Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy

Marie A. BOGOYEVITCH*, Monica B. ANDERSSON, Judith GILLESPIE-BROWN, Angela CLERK, Peter E. GLENNON, Stephen J. FULLER and Peter H. SUGDEN

Cardiac Medicine, National Heart and Lung Institute, Imperial College of Science, Technology and Medicine, Dovehouse Street, London SW3 6LY, U.K.

Phenylephrine and noradrenaline (α -adrenergic agonism) or isoprenaline (β -adrenergic agonism) stimulated protein synthesis rates, increased the activity of the atrial natriuretic factor gene promoter and activated mitogen-activated protein kinase (MAPK). The EC_{50} for MAPK activation by noradrenaline was 2–4 μ M and that for isoprenaline was 0.2–0.3 μ M. Maximal activation of MAPK by isoprenaline was inhibited by the β -adrenergic antagonist, propranolol, whereas the activation by noradrenaline was inhibited by the α_1 -adrenergic antagonist, prazosin. FPLC on a Mono-Q column separated two peaks of MAPK (p42^{MAPK} and p44^{MAPK}) and two peaks of MAPK-activating activity (MEK) activated by isoprenaline or noradrenaline. Prolonged phorbol ester exposure partially down-regulated the activation of MAPK by noradrenaline but not by isoprenaline. This implies a role for protein kinase C in MAPK activation

by noradrenaline but not isoprenaline. A role for cyclic AMP in activation of the MAPK pathway was eliminated when other agonists that elevate cyclic AMP in the cardiac myocyte did not activate MAPK. In contrast, MAPK was activated by exposure to ionomycin, Bay K8644 or thapsigargin that elevate intracellular Ca^{2+} . Furthermore, depletion of extracellular Ca^{2+} concentrations with bis-(*o*-aminophenoxy)ethane-*NNN'*-tetraacetic acid (BAPTA) or blocking of the L-type Ca^{2+} channel with nifedipine or verapamil inhibited the response to isoprenaline without inhibiting the responses to noradrenaline. We conclude that α - and β -adrenergic agonists can activate the MEK/MAPK pathway in the heart by different signalling pathways. Elevation of intracellular Ca^{2+} rather than cyclic AMP appears important in the activation of MAPK by isoprenaline in the cardiac myocyte.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are a family of protein Ser/Thr kinases which are activated in response to peptide growth factors that act through tyrosine kinase receptors [1]. This activation is a result of the phosphorylation of both Tyr- and Thr- residues of MAPK by MAPK kinase or MEK (for MAPK or ERK kinase). G-protein-coupled receptors may also activate the MAPK cascade [1]. In many of these cases this is G_i -dependent (pertussis-toxin sensitive) [2]; however, in the ventricular myocyte both pertussis-sensitive and -insensitive pathways exist [3].

The treatment of cardiac ventricular myocytes with agents such as endothelin, phenylephrine or phorbol esters initiates a programme of changes that typifies the hypertrophic phenotype [4]. These treatments also stimulate MAPK activities [3,5–7] and this may be important in the mediation of the changes in gene expression [8].

Long-term treatment with the β -adrenergic agonist, isoprenaline, also induces some changes that are typical of the hypertrophic response (increased protein synthesis and sarcomere assembly, expression of *c-fos* and *c-jun*) [9,10]. The signalling pathways that lead to this response are unclear. Activation of the cardiac β -adrenergic receptor rapidly increases cyclic AMP concentrations through a G_s -mediated stimulation of adenylate cyclase [11]. This activates cyclic AMP-dependent protein kinase [12] which phosphorylates (among other proteins) the L-type Ca^{2+} channel, thereby increasing Ca^{2+} entry [13]. Alternatively, a

direct G_s -mediated coupling of the L-type Ca^{2+} increases Ca^{2+} entry [13]. The elevation of cyclic AMP in many cell types inhibits MAPK activation and this may explain the inhibition of growth of some cells by agents that elevate cyclic AMP concentrations (reviewed in [14]).

