Dual bradykinin B₂ receptor signalling in A431 human epidermoid carcinoma cells: activation of protein kinase C is counteracted by a G_s-mediated stimulation of the cyclic AMP pathway

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Cell membranes of the human epidermoid cell line A431 express classical bradykinin (BK) B₂ receptors, as assessed by [³H]BK binding studies. Furthermore, stimulation by BK induced a time-dependent modulation of protein kinase C (PKC) activity in A431 cells: a rapid activation (*t*_{1/2} ≈ 1 min) is followed by a slow inhibition (*t*_{1/2} ≈ 20 min) of PKC translocation measured by [³H]phorbol 12,13-dibutyrate binding. In addition, BK stimulated both adenylate cyclase activity in A431 membranes and accumulation of intracellular cyclic AMP (cAMP) in intact cells in a retarded manner. A possible BK-induced activation of the cAMP pathway mediated via PKC, phospholipase D, prostaglandins or Ca²⁺/calmodulin was excluded. A 35 kDa protein was found in A431 membranes to be specifically phosphorylated in the presence of both BK and protein kinase A (PKA). An anti-α_s-antibody, AS 348, abolished stimulation of adenylate cyclase activity in response to BK, cholera toxin and isoprenaline,

strongly suggesting the involvement of G_s proteins in the BK action. The BK-activated cAMP signalling system might be important for the observed inactivation of PKC slowly evoked by BK: the BK-induced rapid activation of PKC is decreased by dibutyryl cAMP, and the slow inhibition of PKC is prevented by an inhibitor of PKA, adenosine 3':5'-monophosphothioate (cyclic, Rp isomer). The inhibition of PKC translocation might be exerted directly at the level of PKC activation, since stimulation of phosphoinositide hydrolysis by BK was affected by neither dibutyryl cAMP nor forskolin. Thus our results provide the first evidence that A431 cells BK is able to activate two independent signal-transduction pathways via a single class of B₂ receptors but two different G proteins. The lagging stimulation of the cAMP signalling pathway via G_s might serve to switch off PKC, which is rapidly activated via G_q-mediated stimulation of phosphoinositide hydrolysis.

INTRODUCTION

The nonapeptide bradykinin (BK) appears to act as a mediator of a wide variety of physiological and pathophysiological responses, e.g. pain, inflammation, vascular permeability, smooth-muscle tone in vascular and other tissues, or neurotransmitter release [1,2]. In addition, there is an emerging role for BK as a modulator of cell proliferation including both mitogenic [3–5] and anti-mitogenic [6] effects.

Two BK receptor types, B₁ and B₂, have been identified [7]. B₂ receptors have a high affinity for BK, whereas [des-Arg⁹]BK, a typical B₁ receptor agonist, is nearly inactive. Both receptors exhibit a different pharmacological profile towards BK antagonists [1,2]. It is commonly accepted that most of the physiological and pathophysiological actions of BK involve B₂ receptors. Starting in 1991, bradykinin B₂ receptors were cloned from different species [8–10] and found to belong to the G-protein-coupled receptor family.

BK B₂ receptor stimulation in many cell types is known to

result in activation of phospholipase C (PLC) or phospholipase A₂ (PLA₂) [2]. Several studies have shown independent activation of PLC and PLA₂ via one individual B₂ receptor class, but different G proteins within the same cell [11,12]. In contrast, up to now there is little evidence for an influence of BK on the adenylate cyclase/cyclic AMP (cAMP) signalling system [2]. Recently, it was shown that in tracheal smooth muscles BK is able to activate adenylate cyclase in an indirect manner via pathways that involve protein kinase C (PKC) and phospholipase D (PLD) [13,14]. We demonstrated a direct inhibitory effect of bradykinin in picomolar concentrations on adenylate cyclase in guinea-pig ileum membranes [15,16]. This effect is mediated via a separate high-affinity B₂ receptor with picomolar affinity towards BK, which occurs additionally to the 'classical' PLC-coupled B₂ receptor with nanomolar affinity towards BK [17].

In the present study we report an increase in adenylate cyclase activity and cAMP concentration in A431 human epidermoid carcinoma cells stimulated by BK. In these cells BK has previously been described to stimulate phosphoinositide metab-

Abbreviations used: BK, bradykinin; Hoe 140, D-Arg[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]BK (Hyp, hydroxyproline; Thi, β-(2-thienyl)-L-alanine; Tic, 1,2,3,4-tetrahydroisoquinolin-2-yl-carboxylic acid; Oic, (3aS,7aS)-octahydroindol-2-yl-carboxylic acid; PLC, phospholipase C; PLA₂, phospholipase A₂; PLD, phospholipase D; PKC, protein kinase C; PKA, protein kinase A; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GTP[S], guanosine 5'-[γ-thio]triphosphate; cAMP, cyclic AMP; dB-cAMP, dibutyryl cAMP; Rp-cAMP-S, adenosine 3':5'-monophosphothioate (cyclic, Rp isomer); PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; IBMX, 3-isobutyl-1-methylxanthine; PGE₂, prostaglandin E₂; G-protein, regulatory heterotrimeric guanine-nucleotide-binding protein; CTX, cholera toxin.

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olism [18] via a pertussis-toxin-insensitive G protein (G_q) and, subsequently, PKC activity [19]. We here describe BK-induced 'lagging' stimulation of the cAMP signalling system, which is obviously mediated via the same class of BK receptors but involves a G_s protein. Furthermore, BK induces specifically in a protein kinase A (PKA)-dependent manner phosphorylation of a 35 kDa protein in A431 membranes, and BK-induced translocation of PKC in A431 cells is inhibited by cAMP. These findings provide evidence for the existence of two independent BK signalling pathways and a 'cross-talk' interaction between adenylate cyclase and PKC in A431 cells.

