Novel bradykinin signalling events in PC-12 cells: stimulation of the cAMP pathway leads to cAMP-mediated translocation of protein kinase C ϵ

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In the rat pheochromocytoma cell line PC-12, bradykinin (BK) stimulated phosphatidylinositol hydrolysis by 4–5-fold and, additionally, intracellular cAMP accumulation by approx. 1.6-fold. EC₅₀ values for BK were 3 nM and 2 nM respectively. The BK-induced increase in cAMP accumulation was paralleled by a 1.6-fold increase in protein kinase A (PKA) activity. The time course of BK-stimulated inositol phosphate formation was rapid ($t_{1/2} < 1$ min), whereas the BK-induced cAMP accumulation was lagging ($t_{1/2}$ approx. 6 min). The effect of BK on the cAMP pathway was independent of pertussis toxin, excluding an indirect stimulation of adenylate cyclase via $\beta\gamma$ -complexes from G_i or G_o proteins. Two different protein kinase C (PKC) inhibitors, bisindolylmaleimide and Ro 31-820, failed to prevent BK-induced cAMP accumulation, and exclude PKC as mediator of BK action on adenylate cyclase. In contrast, the stimulatory effect of BK on cAMP accumulation was completely abolished by two calmodulin antagonists, chlorpromazine and ophiobolin,

suggesting an indirect, Ca²⁺/calmodulin-mediated effect of BK on the cAMP pathway. In addition, exposure of PC-12 cells to BK resulted in a translocation of the PKC isoforms α , δ , ϵ and ζ displaying different kinetics. The BK-induced translocations of the PKCs α and δ were rapid and biphasic, whereas the PKCs ϵ and ζ revealed a slower and slightly transient translocation in response to BK. The BK-elicited translocation of PKC ϵ , but not that of the PKCs α , δ and ζ , was prevented by two different inhibitors of adenylate cyclase, 2',5'-dideoxyadenosine and MDL-12,330A, as well as the PKA inhibitor adenosine 3':5'-monophosphothioate. These findings suggest that the BK-induced translocation of novel (n)PKC ϵ is mediated via the cAMP pathway. Since nPKC ϵ appears to regulate neurite outgrowth in PC-12 cells [Hundke, McMahon, Dadgar and Messing (1995) J. Biol. Chem. 270, 30134–30140] our results provide evidence for a novel signalling mechanism that might be involved in BK-induced neuronal differentiation of PC-12 cells.

INTRODUCTION

The rat pheochromocytoma cell line PC-12 has been widely used to study the signal transduction mechanisms of neurotrophic factors [1]. Proliferation of these cells is stimulated by epidermal growth factor (EGF), whereas neuronal differentiation is induced not only by nerve growth factor (NGF) but also by several neuropeptides, such as vasoactive intestinal peptide (VIP) [2] and bradykinin (BK) [3].

There are several lines of evidence that PC-12 cells exclusively express BK receptors of the B₂ subtype [3,4]. In PC-12 cells, like in most cells and tissues [5], BK stimulates phospholipase C (PLC) leading to accumulation of inositol trisphosphate, intracellular Ca²⁺ and diacylglycerol (DAG) [6]. So far, no alterations in the intracellular cAMP level after exposure of PC-12 cells to BK have been found [5,7]. For the neuronal differentiation of PC-12 cells in response to peptides of the VIP family, in contrast, a cAMP-mediated mechanism has been suggested [2,8]. In PC-12 cells, cAMP was also shown to stimulate the NGF-induced neurite formation [9] and to convert EGF from a proliferative factor to a differentiating one [10]. Only now has BK been demonstrated to be capable of activating adenylate cyclase in tracheal smooth-muscle cells via both a PKC-dependent and a phospholipase D (PLD)-dependent pathway [11,12]. In the human epidermoid carcinoma cell line A431, we have recently

shown that BK stimulates dual signal pathways: PLC via a G_q protein and adenylate cyclase via a G_s protein [13]. Additionally, BK was found to translocate, rapidly, PKC from cytosol to the plasma membrane and to switch off this rapid PKC activation via stimulation of the cAMP pathway [13]. The PKC family consists of multiple isoforms that differ in their regulatory domain, their dependence on Ca²⁺ and their tissue distribution. They are divided at least into three structurally related groups: 'conventional' (c)PKCs (α , β_1 , β_{II} and γ), which are sensitive to Ca²⁺, DAG or tumour-promoting phorbol esters; 'novel' (n)PKCs (δ , ϵ , η and θ), which are regulated by DAG and phorbol esters but are independent of Ca²⁺; and 'atypical' (a)PKCs (ζ , λ and μ), which are insensitive to Ca²⁺, DAG and phorbol esters and may be regulated by phospholipids [14,15].

Recently, an involvement of the PKC isoform ϵ in mediation of neurite outgrowth in PC-12 cells was demonstrated [16], possibly representing a mechanism whereby neuropeptides that produce DAG as second messenger could promote neuronal differentiation. These new data referred to above [9–13,16] prompted us to re-investigate a putative influence of BK on the cAMP system and to study a possible functional link between BK-induced activation of PKC isoforms and the cAMP pathway in PC-12 cells.