A role for Ca^{2+} rather than cyclic AMP has been established in β -adrenergic regulation of myocardial gene expression [10]. We have investigated the adrenergic receptor activation of the MAPK pathway in the ventricular myocyte. Here we report that MAPK is activated by both α - and β -adrenergic agonists. Activation by isoprenaline is dependent on extracellular Ca^{2+} and can be mimicked by agents that elevate intracellular Ca^{2+} but not by agents that elevate cyclic AMP. This provides support for the regulation of the MAPK cascade via Ca^{2+} mobilization.

EXPERIMENTAL

Materials

Sources of reagents have been described previously [3,5,15]. Isoprenaline, noradrenaline and phenylephrine were dissolved in 100 μ M L-ascorbic acid and used fresh daily. The atrial natriuretic factor (ANF) luciferase reporter construct pANF(–638)LA5' and a β -galactosidase expression vector under the control of the human cytomegalovirus promoter, pON249, were provided by K. R. Chien (Department of Medicine, University of California, San Diego, CA, U.S.A.). Recombinant murine p42^{MAPK} was

Abbreviations used: ANF, atrial natriuretic factor; BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; CPT-cyclic AMP, 8-(4-chlorophenylthio)-cyclic AMP; ERK, extracellular signal-regulated protein kinase; GST, glutathione S-transferase; IBMX, 1-methyl-3-isobutylxanthine; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK (or ERK) kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed at: CRC Centre for Cell and Molecular Biology, Chester Beatty Laboratories, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, U.K.

expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli*. This clone and the polyclonal antisera recognizing MAPK or MEK were gifts from C. J. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London, U.K.).

Culture and treatment of cardiac ventricular myocytes

Cardiac myocytes were dissociated from the ventricles of neonatal (1- to 2-day-old) rat hearts and were preplated for 30 min on uncoated culture dishes to remove fibroblasts [3]. The myocytes were finally plated at a density of $(1.0\text{--}1.4) \times 10^3$ cells/mm² on gelatin-precoated dishes and were confluent and spontaneously beating after 18 h. Serum was withdrawn for 24 h before the cells were further treated. When indicated, phorbol 12-myristate 13-acetate (PMA) was added to a final concentration of 1 μ M in the serum-free medium 24 h before exposure of myocytes to agonists. Prelabelling and agonist-stimulated hydrolysis of PtdIns pools were as described elsewhere [3].

Determination of protein synthesis

Following serum withdrawal (after 24 h), myocytes (1×10^6 cells in 35-mm-diam. dishes) were exposed to agonists in culture medium (3 ml) supplemented with 1 mM bromodeoxyuridine, 3 μ Ci of L-[2,6-³H]phenylalanine, 2.5 mM unlabelled phenylalanine and 1.25 mM of other amino acids. The incubation was terminated after 24 h by washing the cells in cold Ca²⁺/Mg²⁺-free Dulbecco's PBS supplemented with 20 μ M cycloheximide. Cells were then scraped into 0.8 ml of this solution and proteins were solubilized by the addition of 200 μ l of 1 M NaOH. BSA and trichloroacetic acid were then added to final concentrations of 0.5% (w/v) and 5% (w/v) respectively. The precipitated protein was collected by centrifugation (300 g, 4 °C, 5 min) and washed three times in ice-cold 5% (w/v) trichloroacetic acid. The precipitate was then solubilized in 1 M NaOH (100 μ l) and the radioactivity determined by liquid scintillation counting. In preliminary experiments, we confirmed that rates of protein synthesis in response to a variety of agonists (PMA, phenylephrine, isoprenaline, endothelin-1 and control) were linear over 24 h.

ANF gene promoter activity

Myocytes were transfected 24–48 h after plating using a calcium phosphate precipitation method. Myocytes (35-mm-diam. plates) were transfected with the ANF reporter construct pANF(–638)LA5' (15 μ g), and pON249 (4 μ g, to control for transfection efficiency) in fresh Dulbecco's modified Eagle's medium/medium 199 (4:1, v/v) supplemented with 4% (v/v) horse serum. Plasmids were diluted to 250 μ l in 0.25 M CaCl₂ and an equal volume of 50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.9, was added. After 20 min incubation, plasmids were added to cells, which were incubated at 37 °C overnight. Cells were washed twice with fresh Dulbecco's modified Eagle's medium/medium 199 (4:1, v/v) supplemented with 10% (v/v) horse serum, twice with serum-free medium and finally incubated in serum-free medium in the presence or absence of agonists. After 48 h, the cells were washed three times with PBS and extracted by incubating in 100 mM potassium phosphate, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol, pH 7.9, for 15 min on ice. Extracts were assayed for luciferase and β -galactosidase [16].

