hormonal factors such as BRCA1/2 (breast cancer 1, early onset gene), p53, oestrogen, progesterone, prolactin, insulin-like growth factors, epidermal growth factor (EGF) and transforming growth factor- β (Biscardi *et al.* 2000, Sachdev & Yee 2001, Wakefield *et al.* 2001, Blackburn & Jerry 2002, Portier 2002, Venkitaraman 2002) are involved in the development of breast cancer and progression of the disease. In normal breast cell proliferation, a crucial role of the extracellular-regulated kinase 1 and 2 (ERK1/2) has been defined, and agonists such as angiotensin II (Ang II) and BK (Greco *et al.* 2002b, 2004) are able to induce proliferation through this pathway. If deregulation occurs, ERK1/2 is still crucial in determining the overall proliferation of the tumour cell by Ang II (Greco *et al.* 2003). In the human breast cancer EFM-192A cell line, BK has a proliferative effect mediated through the activation of the mitogen-activated protein kinase (MAPK) (Drube & Liebmann 2000). We previously reported that in primary cultured epithelial cells, obtained from normal breasts, BK increases cell proliferation by the activation of ERK1/2 (Greco *et al.* 2004). Inhibitors of angiogenesis and of matrix invasion are in development as anti-cancer agents. BK antagonists are involved in the US National Cancer Institute's trials as

drugs for non-small cell lung and prostate cancers, since they are without any evidence of harmful side-effects and, in addition, they have been shown to actively reduce the growth of a broad range of cancer cells, including the ZR-75 breast cancer cells (Stewart 2003).

We here aimed to ascertain whether BK is also a suitable candidate for the proliferative response in human primary cultured epithelial breast cancer cells. To this end, we made primary cell cultures from six cancers in order to investigate whether the BK mitogenic role is still retained in the tumour. Furthermore, the effects of BK were also studied in primary cultured epithelial breast cells obtained from the corresponding histologically proven non-malignant tissue adjacent to the tumour in order to compare results and to specifically evaluate the responsiveness of the cell types obtained from the same patients.

Materials and Methods

Materials

RPMI 1640 medium, antibiotics, glutamine and foetal bovine serum (FBS) were purchased from Celbio (Pero, MI, Italy). Mouse monoclonal antibody for cytokeratin 19 was purchased from Chemicon International (Prodotti Gianni, MI, Italy). Protein kinase C (PKC) and ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Segrate, MI, Italy); Gö6976 and AG1478, conventional PKCs and EGFR inhibitors respectively, were obtained from Calbiochem (Milan, Italy). All others reagents were from Sigma (Milan, Italy).

Primary culture of breast cancer epithelial cells

Six breast cancer tissues and the corresponding histologically proven non-malignant tissue adjacent to the tumour (peritumoural) were obtained after total mastectomies and immediately sent to the histopathology laboratory for the histological diagnosis. All patients gave informed consent to study participation before enrollment. The study protocol was approved by the ethics committee of Lecce University in accordance with the Declaration of Helsinki. All the tumours were invasive intraductal carcinomas from postmenopausal patients who had not received any therapy before surgery. Portions of tissue were placed into transport medium and disaggregated immediately as described previously (Greco *et al.* 2002a). Briefly, breast tissue fragments were digested in RPMI 1640 medium containing 0.010 mg/ml insulin, 10% FBS, 1.0 mg/ml collagenase type I and 100 U/ml hyaluronidase overnight at 37 °C on a rotary platform (200 r.p.m.). After digestion, tissue suspension was pushed through three stainless steel screens (100, 60 and 50 µm mesh size respectively) in order to obtain dispersed cell suspensions that were suspended in 20% FBS growth medium (RPMI 1640

medium, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.0 mM glutamine, 0.005 mg/ml insulin, 5.0 ng/ml EGF, 0.5 µg/ml hydrocortisone, 5.0 µg/ml transferrin, 0.1 µM isoproterenol, 0.01 µM ethanolamine and 0.01 µM o-phosphoethanolamine) and seeded into culture flasks.

After the first passage, cell cultures were maintained at 37 °C in a humidified environment containing 5% CO₂ for up to seven passages in 5% FBS growth medium to avoid fibroblast contamination. The cultured cells exhibited the characteristic features of epithelial cells, i.e. a positive immunocytochemical staining for cytokeratin 19; the contamination from fibroblasts was quantified by using anti-Vimentin antibody (Sigma), showing that their expression was lower than 5% (data not shown).