In the present study we show that in PC-12 cells BK is capable of inducing accumulation of intracellular cAMP via a Ca²⁺/

Abbreviations used: BK, bradykinin; Hoe 140, D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]BK; VIP, vasoactive intestinal peptide; EGF, epidermal growth factor; NGF, nerve growth factor; LPA, lysophosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PKA, cAMP-dependent protein kinase; dB-cAMP, dibutyl cAMP; Rp-cAMP-S, adenosine 3':5'-monophosphothioate (cyclic, Rp isomer); IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; ERK 1, extracellular signal-related protein kinase.

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calmodulin-dependent pathway and activates protein kinase A (PKA). Furthermore, BK was shown to evoke translocation of the PKC isoforms α , δ , ϵ and ζ with different kinetics. The BK-induced translocation of nPKC ϵ was abolished by inhibition of both adenylate cyclase and PKA, whereas the BK effect on translocation of cPKC α , nPKC δ and aPKC ζ was independent in a prior stimulation of the cAMP pathway. Our results provide evidence for a novel BK signalling pathway in PC-12 cells that might be involved in the BK-induced neuronal differentiation.

EXPERIMENTAL

Materials

myo-[2-³H]Inositol (20.5 Ci/mmol), [9,10-³H]palmitic acid (35 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were purchased from New England Nuclear (Dreieich, Germany). The [³H]-cAMP-protein-binding assay kit was obtained from Amersham (Braunschweig, Germany). Cell culture media and supplements were from Gibco (Eggenstein, Germany). Polyclonal antibodies against the human PKC isoforms α , β_1 , β_{II} , γ , δ , ϵ and ζ , as well as against the mitogen-activated protein kinase (MAPK) p44, were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). BK, lysophosphatidic acid (LPA), 3-isobutyl-1-methylxanthine (IBMX), Hepes, dithiothreitol, bacitracin, captopril, leupeptin, pepstatin, aprotinin, PMSF, cAMP, dibutyl cAMP (dB-cAMP), BSA, PMA, chlorpromazine, pertussis toxin (PTX), Tween 20 (polyoxyethylenesorbitan monolaurate), peroxidase-conjugated goat anti-rabbit IgG, myelin basic protein, sodium orthovanadate, Protein A-Sepharose and diagnostic film (Biomax, Eastman Kodak) were obtained from Sigma (Deisenhofen, Germany). Bisindolylmaleimide and Rp-cAMP-S (adenosine 3':5'-monophosphothioate, cyclic, Rp isomer) were obtained from Boehringer (Mannheim, Germany). Ammonium formate and sodium tetraborate were from Serva (Heidelberg, Germany). Ophiobolin, 2',5'-dideoxyadenosine and MDL-12,330A were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). The reagents for SDS/PAGE, Hybond PVDF membranes and the chemiluminescent ECL Western blotting detection system were purchased from Amersham (Braunschweig, Germany). D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]BK (Hoe 140; Incatibant) was kindly provided by Professor B. Schölkens (Hoechst AG, Frankfurt, Germany). Ro 31-8220 was generously given by Dr. D. Bradshaw, Roche Products (Welwyn Garden City, Herts., U.K.).

Cell culture

Rat pheochromocytoma PC-12 cells (DSM, Braunschweig, Germany) were grown in RPMI 1640 supplemented with 10% (v/v) donor horse serum, 5% (v/v) fetal-calf serum, 100 units/ml penicillin, 10 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B in air/CO₂ (19:1). For stimulation experiments, PC-12 cells were grown in 6-well or 24-well dishes as indicated in the Figure legends for the respective assays. Membrane preparations were stored at -80 °C. Protein concentration was determined according to Lowry et al. [17] with BSA as the standard.

Determination of intracellular cAMP

PC-12 cells in 24-well plates were treated with serum-free RPMI overnight. Then, cells were exposed to the agents tested in 500 μ l of serum-free Hepes-buffered RPMI (pH 7.2), supplemented with 100 μ M IBMX and 10 μ M captopril. The reaction was stopped by the addition of 1 ml of ice-cold ethanol (96%, v/v) giving a final concentration of 65% (v/v). The ethanolic cell

extracts were centrifuged at 14000 *g* for 6 min at room temperature. The supernatants containing the extracted cAMP were removed and the pellets were washed with 500 μ l of ethanol (65%, v/v) and centrifuged as above. Supernatants were pooled, evaporated to dryness at 60 °C and resolved in 600 μ l of 0.05 M acetate buffer. The samples were taken for estimation of cAMP by a [³H]cAMP-protein-binding assay from Amersham. For each well, the protein content was determined according to Bradford [18], and the cAMP values were normalized on the basis of the protein contents. For some experiments, PC-12 cells were preincubated with PTX (holotoxin; 2.0 μ g/ml) for 24 h and then assayed as described in the Figure legends, in the absence or presence of BK, as indicated.

Measurement of PKA activity

Subconfluent PC-12 cell cultures in 6-well plates were treated with 1 μ M BK in Hepes-buffered RPMI (pH 7.2), containing 1 mM IBMX and 10 μ M captopril, for the times indicated in the Figure legends. The incubation was stopped by rapid removal of the cells and centrifugation at 10000 *g* for 1 min, rapid aspiration of media and addition of lysis buffer [50 mM Tris/HCl (pH 7.5)/100 mM NaCl/50 mM NaF/5 mM EDTA/200 μ M sodium orthovanadate/0.1 mM PMSF/leupeptin (1 μ g/ml)/1 μ M pepstatin A/1% (v/v) Triton X-100] according to McKenzie and Pouyssegur [19]. Cells were then centrifuged at 15000 *g* for 2 min to pellet non-lysed cells. The supernatants were assayed for PKA activity with [γ -³²P]ATP and with histone 2A as substrate as described previously [20]. Total PKA activity was determined in the presence of 10 μ M cAMP. The activity ratio was calculated by dividing radioactivity (c.p.m.) obtained in the absence of cAMP by that measured in its presence [20].