Characterization of MAPK and MEK activities

Cultured myocytes were exposed to agonists in serum-free medium at 37 °C, then washed with cold Ca²⁺/Mg²⁺-free Dulbecco's PBS and scraped into cold lysis buffer [20 mM β -glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM sodium vanadate, 10 mM benzimidazole, 25 μ g/ml leupeptin, 50 μ g/ml PMSF, 0.3% (v/v) mercaptoethanol (pH 7.5)]. Lysates were centrifuged (10000 g, 4 °C, 10 min) and the supernatants retained for analysis by either the 'direct' phosphorylation of myelin basic protein (MBP) or by 'in gel' MBP phosphorylation in SDS/10%-PAGE gels containing 0.5 mg/ml MBP [3,5,6].

To characterize the MAPK and MEK activities of the ventricular myocytes, extracts were applied to a Mono-Q HR5/5 column (Pharmacia-LKB) [5,6]. Proteins were eluted with a linear NaCl gradient (0–0.3 M NaCl) and fractions (0.5 ml) were assayed for MAPK [6]. One unit of MAPK catalyses the incorporation of 1 pmol of phosphate into MBP/min. MEK activity was assayed using the activation of exogenous GST-p42^{MAPK} [6]. One unit of MEK during 1 min of the preincubation in the presence of unlabelled ATP catalyses the activation of exogenous p42^{MAPK} sufficiently to transfer 1 pmol of phosphate into MBP/min in the subsequent MAPK assay. Fractions with MAPK activity were also assayed by the 'in gel' method [3,5,6], or those with MAPK or MEK activity concentrated 80-fold using Centricon 10 ultrafiltration units and immunoblotted with antibodies to MAPK or MEK using the enhanced chemiluminescence (ECL) method [6].

Assay of cyclic AMP-dependent protein kinase activity

Cultured myocytes were exposed to agonists in serum-free medium at 37 °C then washed with cold Ca²⁺/Mg²⁺-free Dulbecco's PBS and scraped into cold cyclic AMP-dependent protein kinase extraction buffer [10 mM NaH₂PO₄/Na₂HPO₄, 1 mM EDTA (pH 6.8) containing 0.2 mM 1-methyl-3-isobutyl-xanthine (IBMX), 1 mM dithiothreitol, 100 mM NaCl]. Lysates were centrifuged (10000 g, 5 min, 4 °C) and the supernatants assayed using 70 μ M Kemptide in the absence and presence of 2 μ M cyclic AMP [17]. Activity ratios were calculated as the ratio of the cyclic AMP-dependent protein kinase activity in the absence of cyclic AMP to that in the presence of 2 μ M cyclic AMP.

Statistical methods and curve fitting

Results are expressed as means $\pm$ S.E.M. for the number of observations (*n*) indicated. Statistical significance was tested by a two-tailed Student's *t* test. Fitting of concentration-dependence data to sigmoidal curves used the Graph-pad Inplot 4 program (GraphPad Software Inc., San Diego, CA, U.S.A.) with intermediate weighting.

RESULTS

Adrenergic receptor stimulation increases protein synthesis and activation of the ANF gene promoter

The exposure of cultured neonatal ventricular myocytes to either the β -adrenergic agonist, isoprenaline, or the α -adrenergic agonists, phenylephrine or noradrenaline, at concentrations similar to those used routinely by others [9,18–20] produced long-term increases in total protein synthesis rates typical of the hypertrophic response (Table 1). Ventricular re-expression of

Table 1 Adrenergic activation of protein synthesis and the ANF gene promoter

For protein synthesis experiments, ventricular myocytes were exposed for 24 h to phenylephrine, noradrenaline or isoprenaline. Protein synthesis rates were determined by the incorporation of L-[2,6-³H]phenylalanine into protein. For ANF expression experiments, transfected ventricular myocytes were exposed for 48 h to phenylephrine, noradrenaline or isoprenaline. Activation of the ANF gene promoter was determined in the transfected myocytes by the stimulation of luciferase expression and this was normalized for transfection efficiency by using β -galactosidase activity (β -gal). Results (means $\pm$ S.E.M., $n = 3-6$) are expressed relative to control values. Statistical significance: ^a $P < 0.05$ and ^b $P < 0.005$ versus control cells.