Low-density oligonucleotide microarray

Twenty-two genes were chosen using the data in the literature and analysed using oligonucleotide probes. Probes were selected from the 3' end using the public domain software ROSO (<http://pbil.univ-lyon1.fr/roso/>) and checked for alignment with Blast software (<http://www.ncbi.nlm.nih.gov/blast/>). The criteria used to design the oligonucleotide sequences were: (1) Tm difference ± 5 °C; (2) distance of 800–1200 bases from the 3' end; (3) contiguous single nucleotide base repeats <4 nt; (4) potential hairpin structures <9 nt; (5) guanine + cytosine content (GC) between 40 and 55%; and (6) Blast <70% similarity. Oligonucleotide sequences (40 mer) were extracted with a melting temperature of 72.57 ± 2.76 °C and with 46.1 ± 4.47 GC%. Oligonucleotides were synthesised and modified with a C6 amino linker by MWG Biotech Srl (Florence, Italy) and were spotted at 40 pmol/µl in 50% DMSO with the MicroCASTer manual arrayer (Schleicher & Schuell BioScience, Inc., Keene, NH, USA) in duplicate on MWG epoxy slides and kept at 42 °C for 8 h before hybridisation.

Breast cells were grown in complete medium with 5% FBS for 24 h and then total RNAs were extracted by the RNA extraction kit (Promega, Madison, WI, USA). The RNAs were used for the amino-allyl dUTP labelling reaction (Randolph & Waggoner 1997). Briefly, 10 µg total RNA was mixed with 0.5 µg/µl oligo (dT)12–18 primer (Invitrogen, San Giovanni Milanese, MI, Italy) and RNase-free water to 18.2 µl and incubated at 70 °C for 10 min, then snap-frozen in ice for 30 s and 6 µl 5 × Superscript buffer (Invitrogen), 3 µl 0.1 M 1,4-dithio-DL-threitol, 0.6 µl 50 × amino-allyl acid (aa)-dNTP mix (final concentration: 25 mM dATP, 25 mM dCTP, 25 mM dGTP, 15 mM dTTP and 10 mM aa-dUTP) and 2.0 µl Superscript II RT (200 U/µl) (Invitrogen) were added to the mixture. After incubation at 42 °C for 2 h, the RNA was hydrolysed with 10 µl 1 M NaOH and 10 µl 0.5 M EDTA at 65 °C for 15 min; then 10 µl 1 M HCl was added to neutralise the pH. The unincorporated aa-dNTPs and free amines were removed with Genomed

JETquick DNA Clean-up Spin kit (Celbio). The aa-cDNA was speed vacuum dried and resuspended in 4.5 µl 0.1 M Na₂CO₃ for Cy3 Dye Ester coupling (Amersham Bioscience Ltd, Little Chalfont, Bucks, UK). Pre-hybridisation was carried out using 1 × SSC with 0.1% SDS for 1 min with vigorous agitation; then the slides were washed. The speed vacuum dried Cy3-labelled aa-cDNA dissolved in 30 µl hybridisation solution (50% formamide, 1 × SSC and 0.1% SDS) was used for hybridisation. The slides were covered with a sterile coverglass and incubated at 42 °C for 15–18 h in microarray hybridisation chambers (Camlab, Cambridge, Cambs, UK). The slides were then washed in 1 × SSC, 0.2% SDS, then in 0.1 × SSC and 0.2% SDS and finally in 0.1% SSC and centrifuged in a Falcon tube at 800 g for 5 min to dry. Slides were analysed by the Affymetrix 428 laser scanner (MWG Biotech Srl) and Cy3 fluorescence was measured at 532 nm; spot fluorescences were converted in 8 bit images and image analysis with intensities measurements were obtained by the Eisen's free software ScanAnalyze (eisen@genome.stanford.edu).

Enzyme immunoassay (EIA) of oestrogen and progesterone receptors (ER and PgR)

ER EIA and PgR EIA assays (Abbott, Chicago, IL, USA) were carried out in accordance with the manufacturer's instructions.