EXPERIMENTAL

Materials

$[[3,4-^3H_2]Pro^{2,3}]BK$ ($[^3H]BK$) (102 Ci/mmol), $[\alpha-^{32}P]ATP$ (800 Ci/mmol), $(\gamma-^{32}P)ATP$ (3000 Ci/mmol), *myo*- $[2-^3H]inositol$ (20.5 Ci/mmol), and $[^3H(n)]phorbol$ 12,13-Dibutyrate ($[^3H]PDBu$) (21 Ci/mmol) were purchased from New England Nuclear (Dreieich, Germany). $[8-^3H]cAMP$ (26 Ci/mmol), the $[^{125}I]$ -cAMP scintillation proximity assay (SPA) kit and the $[^3H]cAMP$ -protein-binding assay kit were obtained from Amersham (Braunschweig, Germany). BK, $[des-Arg^9]BK$, $D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]BK$, Lys-BK, guanosine 5'- $[\gamma$ -thio]triphosphate (GTP[S]), 3-isobutyl-1-methylxanthine (IBMX), Hepes, Pipes, Tes, dithiothreitol (DTT), 1,10-phenanthroline, bacitracin, captopril, PMSF, cAMP, ATP, phosphocreatine, creatine kinase, BSA, ovalbumin, forskolin, dibutyl cAMP (dB-cAMP), PDBu, phorbol 12-myristate 13-acetate (PMA), PKA, mepacrine, indomethacin, prostaglandin E_2 (PGE_2), angiotensin II and substance P were from Sigma (Deisenhofen, Germany). Leupeptin, pepstatin A, Pefabloc, bisindolylmaleimide and Rp-cAMP-S (adenosine 3':5'-monophosphothioate, cyclic, Rp isomer) were obtained from Boehringer (Mannheim, Germany). Ammonium formate and sodium tetraborate were purchased from Serva (Heidelberg, Germany). $D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK$ (Hoe 140) was kindly provided by Professor B. Schölkens, Hoechst AG (Frankfurt, Germany). The $[D-Phe^7]BK$ analogues $[Thi^{5,8}, D-Phe^7]BK$, $D-Nal[Thi^{5,8}, D-Phe^7]BK$ (Nal, β -2-naphthylalanine) and $[D-Phe^2, D-Phe^7]BK$ were gifts from Dr. J. Stewart (Denver, CO, U.S.A.). All other reagents were of the highest grade commercially available.

Cell culture

A431 human epidermoid carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 4.5 g/l glucose, 2 mM glutamine, 7.5 % foetal-calf serum and antibiotics. Treatment of intact cells with BK and various agents, as outlined in the Figure legends, was performed with nearly confluent cultures.

Membrane preparations

An A431-cell particulate fraction (referred to as 'membranes' throughout the manuscript) was prepared by a procedure modified from the protocol of Akiyama et al. [20]. In brief, cells were grown to near confluence on 625 cm² plates (Nunc). The medium was decanted and the cells were washed twice with an ice-cold buffer containing 20 mM Tris/maleate and 100 mM NaCl, pH 7.2, and once with 20 mM Pipes, pH 7.2. The cells were scraped into Pipes buffer with a rubber policeman and sonicated with a Branson sonifier (1 min, level 3) under cooling. Proteinase inhibitors (leupeptin, 1 μ g/ml; pepstatin A, 1 μ g/ml; pefabloc, 100 μ g/ml; final concns.), EGTA (1 mM) and DTT (10 mM)

were added, and the particulate fractions were pelleted at 48000 rev./min for 30 min in a Sorvall SS34 rotor. The pellet was resuspended in 20 mM Pipes, pH 7.2.

For a comparison of $[^3H]BK$ binding in A431 membranes with that in 'classical' BK B_2 -receptor-containing tissues, crude membrane fractions were prepared from both guinea-pig ileum and rat uterus. Briefly, sections of ileum (approx. 15 cm in length) from Hartley guinea pigs (300–500 g) of either sex, or of uteri from Wistar rats (200 g), were removed immediately after killing the animals. The muscle layers were homogenized with a Polytron (20–30 s) in 25 mM Tes buffer, pH 6.8, containing 1 mM, 1,10-phenanthroline. The homogenates were filtered through two layers of medical gauze, centrifuged at 100000 *g* for 20 min in a Sorvall F-28 rotor, and resuspended in preparation buffer. All membrane preparations were stored at $-80^\circ C$. Protein concentration was determined as described by Lowry et al. [21] or Bradford [22], with BSA as standard.

$[^3H]BK$ binding assay

Binding assays followed the method of Manning et al. [23], with some modifications. Membranes (approx. 200–400 μ g of protein/tube) were incubated with $[^3H]BK$ (in concentrations as indicated in the Figure legends) in 2 ml of a medium containing 25 mM Tes, pH 6.8, 1 mM 1,10-phenanthroline, 140 μ g/ml bacitracin, 1 mM DTT, 10 μ M captopril and 0.1 % BSA. After incubation at $4^\circ C$ for 30 min (equilibrium conditions), the samples were quickly filtered through Whatman GF/B glass-fibre filters pretreated with 0.1 % aq. polyethyleneimine, by using a Brandel harvester. The filters were washed with 3×5 ml of ice-cold preparation buffer, dried, and counted for radioactivity in 5 ml of a toluene-based scintillator. Non-specific binding was determined in the presence of 1 μ M unlabelled BK. Specific binding of $[^3H]BK$ was in the range 40–60 % (A431 cells), 80–90 % guinea-pig ileum and 60–70 % (rat uterus). Saturation data were analysed by using the non-linear regression program LIGAND [24]. Inhibition curves were fitted with the program of Tobler and Engel [25] yielding IC_{50} values. The respective K_i values were calculated by using the equation of Cheng and Prusoff [26].

Adenylate cyclase assay

The activity of adenylate cyclase in A431 membranes was determined as described by Schultz and Jacobs [27], with slight modifications (method A). Briefly, membranes (80 μ g of protein/assay) were incubated for 20 min at $25^\circ C$ in a standard mixture containing $[\alpha-^{32}P]ATP$ (approx. 2×10^7 c.p.m./tube), 5 mM $MgCl_2$, 1 mM IBMX, 0.1 mM cAMP, 0.1 mM ATP, 5 mM phosphocreatine, 150 units of creatine kinase, BSA (1 mg/ml) and 50 mM Hepes, pH 7.2. To inactivate angiotensin-converting enzyme, which degrades BK, the incubation mixture was supplemented with 1 μ M captopril. Variable additions such as NaF (as AlF_4^-), forskolin, GTP[S] or BK were added as indicated, giving a total assay volume of 100 μ l. The samples were preincubated for 5 min at $25^\circ C$, and the reaction was started by adding the membranes. The incubation was terminated by addition of 400 μ l of $ZnCl_2$ solution (125 mM), followed by 500 μ l of Na_2CO_3 solution (125 mM). The samples were centrifuged for 5 min at 10000 *g*, and 800 μ l of the supernatant was transferred to alumina 90 (Merck)-containing columns. After draining of the sample, labelled cAMP was eluted by the subsequent addition of 2×2 ml of 100 mM Tris/HCl buffer, pH 7.5, and ^{32}P was determined by measuring Čerenkov radiation. For each column cAMP recovery was separately

estimated by using [^3H]cAMP. Alternatively, some experiments were performed without labelled ATP by using a modified procedure in accordance with Schmidler et al. [28] (method B). The assay medium contained all reagents as in method A, except [α - ^{32}P]ATP and cAMP. The reaction was started by addition of membranes and stopped by heating (95 °C; 3 min). Then 200 μl of ice-cold distilled water and 30 mg of aluminium oxide were added, followed by centrifugation at 10000 g for 2 min. The cAMP content of the supernatants was measured by a [^3H]cAMP-protein-binding assay.

