Stimulation of phosphatidylinositol hydrolysis, protein kinase C translocation, and mitogen-activated protein kinase activity by bradykinin in rat ventricular myocytes: dissociation from the hypertrophic response

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In ventricular myocytes cultured from neonatal rat hearts, bradykinin (BK), kallidin or BK(1–8) [(Des-Arg^9)BK] stimulated PtdInsP_2 hydrolysis by 3–4-fold. EC_{50} values were 6 nM (BK), 2 nM (kallidin), and 14 μM [BK(1–8)]. BK or kallidin stimulated the rapid (less than 30 s) translocation of more than 80 % of the novel protein kinase C (PKC) isoforms nPKC-δ and nPKC-ϵ from the soluble to the particulate fraction. EC_{50} values for nPKC-δ translocation by BK or kallidin were 10 and 2 nM respectively. EC_{50} values for nPKC-ϵ translocation by BK or kallidin were 2 and 0.6 nM respectively. EC_{50} values for the translocation of nPKC-δ and nPKC-ϵ by BK(1–8) were more than 5 μM. The classical PKC, cPKC-α, and the atypical PKC, aPKC-ζ, did not translocate. BK caused activation and phosphorylation of p42-mitogen-activated protein kinase (MAPK) (maximal at 3–5 min, 30–35 % of p42-MAPK phosphorylated). p44-MAPK was similarly activated. EC_{50} values for p42/p44-MAPK activation by BK were less than 1 nM whereas values for BK(1–8) were more than 10 μM. The order of potency [BK $\approx$ kallidin $\gg$ BK(1–8)] for the stimulation of PtdInsP_2 hydrolysis, nPKC-δ and nPKC-ϵ translocation, and p42/p44-MAPK activities suggests involvement of the B_2 BK receptor subtype. In addition, stimulation of all three processes by BK was inhibited by the B_2 BK receptor-selective antagonist HOE140 but not by the B_1-selective antagonist Leu^{8}BK(1–8). Exposure of cells to phorbol 12-myristate 13-acetate for 24 h inhibited subsequent activation of p42/p44-MAPK by BK suggesting participation of nPKC (and possibly cPKC) isoforms in the activation process. Thus, like hypertrophic agents such as endothelin-1 (ET-1) and phenylephrine (PE), BK activates PtdInsP_2 hydrolysis, translocates nPKC-δ and nPKC-ϵ, and activates p42/p44-MAPK. However, in comparison with ET-1 and PE, BK was only weakly hypertrophic as assessed by cell morphology and patterns of gene expression. This difference could not be attributed to dissimilarities between the duration of activation of p42/p44-MAPK by BK or ET-1. Thus activation of these signalling pathways alone may be insufficient to induce a powerful hypertrophic response.

INTRODUCTION

The actions of vasoactive peptides ET-1 and angiotensin II on the heart have been an area of much research recently. In addition to acting as vasoconstrictors in the coronary vasculature through their actions on vascular smooth muscle, ET-1 (reviewed in [1]) and angiotensin II [2–4] directly influence the contractile state and hypertrophic growth of the ventricular myocyte, probably through the PtdInsP_2 signalling pathway (reviewed in [5]). PtdInsP_2 hydrolysis produces two signalling molecules, diacylglycerol (DG) and InsP_3. DG is the physiological activator of the classical and novel isoforms of protein kinase C (PKC) (reviewed in [6]), whereas InsP_3 regulates intracellular Ca^{2+} movements (reviewed in [5]).

Less attention has been paid to the actions of the vasoactive kinins [the nonapeptide bradykinin (BK) and the decapeptide kallidin (Lys-BK)] on the ventricular myocyte. These peptides are produced locally by the kallikrein–kinin system present in the coronary vasculature and are vasodilatory [7–9]. Two BK receptors (B_1 and B_2) have been cloned and both belong to the G-protein-coupled heptahelical superfamily [10–12]. In ventricular myocytes cultured from neonatal rats, the B_2 receptor is principally responsible for BK binding and, like the ET-1 and angiotensin II receptors, is coupled to the hydrolysis of membrane PtdInsP_2 [13].

Because it is cardioprotective (reviewed in [14,15]) and might improve cardiac function [16], there is considerable interest in the role of BK in the heart. The signalling pathways distal to BK-activated PtdInsP_2 hydrolysis are relatively poorly characterized in this tissue. We therefore studied the effects of BK and related peptides on the hydrolysis of PtdInsP_2, translocation of PKC isoforms and stimulation of p42-MAPK and p44-MAPK activities (where MAPK is mitogen-activated protein kinase) in neonatal rat ventricular myocytes. Stimulation of PtdInsP_2 hydrolysis, and PKC and p42/p44-MAPK activities, may mediate the hypertrophic response of these myocytes to agonists (reviewed in [17,18]). Thus we also investigated whether BK caused any of the alterations associated with the development of the hypertrophic phenotype.

EXPERIMENTAL

General materials

Radiochemicals, prestained molecular mass standard proteins, horseradish peroxidase-linked donkey anti-rabbit immuno-

Abbreviations used: ANF, atrial natriuretic factor; BK, bradykinin; BK(1–8), [Des-Arg^9]BK; DG, diacylglycerol; ET-1, endothelin-1; ECL, enhanced chemiluminescence; InsP_x, pooled inositol mono-, bis- and tris-phosphates; LUX, luciferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MHC, myosin heavy chain; PBST, PBS/Tween-20; PE, phenylephrine; PKC, the protein kinase C family consisting of classical (cPKC), novel (nPKC) and atypical (aPKC) subfamilies; PMA, phorbol 12-myristate 13-acetate; SkM, skeletal muscle; SRE, serum response element; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. We have used the p42-MAPK and p44-MAPK terminology throughout this paper. It should be noted that p44-MAPK is the same enzyme as extracellular signal-regulated protein kinase (ERK1) and p42-MAPK is the same enzyme as ERK2.

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globulin, the ECL Western blotting detection reagents, autoradiography film (Hyperfilm MP) and intensifying screens were from Amersham International (Amersham, Bucks., U.K.). SDS/PAGE reagents, AG1 × 8 ion exchange resin (formate form) and the reagent for the assay of protein by the Bradford method [19] were from Bio-Rad (Hemel Hempstead, Herts., U.K.). Schleicher & Schuell nitrocellulose (0.45 μm) was obtained from Anderman & Co. (Kingston-upon-Thames, Surrey, U.K.) The peptide inhibitor of cAMP-dependent protein kinase and HOE140 were from Bachem (Saffron Walden, Essex, U.K.). Otherwise, agonists, Leu^{8}BK(1–8) (Leu^8[Des-Arg^9]BK), medium 199, Dulbecco's modified Eagle's medium, protease inhibitors and other biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Falcon Primaria culture dishes were from Marathon Laboratory Supplies (London, U.K.). Chamber slides and additional tissue culture products were from Life Technologies (Paisley, Scotland, U.K.). Fluorescent mounting medium was from Dako (High Wycombe, Bucks., U.K.). Other laboratory chemicals were from Merck (Lutterworth, Leics., U.K.). PE was dissolved in 100 μM L-ascorbic acid and used fresh daily. Stock solutions of agonists were diluted in serum-free medium before use.