Agonist	Protein synthesis (% of control)	ANF-luciferase/ β -gal (% of control)
Isoprenaline (50 μ M)	123 $\pm$ 7 ^a	430 $\pm$ 110 ^a
Noradrenaline (50 μ M)	144 $\pm$ 4 ^b	1030 $\pm$ 125 ^b
Phenylephrine (50 μ M)	138 $\pm$ 7 ^b	1050 $\pm$ 270 ^a

ANF is a specific marker of the hypertrophic response [21]. Isoprenaline, phenylephrine or noradrenaline increased activation of the ANF-luciferase normalized to β -galactosidase (Table 1). The development of the hypertrophic phenotype is also characterized by increased cell size and myofibrillar organization [4,9]. We confirmed that exposure to 50 μ M isoprenaline (24 h) elicited these changes in the ventricular myocytes (A. Clerk and P. H. Sugden, unpublished work). The mechanism by which β -adrenergic stimulation causes hypertrophy of the ventricular myocyte has not been resolved. The following experiments were performed to investigate common intracellular signalling pathways activated upon exposure to α - and β -adrenergic agonists and the mechanism(s) of this activation in the myocyte.

Activation of MAPK in cytosolic extracts of cardiac myocytes

Because activation of MAPK has been associated with cell growth, we examined this signalling pathway in the ventricular myocyte exposed to adrenergic agonists. Noradrenaline or isoprenaline activated MAPK in crude cytosolic extracts (Figure 1). Maximum activation was observed after 5 min and declined within 1 h. MAPK activity was also stimulated by PMA (Figure 1), this was sustained (> 300% of control for 1 h; see also [6]).

The involvement of protein kinase C (PKC) and PtdIns hydrolysis in the adrenergic stimulation of MAPK

The activation of MAPK by PMA implies a PKC-dependent pathway for the activation of MAPK in the myocyte. Many PKC isotypes are activated by diacylglycerols produced by the hydrolysis of membrane PtdIns [22] and we investigated the role of PtdIns hydrolysis and PKC in the activation of MAPK in the cardiac myocyte. As assayed by the phospho[³H]inositol prelabelling technique, exposure of myocytes to 50 μ M noradrenaline or 50 μ M phenylephrine stimulated rates of PtdIns hydrolysis (440 $\pm$ 90% and 300 $\pm$ 20% respectively after 10 min, $n = 3$; $P < 0.05$ versus control at 10 min). In contrast, exposure to 50 μ M isoprenaline did not significantly increase PtdIns hydrolysis (120 $\pm$ 20% of control after 10 min) until after 40 min of exposure (270 $\pm$ 40% of control at 40 min, $n = 3$; $P < 0.05$).

Pretreatment with 1 μ M PMA for 24 h depletes cardiac myocytes of PKC- α , PKC- δ and PKC- ϵ but not PKC- ζ [6]. The stimulation of MAPK in crude extracts of myocytes subsequently re-exposed to 1 μ M PMA for 5 min was decreased by 86% (Table 2). The activation by 50 μ M noradrenaline or 50 μ M

Figure 1 Time courses for the activation of MAPK in cardiac myocytes

Ventricular myocytes were stimulated for the times indicated with 50 μ M isoprenaline (●), 50 μ M noradrenaline (○) or 1 μ M PMA (■). MAPK activity in soluble extracts of these cells was assayed as described in the Experimental section. Results (means $\pm$ S.E.M., $n = 3-6$ independent observations) are expressed relative to MAPK activities in control cells.