Determination of intracellular cAMP

A431 cells in 24-well plates were treated with serum-free DMEM overnight, and then the medium was changed to Hepes-buffered serum-free DMEM, which had been adjusted to pH 7.2 just before addition. Then, cells were exposed to the agents tested in 500 μl of serum-free medium supplemented with 100 μM IBMX and 10 μM captopril. The reaction was stopped by addition of 1 ml of ice-cold ethanol (96%, v/v) giving a final concentration of 65% (v/v). The cells were scraped off the plates, and the ethanolic extract was centrifuged at 14000 g for 6 min at room temperature. The supernatants containing the extracted cAMP were collected separately, and the pellets were washed with 500 μl of ethanol (65%, v/v) and centrifuged as above. Each supernatant was pooled with that from the first centrifugation step, evaporated to dryness at 60 °C and redissolved in 600 μl of 0.05 M acetate buffer. Two samples were taken for estimation of cAMP by the cAMP [^{125}I]scintillation proximity (non-acetylation) assay from Amersham. For each well, the protein content was determined [22]. The cAMP values were normalized on the basis of the protein contents, which varied between 52 and 58 μg per well.

Measurement of PKC translocation and [^3H]PDBu binding

Sub-confluent cultures of A431 cells were subjected to a change of medium to serum-free DMEM, pH 7.2, containing 10 μM captopril. Cells were then exposed to BK (1 μM) at room temperature for various times as indicated in the Figure legends. For several experiments, cells were pretreated either with dB-cAMP (10 μM) for 15 min or with the PKA inhibitor Rp-cAMP-S (5 μM) for 60 min. Then BK (1 μM) was added for the incubation times as indicated. The incubation was terminated by aspirating the medium and preparing A431 membranes as described above. The measurement of [^3H]PDBu binding to A431 membranes was based on a modification of the technique described previously [29,30]. Binding assays were conducted at 37 °C in a total volume of 1 ml of 20 mM Hepes, pH 7.4, containing bacitracin (100 $\mu\text{g}/\text{ml}$), PMSF (0.1 mM), trypsin inhibitor (10 $\mu\text{g}/\text{ml}$) and [^3H]PDBu (2 nM), with or without unlabelled PDBu (1 μM), for 5 min at 37 °C. The reaction was terminated by filtration through Whatman GF/B glass-fibre filters, pretreated with 0.1% (v/v) aq. polyethyleneimine, by using a Brandel Harvester. The filters were washed with 3 $\times$ 5 ml of 10 mM Hepes buffer, pH 7.2, dried, and counted for radioactivity by liquid scintillation.

Determination of total inositol phosphates

BK-induced formation of inositol phosphates in A431 cells was determined as described by Tilly et al. [18]. Briefly, cells were prelabelled with 4 μCi of [^3H]inositol/ml for 24 h in inositol-free DMEM. At 2 h before stimulation, the medium was changed to serum-free DMEM containing 20 mM Hepes (pH 7.2). Then the

cells were stimulated for 10 min with BK at the concentrations indicated in the presence of 10 mM LiCl. The reaction was terminated by replacing the medium with 1 ml of 10% trichloroacetic acid. The inositol phosphate-containing extracts were washed with 4 $\times$ 2 vol. of water-saturated diethyl ether, neutralized by adding Tris base, diluted to 4 ml with distilled water and placed on AG 1 $\times$ 8 columns (200–400 mesh, formate form; Bio-Rad). The columns were subsequently eluted with 2 ml of water and 5 ml of 60 mM ammonium formate/5 mM sodium tetraborate, followed by 5 $\times$ 2 ml of 1.0 M ammonium formate/0.1 M formic acid yielding the inositol phosphate fraction.

Immunoblotting experiments

Antisera were raised against peptides corresponding to the specific regions of G-protein α subunits and characterized as described elsewhere [31,32]. Membrane proteins were separated by SDS/PAGE on gels containing 10% (w/v) acrylamide and transferred on to nitrocellulose filters. After blocking the filters with 3% (w/v) ovalbumin in PBS, pH 7.4, and washing in PBS/Tween 20 (0.05%, v/v), strips of the nitrocellulose were incubated for 1 h with the following antisera: AS 348/2 (anti- α_s ; 1:150); AS 368/2 (anti- $\alpha_{q/11}$; 1:150); AS 266/4-9 (anti- $\alpha_{1\text{common}}$; 1:150); and AS 6/3 (anti- α_{ocommon} ; 1:200). Incubation was followed by five washes in PBS containing 0.05% Tween 20. Filters were then incubated with goat anti-(mouse IgG)-peroxidase (Sigma) for 1 h and subsequently washed as described above. Secondary antibodies were detected by chemiluminescence (ECL) Western-blotting detection system (Amersham).

Membrane phosphorylation procedure, SDS/PAGE and autoradiography

A431-cell membranes (80 μg of protein/assay) were phosphorylated in 50 mM Hepes buffer, pH 7.1, containing 10 mM MgCl_2 , 1 mM IBMX, 10 μM captopril, 10 μM ATP and [γ - ^{32}P]ATP (5 $\mu\text{Ci}/\text{assay}$). BK (1 μM), Hoe 140 (1 μM) in the absence or presence of PKA (50 units/assay) were added as indicated. The reaction was started by the addition of membranes (30 μl) and terminated by the addition of an equivalent volume (100 μl) of a solution containing SDS (6%, w/v), Bromophenol Blue (0.1%), 2-mercaptoethanol (30%, v/v) and glycerol (40%, v/v), followed by boiling at 100 °C for 5 min. After cooling, the samples were subjected to SDS/PAGE on 10% acrylamide gels (30 mA, 600 V, 4 h). The resolved gels were fixed, stained, dried, and autoradiographed at -70 °C by using Reflection autoradiography film (Du Pont) and an intensifying screen. Typical exposure times ranged between 5 and 12 h. Autoradiograms were scanned and analysed quantitatively by using the program NIH Image 1.51.