Antisera and antibodies

With the exception of anti-PKC-α, anti-PKC antisera were against oligopeptide sequences corresponding to sequences in the C-terminal V_5 regions of rat PKC isoforms. Antisera against the nPKC-δ sequence VNPKYEQFLE and the nPKC-ϵ sequence KGFSYFGEDLMP were from Life Technologies. A mouse monoclonal antibody against an 18 kDa fragment of rat brain cPKC-α (residues 270–427) from Transduction Laboratories (Lexington, KY, U.S.A.) was obtained through Affiniti Research Products (Exeter, Devon, U.K.). Antiserum against the aPKC-ζ sequence INPLLLSAEESV was a gift from Dr. P. J. Parker, Imperial Cancer Research Fund Laboratories, London, U.K. Antiserum 124 raised against the C-terminal peptide sequence KEKLKELIFEETAR from mouse p42-MAPK [20] was a gift from Professor C. J. Marshall, Chester Beatty Laboratories, Institute of Cancer Research, London, U.K. For immunostaining, the mouse monoclonal antibody to rat β-myosin heavy chain (βMHC) was from Novocastra (Newcastle-upon-Tyne, Tyne and Wear, U.K.), and biotinylated anti-mouse IgG and streptavidin–Texas Red was from Amersham International.

Fusion gene reporter plasmids

The reporter plasmid pANF(−638)LΔ5′, which contains nt −638 to +62 of 5′ sequence flanking the transcriptional initiation site of the atrial natriuretic factor (ANF) gene inserted into the luciferase (LUX) reporter plasmid pSV0ALΔ5′ [21], was a gift from Dr. K. R. Chien, Department of Medicine, University of California, San Diego, CA, U.S.A. The reporter construct for βMHC consisted of nt −667 to +38 of the rat βMHC gene cloned into the LUX reporter expression vector pXP1 [22]. The skeletal muscle (SkM) α-actin reporter construct contained nt −394 to +24 of the chicken SkM α-actin gene cloned into pXP1 [22–24]. The c-*fos* serum response element (SRE) reporter construct contained nt −318 to −291 centred on the CC(A/T)$_6$GG (nt −309 to −300) sequence of the murine c-*fos* SRE that was placed upstream of a neutral murine c-*fos* promoter (nt −56 to +109) [25] and subcloned into a pXP2 LUX reporter expression vector [24]. The βMHC, SkM α-actin and c-*fos* SRE reporter constructs were gifts from Dr. M. D. Schneider, Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX, U.S.A. Plasmid pON249 [26] (also from Dr. K. R. Chien) in which β-galactosidase expression is controlled by a constitutive cytomegalovirus promoter was co-transfected to control for transfection efficiency.

Ventricular myocyte cultures

Myocytes were isolated from the ventricles of 1–2-day-old rats and were cultured by a method based on that of Iwaki et al. [27]. Myocytes were dissociated with 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in 116 mM NaCl, 20 mM Hepes, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$ (pH 7.35). The cells were resuspended in Dulbecco's modified Eagle's medium/medium 199 (4:1, v/v) supplemented with 10 % (v/v) horse serum, 5 % (v/v) fetal calf serum, and penicillin and streptomycin (each at 100 i.u./ml). Cells were preplated for 30 min on uncoated 60 mm culture dishes to reduce fibroblast contamination. Unless stated otherwise, myocytes were plated at a final density of 1.4×10^3 cells/mm^2 on 60 mm gelatin-precoated dishes in 4 ml of medium. After 18 h, myocytes were confluent and beating spontaneously. Serum was then withdrawn for 24 h and the cells were exposed to agonists in serum-free medium.

Preparation of the soluble and particulate fractions of myocytes

Cells were washed three times in 1 ml of ice-cold PBS and lysed in 150 μl of Buffer A [12.5 mM Tris/HCl, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM dithiothreitol, 300 μM PMSF, 120 μM pepstatin A, 200 μM leupeptin, 10 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, pH 7.4] containing 0.05 % digitonin [28,29]. The extracts were incubated for 5 min at 4 °C. For the determination of subcellular distribution, extracts were centrifuged (Eppendorf 5414 microcentrifuge, at 10000 ***g*** for 15 min at 4 °C). The soluble fraction was retained. The particulate fraction was washed in 150 μl of Buffer A, centrifuged and finally resuspended in the same volume of Buffer A containing 1 % (v/v) Triton X-100. Protein concentrations were determined by the Bradford method [19].

Hydrolysis of PtdInsP_2

Myocytes (1.4×10^3 cells/mm^2, 35 mm dishes) were incubated with serum-free medium (2 ml) containing 5 μCi of [^{3}H]inositol for 24 h. They were then exposed to agonists and antagonists in serum-free medium containing 10 mM LiCl. The incubation was terminated by the removal of medium and addition of 0.8 M $HClO_4$ (0.5 ml). Each plate was scraped and then rewashed with 0.8 M $HClO_4$ (0.5 ml). After removal of the precipitated protein by centrifugation at 2000 ***g***, the supernatant fractions were neutralized with 5 M KOH/0.5 M Tris base and were applied to columns of AG1 × 8 ion-exchange resin (formate form). Purification of pooled inositol mono-, bis- and tris-phosphates (InsP_x) was done with a method based on that of Berridge et al. [30], which we have described previously [31].

Immunoblotting

Soluble fractions from myocytes were heated at 100 °C with 0.33 vol. of SDS sample buffer [10 % (w/v) SDS, 13 % (v/v) glycerol, 300 mM Tris/HCl, 130 mM dithiothreitol, 0.2 % Bromophenol Blue, pH 6.8] whereas particulate fractions were solubilized by heating in a volume of SDS sample buffer equal to the volume of the original extract. Proteins (20–40 μg) were separated by SDS/PAGE on a 10 % (w/v) polyacrylamide resolving gel with a 6 % (w/v) stacking gel and were transferred electro-

phoretically to nitrocellulose [29]. Non-specific binding sites were blocked with 5% (w/v) non-fat milk powder in PBST [PBS containing 0.05% (v/v) Tween-20, pH 7.5] for 30 min at room temperature. Primary antibodies were diluted in blocking solution (1:250 for anti-PKC-α, 1:500 for anti-PKC-δ and anti-PKC-ϵ, 1:2000 for anti-PKC-ζ and anti-p42-MAPK). Nitrocellulose was incubated with primary antibodies overnight at 4 °C. Preliminary experiments established the specificity of immunoreactivity by the use of the appropriate competing peptide antigen (2 μg/ml). After washing in PBST (three times for 5 min each), nitrocellulose was incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody [1:5000 in 1% (w/v) non-fat milk powder in PBST]. After repeating the washing procedure described above, bound antibody was detected by the enhanced chemiluminescence (ECL) method with exposure to Hyperfilm for 1–15 min (depending on signal strength). Immunoblots were quantified by laser scanning densitometry.

Detection of p42-MAP and p44-MAPK activities by the 'in gel' phosphorylation of myelin basic protein (MBP)

Denatured soluble or particulate fractions in SDS sample buffer were prepared as described above. Phosphorylation of MBP in 10% (w/v) polyacrylamide gels containing 0.5 mg/ml MBP after SDS/PAGE was studied by a previously described adaptation [32–34] of the method of Kameshita and Fujisawa [35]. Quantification was by laser scanning densitometry.