Table 2 Effect of chronic PMA pretreatment on the stimulation of MAPK activity

Ventricular myocytes were preincubated in serum-free medium in the absence or presence of 1 μ M PMA for 24 h. The medium was then replaced with medium containing either PMA, isoprenaline, noradrenaline, phenylephrine or no agonist (control). After 5 min of exposure, extracts were prepared and directly assayed for MAPK activity using MBP. Results (means $\pm$ S.E.M., $n = 5$) are expressed relative to MAPK activities in extracts from control cells that had not been preincubated with PMA. Statistical significance: ^a $P < 0.10$, ^b $P < 0.05$ and ^c $P < 0.001$ preincubated cells (+PMA preincubation) versus cells without PMA preincubation (−PMA preincubation).

Acute treatment	MBP kinase activity (% of control)	
	(− PMA preincubation)	(+ PMA preincubation)
Control	100 $\pm$ 0	123 $\pm$ 10
PMA (1 μ M)	490 $\pm$ 21	155 $\pm$ 22 ^c
Isoprenaline (50 μ M)	162 $\pm$ 17	168 $\pm$ 24
Noradrenaline (50 μ M)	262 $\pm$ 14	201 $\pm$ 20 ^b
Phenylephrine (50 μ M)	227 $\pm$ 16	166 $\pm$ 24 ^a

phenylephrine for 5 min was also decreased by 38% or 48% respectively (Table 2). Activation by isoprenaline was not significantly affected ($P = 0.84$, Table 2). Thus, unlike the responses to PMA, noradrenaline or phenylephrine, the activation of MAPK by isoprenaline appears to be independent of PKC- α , - δ and - ϵ .

α - and β -Adrenergic receptor activation of MAPK

When samples of cytosolic extracts from isoprenaline- or noradrenaline-treated myocytes were examined by MAPK assays using 'in gel' assays, MBP kinases of 107, 65, 44 and 42 kDa were detected (Figure 2). Only the bands at 42 and 44 kDa (as indicated by arrows in Figure 2) changed in intensity with increasing agonist concentration. These bands were co-ordinately regulated in response to either α - or β -adrenergic stimulation, although the 44 kDa band was always less intense than the

Figure 2 Dependence of MAPK activation on isoprenaline or noradrenaline concentration

Ventricular myocytes were exposed for 5 min at 37 °C to increasing concentrations of (top two panels) isoprenaline or (bottom two panels) noradrenaline. MBP kinases were assayed by the 'in gel' assay. The numbers to the right of each upper panel refer to the molecular masses (kDa) of marker proteins. The arrows to the left of each of these panels indicate the positions of p42^{MAPK} and p44^{MAPK}. For the lower panels, autoradiographs were scanned using a laser densitometer and the results expressed as arbitrary absorbance units (dashed lines, p42^{MAPK}; dotted lines, p44^{MAPK}). The results are shown from a single experiment. Similar results were obtained in five separate experiments.

Table 3 The effect of α - and β -adrenergic antagonists on the stimulation of MAPK

Cardiac myocytes were exposed to 50 μ M isoprenaline, 50 μ M noradrenaline, or no agonist (control) together with either no addition, 0.1% (v/v) DMSO (control for addition of solvent), 20 μ M DL-propranolol or 5 μ M prazosin. After 5 min of exposure, extracts were prepared and directly assayed for MAPK activity using MBP. Results (means $\pm$ S. E. M., $n = 5$) are expressed relative to MAPK activities in extracts from control cells. Statistical significance: ^a $P < 0.02$, ^b $P < 0.001$ no addition versus addition.

Treatment	MBP kinase activity (% of control)			
	No addition	+ DMSO	+ Propranolol	+ Prazosin
Isoprenaline (50 μ M)	171 $\pm$ 16	199 $\pm$ 46	125 $\pm$ 9 ^a	162 $\pm$ 18
Noradrenaline (50 μ M)	335 $\pm$ 29	359 $\pm$ 39	297 $\pm$ 35	153 $\pm$ 16 ^b

42 kDa band. These two bands correspond to the 42 kDa (ERK2) and 44 kDa (ERK1) isoforms of MAPK [5,6]. For isoprenaline (Figure 2, top two panels), the EC_{50} values for the stimulation of p42^{MAPK} and p44^{MAPK} were 0.3 and 0.2 μ M respectively, and the values for extrapolated maximal stimulation of the two MAPK isoforms were 436 and 447% respectively (pooled data from five experiments). For noradrenaline (Figure 2, bottom two panels), the EC_{50} values for the stimulation of p42^{MAPK} and p44^{MAPK} were 4 and 2 μ M respectively, and the values for extrapolated maximal stimulation of the two MAPK isoforms were 1450 and 1420% respectively (pooled data from five experiments). Thus, although the 44 kDa band is less intense than the 42 kDa band, its relative stimulation is similar to that of the 42 kDa band.