Determination of total inositol phosphates

BK-induced formation of inositol phosphates in PC-12 cells grown in 24-well dishes was determined as described previously [13]. Briefly, cells were prelabelled with 4 μ Ci of *myo*-[³H]-inositol/ml for 24 h in inositol-free RPMI containing 20 mM Hepes (pH 7.2). Then the cells were stimulated for 10 min with BK at the concentrations indicated in the Figure legends in the presence of 10 mM LiCl. For some experiments, pretreatment with PTX was performed as described for cAMP determination. The reaction was terminated by replacing the medium with 1 ml of 10% (v/v) trichloroacetic acid. Inositol phosphate-containing extracts were washed 4 times with 2 vol. of water-saturated diethyl ether, neutralized by adding Tris base, diluted to 4 ml with distilled water and placed on AG1 $\times$ 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/0.1 M formic acid five times, yielding the inositol phosphate fraction. Radioactivity was measured using a Flo-Scint scintillator (Packard).

PLD assay

Subconfluent PC-12 cells were preincubated with [³H]palmitate (2 μ Ci/ml) in RPMI containing 1% fetal-calf serum and 1% donor horse serum for 14 h. These conditions have been shown to result in the selective incorporation of radioactivity into phosphatidylcholine [21]. PLD activity was determined using a transphosphatidylase assay [21]. Briefly, [³H]palmitate-labelled cells were washed and resuspended in Hepes-buffered RPMI (pH 7.2) containing captopril (1 μ M), 0.3% (v/v) butan-1-ol and BK (1 μ M), PMA (100 nM) or dB-cAMP (1 μ M), as indicated in the legend to Table 5. Incubation was terminated by

removal of the medium (centrifugation at 5000 *g* for 1 min) and lipids were extracted subsequently with 200 μ l of ice-cold methanol and 200 μ l of chloroform. Dried lipid extracts were resolved in 100 μ l of chloroform/methanol (19:1, v/v). The non-metabolizable [3 H]phosphatidylbutanol, which is a direct indicator of PLD activity, was separated from other lipids by TLC in a solvent (upper phase) consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, by vol.) [21]. [3 H]Phosphatidylbutanol, which routinely migrated with an R_F of 0.35, was quantified by excising the appropriate areas and counting for associated radioactivity.

Measurement of p44^{mapk} activity

PC-12 cells, with or without PTX pretreatment (200 ng/ml), were transferred to serum-free Hepes-buffered RPMI and stimulated with BK or LPA for 5 min at 37 °C, as described in the Figure legends. Then, the cells were centrifuged for 1 min at 5000 *g*, the medium was removed and the cells were lysed in 1 ml of lysis buffer [20 mM Hepes (pH 7.5)/10 mM EGTA/40 mM β -glycerophosphate/1% Triton X-100/2.5 mM MgCl₂/2 mM orthovanadate/1 mM dithiothreitol/1 mM PMSF/aprotinin (20 μ g/ml)/leupeptin (20 μ g/ml)]. After a 30-min incubation on ice, the lysates were centrifuged (10 min, 4 °C, 15000 *g*) to pellet insoluble material. The supernatants were transferred into new microtubes and endogenous p44^{mapk} [extracellular signal-related protein kinase (ERK 1)] was immunoprecipitated using a rabbit polyclonal antibody from Santa Cruz Biotechnology. Assays were performed in duplicate using 1 ml of cell lysate and 1 μ g of antibody. Immunoprecipitates were subsequently washed with PBS, containing 1% Triton X-100 and 2 mM orthovanadate, Tris/HCl, pH 7.5, containing 0.5 M LiCl, and kinase buffer [12.5 mM Mops (pH 7.5)/12.5 mM β -glycerophosphate/7.5 mM MgCl₂/0.5 mM EGTA/0.5 mM sodium fluoride/0.5 mM vanadate]. Phosphorylation of immunoprecipitates was performed in 30 μ l of kinase buffer, supplemented with 1 μ Ci of [γ - 32 P]ATP, 20 μ M ATP, 1.5 mg/ml myelin-basic protein and 3.3 μ M dithiothreitol. After 20 min at 30 °C, the reaction was terminated by addition of 10 μ l of SDS/PAGE buffer. The samples were boiled for 5 min and analysed by SDS gel electrophoresis on 12% (w/v) gels. The dried gels were autoradiographed, and the radioactivity incorporated into myelin basic protein was quantified using a Phosphorimager (NIH Image 1.57).