Measurement of intracellular Ca²⁺ [Ca²⁺]_i

Serum-starved breast cells were loaded with 5 µM Fura 2-AM for 45 min at 37 °C in HEPES-buffered Krebs-Ringer solution (KRH; 140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl₂, 2.0 mM CaCl₂, 6.0 mM glucose and 10 mM HEPES, pH 7.4) containing 0.2% Pluronic F-127 (Molecular Probes, Leiden, The Netherlands) and 0.1% bovine serum albumin (BSA) (Greco *et al.* 2002a). Loaded cells were washed and 7 × 10⁶ cells/ml were used for fluorimetric measurement using the spectrofluorometer JASCO FP 750 (Jasco Corporation, Tokyo, Japan). Excitation monochromators were set at 340 and 380 nm, with a chopper interval of 0.5 s, and the emission monochromator was set at 510 nm. [Ca²⁺]_i was calculated according to the equation of Grynkiewicz *et al.* (1985), using the software Spectra Manager provided by Jasco. The basal levels of [Ca²⁺]_i and the maximal increase evoked by agonists were calculated according to the formula: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](Sf_2/Sb_2)$ where R_{max} and R_{min} values were determined by inclusion of 20 µl Triton X-100 (0.01% final concentration) and 20 µl EGTA (5 mM final concentration) respectively and R is the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm; Sf₂ and Sb₂ are the fluorescence proportionality coefficients obtained at 380 nm under R_{min} and R_{max} conditions respectively.

Proliferation assay by cell count

Breast cells were seeded at a density of 1 × 10⁴ cells/well in 96-well plates in RPMI growth medium with 5% FBS and incubated overnight at 37 °C in a humidified environment containing 5% CO₂ to allow adherence. The medium was changed to FBS-free growth medium for 18 h to induce quiescence. Agonists and inhibitors were diluted in FBS-free growth medium. Cells were seeded at 2.5 × 10⁴ cells/well on 24-well plates, and cells were counted in a Burkert cell chamber (Sigma) 24 h after treatment.

Immunoblot analysis

Cells in flasks were incubated with agonist and/or inhibitors in RPMI medium without FBS for the required periods at 37 °C. The stimulation was stopped by transferring the flasks onto ice. The cells were extracted with lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.25 M sucrose, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and sonicated on ice (3 × 10 s cycles). The mixture was centrifuged for 10 min at 800 g and the supernatant was saved and centrifuged at 100 000 g for 1 h; the supernatant was taken as the cytosol fraction. The pellet was resuspended in lysis buffer plus 1% Triton X-100 and centrifuged as before; the supernatant was collected as the membrane fraction. Cellular lysates were used to quantify the ERK1/2 phosphorylation; cytosols and membrane fractions were collected for detecting PKC isozyme 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 immunoblotting. The enrichment factors (enzyme activities of final purified membrane pellet and cytosol compared with those of the initial homogenate) were 29.1 ± 3.2 and not determined (ND) and 35 ± 2.2 and ND, in peritumour and tumour cells 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 electrotransfer onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Amersham, Bucks, UK). We used the rabbit antibodies against PKC isozymes and the monoclonal mouse antibody anti-phosphorylated ERK1/2. Antibody anti-PKC-α was diluted 1:5000, while the other anti-PKC antibodies were diluted 1:2000 and the anti-phosphorylated ERK1/2 1:500. The filter was incubated with the appropriate primary antibody and then with peroxidase-conjugated secondary antibodies diluted 1:10 000. Equal protein loading was confirmed with duplicate blots probed with antiserum against total ERK1/2 (Promega). Proteins were detected using the enhanced chemiluminescence ECL (Amersham Bioscience Ltd). The intensity of the bands was quantified by

Table 1 Description and relative intensity folds of the low-density oligo microarray