Immunocytochemistry

Permanox eight-well (1 cm square wells) tissue culture chamber slides (precoated with gelatin) were treated with laminin (20 μg/ml in PBS) for 3 h at room temperature. Unbound laminin was removed by washing three times with PBS. Myocytes were plated at a density of 5×10^4 cells per well in 0.5 ml of medium. Serum was withdrawn after 18 h; after 24 h in serum-free medium, myocytes were exposed to agonists for 24 h. The cells were washed three times in PBS and fixed in 4% (v/v) formaldehyde for 10 min. The cells were permeabilized with 0.3% (v/v) Triton X-100 (10 min, room temperature) and non-specific binding was blocked with 1% (w/v) BSA in 0.3% Triton X-100 (10 min, room temperature). Antibodies etc. were diluted in 0.3% Triton X-100 in PBS before use and myocytes were washed three times in PBS after each stage of the immunostaining procedure. βMHC was detected with a mouse monoclonal anti-βMHC primary antibody (1:40 dilution, 1 h at 37 °C), a biotinylated anti-mouse IgG secondary antibody (1:200 dilution, 30 min at 37 °C), and streptavidin–Texas Red (1:200 dilution, 15 min at room temperature).

Transient transfection

At 24 h after the initial plating, myocytes (350 cells/mm², 60 mm dishes, 4 ml of medium) were transfected [36] with 15 μg of LUX reporter plasmid plus 4 μg of pON249 per dish by a calcium phosphate precipitation method. Plasmids were diluted in 0.25 M $CaCl_2$ and an equal volume of 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid, pH 6.9, containing 280 mM NaCl and 1.5 mM Na_2HPO_4 was added. After 20 min, cells were transfected with this suspension (1 ml per 60 mm dish). After transfection for 16–20 h, cells were washed in maintenance medium containing 10% horse serum, then twice with maintenance medium. Cells were incubated for 48 h in 4 ml of maintenance medium containing agonists as appropriate, washed twice with ice-cold PBS and extracted on ice with 0.4 ml of 0.1 M potassium phosphate, pH 7.9, containing 0.5% (v/v) Triton X-100 and 1 mM dithiothreitol for 15 min. LUX activity was assayed in 0.5 ml of 100 mM Tricine, pH 7.8, containing 10 mM $MgSO_4$, 2 mM EDTA, 75 μM luciferin and 5.5 mM ATP. Light emitted was measured with an LKB 1219 RackBeta liquid-scintillation counter with the photomultipliers set out of coincidence. β-Galactosidase was assayed in 0.3 ml of 66 mM Na_2HPO_4/NaH_2PO_4, pH 7.3, containing 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 4.4 mM *o*-nitrophenyl β-δ-galactopyranoside at 37 °C for 1 h, after which 0.5 ml of 0.5 M Na_2CO_3 was added and the absorbance at 410 nm measured. To assess transfection efficiency, myocytes were washed twice with ice-cold PBS, fixed with 4% formaldehyde for 10 min, and stained with 0.2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. The number of blue cells in 100 fields was counted for each treatment.

Statistical methods and curve fitting

Results are presented as means ± S.E.M. Statistical significance was tested by a two-tailed Student's *t* test and was taken as being established at $P < 0.05$. Fitting of logarithmic concentration-dependence data to sigmoid curves used the GraphPad Inplot 4 program (GraphPad Software Inc., San Diego, CA, U.S.A.).

RESULTS

Stimulation of PtdInsP_2 hydrolysis by BK, kallidin and BK(1–8)

BK, kallidin and BK(1–8) stimulated PtdInsP_2 hydrolysis in a concentration-dependent manner (Figure 1). There was a marked difference between log EC_{50} values for BK (6 nM) and kallidin (3 nM) on the one hand, and BK(1–8) (14 μM) on the other (Table 1). In separate experiments, the maximal rates of PtdInsP_2 hydrolysis were measured in 10 min incubations with four separate preparations of myocytes at high concentrations of agonists. Values were similar for all three agonists. At 1 μM BK ($170 \times EC_{50}$), the maximal stimulation of PtdInsP_2 hydrolysis relative to the control (no agonist) was 3.73 ± 0.47. The corresponding values for 0.1 μM kallidin ($33 \times EC_{50}$) and 100 μM BK(1–8) ($7 \times EC_{50}$) were 3.34 ± 0.28 and 2.99 ± 0.53 respectively. Interestingly, the maximal stimulation of PtdInsP_2 by BK, kallidin or BK(1–8) was significantly less ($P < 0.001$) than with high concentrations [0.1 μM; $67 \times EC_{50}$ (M. A. Bogoyevitch and P. H. Sugden, unpublished work)] of ET-1, where the stimulation relative to the control was 9.87 ± 1.06.

The order of potency for stimulation of PtdInsP_2 hydrolysis [i.e. BK ≈ kallidin ≫ BK(1–8)] is consistent with stimulation through the B_2 receptor and this was confirmed by the use of selective antagonists [37–40]. At a BK concentration of 1 μM, the B_2 receptor-selective antagonist HOE140 inhibited the stimulation of PtdInsP_2 hydrolysis by 46 ± 9% at a HOE140 concentration of 1 μM and by 88 ± 6% at a HOE140 concentration of 10 μM. In contrast, 50 μM Leu^{8}BK(1–8) (a B_1 receptor-selective antagonist) did not significantly inhibit the stimulation of PtdInsP_2 hydrolysis by 1 μM BK. Three separate preparations of myocytes were used in these experiments. Initial control experiments showed that HOE140 or Leu^{8}BK(1–8) alone did not inhibit basal rates of PtdInsP_2 hydrolysis.

Translocation of PKC isoforms

Myocytes were exposed to high concentrations (1 μM) of BK, then soluble and particulate fractions were prepared and immuno-

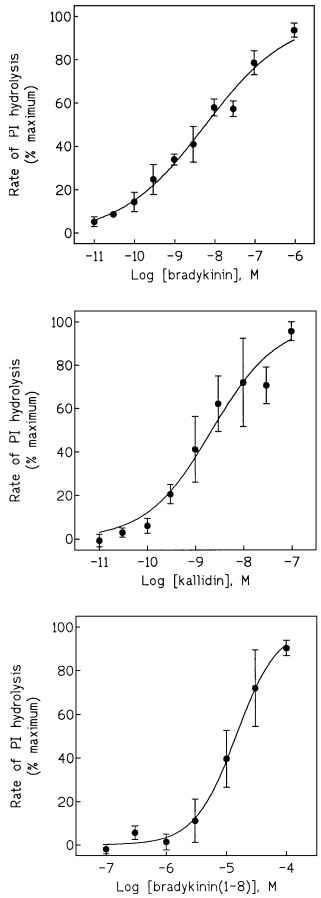

Figure 1 The dependence of the stimulation of PtdInsP_2 hydrolysis on the concentration of kinins

Myocytes were prelabelled with [^{3}H]inositol for 24 h, exposed to agonists for 10 min, and PtdInsP_2 hydrolysis was measured at various concentrations of BK (top panel), kallidin (middle panel) and BK(1–8) (bottom panel) as described in the Experimental section. The results (in terms of d.p.m. of InsP_x formed) from an individual experiment were fitted to a sigmoid curve (GraphPad InPlot 4 software). From the individual extrapolated maxima and minima of d.p.m. of InsP_x formed, the rates of PtdInsP_2 hydrolysis were calculated as percentages of the maximal rates, and means ± S.E.M. (for four separate myocyte preparations) were subsequently calculated.

blotted with isoform-specific antisera against cPKC-α, nPKC-δ, nPKC-ϵ or aPKC-ζ (Figure 2A). The isoforms nPKC-δ and nPKC-ϵ translocated rapidly (essentially complete in less than 30 s) from the soluble to the particulate fraction (Figures 2A and 2B). Approx. 80 % of the soluble nPKC-δ and 90 % of the soluble nPKC-ϵ translocated (Figure 2B). Translocation was almost completely reversed by 3 min (Figure 2B). There was no evidence that cPKC-α or aPKC-ζ translocated (Figure 2A).