Stimulation of PC-12 cells and membrane preparation for the different PKC determinations

For the measurement of PKC translocation, PC-12 cells were grown in 6-well dishes and subjected to serum-free Hepes-buffered RPMI (pH 7.2), containing 10 μ M captopril for 1 h before stimulation. Cells were then exposed to BK (1 μ M) at 37 °C for various times, as indicated in the Figure legends. For several experiments, cells were pretreated with the PKA inhibitor Rp-cAMP-S (5 μ M) for 15 min, or with the adenylate cyclase inhibitors 2',5'-dideoxyadenosine (500 μ M) or MDL-12,330A (500 μ M) for 20 min. Then BK was added for the incubation times indicated. Stimulation with dB-cAMP (1 μ M) was performed for 15 min. Then, incubation was terminated by removing the cells and centrifugation at 20000 *g* for 2 min (4 °C). The sedimented cells were washed with 2 ml of 50 mM Hepes, pH 7.4, and recentrifuged at 100000 *g* for 20 min (4 °C). The pellets were resuspended in 50 mM Hepes, pH 7.4, containing bacitracin (100 μ g/ml), PMSF (0.1 mM), pepstatin A (1 μ g/ml) and leupeptin (2 μ g/ml), and were stored at -80 °C. Protein concentration was determined according to Lowry et al. [17] or Bradford [18] with BSA as the standard. For preparation of

whole-cell lysates, non-treated PC-12 cells were collected by low-speed centrifugation (1000 *g*, 5 min, 4 °C), washed with PBS, pH 7.4, and centrifuged again. The pellet was treated with lysis buffer [PBS, containing bacitracin (100 μ g/ml), PMSF (0.1 mM), pepstatin A (1 μ g/ml), leupeptin (2 μ g/ml), 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate and 0.1% (w/v) SDS] for 30 min at 4 °C, resuspended and centrifuged at 4 °C and 30000 *g* for 10 min. Immunoprecipitation experiments with Protein A-Sepharose-coupled PKC-isoform-specific antibodies was performed according to the manufacturer's instructions (Santa Cruz).

Western-blot analysis

Proteins of PC-12 cell lysates or membranes from stimulated PC-12 cells were separated on 7.5% gels by SDS/PAGE and transferred to Hybond PVDF membranes (Amersham). After blocking in 1% (w/v) BSA/1% (w/v) non-fat dried milk powder overnight, the PVDF strips were incubated for 1 h with the various PKC antibodies (Santa Cruz; 1 μ g/ml, in blocking solution) as indicated in the Figure legends. The strips were washed twice with Tris-buffered saline, pH 7.6, containing 0.05% (v/v) Tween 20 washing buffer, incubated for 45 min with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz), and washed again four times as described above. Secondary antibodies were then detected using a chemiluminescence (ECL) Western blotting detection system (Amersham) by exposure to Biomax films.

RESULTS

BK stimulates accumulation of intracellular cAMP and PKA activity in PC-12 cells

In intact PC-12 cells, we first measured the BK-induced inositol phosphate formation, which revealed an EC₅₀ value of approx. 3 nM (Figure 1A). Similarly, BK elicited a concentration-dependent increase in intracellular cAMP accumulation with an EC₅₀ value of approx. 2 nM (Figure 1B). The very similar shape

Figure 1 Effects of BK on accumulation of inositol phosphates (A) and intracellular cAMP (B) in intact PC-12 cells

PC-12 cells were incubated at increasing concentrations of BK for 10 min (A) or 20 min (B). The incubations and the determinations of total inositol phosphates (A) or cAMP (B) were performed as described in the Experimental section. Data are means $\pm$ S.E.M. for three separate experiments in duplicate (A) or triplicate (B) determinations. Insets: Time-dependent accumulation of inositol phosphates (A) or cAMP (B) in response to 1 μ M BK. Data are means of two independent experiments in duplicate.

Table 1 Effects of the BK B₂ receptor antagonist Hoe 140 and of PTX on BK-induced inositol phosphate formation and cAMP accumulation in PC-12 cells

Where indicated, PC-12 cells were pretreated with Hoe 140 (1 μ M) for 15 min and then stimulated with 1 μ M BK for 20 min at 37 °C. In another experiment PC-12 cells were incubated with PTX (2 μ g/ml) for 24 h, followed by stimulation with 1 μ M BK for 20 min at 37 °C. All assays were performed in the presence of 1 μ M captopril as described in the Experimental section. Results are expressed as the percentage over control and are means $\pm$ S.E.M. of three separate experiments in duplicate or triplicate. *Significantly higher versus control ($P < 0.05$; Student's t test); **significantly lower versus BK alone ($P < 0.05$; Student's t test).

Treatment	Inositol phosphate formation (%)	cAMP accumulation (%)
None (Control)	100	100
BK (1 μ M)	321 $\pm$ 31*	156 $\pm$ 13*
Hoe 140 (1 μ M)	103 $\pm$ 10	98 $\pm$ 7
Hoe 140 + BK	120 $\pm$ 22**	106 $\pm$ 12**
PTX (2 μ g/ml)	102 $\pm$ 10	115 $\pm$ 9
PTX + BK	325 $\pm$ 26*	160 $\pm$ 15*

Table 2 Effect of BK on the activity of the cAMP-dependent protein kinase (PKA) in PC-12 cells

PC-12 cells were treated with 1 μ M BK in the absence or presence of the BK B₂ receptor antagonist Hoe 140 (1 μ M; 10 min pretreatment) and then processed as described in the Experimental section. The activity ratio was determined by assaying the PC-12 cell extracts without or with 10 μ M cAMP and with 10 mg of histone 2A/ml as substrate. Results are means $\pm$ S.E.M. of four to eight (numbers in parentheses) separate experiments in triplicate determinations: *significantly different from control ($P < 0.05$; Student's t test).