	GenBank ID	Function	N (a)	T (b)	PT (c)	PT/T	T/N	P
Gene name								
β-actin	NM 001101	Housekeeping	1.0	1.0	1.0	1.0	1.0	
CCND1	M64349	Cell cycle	1.1	3.6	2.0	0.5	3.6	a vs b, a vs c, b vs c: <0.05
CCNE2	AF106690	Cell cycle	1.1	4.2	3.0	0.7	3.8	a vs b, a vs c, b vs c: <0.05
CCNA1	NM 003914	Cell cycle	1.3	4.0	2.6	0.6	3.1	a vs b, a vs c, b vs c: <0.05
EGFR	NM 005228	Growth factor	1.7	6.7	6.0	0.9	3.9	a vs b, a vs c: <0.05; b vs c: >0.05
erbB-2	X03363	Growth factor	1.5	8.4	7.9	0.9	5.6	a vs b, a vs c: <0.05; b vs c: >0.05
p53	NM 000546	Cell cycle	1.8	2.8	2.5	0.9	1.5	a vs b, a vs c: <0.05; b vs c: >0.05
c-KIT	NM 000222	Tumorigenesis	1.1	3.8	3.2	0.8	3.4	a vs b, a vs c: <0.05; b vs c: >0.05
GATA3	X55037		1.1	2.6	2.2	0.8	2.4	a vs b, a vs c: <0.05; b vs c: >0.05
JNKK2	AF022805	Tumorigenesis	1.0	3.7	3.1	0.8	3.7	a vs b, a vs c: <0.05; b vs c: >0.05
TFF1	NM 005423	Hormone sensitivity	2.7	4.1	3.9	0.9	1.5	a vs b, a vs c: <0.05; b vs c: >0.05
ABCB	NM 004827	Multidrug resistance	1.1	2.5	2.1	0.8	2.3	a vs b, a vs c: <0.05; b vs c: >0.05
CASP9	AB020979	Apoptosis	1.3	2.4	2.0	0.8	1.8	a vs b, a vs c: <0.05; b vs c: >0.05
ABCC1	L05628	Multidrug resistance	1.2	2.6	2.3	0.9	2.2	a vs b, a vs c: <0.05; b vs c: >0.05
MDR1	AF016535	Drug resistance	1.1	5.4	5.1	0.9	4.9	a vs b, a vs c: <0.05; b vs c: >0.05
Survivin	AF077350	Tumorigenesis	0.5	6.3	6.9	1.1	12	a vs b, a vs c: <0.05; b vs c: >0.05
ABCG	NM 004827	Drug resistance	1.1	2.6	2.2	0.8	2.4	a vs b, a vs c: <0.05; b vs c: >0.05
Ki67	X65550	Proliferation	1.3	4.6	4.0	0.8	3.5	a vs b, a vs c: <0.05; b vs c: >0.05
ESR1	NM 000125	Hormone sensitivity	2.4	6.2	7.1	1.1	2.6	a vs b, a vs c: <0.05; b vs c: >0.05
ESR2	NM 001437	Hormone sensitivity	1.5	3.2	4.1	1.3	2.1	a vs b, a vs c: <0.05; b vs c: >0.05
PGR	AF016381	Hormone sensitivity	1.9	4.3	5.0	1.2	2.3	a vs b, a vs c: <0.05; b vs c: >0.05
LRP	X79882	Drug resistance	0.8	1.2	1.0	0.8	1.5	a vs b, a vs c, b vs c: >0.05
MYC	M14206	Cell cycle	1.1	4.3	4.0	0.9	3.9	a vs b, a vs c: <0.05; b vs c: >0.05
CTSD	M11233	Proteolysis	1.3	5.3	8.3	1.2	4.1	a vs b, a vs c, b vs c: <0.05

Relative fold (median intensity of each gene/median intensity of β-actin) in N, cultured peritumoural breast cells; T, cultured tumoural breast cells; PT, primary tumour fragments; PT/T and T/N, fold between the intensities of each measured gene. *P*, statistical significance by Student's *t*-test.

scanning densitometry using the NIH Image 1.62 software (NIH, Bethesda, MD, USA).

Statistics

Experimental points represent the means ± S.D. of three replicates. Statistical analysis was carried out using Student's *t*-test for unpaired samples and ANOVA with the Fisher's PLSD test. *P* < 0.05 was chosen as the level of significance.

Results

Since (1) ER and PgR concentrations, (2) specific markers of epithelial origin and (3) BK-induced $[Ca^{2+}]_i$ increase did not change significantly until the fourth culture passages (data not shown), we used, for all the experiments shown herein, cultured breast epithelial cells at passages two to three.

Characterisation of tumoural breast epithelial cells in primary culture

The tumoural origin of cell cultures was assessed by low-density oligonucleotide microarrays performed in primary tumour fragments and in the resultant cultured cells.

The glass slides for low-density oligonucleotide microarrays were spotted with 40 mer oligonucleotides of 23 genes, covering cell cycle regulation, ER and PgR, multidrug resistance and metastatic/invasive phenotyping, and β-actin as the housekeeping gene (Table 1). The fluorescence of each spot was normalised against β-actin and the relative folds for cultured tumour cells and primary tumours are shown. Primary tumours and resultant cultured cells showed a similar gene pattern for most of the genes (*P* > 0.05, Student's *t*-test), except for the cathepsin D and the cyclin genes (*P* < 0.05, Student's *t*-test) (Table 1). The gene expression pattern of cultured tumour cells was then compared with that of the cultured peritumoural cells. Sixteen out of the 22 genes were significantly overexpressed in cancer compared with peritumour cells (*P* < 0.05, Student's *t*-test) (Table 1).