Translocation of nPKC-δ or nPKC-ϵ was dependent on BK concentration (Figure 3A). The log EC_{50} value (Table 1) for the translocation of nPKC-δ from the soluble fraction by BK (EC_{50} 10 nM) was significantly greater ($P < 0.05$) than that for nPKC-ϵ (EC_{50} 2 nM). Kallidin (Figure 3B) also caused a concentration-dependent translocation of both nPKC-δ (EC_{50} 2 nM) and nPKC-ϵ (EC_{50} 0.6 nM) although the log EC_{50} values were not significantly different (Table 1). The EC_{50} values for the translocation of nPKC-δ and nPKC-ϵ by BK(1–8) were at least three orders of magnitude greater and again nPKC-ϵ translocated more readily than nPKC-δ (Table 1).

Table 1 Log EC_{50} values for BK, kallidin and BK(1–8)

Log EC_{50} values were calculated from sigmoid curve fitting of results from individual experiments (for composite data, see Figure 1 for PtdInsP_2 hydrolysis, Figures 3A and 3B for PKC translocation, and Figures 6A and 6B for p42/p44-MAPK activation). The individual log EC_{50} values were averaged and are expressed as means ± S.E.M. for three to five separate preparations of myocytes). Exceptions to this general protocol were as follows. For the translocation of PKC-δ or PKC-ϵ by BK(1–8), fitting of the three experiments to sigmoid curves was unsuccessful so the composite curves were used. For the activation of p42-MAPK and p44-MAPK by BK(1–8), two experiments were performed. Abbreviation: n.d., not determined.

	log [EC_{50} (M)]		
Agonist ...	BK	Kallidin	BK(1–8)
PtdInsP_2 hydrolysis	−8.20 ± 0.19	−8.58 ± 0.33	−4.85 ± 0.21
Translocation of PKC-δ	−8.02 ± 0.17	−8.63 ± 0.49	−4.27
Translocation of PKC-ϵ	−8.65 ± 0.11	−9.22 ± 0.39	−5.07
Activation of p42-MAPK	−9.42 ± 0.18	n.d.	−4.06, −5.14
Activation of p44-MAPK	−9.07 ± 0.10	n.d.	−4.79, −5.03

In myocytes exposed to 1 μM BK, the translocation of nPKC-δ was inhibited by approx. 60–70 % by 10 μM HOE140 and was completely inhibited by 50 μM HOE140 (Figure 4A). The BK-induced translocation of nPKC-ϵ was inhibited by approx. 60 % by 50 μM HOE140 (Figure 4B). This less extensive inhibition presumably reflects the lower EC_{50} of nPKC-ϵ translocation for BK (Table 1). Leu^{8}BK(1–8), at concentrations up to 50 μM, did not inhibit translocation of either nPKC-δ or nPKC-ϵ (Figures 4A and 4B). The spectrum of agonism and antagonism shows that the translocation of nPKC-δ and nPKC-ϵ is mediated through the B_2 receptor.

Activation and phosphorylation of p42-MAPK and p44-MAPK

'In gel' MAPK assays showed that 1 μM BK maximally activated p42-MAPK and p44-MAPK within 2–3 min (Figure 5A). After their phosphorylation, the mobilities of p42-MAPK and p44-MAPK are slightly decreased on SDS/PAGE [34], enabling the proportion of the total MAPK pool phosphorylated to be measured by immunoblotting. The phosphorylation of p42-MAPK (Figure 5B) was maximal within 3 min, reaching approx. 30–35 % of the total p42-MAPK pool. The anti-p42-MAPK antiserum used (antiserum 124) cross-reacts with the p44-MAPK [20]. Although antiserum 124 detected unphosphorylated p44-MAPK, no decreased-mobility band was detected on exposure of myocytes to BK (Figure 5B). The likely explanation is that anti-p42-MAPK antiserum did not cross-react well with phosphorylated p44-MAPK [34].

The EC_{50} values for the activation of p42-MAPK and p44-MAPK by BK as determined by 'in gel' MAPK assays (Figure 6A) were 0.4 and 0.9 nM respectively (Table 1). In contrast, BK(1–8) was at least four orders of magnitude less potent (Figure 6B and Table 1). Activation of p42-MAPK and p44-MAPK by 1 μM BK was inhibited by 75–95 % by 50 μM HOE140 whereas 50 μM Leu^{8}BK(1–8) did not inhibit activation (Figure 6C).

The participation of PKC in the pathway of activation of p42/p44-MAPK by BK was examined in PKC down-regulation experiments. cPKC-α, nPKC-δ, nPKC-ϵ and aPKC-ζ are present

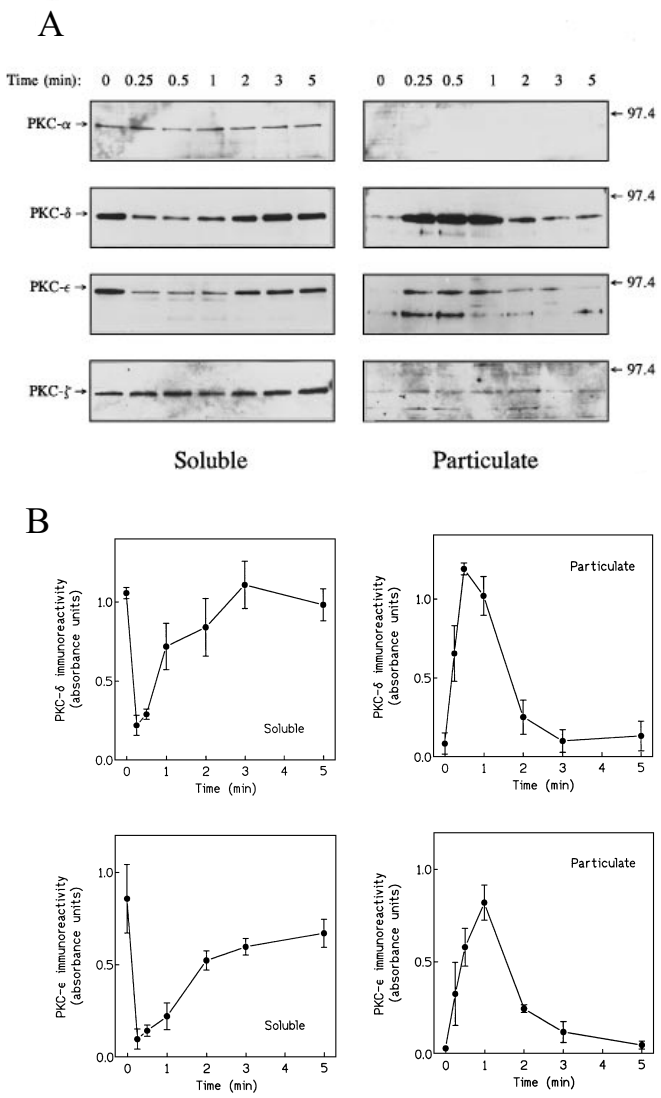

Figure 2 Effects of BK on the translocation of PKC isoforms from the soluble to the particulate fraction

Myocytes were exposed to 1 μM BK for the times indicated, and denatured soluble and particulate fractions were prepared as described in the Experimental section. For SDS/PAGE, protein loading was 20 μg per lane for soluble fractions and 40 μg per lane for particulate fractions. After SDS/PAGE and transfer of protein to nitrocellulose, immunoblotting was performed as described in the Experimental section with ECL detection. (**A**) Immunoblots for cPKC-α, nPKC-δ, nPKC-ϵ and aPKC-ζ. Experiments were performed with three separate myocyte preparations with similar results. (**B**) Immunoblots for the soluble and particulate fractions of nPKC-δ (upper panels) or nPKC-ϵ (lower panels) quantified by laser scanning densitometry. Results are means $\pm$ S.E.M. for three separate myocyte preparations.