Treatment	Activity ratio
None (control) (8)	0.46 $\pm$ 0.08
BK (1 μ M) (6)	0.72 $\pm$ 0.05*
Hoe 140 (1 μ M) (4)	0.51 $\pm$ 0.04
Hoe 140 + BK (4)	0.50 $\pm$ 0.06

of both concentration–response curves and the ability of the BK B₂ receptor antagonist Hoe 140 to prevent the BK action on inositol phosphate formation, as well as cAMP accumulation (Table 1), suggest that both effects are mediated via the same type of B₂ receptor.

With respect to the time course of BK effects on both pathways, a clear discrepancy can be observed. Compared with the rapid effect of BK on inositol phosphate formation ($t_{1/2} < 1$ min; Figure 1A, inset), the BK-induced increase in cAMP accumulation is delayed ($t_{1/2}$ approx. 6 min; Figure 1B, inset). This finding indicates an indirect effect of BK on the cAMP pathway rather than a direct activation of adenylate cyclase via a G_s protein.

The time course of the BK-induced increase in intracellular cAMP accumulation is paralleled by the increase in PKA activity in response to BK (1 μ M) ($t_{1/2}$ 6–8 min; results not shown). Measured at the time of maximal activation (20 min after BK addition; time equilibrium) the PKA activity ratio (activity of free catalytic subunit released by BK-induced cAMP divided by total kinase activity in the presence of 10 μ M cAMP) [20] was significantly increased by BK compared with the control (Table 2). In the presence of the BK B₂ receptor antagonist Hoe 140, BK failed to activate PKA in PC-12 cells.

Table 3 Effects of different PKC inhibitors or PMA on BK-induced intracellular cAMP accumulation in PC-12 cells

Where indicated, cells were pretreated with the PKC inhibitors bisindolylmaleimide (BIS) (1.25 μ M) or Ro 31-8220 (30 μ M) for 30 min, followed by stimulation with 1 μ M BK and/or 0.1 μ M PMA for 20 min. cAMP was measured as described in the Experimental section. Results are means $\pm$ S.E.M. from two independent experiments in quadruplicate 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	pmol of cAMP/well
None (control)	56.5 $\pm$ 2.0
BK (1 μ M)	66.5 $\pm$ 2.1*
PMA (100 nM)	66.0 $\pm$ 2.6*
BIS (1.25 μ M)	56.1 $\pm$ 1.5
Ro 31-8220 (30 μ M)	55.4 $\pm$ 2.8
BIS + BK	67.8 $\pm$ 3.2*
BIS + PMA	57.1 $\pm$ 1.9
Ro 31-8220 + BK	74.6 $\pm$ 7.4*
Ro 31-8220 + PMA	54.8 $\pm$ 3.0
PMA + BK	76.0 $\pm$ 5.2**

Table 4 Effects of calmodulin antagonists on BK-elicited cAMP accumulation

PC-12 cells were pretreated with the calmodulin antagonists chlorpromazine (30 μ M) or ophiobolin (100 μ M) for 30 min, followed by stimulation with 1 μ M BK for 20 min. Results are means $\pm$ S.E.M. from two independent experiments performed in quadruplicate determinations: *significantly different from control ($P < 0.05$; Student's t test).

Treatment	pmol of cAMP/well
None (control)	56.5 $\pm$ 2.0
BK (1 μ M)	66.5 $\pm$ 2.1*
Chlorpromazine (30 μ M)	56.8 $\pm$ 6.4
Chlorpromazine + BK	58.7 $\pm$ 5.1
Ophiobolin (100 μ M)	53.5 $\pm$ 2.4
Ophiobolin + BK	51.3 $\pm$ 3.5

Influence of various inhibitors and modulators of signal transduction on BK-induced activation of the cAMP pathway

Concerning the time course of BK-induced cAMP accumulation we investigated several routes that may be involved in the indirect activation of different adenylate cyclase isoforms. Treatment of PC-12 cells with either BK or PMA resulted in an equivalent increase in intracellular cAMP accumulation (Table 3). The combination of BK and PMA resulted in an additive cAMP accumulation, suggesting that both agents act via different pathways, i.e. the effect of BK on adenylate cyclase activity is independent of PKC. This result is supported by the findings that the PKC inhibitors bisindolylmaleimide or Ro 31-8220 failed to suppress the BK-induced cAMP accumulation in PC-12 cells, whereas the cAMP accumulation elicited by PMA was prevented by these inhibitors (Table 3). In contrast, the stimulatory effect of BK on cAMP accumulation was completely abolished after pretreatment of PC-12 cells with the calmodulin antagonists chlorpromazine or ophiobolin (Table 4). These findings indicate that calmodulin is essentially involved in the BK-induced increase in adenylate cyclase activity in PC-12 cells.

Effects of BK on both the phosphatidylinositol pathway and the cAMP pathway are mediated via a PTX-insensitive G-protein

The effects of BK on inositol phosphate formation and on cAMP

Figure 2 Comparison of the effects of BK and LPA on the activity of MAPK in PC-12 cells: dependence on pretreatment of PC-12 cells with PTX

Serum-starved PC-12 cells were pretreated for 24 h with PTX (200 ng/ml) and then stimulated by either LPA (2.5 μ M; 5 min) or BK (1 μ M; 10 min). Cell lysates were processed and MAPK activity was determined using myelin basic protein as the substrate, as described in the Experimental section. Experiments were performed twice with similar results. Phosphorylated myelin basic protein was visualized by autoradiography and quantified with a phosphorimager.