RESULTS

Characterization of the A431 BK receptor

As shown in Figure 1, [^3H]BK labelled a single affinity class of sites in A431-cell membranes. A Scatchard plot, as well as computer curve fitting, of saturation data revealed a K_d value of 0.9 ± 0.3 nM and a B_{max} value of 110 ± 40 fmol/mg of protein. Competitive binding experiments using 0.3 nM [^3H]BK showed that the BK receptor in A431 cells possesses a high affinity for BK and kallidin ([Lys]BK), as well as the B_2 -receptor antagonist Hoe 140. In contrast, the receptor exhibited a very low affinity towards the classical B_1 -receptor agonist [des-Arg 9]BK (Table 1). We also measured binding competition by other peptides,

Figure 1 Saturation binding and Scatchard plot (inset) of $[^3\text{H}]\text{BK}$ binding to A431-cell membranes

Increasing concentrations of $[^3\text{H}]\text{BK}$ (20 pM–2 nM) were incubated with A431-cell membranes (0.3–0.4 mg of protein/assay) as described in the text. The graphs are representative of single experiments assayed in duplicate, which were repeated twice with comparable results. B, bound; F, free.

Table 1 Comparison of binding properties of BK B_2 receptors in A431-cell membranes with those in guinea-pig ileum (GPI) and rat uterus (RUT) membranes

Inhibition data obtained from the displacement of $[^3\text{H}]\text{BK}$ (0.1–0.3 nM) by various BK analogues were determined as described in the Experimental section. The K_i values represent the means from two separate experiments performed in triplicate determinations. K_H and K_L denote high- and low-affinity binding sites [17].

	K_i (nM)		
	A431	GPI	RUT
Agonists			
BK	1.1	$K_H = 0.005$ $K_L = 0.9$	$K_H = 0.012$ $K_L = 1.2$
Lys-BK	1.9	0.5	0.1
[des-Arg ⁹]BK	> 10 000	> 10 000	> 10 000
Antagonists			
Hoe 140	0.9	0.11	0.09
D-Arg[Hyp ³ ,Thi ^{5,8} , D-Phe ⁷]BK	4.1	8.6	$K_H = 0.02$ $K_L = 1.1$
[Thi ^{5,8} ,D-Phe ⁷]BK	48	9.4	12
[D-Nal ¹ ,Thi ^{5,8} ,D-Phe ⁷]BK	> 3000	> 3000	480
[D-Phe ² ,D-Phe ⁷]BK	1000	1080	820

including angiotensin II and substance P. These peptides affected the binding of $[^3\text{H}]\text{BK}$ only at concentrations higher than 1 μM , thus confirming its specificity (Table 1). We further examined the ability of several [D-Phe⁷]BK analogues which are known to be ‘classical’ bradykinin B_2 -receptor antagonists [2] to displace $[^3\text{H}]\text{BK}$ from the A431 receptor. The antagonist affinity profile is more similar to the affinities found for the bradykinin B_2 receptors in guinea-pig ileum membranes compared with that in rat uterus membranes (Table 1). Slight differences may be explained by species differences which exist between bradykinin B_2 receptors [2]. An additional high-affinity binding site with picomolar affinity

Figure 2 Stimulation of adenylate cyclase activity in A431-cell membranes by increasing concentrations of BK

Adenylate cyclase activity was measured by method A as described in the Experimental section. Points represent means $\pm$ S.E.M. from three to four separate experiments or from two experiments (*) performed in quadruplicate determinations. Inset: effects of the BK- B_2 -receptor antagonists Hoe 140 (B; 10 μM) and D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]BK (C; 10 μM) on BK (A; 1 μM)-induced stimulation of A431-cell adenylate cyclase activity. The BK- B_1 -receptor agonist [des-Arg⁹]BK (D; 1 μM) did not significantly change the adenylate cyclase activity in A431-cell membranes. Bars represent means $\pm$ S.E.M. of three separate experiments in quadruplicate determinations. The BK-induced increase in adenylate cyclase activity is statistically significant from the control (*) and the effect is significantly (**) different as compared with A ($P < 0.05$; Student's t test).

towards BK, which has been found in the rat uterus and the guinea-pig ileum [17], does not occur in A431 membranes.

BK stimulates adenylate cyclase activity and accumulation of intracellular cAMP in A431 cells

As shown in Figure 2, BK caused a concentration-dependent stimulation of the basal activity of adenylate cyclase (4.6 ± 0.3 pmol/mg of protein) in A431 membranes up to approx. 184% compared with the control [forskolin (0.1 mM), 146%; GTP[S] (10 μM), 120%; NaF (0.1 mM), 293%]. Half-maximal and maximal stimulations were observed at 180–200 nM and 1–2 μM BK respectively. At concentrations higher than 3 μM BK the stimulation of adenylate cyclase was attenuated. The effect of BK was almost completely prevented by the two potent B_2 antagonists Hoe 140 and D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]BK (Figure 2, inset). The B_1 receptor agonist [des-Arg⁹]BK failed to influence the adenylate cyclase activity in A431 membranes (Figure 2, inset). These findings suggest that BK activation of adenylate cyclase activity is mediated via a bradykinin B_2 receptor. In order to see whether the effect of BK on adenylate cyclase might be indirect, the influence of various relevant signal-transduction inhibitors was studied. Although the adenylate cyclase activity in A431 membranes was stimulated by PGE₂,

Table 2 Influence of various signal-transduction inhibitors on BK-induced stimulation of adenylate cyclase activity in A431-cell-membranes

Adenylate cyclase activity was measured as described in the Experimental section (method A). The results are expressed as means $\pm$ S.E.M. of three to six experiments (numbers in parentheses) in duplicates with membranes from different cell preparations: * significantly different from control ($P < 0.05$; Student's t test); † not significantly different from BK alone (Student's t test).