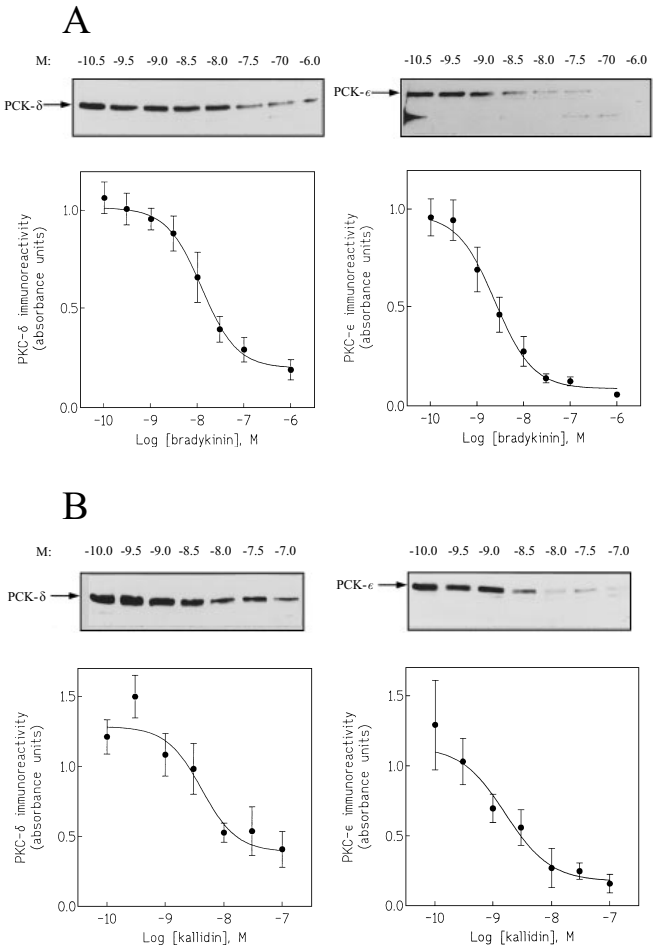

Figure 3 Dependence of the translocation of nPKC-δ and nPKC-ε on BK or kallidin concentration

Myocytes were exposed to (**A**) BK or (**B**) kallidin for 30 s, and denatured soluble fractions were prepared as described in the Experimental section. For SDS/PAGE, protein loading was 20 μg per lane. After SDS/PAGE and transfer of protein to nitrocellulose, immunoblotting of nPKC-δ or nPKC-ϵ was performed as described in the Experimental section with ECL detection. The immunoblots are shown in the upper panels. The numbers above the immunoblots in (**A**) are log [bradykinin] (M) and in (**B**) are log [kallidin] (M). Experiments were performed with five (BK) or three (kallidin) separate myocyte preparations with similar results. In the lower panels, immunoblots were quantified by laser scanning densitometry. Results are means $\pm$ S.E.M.

in cultured neonatal ventricular myocytes and all except aPKC-ζ are down-regulated by exposure to 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h [33,41]. After PKC down-regulation, the stimulation of p42-MAPK and p44-MAPK by BK was inhibited by at least 85% (Figure 6C), implying that cPKC-α, nPKC-δ and/or nPKC-ϵ (or additional PKCs that were down-regulated but not studied in these experiments) participate in the signal transduction process.

Using 'in gel' MAPK assays, we also examined the activation of p42-MAPK and p44-MAPK by BK (Figure 7A) over a prolonged time (up to 8 h) and compared it with their activation by ET-1 (Figure 7B). With BK, p42-MAPK and p44-MAPK activities had largely returned to control values within 2 h (Figure 7A). Although the activation of p42-MAPK and p44-MAPK was slightly greater with ET-1 (Figure 7B), the time course was similar to that for BK (Figure 7A).

Is BK a hypertrophic agonist?

The stimulation of PtdInsP_2 hydrolysis (Figure 1), the translocation of nPKC-δ and nPKC-ϵ (Figures 2 and 3), and the activation of p42-MAPK and p44-MAPK by BK (Figures 5, 6 and 7A) are similar to the responses induced by ET-1 and PE [34], two well-established hypertrophic agonists [21,42–44]. We investigated whether BK was hypertrophic by using two approaches. First, we examined the cell morphology and myofibrillar organization by immunocytochemical localization of βMHC

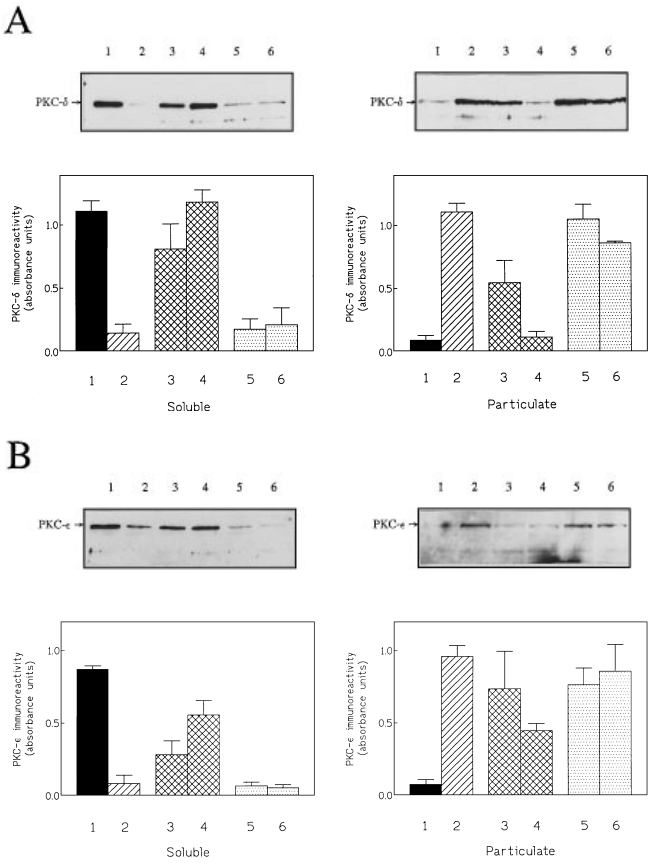

Figure 4 Effects of B_1 or B_2 receptor antagonists on the BK-stimulated translocation of nPKC-δ or nPKC-ε

Myocytes were exposed to 1 μM BK for 30 s in the absence or presence of B_1 or B_2 receptor antagonists, and denatured soluble and particulate fractions were prepared as described in the Experimental section. For SDS/PAGE, protein loading was 20 μg per lane for soluble fractions and 40 μg per lane for particulate fractions. After SDS/PAGE and transfer of protein to nitrocellulose, nPKC-δ (**A**) or nPKC-ϵ (**B**) was immunoblotted as described in the Experimental section, with ECL detection. Lanes are labelled as follows: lane 1, control (no addition); lane 2, 1 μM BK; lane 3, 1 μM BK plus 10 μM HOE140; lane 4, 1 μM BK plus 50 μM HOE140; lane 5, 1 μM BK plus 10 μM Leu^{8}BK(1–8); lane 6, 1 μM BK plus 50 μM Leu^{8}BK(1–8). Immunoblots are shown in the upper panels. Experiments were performed with three separate myocyte preparations with similar results. In the lower panels, immunoblots were quantified by laser scanning densitometry. Results are means $\pm$ S.E.M.