ER and PgR concentrations were measured in cytosols obtained from cultured cells and from primary tumours fragments by EIA. No differences in ER and PgR concentrations between tumoural-derived cells and primary tumour fragments were obtained (*P* > 0.05, Student's *t*-test), whilst significant differences were found between primary tumour (or tumour) cells and the peritumour-derived cells (*P* < 0.05 for both, Student's *t*-test) (Table 2).

Cultured tumoural and peritumoural cells also had a different time-course proliferation curve and a different PKC isozyme expression. Cell proliferation rate was evalu-

Table 2 ER and PgR concentrations in cytosols from primary tumours (PT), tumoral (T) and peritumoural (N)-derived cell cultures

	PT (a)	T (b)	N (c)	P
ER (fmol/mg protein)	236±12	289±21	67±8	a vs b: >0.05; a vs c, b vs c: <0.05
PgR (fmol/mg protein)	122±10	156±21	33±10	a vs b: >0.05; a vs c, b vs c: <0.05

ated by cell counting starting from 2.5×10^4 seeded cells/well in complete growth medium at 24, 48 and 72 h (Fig. 1A). Results indicate that tumour cells had a higher proliferation rate than peritumour cells ($P < 0.0001$, ANOVA). Finally, the expression of eight PKC isozymes (PKC- α , - β , - δ , - ϵ , - η , - ι , - θ and - ζ) was investigated in crude cell lysates by SDS-PAGE and Western blotting analysis (Fig. 1B). All the isozymes studied were present in cultured breast cells, with the expression in tumour higher than in peritumour cells.

These data indicated that tumour-derived epithelial cells retained some biological features of the source primary tumours and that tumour and peritumour cells obtained from the same patient were different.

Figure 1 (A) Cell proliferation measured by cell count in tumour (solid bars) and peritumour (open bars) cells in complete growth medium at 24, 48 and 72 h. The data are means $\pm$ S.D. of four different experiments run in eight replicates and are presented as cell number. (B) Expression of PKC isozymes evaluated in tumour and peritumour crude cell lysates by SDS-PAGE and Western blotting analysis in samples obtained from six breasts. Densitometry analysis of bands are also represented as histograms for tumour (solid bars) and peritumour (open bars). Different letters indicate statistical differences by Fisher's PLSD test.

We also evaluated the time-course proliferation curve, the PKC isozyme expression, ER and PgR concentrations and the gene pattern in cell culture extracts obtained from breast reductions (normal samples) used in a previous study (Greco *et al.* 2004). The results showed no statistically significant differences between normal and peritumoural samples (data not shown), further indicating the non-cancerous origin of the cultured peritumour cells.

BK stimulates the proliferation of breast epithelial cells

Cells were stimulated with increasing concentrations of BK (0, 0.001, 0.01, 0.1, 1.0 and 10 μ M) and it was found that BK stimulated the 24-h cell proliferation in a dose-dependent manner, starting at 0.001 and reaching the maximal level at 1.0 μ M BK ($P < 0.0001$, ANOVA). The effect of BK was higher in tumour than in peritumour cells ($P < 0.001$, Student's *t*-test) (Fig. 2).

Figure 2 Serum-starved cells were incubated with increasing concentrations of BK for 24 h. Cell proliferation was measured by cell count and results were compared with control (C), cells incubated in medium only. The data are means $\pm$ S.D. of four different experiments run in eight replicates and are presented as cell number. Different letters indicate statistical differences by Fisher's PLSD test.

Figure 3 (A) Effect of 1 μ M BK on $[Ca^{2+}]_i$ in tumour (T) and peritumour (PT) cells in the presence of extracellular Ca^{2+} . The arrow indicates the time-point at which BK was added. (B) Dose-dependent response following stimulation of serum-starved tumour (circles) and peritumour (squares) cells with 0, 0.001, 0.01, 0.1, 1 and 10 μ M BK. $\Delta[Ca^{2+}]_i$ indicates the $[Ca^{2+}]_i$ concentration above the basal level. Different letters indicate statistical differences by Fisher's PLSD test. Results are representative of triplicate cell cultures from six different patients.