Treatment	Specific activity of adenylate cyclase (% of control)
Control	100
BK (1 μ M)	184 $\pm$ 20* (6)
PGE ₂ (100 nM)	149 $\pm$ 11* (6)
Mepacrine (0.1 mM)	102 $\pm$ 3 (4)
Indomethacin (0.1 mM)	97 $\pm$ 11 (5)
Mepacrine + BK	155 $\pm$ 18† (4)
Indomethacin + BK	156 $\pm$ 9† (5)
Bisindolylmaleimide (1.25 mM)	99 $\pm$ 9 (6)
Bisindolylmaleimide + BK	216 $\pm$ 44† (6)
Chlorpromazine (30 μ M)	103 $\pm$ 7 (4)
Chlorpromazine + BK	173 $\pm$ 12† (4)

Figure 3 Concentration-dependent cAMP accumulation in response to BK

A431 cells were incubated at increasing concentrations of BK for 20 min. The results are expressed as pmol of cAMP/well and represent means $\pm$ S.E.M. from three experiments.

neither the PLA₂ inhibitor mepacrine nor the cyclo-oxygenase inhibitor indomethacin significantly affected the BK-induced increase in adenylate cyclase activity (Table 2). We also investigated the ability of BK to increase adenylate cyclase activity in presence of the PKC inhibitor bisindolylmaleimide [33] or the calmodulin inhibitor chlorpromazine. Both compounds did not significantly change the BK-induced stimulation of adenylate cyclase (Table 2).

In intact A431 cells, BK induced a concentration-dependent increase in intracellular cAMP (EC_{50} approx. 2 nM; Figure 3). With respect to time-course, BK elicited a slow increase ($t_{1/2}$ approx. 10 min) in intracellular cAMP compared with the very rapid effect induced by isoprenaline ($t_{1/2} \leq 1$ min) (Figure 4). The

Figure 4 Time-dependent accumulation of intracellular dAMP in response to BK or isoprenaline

A431 cells were incubated in the presence of either 1 μ M BK (●) or 10 μ M isoprenaline (□) for the times indicated. The cAMP contents were measured as described in the Experimental section. The results are expressed as pmol of cAMP/well and represent means $\pm$ S.E.M. from three independent experiments in duplicate determinations.

Table 3 Effects of PMA and BK on intracellular cAMP levels in A431 cells

Where indicated, cells were pre-treated with the PKC inhibitor bisindolylmaleimide (1.25 μ M) for 30 min. cAMP was measured as described in the Experimental section. The results are expressed as pmol of cAMP/well and are means $\pm$ S.E.M. of combined data from three experiments in duplicate determinations: * significantly different from control ($P < 0.05$; Student's t test); † significantly different from BK or PMA alone ($P < 0.05$; Student's t test)

Treatment	cAMP (pmol/well)
Control	34.5 $\pm$ 1.7
BK (1 μ M)	47.2 $\pm$ 1.5*
PMA (0.1 μ M)	46.2 $\pm$ 6.2*
BK + PMA	60.4 $\pm$ 7.2†
Bisindolylmaleimide (1.25 μ M)	36.0 $\pm$ 3.5
Bisindolylmaleimide + BK	45.2 $\pm$ 5.4*

BK-induced cAMP accumulation reached a maximum between 20 and 30 min and declined rapidly thereafter (results not shown). Stimulation with BK typically resulted in a maximal increase in cAMP accumulation up to 137% above basal (100%) at 20 min of stimulation. Treatment of A431 cells with either BK (1 μ M) or PMA (0.1 μ M) resulted in an almost equivalent increase in intracellular cAMP accumulation (Table 3). The effects of BK and PMA together on the cAMP response were clearly additive, indicating that both agents act via different pathways, i.e. the cAMP response evoked by BK is independent of PKC. This result is further supported by the findings that neither the BK-induced adenylate cyclase activation in A431 membranes (Table 2) nor the cAMP accumulation by BK in intact A431 cells (Table 3) was blocked by the PKC inhibitor bisindolylmaleimide. The inhibitor concentration used (1.25 μ M) [33] inhibited completely the PMA (1 μ M)-induced stimulation of adenylate cyclase activity in A431 membranes (results not shown).

Table 4 Effect of butan-1-ol on BK-stimulated cAMP accumulation

A431 cells were pre-treated for 5 min with 0.3% (v/v) (final concn.) butan-1-ol, followed by stimulation with bradykinin (1 μ M). These results are expressed as pmol of cAMP/well and are means $\pm$ S.E.M. from three independent experiments in duplicate determinations: * significantly different from control ($P < 0.05$; Student's t test); † significantly different from non-treated cells ($P < 0.05$; Student's t test).

Cells	Control	+ BK
Non-treated	36.1 $\pm$ 6.3	50.8 $\pm$ 4.7*
Butan-1-ol-treated	44.0 $\pm$ 1.4†	55.1 $\pm$ 7.8*

Table 5 Effect of cholera toxin on cAMP production in intact A431 cells

A431 cells were stimulated by the agents indicated, and the cAMP level was measured as described in the Experimental section. The results are expressed as pmol of cAMP/well and represent means $\pm$ S.E.M. from three separate experiments performed in duplicate determinations: * not significantly different from CTX or BK alone (Student's t test).

Treatment	cAMP (pmol/well)
Control	36.1 $\pm$ 6.3
CTX (10 nM)	50.4 $\pm$ 2.2
BK (1 μ M)	50.8 $\pm$ 4.7
CTX + BK	51.2 $\pm$ 6.3*

Figure 5 Identification of G-proteins present in A431-cell membranes

Immunoblotting was performed as described in the Experimental section. Shown are the autoluminograms of the nitrocellulose strips. The position of the 45 kDa marker protein is shown on the right. Each lane corresponds to a strip incubated with the following antisera: lane 1, AS 348 (anti- α_s ; 1:150); lane 2, AS 368 (anti- $\alpha_{q/11}$; 1:150); lane 3, AS 266 (anti- $\alpha_{i\text{common}}$; 1:150); lane 4, AS 6 (anti- $\alpha_{o\text{common}}$; 1:200); lane 5, AS 6 and guinea-pig brain membranes (for comparison).