Figure 3 Time course for the BK-induced translocation of the PKC isoforms α , β , δ , ϵ and ζ

PC-12 cells were exposed to 1 μ M BK for the times indicated and particulate fractions were prepared as described in the Experimental section. After SDS/PAGE and transfer of protein to PVDF membranes, immunoblotting was performed using antisera against the different PKC isoforms (1 μ g/ml; Santa Cruz). The immunoblots were subjected to background smoothing and quantification using the program NIH Image 1.57. Molecular masses, related to respective marker proteins, are indicated on the right. The results are representative of two or three independent experiments.

accumulation are not inhibited by pretreatment of PC-12 cells with PTX (Table 1), suggesting that no G_i or G_o proteins are involved. In an alternative approach, we studied the activation of the MAPK (ERK 1) by BK and, for control, by LPA. LPA has been reported to activate MAPK in PC-12 cells in a PTX-sensitive, G_i -dependent pathway [22]. Figure 2 shows that MAPK activation by LPA was inhibited by pretreatment with PTX (200 ng/ml), whereas the BK-induced activation of MAPK in PC-12 cells was not affected by PTX. These results suggest that in PC-12 cells a PTX concentration of 200 ng/ml is sufficient to block G_i proteins and, therefore, that the signal transduction of BK includes a G-protein of the $G_{q/11}$ family. Indeed, immunoblotting with specific antibodies against α -subunits of various

Figure 4 Effects of the BK B_2 receptor antagonist, Hoe 140, on the BK-stimulated translocation of the PKC isoforms α , δ , ϵ and ζ

PC-12 cells were exposed to 1 μ M BK for 10 min in the absence or presence of the B_2 receptor antagonist Hoe 140 (1 μ M). Membranes were prepared and analysed by immunoblotting as described in the Experimental section. Representative immunoblots are shown after background smoothing and quantification with the program NIH Image 1.57 of experiments repeated three times with similar results. Molecular masses, related to respective marker proteins, are indicated on the right.

G-proteins revealed the presence of at least G_s , G_i , G_o and $G_{q/11}$ proteins in PC-12 cell membranes (results not shown).

Translocation of the PKC isoforms α , δ , ϵ and ζ after exposure of PC-12 cells to BK

Western blotting of whole-cell extracts established that PC-12 cells contain five PKC isoenzymes (α , β , δ , ϵ and ζ), whereas the PKCs β_{II} and γ do not occur in significant amounts (results not shown). For the classical and novel PKC isoforms, increased association with the particulate fraction (translocation from cytosol to the plasma membrane) is equated with activation [23]. As shown in Figure 3, after exposure of PC-12 cells to 1 μ M BK for various times, only a small increase in the association of PKC β with the particulate fraction was detected, which was not significant throughout the repeated experiments. In contrast, the PKC isoforms α , δ , ϵ and ζ were significantly translocated by BK, but their translocation followed different kinetics. The translocation of the PKCs α and δ is rapid, showing an elevated membrane level after 1 min of treatment with BK and a second increase after 20 min. The membrane levels of PKC ζ are also elevated after 1–3 min of BK stimulation, but show a further increase after long-term treatment. In comparison, PKC ϵ is slowly translocated. An increase in membrane-associated PKC ϵ was observed within only 3–5 min of BK stimulation. To verify the specificity of the BK effect on PKC translocation, PC-12 cells were treated with 1 μ M BK in the absence or presence of 1 μ M BK B_2 receptor antagonist Hoe 140 for 10 min. As shown in Figure 4, Hoe 140 significantly inhibited the effect of BK on PKC translocation, suggesting that the BK-induced translocation of the PKC isoforms α , δ , ϵ and ζ in PC-12 cells is mediated via the B_2 receptor. In Figure 5, the specificity of the respective isoform-specific PKC antibodies is demonstrated.

Figure 5 Specificity of PKC antisera

PC-12 cells were lysed and subjected to immunoprecipitation with the PKC antisera as indicated, followed by SDS/PAGE and immunoblotting with the respective PKC isoform-specific antibodies, as described in the Experimental section. Immunoprecipitation was performed according to the instructions of the manufacturer in the absence and in the presence of a 10-fold excess of the antigenic (control) peptides supplied by Santa Cruz (sc-208P for PKCα, sc-213P for PKCδ, sc-214P for PKCε and sc-216P for PKCζ).

Figure 6 Influence of the PKA inhibitor (PKA-I), Rp-cAMP-S, on the stimulation of PKC isoforms by BK, and dB-cAMP-induced translocation of PKC isoforms

PC-12 cells were pretreated with Rp-cAMP-S (5 μM) for 15 min, followed by stimulation with BK (1 μM) for 10 min. In another set of experiments, PC-12 cells were exposed to 1 μM dB-cAMP for 15 min. Membrane preparation and immunoblotting were performed as described in the Experimental section. Immunoblots were subjected to background smoothing and quantification using the program NIH Image 1.57. Experiments were carried out with five to eight separate PC-12 cell preparations giving similar results. Molecular masses, related to respective marker proteins, are indicated on the right.