BK mediates changes in $[Ca^{2+}]_i$

Ca^{2+} mediates the expression of immediate early genes involved in cell proliferation (Ransone & Verma 1990). The effects of BK on $[Ca^{2+}]_i$ were evaluated in tumour and peritumour cells. The resting $[Ca^{2+}]_i$ was 96.5 ± 11 and 92 ± 12 nM in tumour and peritumour cells respectively ($n=8$; $P>0.05$, Student's *t*-test). The $[Ca^{2+}]_i$ response to BK was similar in shape in both cell types: BK caused an increase in $[Ca^{2+}]_i$ in a dose-dependent manner, showing maximal effect at 1.0 μ M BK. BK at 1 μ M induced a $[Ca^{2+}]_i$ increase with a 10–15 s delay to a peak of 448 ± 53 nM above resting level in peritumour and to a peak of 600 ± 48 in tumour cells ($P<0.01$ for both cell types, Student's *t*-test) (Fig. 3).

The phospholipase C (PLC)- β activity was inhibited by incubating serum-starved cells for 45 min with 1.0 μ M U73122, a specific PLC- β inhibitor; cells were then stimulated with 1.0 μ M BK for 24 h. U73122 significantly reduced the BK-induced proliferation in both cells ($P<0.0005$ for both cell types, Student's *t*-test) (Fig. 4), indicating a role of phospholipid hydrolysis in this process.

Serum-starved cells were pretreated for 45 min with 10 μ M of either B_2 or $B-1$ inhibitor, Hyp3-BK (B2-I) and Lys (des-arg-leu)-BK (B1-I) respectively, and the 24-h proliferation induced by 1.0 μ M BK was assessed. In the presence of B_2 inhibitor, BK-induced cell proliferation was completely blocked ($P<0.0005$ for both cell types, Student's *t*-test), whilst Lys (des-arg-leu)-BK did not have any effect ($P>0.05$ for both cell types, Student's *t*-test)

(Fig. 4). These results indicated that BK leads to cell proliferation through B_2 receptor activation.

BK activates PKC isozymes

The effects of BK on PKC isozymes were studied by stimulating serum-starved cells with 1.0 μ M BK for 1, 5, 25 and 60 min. SDS-PAGE-separated cytosol and membrane proteins were immunoblotted using specific antibodies to PKC- α , - β , - δ , - ϵ , - η , - ι , - θ and - ζ isozymes. All the isozymes were expressed, but only the

Figure 4 Serum-starved tumour (solid bars) and peritumour (open bars) cells were preincubated for 45 min with 1.0 μ M U73122 or 1.0 μ M Hyp3-BK (B2-I) or Lys (des-arg-leu)-BK (B1-I) and then stimulated with 1.0 μ M BK. Cell proliferation was measured by cell count and results were compared with cells incubated with BK only (C). The asterisks indicate statistical significance (by Student's *t*-test) compared with control cells treated with BK only.

Figure 5 Fold translocation of PKC isozymes provoked by 1.0 μ M BK at various incubation times (cancer cells; time in min) and at 5 min (peritumour cells (PT; solid bars)). PKC isoforms from control cells were considered to be 1.0-fold activated. Different letters indicate statistical differences by Fisher's PLSD test. The asterisks indicate statistical significance (by Student's *t*-test) between 5 min (25 min for PKC- η) translocation in tumour and peritumour cells.

conventional PKC- α and - β and the novel PKC- δ , - ϵ and - η isozymes translocated from the cytosol to the membrane. The maximal effects were obtained at 5 min for PKC- α , - β , - δ and - ϵ , and at 25 min for PKC- η in both cell types (Fig. 5) with higher translocations in tumour than in peritumour cells ($P < 0.001$ for PKC- α , - β and - δ and $P < 0.01$ for PKC- ϵ and - η , Student's *t*-test) (Fig. 5).