Recently, it has been demonstrated that pretreatment of tracheal smooth-muscle cells with 0.3% (v/v) butan-1-ol, which traps the PLD-derived phosphatidate, completely blocked the PLD-mediated BK-induced increase in cAMP accumulation [13]. In A431 cells, in contrast, 0.3% butan-1-ol did not affect the BK-induced increase in cAMP accumulation (Table 4). Finally, cholera toxin (CTX) caused significant stimulation of cAMP production in A431 cells (Table 5). The combination of CTX and BK resulted in a cAMP accumulation that was quantitatively the same as that induced by CTX or BK alone (Table 5). This finding indicates that both agents probably act via the same pathway.

Effect of anti- G_{sz} antiserum on BK-induced stimulation of adenylate cyclase activity

To investigate further the importance of the CTX substrate G_{sz} for the BK-induced adenylate cyclase stimulation in A431

Table 6 Effect of anti- G_{sz} antiserum on BK-induced stimulation of adenylate cyclase activity in A431-cell membranes

Membranes from A431 cells were pre-treated for 1 h at 37 °C in presence of either AS 348 or non-immune serum (control) at a final dilution of 1:30. After this period, adenylate cyclase activity in response to BK, isoprenaline, CTX or forskolin was determined by use of method B as described in the Experimental section. Results are means $\pm$ S.E.M. for assays in duplicate determinations from four separate experiments with three different cell preparations (percentage values were calculated versus the respective basal activities): * significantly different versus response in control ($P < 0.05$; Student's t test).

Treatment	Adenylate activity (pmol of cAMP/20 min per mg of protein)	
	Non-immune serum	Anti- G_{sz} antiserum AS 348
Basal	85.9 $\pm$ 2.5 (100%)	95.4 $\pm$ 3.5 (100%)
BK (1 μ M)	113.6 $\pm$ 5.6 (132 $\pm$ 4.9%)	98.4 $\pm$ 3.8 (103 $\pm$ 4%)*
Isoprenaline (10 μ M)	111.7 $\pm$ 3.8 (130 $\pm$ 4%)	105.3 $\pm$ 3.7 (110 $\pm$ 3.5%)
CTX (1 μ M)	111.3 $\pm$ 2.0 (129 $\pm$ 1.8%)	96.5 $\pm$ 5.6 (101 $\pm$ 5.8%)*
Forskolin (0.1 mM)	347 $\pm$ 25 (404 $\pm$ 7.1%)	331 $\pm$ 2.9 (367 $\pm$ 10%)*

membranes, we studied the effects of the anti- α_s antiserum AS 348 to attenuate the BK action. AS 348 was raised against the C-terminal region of G_{sz} , corresponding to amino acids 385–394 [31]. To detect the presence of G proteins in A431 membranes and to establish the specificity of AS 348, Western blots with this and several other G-protein-specific antibodies were performed (Figure 5). Antisera AS 348 and AS 368 (anti- $\alpha_{q/11}$) yielded strong bands of the expected size (as indicated), whereas AS 266 (anti- $\alpha_{i\text{common}}$) gave only a weak reaction. AS 6 (anti- $\alpha_{o\text{common}}$) failed to detect G_o proteins in A431 membranes, although it recognized, for comparison, a broad 39 kDa band in pig brain membranes. Pre-treatment of A431 membranes with AS 348 at a dilution of 1:30 (v/v) completely abolished the BK-induced adenylate cyclase activation (Table 6), whereas non-immune serum had no effect under similar conditions. Also, the stimulation of adenylate cyclase evoked by CTX or isoprenaline was clearly decreased by AS 348 (Table 6). In contrast, stimulation by 0.1 mM forskolin was only slightly affected by AS 348 at the dilution used.

The BK-induced modulation of PKC translocation is dependent on the presence of cAMP

Stimulation of phosphoinositide breakdown and subsequent activation of PKC by BK in A431 cells has been well documented [18,19]. The activation of PKC has been shown to correlate with its translocation from the cytosol to the plasma membrane and a subsequent increase in the binding of labelled phorbol ester to intact cells [29,30]. Specific binding of [3 H]PDBu to whole cells has been found to reflect the amount of PKC that is associated with the plasma membrane [29,30]. We therefore employed [3 H]PDBu binding to A431-cell membranes for measurement of BK-induced PKC activation. In initial studies we examined the kinetics of [3 H]PDBu binding to A431 membranes at 37 °C. A steady-state level (control) of [3 H]PDBu binding of approx. 300 fmol/mg of protein was reached within 1 min and was maintained for at least 10 min (results not shown). To determine

Figure 6 Time course for the effect of BK on PKC translocation in A431 cells

BK (1 μ M) was added to A431 cells in culture and the reaction was stopped at the times indicated. Membrane preparations and measurement of specific [3 H]PDBu binding were performed as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. Broken line indicates basal level of [3 H]PDBu binding.

Table 7 Effects of dB-cAMP and Rp-cAMP-s on BK-induced changes in PKC translocation

A431 cells were pre-treated with dB-cAMP for 15 min or with the PKA inhibitor Rp-cAMP-S for 60 min, followed by incubation with BK (1 μ M) for the times indicated. Membrane preparation and measurement of specific [3 H]PDBu binding were performed as described in the Experimental section. Values represent means $\pm$ S.E.M. of three separate experiments in triplicate determinations: * significantly different from the BK effect in absence of dB-cAMP or Rp-cAMP-S ($P < 0.05$; Student's t test).

Treatment	Incubation time (min)	[3 H]PDBu bound (fmol/mg of protein)
Control		308 $\pm$ 5
dB-cAMP (10 μ M)	15	338 $\pm$ 5
BK (1 μ M)	3	426 $\pm$ 3
dB-cAMP + BK	15 + 3	353 $\pm$ 5*
BK (1 μ M)	5	395 $\pm$ 2
dB-cAMP + BK	15 + 5	348 $\pm$ 4*
Rp-cAMP-S (5 μ M)	60	306 $\pm$ 15
BK (1 μ M)	20	213 $\pm$ 7
Rp-cAMP-S + BK	60 + 20	341 $\pm$ 10*

the time course of BK-induced PKC activation, A431 cells were incubated with 1 μ M BK for various times, immediately followed by membrane preparation and measurement of [3 H]PDBu binding. As shown in Figure 6, BK induced a rapid increase in the specific binding of [3 H]PDBu to A431-cell membranes. The increase reached a peak value (approx. 140% of control) 3 min after the addition of BK. The response was strongly dependent on the duration of BK incubation and returned almost to basal