Figure 7 Effects of different adenylate cyclase inhibitors on BK-induced translocation of the PKC isoforms ε and ζ

PC-12 cells were pretreated with the adenylate cyclase inhibitors 2',5'-dideoxyadenosine (DDA; 500 μM) or MDL-12,330A (MDL; 500 μM) for 20 min and then stimulated with BK (1 μM) for 10 min. PKC translocation was determined as described in the Experimental section. Immunoblots were processed using the NIH Image 1.57 program. Experiments were replicated three times yielding comparable results. Molecular masses, related to respective marker proteins, are indicated on the right.

Table 5 Formation of [³H]phosphatidylbutanol in PC-12 cells

[³H]Palmitate-labelled PC-12 cells were treated with BK (1 μM), PMA (100 nM) or dB-cAMP (1 μM) for 20 min in the presence of butan-1-ol (0.3%, v/v). Quantification of [³H]phosphatidylbutanol was performed as described in the Experimental section. The c.p.m./well data are from a representative experiment performed in duplicate determinations. The ratio data are pooled across four separate experiments: *significantly different from control (*P* < 0.05; Student's *t* test).

Treatment	[³ H]Phosphatidylbutanol	
	c.p.m./well	Ratio (stimulated/basal)
Basal	3196	1.0
BK (1 μM)	4783	1.38 ± 0.09*
PMA (100 nM)	8276	2.38 ± 0.31*
dB-cAMP (1 μM)	3381	1.02 ± 0.08

Influence of pretreatment of PC-12 cells with inhibitors of adenylate cyclase or PKA on BK-induced PKC translocation

Considering the modulatory action of the PKA inhibitor Rp-cAMP-S on [³H]phorbol 12,13-dibutyrate binding in A 431 cells [13], we firstly investigated the effect of BK on translocation of the PKC isoenzymes α, δ, ε and ζ after pretreatment with Rp-cAMP-S. Figure 6 demonstrates that inhibition of endogenous PKA by Rp-cAMP-S clearly prevented the BK-induced translocation of PKCε, whereas the translocation of the PKCs α, δ and ζ evoked by BK remained almost unchanged. The inhibitor, Rp-cAMP-S, did not significantly modulate the membrane-associated PKC levels. In another set of experiments, PC-12 cells were pretreated with two different inhibitors of adenylate cyclase, 2',5'-dideoxyadenosine or MDL-12,330A, before stimulation with BK. In both cases we observed a specific inhibition of BK-induced translocation of the PKC isoform ε (Figure 7). The effect of BK on PKCζ, which appeared to be slightly decreased in the presence of Rp-cAMP-S (Figure 6) was not significantly modified after inhibition of adenylate cyclase. These results suggest that in PC-12 cells the BK-induced increase in cAMP accumulation represents an upstream event of the BK-induced translocation of PKCε. Compared with the respective control cells we observed an increase in the membrane levels of PKCε and also, very surprisingly, an increase in membrane-associated PKCα, PKCδ and PKCζ after treatment of PC-12 cells with dB-cAMP (1 μM). This finding indicates an effect of cAMP on the translocation of the PKCs α, δ and ζ that is independent of the BK pathway.

cAMP has no effect on the activity of PLD

In order to prove whether or not the intracellular cAMP accumulation in response to BK has an effect on phospholipid signalling, especially the generation of phosphatidic acid in PC-12 cells, we investigated the influence of dB-cAMP on the activity of PLD using a transphosphatidyl assay. In Table 5, the PLD-catalysed formation of [³H]phosphatidylbutanol after stimulation of [³H]palmitate-labelled PC-12 cells with BK, PMA or dB-cAMP is summarized. BK, as well as PMA, induced a substantial increase in PLD activity with 1.38-fold and 2.38-fold respectively over basal. In contrast, there is an apparent lack of PLD stimulation by dB-cAMP.

DISCUSSION

In this study, we present evidence that in PC-12 cells BK is capable of stimulating the cAMP/PKA pathway secondary to the phosphatidylinositol pathway. Furthermore, it is demon-

strated that BK evokes the translocation of the PKC isoforms α , δ , ϵ and ζ and that specifically the BK-induced translocation of nPKC ϵ is mediated via the cAMP pathway.

In intact PC-12 cells, BK increased the accumulation of intracellular cAMP, yielding an EC₅₀ value of approx. 2 nM. This value correlates very well with both the EC₅₀ value of approx. 3 nM for the BK-induced increase in inositol phosphate formation and the K_d value of 4 nM reported for the binding of [³H]BK to BK B₂ receptors expressed in PC-12 cells [4]. In addition, it was demonstrated that BK stimulates the activity of PKA in PC-12 cells. These results are contradictory to a previous report that exposure of PC-12 cells to BK did not increase intracellular cAMP levels [7].