Novel PKC isozymes have a role in BK-dependent cell proliferation

Serum-starved cells were incubated for 45 min with 0.1 and 1.0 μ M Gö6976, a Ca^{2+} -dependent PKC isozyme inhibitor, or with GF109203X, an inhibitor of all PKCs, before stimulation for 24 h with 1.0 μ M BK. Figure 6 shows that Gö6976 did not affect the mitogenic effect of

Figure 6 Tumour (solid bars) and peritumour (open bars) serum-starved cells were treated with 0.1 and 1.0 μ M Gö6976 or GF109203X (GFX) for 45 min and then stimulated for 24 h with 1.0 μ M BK. Cell proliferation was measured by cell count and results were compared with cells incubated with BK only (C). The data are means $\pm$ s.d. of four different experiments run in eight replicates. The asterisks indicate statistical significance (by Student's *t*-test) compared with control cells treated with BK only.

Figure 7 (A) Tumour and (B) peritumour breast cells were treated without or with $1.0 \mu\text{M}$ BK for 5, 20 and 45 min. Cell lysates were subjected to SDS-PAGE and probed with total ERK1/2 or anti-phospho-ERK1/2 (pERK1/2). Representative autoradiographs are shown and results from densitometry are expressed as the percentage ratio over cells cultured in serum-free medium only (C). Different letters indicate statistical differences by Fisher's PLSD test.

BK, either in tumour or in peritumour (Fig. 6) cells ($P > 0.05$ for both cell types, Student's *t*-test). On the contrary, GF109203X inhibited the mitogenic effect of BK in both cells (Fig. 6) ($P < 0.0005$ for both cell types, Student's *t*-test), indicating that novel PKC isozymes participate in the effects of BK.

BK activates ERK1/2 and its role in cell proliferation

Serum-starved cells were treated with $1.0 \mu\text{M}$ BK for 5, 20 and 45 min, and cell lysates were blotted and incubated with anti-phospho-ERK1/2 antibody. Figure 7 shows that BK induces phosphorylation of ERK1/2 ($P < 0.0005$, ANOVA), with phosphorylation higher in tumour than in peritumour cells ($P < 0.005$, Student's *t*-test).

Serum-starved cells were treated for 45 min with increasing concentrations (0.01 , 1.0 and $30 \mu\text{M}$) of PD98059, an inhibitor of the mitogen-activated protein kinase kinases (MEK) upstream enzyme of the MAPK cascade (Alessi *et al.* 1995), before stimulation for 5 min with $1.0 \mu\text{M}$ BK. PD98059 had a dose-dependent inhibitory effect on BK-mediated cell proliferation in both cell types ($P < 0.001$ for both cell types, ANOVA) (Fig. 8), suggesting that MEK is required for the proliferative effect of BK.

Discussion

We have recently shown a role for BK in the proliferation of the primary cultured human epithelial breast cells (Greco *et al.* 2004), which is mediated by the activation of MAPK. Here we have shown that BK also retains a similar behaviour in tumoural-derived cell cultures, more relevant than in peritumoural-derived cells. The complexity of

obtaining a tumoural cell culture from primary tumours has prompted us to investigate if cells after culture manipulation still retain the same tumoural characteristics normally shown in primary tumours. With this in mind, we compared the expression pattern of some genes of relevance in breast cancer, e.g. ER, PgR, EGF receptor, ErbB-2 and Survivin. We found that tumoural-derived cells and primary tumours have a similar gene expression pattern evaluated by 'low-density' oligonucleotide microarray, indicating that cells in cultures retain the original tumoural characteristics (Table 1). Moreover, the overall different gene pattern between tumoural- and peritumoural-derived cells indicated that there was not a cell contamination.

That BK is a growth factor for breast epithelial cells is of relevance, since BK (and prostaglandin I_2) is one of the

Figure 8 Tumour (solid bars) and peritumour (open bars) serum-starved cells were treated with increasing concentrations of PD98059, before treatment for 5 min with $1.0 \mu\text{M}$ BK. Cell proliferation was measured by cell count and results were compared with control (C) cells incubated in medium only. Different letters indicate statistical differences by Fisher's PLSD test.

major mediators of the initial acute phase of inflammation and the breast may develop mastitis, during lactation and at any age in relation to congenital lesions such as duct ectasia, chronic disseminated infections, or during granulomatous, autoimmune or malignant processes (Michie *et al.* 2003). In addition, regular use of non-steroidal anti-inflammatory drugs may have a chemopreventive effect against the development of breast cancer (Harris *et al.* 2003). The data shown herein regarding the over-responsiveness of breast cancer cells to BK is important inasmuch as it points to BK as an essential extracellular factor for the maintenance of the tumorigenic growth.