Figure 7 BK-induced inositol phosphate accumulation and effects of cAMP and forskolin

myo-[3 H]inositol-pre-labelled A431 cells were incubated with increasing concentrations of BK for 10 min. The incubation and the determination of total inositol phosphates were performed as described in the Experimental section. Inset: Effects of BK- B_2 -receptor antagonist Hoe 140 (1 μ M; C), dB-cAMP (10 μ M; D) and forskolin (100 μ M; E) on BK-induced accumulation of inositol phosphates (1 μ M; B) compared with the control (A). Cells were pre-treated with dB-cAMP or forskolin for 20 min before addition of BK. Hoe 140, dB-cAMP and forskolin at the concentrations used had no influence on the inositol phosphate level in A431 cells (results not shown). All data are expressed as means $\pm$ S.E.M. for four to six separate experiments.

levels within 8 min. Incubation with BK for longer than 10 min resulted in a significant decrease of [3 H]PDBu binding compared with the control (approx. 50% after 30 min). Pre-treatment of cells with the competitive inhibitor of PKA, Rp-cAMP-s, prevented the BK-induced inhibition of PKC. In addition, pre-treatment of A431 cells with dB-cAMP significantly diminished the BK-induced rapid activation of PKC (Table 7). Thus, the slow inhibitory effect of BK on PKC activity in A431 cells appears to be mediated via the cAMP signalling system.

cAMP has no effect on the BK-induced formation of inositol phosphates

To study whether the cAMP-mediated inhibition of PKC observed in membranes is due to affecting BK-induced phosphoinositide hydrolysis, the latter was studied in intact cells. In our experimental setting, half-maximal stimulation of inositol phosphate formation in A431 cells occurred at a BK concentration of approx. 40 nM. At a saturating concentration of 1 μ M BK, the inositol phosphate level was 3-fold that of the control. Tilly et al. [18] reported a similar increase in inositol phosphate formation, but a 10-fold higher sensitivity towards BK (half-maximal stimulation at approx. 4 nM BK). This difference may be due to experimental conditions. The BK-induced inositol phosphate accumulation was clearly prevented by Hoe 140, suggesting that the B_2 type of BK receptors is involved. Preincubation of A431 cells with dB-cAMP or forskolin, in contrast, did not significantly affect bradykinin action on inositol phosphate formation (Figure 7, inset). Thus inhibition of PKC activation via the cAMP pathway should occur downstream of the phosphoinositide hydrolysis.

Figure 8 Effects of BK and PKA on the phosphorylation pattern of A431-cell membrane proteins

Phosphorylation of A431-cell membranes in the presence of [γ - 32 P]ATP (5 μ Ci/assay) was performed as described in the Experimental section. The phosphorylated proteins were separated by SDS/PAGE and revealed by autoradiography. Lanes: 1, membranes without additions (control); 2, BK (1 μ M); 3, BK (1 μ M) + PKA (50 units); 4, BK (1 μ M) + PKA (50 units) + Hoe 140 (10 μ M); 5, PKA without A431-cell membranes. Molecular-mass markers (kDa) are shown on the right. Shown is a typical experiment out of three.

PKA-dependent and BK-specific phosphorylation of a 35 kDa protein in A431-cell membranes

In order to test under conditions *in vitro* whether activation of adenylate cyclase by BK might result in a PKA activation in A431 cells, and subsequently in a PKA-dependent phosphorylation of membrane proteins, cell membranes were treated with BK in the presence of exogenous PKA and [γ - 32 P]ATP. The autoradiogram presented in Figure 8 shows that, in the presence of both BK (1 μ M) and exogenously added PKA (50 units), a 35 kDa protein band was phosphorylated (lane 3). The phosphorylation of the 35 kDa protein did not occur without BK (lane 1), nor in the presence of BK and in the absence of PKA (lane 2), nor was the phosphoprotein band a part of the phosphorylation pattern of PKA itself (lane 5). Phosphate incorporation into this 35 kDa phosphoprotein was suppressed by 85% in the presence of the bradykinin- B_2 -receptor antagonist Hoe 140 (lane 4). These findings demonstrate that the phosphorylation was specifically induced by BK in the presence of PKA. The BK-induced phosphorylation was time-dependent, occurring after prolonged incubation with BK for at least 30 min (results not shown).

DISCUSSION

In cell membranes of the human epidermoid carcinoma cell line A431, both saturation and inhibition studies with [3 H]BK revealed a single class of binding sites, indicating the existence of a homogeneous receptor population. The binding affinity of approx. 1 nM corresponds well to those reported for most bradykinin B_2 receptors in various tissues [1,2]. The BK binding site in A431-cell membranes exhibits the characteristic antagonist affinity profile, qualifying it as a classical bradykinin B_2 receptor, and failed to bind the bradykinin B_1 receptor agonist [des-Arg⁹]BK with high affinity.

In addition to the well-known BK-induced stimulation of inositol phosphate formation in A431 cells [18], we observed both a stimulation of adenylate cyclase activity in A431 membranes and an increase in cAMP accumulation in intact A431 cells

by BK. In intact cells the dose-response curve of the BK-induced accumulation of cAMP showed an EC_{50} value of approx. 2 nM, which matches quite well to the K_d value of the B_2 receptor. In addition, the dose-response curve of the BK-induced cAMP accumulation is similar to that for BK-induced inositol phosphate formation ([18]; Figure 7 of the present work), indicating that in A431 cells BK stimulates both PLC and adenylate cyclase via the same receptor. In membrane assays a discrepancy between the EC_{50} value of approx. 180 nM obtained from the concentration-response curve of BK on adenylate cyclase activity and the affinity constant (K_d) obtained from the binding experiments was obvious. To understand this discrepancy, one has to consider that binding studies have been routinely performed in accordance with Manning et al. [23], using Tes buffer at pH 6.8. We recently reported that adenylate cyclase is almost inactive under these conditions [15]. However, at pH 8.0, which is routinely employed for determination of adenylate cyclase activity, BK does not bind to its receptor [15]. To study effects of BK on adenylate cyclase we therefore had to use 'compromise' assay conditions, i.e. which are most likely the reason for the observed low EC_{50} value of BK displayed in the adenylate cyclase assay compared with the binding assay, which was performed under optimal binding conditions. Comparing the time courses of BK effects on cAMP accumulation in intact cells with that on phosphoinositide hydrolysis revealed a clear difference. Whereas inositol phosphates accumulated very rapidly following stimulation by BK, reaching a peak within 30–60 s [18], BK-induced cAMP accumulation is obviously delayed and reaches its maximum after 10–15 min. The retarded kinetics of BK-induced cAMP accumulation in A431 cells also becomes obvious in comparison with the rapid stimulation of cAMP accumulation induced by isoprenaline in these cells under identical assay conditions (Figure 4). This finding might suggest that the BK action on cAMP production represents a secondary event which is mediated via other second-messenger systems. We therefore investigated various putative mechanisms by which adenylate cyclase is stimulated in an indirect manner.