We confirmed that the activation of MAPK by isoprenaline was mediated through a β -adrenergic receptor. Exposure of cells to 50 μ M isoprenaline and the β -adrenergic antagonist DL-propranolol decreased the activation of MAPK by isoprenaline by 65%, whereas the exposure to 50 μ M isoprenaline and the α_1 -adrenergic antagonist prazosin did not decrease the response (Table 3). In contrast, the response to 50 μ M noradrenaline was decreased by 77% by prazosin but was not inhibited (16%) by DL-propranolol. The presence of DMSO did not alter the responses to either isoprenaline or noradrenaline (Table 3).

α - and β -Adrenergic receptor activation of MEK

Activation of MAPK and its upstream regulator, MAPK kinase (MEK), was further studied by FPLC of extracts of cardiac myocytes on Mono-Q [6]. Two major MBP kinase peaks were observed after exposure of the cells to 50 μ M isoprenaline (Figure 3a) or 50 μ M noradrenaline (Figure 3b). 'In gel' MBP kinase assays confirmed that peak K1 contained activated renaturable 42 kDa MAPK and peak K2 contained activated renaturable 44 kDa MAPK (results not shown). Peak K2 also contained a renaturable 65 kDa band that was not activated after isoprenaline exposure. The identity of this uncharacterized MBP kinase is unknown. We confirmed the identity of these peaks by the immunoblotting of peak fractions with antiserum that recognizes both 42 and 44 kDa MAPKs [23]. Both 42 and 44 kDa proteins were detected in peak K1 and a weaker, slightly retarded 44 kDa band was detected in peak K2 (results not shown).

FPLC of extracts from isoprenaline- (Figure 3a) or noradrenaline- (Figure 3b) treated cardiac myocytes on Mono-Q revealed two peaks of MEK activity (KK1 and KK2) that activated recombinant p42^{MAPK}. In both cases, MEK peak KK1 was greater than peak KK2. Antiserum raised against recombinant

Figure 3 Activation of MAPK and MEK in cardiac myocytes exposed to isoprenaline or noradrenaline

Ventricular myocytes were exposed for 5 min to 50 μ M isoprenaline (a) or 50 μ M noradrenaline (b). Extracts were prepared and subjected to FPLC using a Mono-Q column with elution by a linear salt gradient (0–0.3 M NaCl, straight dotted line). MAPK (dashed line: ■, +agonist; □, control) and MEK (solid line: ●, +agonist; ○, control) activities in fractions were determined. Two peaks of MAPK are labelled K1 and K2 and the two peaks of MEK activity are labelled KK1 and KK2.

rabbit MEK-1 recognized a 50 kDa protein in fractions corresponding to peaks KK1 and KK2 (results not shown). These results confirm that isoprenaline and noradrenaline activate MEK within the MAPK cascade.

Activation of MAPK by Ca^{2+} -dependent pathways

Ca^{2+} mobilization is a feature of stimulation of β -adrenergic (through the L-type Ca^{2+} channel [13]) and α_1 -adrenergic (through PtdIns hydrolysis [24]) receptors. The role that Ca^{2+} mobilization may play in the activation of MAPK in myocytes was investigated by the use of Bay K8644, thapsigargin or ionomycin, agonists that elevate intracellular Ca^{2+} concentrations via different mechanisms. Each of these agonists significantly increased MAPK activity to levels comparable with that achieved after exposure to isoprenaline (Table 4). Thapsigargin or ionomycin did not activate PtdIns hydrolysis ($103 \pm 11\%$ or $110 \pm 19\%$ of control values at 10 min exposure; mean $\pm$ S.E.M., $n = 4$ –5 independent observations). FPLC of extracts of thapsigargin-treated (1 μ g/ml, 5 min) cardiac myocytes on Mono-Q revealed two peaks of MEK and two peaks of MAPK (results not shown).