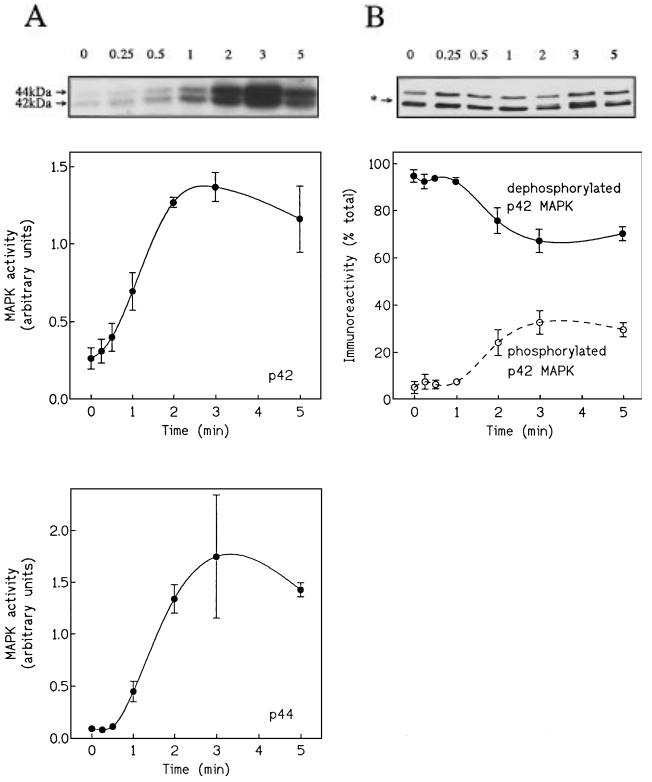

Figure 5 Stimulation of the activities of p42-MAPK and p44-MAPK, and of the phosphorylation of p42-MAPK by BK

Myocytes were exposed to 1 μM BK for the times indicated, as described in the Experimental section. Denatured soluble fractions were prepared and applied (20 μg of protein per lane) to SDS/polyacrylamide gels formed in (**A**) the presence or (**B**) the absence of MBP. After SDS/PAGE, 'in gel' MAPK assays (**A**) or immunoblotting (**B**) were performed as described in the Experimental section. In (**A**) the upper panel shows a typical autoradiograph of an 'in gel' MAPK assay. The corresponding lower panels show quantification (means $\pm$ S.E.M. for three individual separate preparations) of p42-MAPK and p44-MAPK activities obtained by laser scanning densitometry of the autoradiographs. In (**B**) the upper panel shows a typical immunoblot (ECL detection). The asterisk marks the position of phosphorylated p42-MAPK. The lower panel shows the quantification (means $\pm$ S.E.M., expressed as a percentage of total p42-MAPK immunoreactivity, for three separate myocyte preparations) of phosphorylated p42-MAPK (○, broken lines) and dephosphorylated p42-MAPK (●, solid lines) obtained by laser scanning densitometry of immunoblots.

(Figure 8). Secondly, as shown in Table 2, we measured the activation of promoters for genes whose induction is indicative of the hypertrophic response (reviewed in [45]).

Myocytes cultured in serum-free medium in the absence of agonists were small with disorganized myofibrils, and cell–cell contacts were absent (Figure 8A). After culture for 24 h in the presence of 0.1 μM ET-1 (Figure 8B) or 50 μM PE (Figure 8C), myocytes were large, cell–cell contacts were frequent and there was strong staining of βMHC in well-organized, cross-striated myofibrils. Some of the cells cultured in 10 μM BK for 24 h (Figure 8D) displayed a greater myofibrillar density and degree of organization than unstimulated cells (Figure 8A) but the cellular response was not uniform and differed distinctly from cells exposed to ET-1 (Figure 8B) or PE (Figure 8C).

The development of the hypertrophic phenotype in the ventricular myocyte involves a reversion to a foetal pattern of gene expression (reviewed in [45]). Genes whose expression is up-regulated include ANF, βMHC, c-*fos* (transiently), and SkM α-actin. Myocytes were transfected with reporter constructs containing these promoters. In preliminary experiments, we showed (by X-gal staining of fixed cells) that there were no differences in transfection efficiencies (always approx. 2% of cells) between experiments. Promoter activities were therefore expressed as LUX activities relative to control cultures (Table 2). Although the activities of promoters for ANF, βMHC, SRE-regulated c-*fos* and SkM α-actin were up-regulated after exposure of cells to 10 μM BK for 48 h, the stimulation was small (less than 3-fold) and was much less than that with 0.1 μM ET-1 (10–30-fold) or 100 μM PE (28–221-fold). The conclusions from this experiment were not altered when LUX activity was expressed relative to β-galactosidase activity (results not shown).

It could be argued that our failure to detect a strong hypertrophic response with BK was caused by its loss from the culture medium through degradation, among other factors. The concentrations of BK, ET-1 and PE initially present in the culture media were sufficient to stimulate PtdInsP_2 hydrolysis maximally (Table 1) [31]. In our studies of the morphological and