Firstly, in several tissues BK is able to stimulate arachidonic acid release, followed by PGE_2 -mediated stimulation of adenylate cyclase [2]. This pathway was excluded for A431 cells, because the BK-induced activation of adenylate cyclase was not blocked by either the PLA_2 inhibitor mepacrine or the cyclo-oxygenase inhibitor indomethacin. Secondly, the type II and IV isoforms of adenylate cyclase are activated by PKC, and the type I and III isoforms may be activated by Ca^{2+} /calmodulin [34,35]. Both PKC activation and Ca^{2+} release are signals integrated in the rapid activation of the phosphoinositide system by BK. The PKC inhibitor bisindolylmaleimide inhibited neither the BK-induced activation of adenylate cyclase nor the cAMP accumulation in intact cells after stimulation with BK. Furthermore, BK causes an increase in cAMP in A431 cells that is substantially augmented by simultaneous treatment with the phorbol ester PMA. This additive effect suggests that both agents probably use different pathways to stimulate adenylate cyclase. The possibility that the BK effect is mediated via Ca^{2+} /calmodulin is also unlikely. Experiments with the calmodulin inhibitor chlorpromazine failed to affect the BK-induced adenylate cyclase activation. Thirdly, recent findings in cultured tracheal smooth-muscle cells indicated a link between BK-activated PLD, phosphatidate production and adenylate cyclase activation [13,14]. Pre-treatment of these cells with butan-1-ol (0.3%, v/v) which traps phosphatidate, completely abolished the BK-stimulated cAMP accumulation [13,14]. In contrast with tracheal smooth-muscle cells, in A431 cells butan-1-ol failed to affect BK-induced cAMP accumulation, but rather slightly increased the basal intracellular cAMP level.

These data indicate that the BK-induced cAMP response is not mediated via PLD stimulation in A431 cells. Finally, the adenylate cyclase isoforms II and IV may be also indirectly stimulated by $\beta\gamma$ complexes derived from pertussis-toxin-sensitive G_i or G_o proteins [34,35]. Although A431 membranes express G_i proteins (Figure 5), their participation in the BK-induced adenylate cyclase activation is unlikely, since the rapid stimulation of phosphoinositide hydrolysis by BK is clearly mediated via pertussis-toxin-insensitive G proteins [18]. Taken together, these data indicate that a number of pathways known to activate adenylate cyclase indirectly in response to BK in other cell systems are apparently not involved in BK-induced cAMP accumulation in A431 cells. We therefore attempted to investigate whether B_2 receptors functionally couple G_s proteins. Support came from the observation that, when A431 cells were treated with BK and CTX simultaneously, no additive effect on cAMP accumulation was observed, suggesting that both agents probably act via the same pathway. This prompted us to study the effect of an anti- α_s antibody on BK-induced adenylate cyclase activation. Since the C-terminus of G protein α -subunits is important for their activation by receptors, antibodies directed against this region suppress G_s function and were recently successfully used as tools for studying the functional importance of G_s [28,36] or G_i [37,38] proteins in agonist-regulated adenylate cyclase activity. The specificity of the anti- α_s antiserum AS 348 was shown previously [31,32]. Pretreatment of A431 membranes with AS 348, which is selective for the C-terminal region of α_s [32], did in fact significantly decrease the stimulation of adenylate cyclase by CTX and isoprenaline, which are known to act via G_s . Obviously antibodies directed against the C-terminus of G_s , which is responsible for the interaction with the receptor, also affect the CTX target site [28]. The stimulatory effect of forskolin, which directly activates the catalytic subunit of adenylate cyclase, was only slightly affected by AS 348 in the concentration used. In summary, our data strongly suggest direct coupling of bradykinin B_2 receptors in A431 cells to G_s and in turn activation of adenylate cyclase in A431 cells.

At present, it remains to answer the question why the adenylate cyclase activation by BK is rather slow compared with the rapid activation of PLC. Obviously, the G_s protein in A431 cells is available, because isoprenaline is able to induce a very rapid activation of cAMP accumulation. One may therefore assume that the BK receptor/ $G_{s\alpha}$ coupling is negatively controlled by an unknown mechanism which is turned off with the increasing stimulation of the phosphoinositide system by BK. This mechanism requires further investigation. Also, from our experiments there is no indication of which isoform of adenylate cyclase may be targeted by BK.

So far, we have two indications for molecular targets of cyclic AMP in relation to BK. Firstly, the BK-induced rapid translocation of PKC is followed by a BK-dependent inactivation of PKC. The time course of the PKC inhibition correlates with the time course of BK-induced cAMP accumulation. The rapid activation of PKC is significantly decreased by pre-treatment with dB-cAMP, whereas the PKC inactivation after long-term incubation with BK is abolished by pre-treatment with the PKC inhibitor Rp-cAMP-S. In contrast, the BK-induced stimulation of PLC is affected by neither dB-cAMP nor forskolin. It is tempting to speculate that in A431 cells BK is able to modulate its own stimulatory effect on PKC via the cAMP pathway. Secondly, we found a 35 kDa protein in A431 membranes which is specifically phosphorylated only in the presence of both BK and exogenously added PKA. The identity of this phosphoprotein remains to be elucidated. In addition, it is likely that perhaps other BK- and PKA-dependent phosphorylation targets exist in

A431 cells. There is an increasing amount of evidence that cAMP is able either to inhibit [39] or to activate [40] the MAPK (mitogen-activated protein kinase) kinase pathway in various cells.

Our results provide the first evidence that in A431 cells BK activates dual pathways, i.e. the PLC/PKC pathway and independently the cAMP/PKA pathway. In A431 cells cAMP has been reported to inhibit cell growth [41], and CTX was found to enhance the growth-inhibitory effect of epidermal growth factor [42]. On the basis of our findings, it will be highly interesting to investigate further the 'cross-talk' between the BK and the EGF signal-transduction systems in A431 cells.

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