The lagging time course of BK-induced cAMP accumulation in PC-12 cells suggests that the BK action on the cAMP pathway might be a secondary event. The lack of different PKC inhibitors to prevent the BK-induced increase in cAMP accumulation, as well as the additive effect of PMA and BK, indicate that the stimulation of adenylate cyclase by BK is not mediated via PKC. The effect of BK on cAMP accumulation is insensitive to PTX, excluding that $\beta\gamma$ -complexes from G_i proteins may be activators of adenylate cyclase in PC-12 cells. In contrast, the BK-induced cAMP accumulation was clearly inhibited by the calmodulin antagonists chlorpromazine or ophiobolin, suggesting the involvement of a Ca²⁺/calmodulin-sensitive adenylate cyclase. Among the various isoforms of adenylate cyclase identified up to now, the type I and VIII isoenzymes (and type III to a lesser extent) may be activated by Ca²⁺ in the presence of calmodulin. In addition, type III adenylate cyclase has been demonstrated to be inhibited due to phosphorylation by calmodulin kinase [24,25]. It is assumed therefore that in PC-12 cells adenylate cyclase type I, III or VIII may be stimulated by BK, because of its effect on the phosphatidylinositol pathway to enhance the intracellular Ca²⁺ concentration. However, it was not the aim of the present work to characterize the adenylate cyclase isoform involved in BK action in detail. Instead we focused our interest on the putative biological importance of the BK-induced cAMP accumulation in PC-12 cells.

In PC-12 cells, not only the cAMP-elevating peptides VIP [2] or the VIP similar pituitary adenylate cyclase-activating polypeptide [26], but also PKC activation by phorbol esters [27], have been shown to promote neurite outgrowth, as does BK. In A431 cells, we identified a feedback inhibition of BK-induced PKC translocation, by a lagging activation of the cAMP/PKA pathway, evoked by BK [13,28]. Furthermore, there are several reports about the activation of different PKC isoforms by BK [29–31]. These data suggested a possible link between cAMP and PKC in BK signalling.

As assessed by immunoblotting, PC-12 cells express the PKC isoforms α , β , δ , ϵ and ζ (results not shown). The PKCs β , δ and γ were not detectable in significant amounts. It cannot be excluded, however, that still other PKC isoforms, such as η , θ , λ or μ , may also be expressed in PC-12 cells. Among the PKC isoforms detected, the translocation of PKCs α , δ , ϵ and ζ was significantly stimulated by BK with different kinetics. For the BK-induced translocation of the PKCs α and δ , we observed rapid and biphasic kinetics with a second increase after long-term stimulation. The PKCs ϵ and ζ , in contrast, revealed a slower and slightly transient translocation after stimulation with BK. Similar biphasic kinetics for translocation of the PKC isoforms α , ϵ and ζ in response to BK were found in fibroblasts [29], whereas in rat myocytes BK evoked only a rapid and transient translocation of the PKCs δ and ϵ [31]. In PC-12 cells, the BK-induced translocation of PKC ϵ is significantly diminished after pretreatment with inhibitors of adenylate cyclase as well as of PKA. The

translocation of the PKCs α and δ in response to BK remained unchanged when the endogenous cAMP system was inhibited before the addition of BK. The BK-induced translocation of PKC ζ appeared to be slightly affected in the presence of the PKA inhibitor, Rp-cAMP-S, but inhibition of adenylate cyclase failed to decrease the BK effect in a significant manner. Thus, our findings suggest that the BK-induced translocation of nPKC ϵ may be mediated via the cAMP pathway. Surprisingly, stimulation of PC-12 cells with 1 μ M dB-cAMP significantly increased the membrane-associated levels not only of PKC ϵ , but also of the PKCs α , δ and ζ , independently of BK. Since PKC translocation to the membrane requires a lipid source, we studied whether dB-cAMP might be capable of stimulating the activity of PLD resulting in the formation of phosphatidic acid or, indirectly, DAG for the activation of PKC. In PC-12 cells both BK and the phorbol ester PMA clearly increased the concentration of [³H]-phosphatidylbutanol, indicating a stimulation of PLD activity. The order of magnitude of PLD stimulation is comparable with those previously published by Purkiss et al. [32]. In contrast, we obtained no evidence of dB-cAMP stimulation of PLD activity in PC-12 cells, suggesting that cAMP does not act on PLD. It may be assumed that various PKC isoforms may be translocated alternatively by cAMP, depending on, for instance, cAMP concentration, cell compartmentalization, the absence or presence of other second messengers or the kinetics of second-messenger generation within dual signalling pathways.

The translocation of PKC depends on two equally important regulatory mechanisms. Firstly, the enzyme is rendered catalytically competent by a transphosphorylation, followed by two autophosphorylations, that release the protein into the cytosol. Secondly, the generation of DAG (or probably other lipid-derived second messengers) results in membrane translocation, pseudosubstrate release from the substrate binding site and substrate phosphorylation (for a review see [33,34]). The primary transphosphorylation is mediated via a putative PKC kinase. It may be speculated that the BK-activated PKA might act as a heterologous PKC kinase that induces the first phosphorylation event in the activation process of PKC ϵ . We are currently examining a putative role for PKA for transphosphorylation of PKC isoforms.

In conclusion, our results suggest that in PC-12 cells, BK is capable of activating the phosphoinositide pathway and, subsequently, the cAMP pathway. Among four different PKC isoforms which are translocated by BK in PC-12 cells, the translocation of nPKC ϵ is mediated via the cAMP/PKA pathway. Obviously, multiple pathways may be involved in proliferation and/or neuronal differentiation of PC-12 cells, as evidenced by activation of MAP kinase [8,35], activation of c-Jun N-terminal kinases [36], or activation of the proline-rich tyrosine kinase PYK2 [37]. Very recently, nPKC ϵ was demonstrated to be capable of activating Raf kinase, upstream of MAP kinase [38]. Our results define a novel proximal effector system that might link the cAMP pathway with the MAP kinase pathway in PC-12 cells.

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