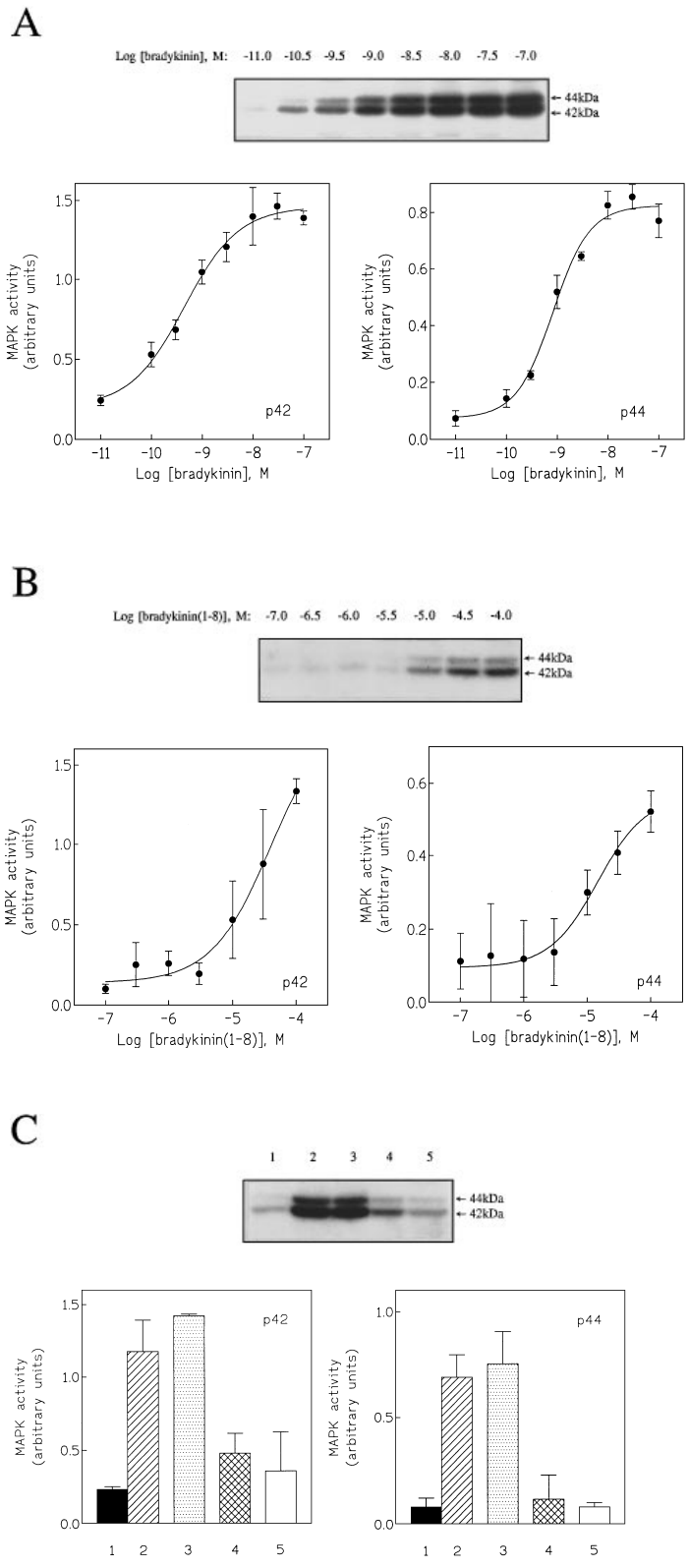

Figure 6 Dependence of the activation of p42-MAPK or p44-MAPK on BK or BK(1–8) concentration, and the effects of BK receptor antagonists or PKC down-regulation on the activation of p42-MAPK and p44-MAPK by BK

Myocytes were exposed to various concentrations of (**A**) BK or (**B**) BK(1–8), or (**C**) to 1 μM BK in the absence or presence of B_1 or B_2 receptor antagonists for 3 min, as described in the Experimental section. Denatured soluble fractions were prepared and applied (20 μg of protein per lane) to SDS/polyacrylamide gels formed in the presence of MBP. After SDS/PAGE, 'in gel' MAPK assays were performed as described in the Experimental section. The upper panels in (**A**), (**B**) and (**C**) show typical autoradiographs of 'in gel' MAPK assays. The corresponding lower panels show quantification of p42-MAPK and p44-MAPK activities obtained by laser scanning densitometry of the autoradiographs {means $\pm$ S.E.M. for three [BK] or two [BK(1–8)] individual preparations of myocytes}. In (**C**) lanes are labelled as follows: lane 1, control (no addition); lane 2, 1 μM BK; lane 3, 1 μM BK plus 50 μM Leu^{8}BK(1–8); lane 4, 1 μM BK plus 50 μM HOE140. In lane 5, myocytes were exposed to 1 μM PMA for 24 h and the medium was replaced with one containing 1 μM BK.

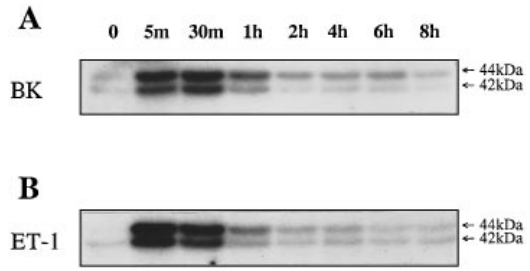

Figure 7 Stimulation of p42-MAPK and p44-MAPK by BK and ET-1 over an 8 h period

Myocytes were exposed to (**A**) 1 μM BK or (**B**) 0.1 μM ET-1 for 0 min, 5 min, 30 min, 1 h, 2 h, 4 h, 6 h or 8 h, as described in the Experimental section. Denatured soluble fractions were prepared and applied (20 μg of protein per lane) to SDS/polyacrylamide gels formed in the presence of MBP. After SDS/PAGE, 'in gel' MAPK assays were performed as described in the Experimental section. This experiment was repeated with similar results.

Table 2 Stimulation of promoter activities

Myocytes were transfected with LUX reporter plasmids as described in the Experimental section. They were exposed to 10 μM BK, 0.1 μM ET-1 or 100 μM PE for 48 h. The LUX activities in extracts of these cells are expressed as means $\pm$ S.E.M. for three separate myocyte preparations, relative to controls in the absence of agonist.

	Agonist ...	LUX activity (fold of control)	
Reporter construct	BK	ET-1	PE
ANF	2.1 $\pm$ 0.1	33 $\pm$ 9	221 $\pm$ 54
βMHC	1.9 $\pm$ 0.5	13 $\pm$ 3	88 $\pm$ 32
SkM α-actin	2.5 $\pm$ 1.0	10 $\pm$ 4	29 $\pm$ 1
c-*fos* SRE	1.3 $\pm$ 0.2	3.5 $\pm$ 0.9	28 $\pm$ 5

transcriptional features of the hypertrophic response (Figure 8 and Table 2, respectively), the more prolonged experiments (48 h) were the transient transfections; these were also performed at the higher ratio of cell number to volume of medium (see the Experimental section). We therefore incubated cells with agonists under these conditions, removed the culture media after 48 h and examined their ability to stimulate PtdInsP_2 hydrolysis when applied for 10 min to fresh cultures of myocytes prelabelled with [^{3}H]inositol. For BK, ET-1 and PE there was no difference between the rate of PtdInsP_2 hydrolysis induced by fresh medium or by medium that had been in contact with myocytes for 48 h (results not shown). The overall conclusion is that BK, although weakly hypertrophic, is not as powerful a hypertrophic agonist as ET-1 or PE in spite of its comparable effects on PKC and p42/p44-MAPK.

DISCUSSION

BK is cardioprotective (reviewed in [14,15]). Myocardial ischaemia increases the outflow of BK from the heart [46,47] and its local production may limit the damage after reperfusion of the infarcted myocardium [48–51]. BK may also be partly responsible for the 'ischaemic preconditioning' of the myocardium ([52–54];

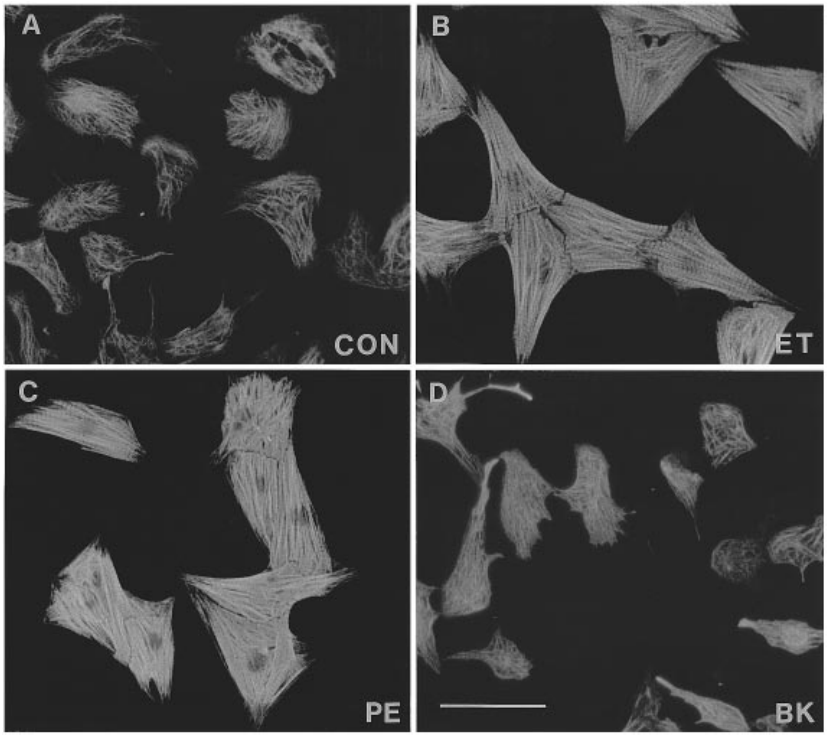

Figure 8 The effects of ET-1, PE or BK on ventricular myocyte size and myofibrillar organization