The progression from normal breast epithelium to breast cancer is a complex multistep process resulting from the uncoupling of the systems controlling cell proliferation and differentiation, thus leading to extensive cellular growth. Research in the human breast field regarding the control of proliferation has stressed the functional implication of oestrogens and progesterone, EGF, insulin-like growth factor, fibroblast growth factor, nerve growth factor (Ethier 1995, Descamps *et al.* 1998, Xing & Imagawa 1999, Nurcombe *et al.* 2000, Dupont & Le Roith 2001) and, more recently, Ang II (Greco *et al.* 2002b, 2003). Relationships between the physiology of the epithelial breast cell and the components of the kallikrein–BK system are poorly defined.

This study has explored for the first time the mitogenic effects of BK in primary cultured epithelial breast cells obtained from six cancerous human breasts. These mitogenic effects were compared with those achieved in primary cultured epithelial breast cells obtained from corresponding histologically proven non-malignant tissue adjacent to the tumour; this in order to specifically evaluate the responsiveness of the cell types obtained from the same patients. We demonstrated here that, in breast cancer cells, BK stimulated cell proliferation through the B₂ receptor; the proliferative effects of BK was higher in tumour with respect to peritumour cells (Fig. 3).

It is known that MAPK is a key signal-transducing protein which transmits signals involved in cell proliferation, and BK has been found to elicit mitogenic responses through the activation of MEK/MAPK pathways in other cell types (Velarde *et al.* 1999, Luo *et al.* 2000), including normal epithelial breast cells (Greco *et al.* 2004). We have here confirmed that the proliferation of epithelial breast cancer cells was also sustained by ERK1/2 activation (Fig. 7). Upstream regulators of MAPK, such as the oncogene products *ras* (Janes *et al.* 1994) and *Raf-1* (Callans *et al.* 1995), as well as PKC (Arteaga *et al.* 1991), have been associated with breast cancer. Most of the biological actions of the B₂ receptor are mediated via G_{q/11} protein leading to an increase in [Ca²⁺]_i and PKC activation in different cell types (Enomoto *et al.* 1995, Ankorina-Stark *et al.* 1997, Wiernas *et al.* 1998). In breast cells, BK induced a [Ca²⁺]_i increase higher in tumour than in peritumour cells (Fig. 2). Ca²⁺ is an important mediator of

the expression of immediate early genes such as *c-fos*, *c-jun* and *c-myc* involved in the regulation of cell proliferation (Curran & Morgan 1987, Ransone & Verma 1990). Nevertheless, in tumour, peritumour and normal cells (Greco *et al.* 2004) the BK-dependent Ca²⁺-dependent PKCs were not responsible for the mitogenic stimulus of BK, since their inhibition by Gö6976 did not affect the proliferative effect of BK (Fig. 6). On the other hand, [Ca²⁺]_i regulated the Ang II-provoked proliferation of breast tumour cells in primary culture (Greco *et al.* 2003). These discrepancies could be explained by the different kinetics between the PKC activation provoked by Ang II and BK; actually, Ang II stimulated translocation of PKC- α and - β isozymes at 25 min, whereas BK did so at 5 min (Fig. 5). However, in the mitogenic effects of BK, the contribution of PLC was demonstrated by U73122 (Fig. 4). As a result of [Ca²⁺]_i increase and PLC activation, the B₂ receptor provoked the translocation from the cytosol to the membrane of five PKC isozymes (PKC- α , - β , - δ , - ϵ and - η) in tumour cells (Fig. 5).

In conclusion, this study has shown for the first time that BK has mitogenic effects in epithelial breast cancer cells and in the normal peritumour cells in primary culture. It has been shown that kinins are able to increase vascular permeability and cell proliferation, thus facilitating tumour metastasis (Robert & Gulick 1989, Marceau 1995). Moreover, after infiltration in normal adjacent tissues, many tumour cells can chemotactically attract inflammatory cells (Dlamini *et al.* 1999) thereby regulating angiogenesis (Plendl *et al.* 2000). In this regard, BK represents an influential mitogenic agent in normal breast glandular tissue, whose effects are also retained in peritumour and tumour cells. Hence, it is likely that BK has an important role in cancer endorsement and progression.

Acknowledgements

We are grateful to the Italian MIUR for project grant support (Ricerca di Base). We also would like to thank M Giuseppe A Pede for skilful technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 12 April 2005

Accepted 20 May 2005

Made available online as as Accepted Preprint
10 June 2005