Myocytes were exposed to (**A**) serum-free medium, or to serum-free medium containing (**B**) 0.1 μM ET-1, (**C**) 50 μM PE or (**D**) 10 μM BK for 24 h. βMHC was localized by indirect immunostaining as described in the Experimental section. Scale bar, 50 μm. Similar results were obtained with a total of four separate myocyte preparations.

reviewed in [55,56]) where a brief period of ischaemia protects the heart against a subsequent ischaemic episode [57–59]. There is evidence that activation of PKC is involved in this phenomenon [60–62]. Furthermore some of the beneficial effects of angiotensin-converting enzyme inhibitors, frequently used in the treatment of cardiovascular disease, can be ascribed to their ability to inhibit BK degradation as well as inhibiting the production of angiotensin II [15,16].

Two BK receptor subtypes (B_1 and B_2) have been identified. B_1 and B_2 receptor-mediated responses can be distinguished pharmacologically on the basis of agonist potencies or by the use of receptor-selective antagonists (reviewed in [37–40]). For the B_2 receptor, the order of potency is BK $\approx$ kallidin $<$ BK(1–8), whereas for the B_1 receptor the order is reversed [BK(1–8) $\approx$ kallidin $<$ BK]. HOE140 is a selective antagonist for the B_2 receptor whereas Leu^{8}BK(1–8) is a selective antagonist for the B_1 receptor. For PtdInsP_2 hydrolysis in ventricular myocytes, the order of potency for agonists (Figure 1 and Table 1) and antagonists (given in the text) indicates coupling to the B_2 receptor (see also [13]).

As assessed by immunoblotting, neonatal rat ventricular myocytes in culture express cPKC-α, nPKC-δ, nPKC-ϵ and aPKC-ζ [33,41,63,64]. cPKC-$\beta_{1/2}$ and cPKC-γ are much more difficult to detect. The pattern of expression of PKC isoforms in these cells has not yet been fully characterized. Thus other PKC isoforms {nPKC-η, nPKC-θ, aPKC-$\lambda(\iota)$, n/aPKC-μ; reviewed in [6]} may be present. As for PtdInsP_2 hydrolysis, the translocation of nPKC-δ and nPKC-ϵ is coupled to the B_2 receptor (Figures 3 and 4; Table 1). The time courses and extent of translocation of nPKC-δ and nPKC-ϵ resemble those induced by ET-1 rather than PE [34]. Like ET-1, BK causes essentially complete (more than 80 %) translocation of nPKC-δ and nPKC-ϵ from the soluble to the particulate fraction in less than 30 s and the translocation event is reversed by 3–5 min (Figures 2A and 2B). Similarly EC_{50} for the BK-induced translocation of nPKC-ϵ is significantly less than that for nPKC-δ (Figure 3A and Table 1). PE only translocates nPKC-ϵ in our hands [34]. As with ET-1 or PE [34], BK does not cause any convincing translocation of cPKC-α (Figure 2A). However, PMA does cause translocation of cPKC-α in these cells [41,63], indicating that the isoform is susceptible to translocation. cPKC-α might form only a relatively loose association with the particulate fraction and this could be influenced by the nature of the phospholipids present [65,66]. Our cell lysis conditions might have been too harsh to detect translocation of cPKC-α. However, we failed to detect translocation even when cells were lysed by freeze–thawing in the absence of detergent (results not shown). In common with other agonists [34,41,63,64], BK did not translocate aPKC-ζ (Figure 2A). The regulation of aPKC-ζ is not well understood although it has been suggested that the enzyme might be activated by second messengers other than DG [67-69].

The activation of p42- and p44-MAPK is also coupled to the B_2 receptor (Figure 6). As for ET-1 or α_1-adrenergic agonists [33], the activation of p42-MAPK and p44-MAPK by BK is prevented by pre-exposure of myocytes to 1 μM PMA for 24 h (Figure 6C). Under these conditions there is essentially a complete loss of cPKC-α, nPKC-δ and nPKC-ϵ immunoreactivity from the myocyte in the absence of any loss of aPKC-ζ [33,41]. Assuming that translocation is a prerequisite for activation of PKC (and this may not be true, particularly for aPKC-ζ), these results indicate that neither cPKC-α nor aPKC-ζ can be responsible for the events leading to the activation of p42/p44-MAPK by BK. nPKC-δ and/or nPKC-ϵ might mediate activation of p42/p44-MAPK but, because the regulation of nPKC-η, nPKC-θ, aPKC-$\lambda(\iota)$ and nPKC-μ has not been fully characterized in the ventricular myocyte, participation of these isoforms cannot be excluded. The maximum extent of phosphorylation of p42-MAPK elicited by BK corresponds to approx. 30–35 % of the total p42-MAPK pool (Figure 5B). This response lies between that for PE (20–25 %) and ET-1 (50–60 %) [34]. The overall conclusion is that although the patterns of translocation of PKC isoforms and the activation and phosphorylation of p42/p44-MAPK by BK, ET-1 and PE are similar, there may be important differences in the quantitative nature of the responses.

Studies with phorbol esters and transient transfections with constitutively activated constructs have indicated that PKC is important in the development of the hypertrophic phenotype (reviewed in [17,18]). Equally, the hypothesis that p42/p44-MAPK is involved in some aspects of the hypertrophic response, first suggested on the basis of correlative studies [32,33], has recently been directly supported by transient transfection and antisense oligodeoxynucleotide approaches [36,70,71]. Both PE and ET-1 are powerful hypertrophic agonists [21,42–44]. It is therefore perplexing that BK only weakly induces the characteristic changes in morphology (Figure 8D) and gene expression (Table 2). In PC12 cells the duration of the activation of p42/p44-MAPK is critical in determining the cellular response (reviewed in [72]). Thus treatment of PC12 cells with epidermal growth factor causes a relatively transient activation of p42/p44-MAPK (which returns to control values in 1–2 h) and a proliferative response [73]. In contrast, nerve growth factor causes a prolonged activation of p42/p44-MAPK and leads to differentiation of the cells and neurite outgrowth [73]. We considered the possibility that the hypertrophic response in cardiac myocytes might similarly depend on the duration of p42/p44-MAPK activation. However, there was no significant difference in the time course of activation of p42-MAPK and p44-MAPK by BK (Figure 7A) and ET-1 (Figure 7B). It could be that other signalling pathways need to be activated in addition to the PtdInsP_2 hydrolysis $\rightarrow$ DG $\rightarrow$ PKC $\rightarrow$ $\rightarrow$ p42/p44-MAPK pathway or that the importance of this pathway has been overemphasized. Alternatively, BK may stimulate other pathways that prevent the hypertrophic response. We are currently examining the reasons for this difference, particularly with respect to membrane lipid-dependent signalling.

We thank Nicola Haward for preparing the cultures of ventricular myocytes and for other assistance. This work was supported grants from the British Heart Foundation and the Biotechnology and Biological Sciences Research Council. S.J.F. is a British Heart Foundation Lecturer in Basic Sciences.

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Received 13 December 1995/1 March 1996; accepted 7 March 1996