Table 4 The effect of elevation of Ca^{2+} or cyclic AMP on the stimulation of MAPK

Cardiac myocytes were exposed to isoprenaline, noradrenaline, BAY K8644, thapsigargin, ionomycin, IBMX, CPT-cyclic AMP, glucagon, forskolin, or no agonist (control). After 5 min of exposure, extracts were prepared and directly assayed for MAPK activity using MBP. Results (means $\pm$ S.E.M., $n = 4$ –6) are expressed relative to MAPK activities in extracts from control cells. Statistical significance: $^aP < 0.005$, addition versus control.

Treatment	MBP kinase activity (% of control)
Isoprenaline (50 μ M)	199 $\pm$ 17 ^a
Noradrenaline (50 μ M)	289 $\pm$ 24 ^a
BAYK8644 (10 μ M)	163 $\pm$ 14 ^a
Thapsigargin (1 μ g/ml)	189 $\pm$ 10 ^a
Ionomycin (100 nM)	149 $\pm$ 10 ^a
IBMX (10 μ M)	114 $\pm$ 8
CPT-cyclic AMP (100 μ M)	85 $\pm$ 7
Glucagon (1 μ M)	123 $\pm$ 10
Forskolin (1 μ M)	96 $\pm$ 11

Figure 4 Activation of cyclic AMP-dependent protein kinase in cardiac myocytes

Ventricular myocytes were exposed for 1, 3 or 5 min to 50 μ M isoprenaline (ISO), 100 μ M CPT-cyclic AMP (CPT) or 10 μ M IBMX. Extracts were prepared and cyclic AMP-dependent protein kinase activity ratios were assayed using Kemptide as a substrate. Results are means $\pm$ S.E.M., ($n = 4$ –6 independent observations).

Elevation of cyclic AMP activates cyclic AMP-dependent protein kinase but not MAPK

Isoprenaline rapidly activates cyclic AMP-dependent protein kinase in the cultured ventricular myocyte (Figure 4). Agents such as IBMX and the cell membrane-permeating cyclic AMP analogue, 8-(4-chlorophenylthio)-cyclic AMP (CPT-cyclic AMP) can also activate the cyclic AMP-dependent protein kinase (Figure 4). However, IBMX or CPT-cyclic AMP failed to activate MAPK in the ventricular myocytes (Table 4). Other agents such as glucagon or forskolin that also elevate cyclic AMP in the heart [17] also failed to activate MAPK (Table 4). Prolonged exposure (up to 1 h) to these agents which activate cyclic AMP-dependent protein kinase did not activate the MAPKs (results not shown).

Extracellular Ca^{2+} is essential for the activation of MAPK by isoprenaline

Because mobilization of Ca^{2+} mimicked the activation of MAPK

Table 5 Stimulation of MAPK by isoprenaline is dependent on Ca^{2+}

Cardiac myocytes were exposed to isoprenaline, noradrenaline, or PMA in the presence of no addition, 10 μM nifedipine, 10 μM verapamil or 3 mM BAPTA. After 5 min of exposure, extracts were prepared and directly assayed for MAPK activity using MBP. Results (means $\pm$ S.E.M., $n = 4-6$) are expressed relative to MAPK activities in extracts from control cells. Statistical significance: $^bP < 0.05$, no addition versus addition of nifedipine, verapamil or BAPTA; $^aP < 0.05$ versus control cells.

Treatment	MBP kinase activity (% of control)			
	No addition	+ Nifedipine	+ Verapamil	+ BAPTA
Isoprenaline (50 μM)	189 $\pm$ 26 ^b	114 $\pm$ 9 ^a	120 $\pm$ 7 ^a	115 $\pm$ 10 ^a
Noradrenaline (50 μM)	268 $\pm$ 24 ^b	199 $\pm$ 23 ^b	245 $\pm$ 25 ^b	239 $\pm$ 18 ^b
PMA (1 μM)	494 $\pm$ 37 ^b	443 $\pm$ 33 ^b	484 $\pm$ 46 ^b	478 $\pm$ 31 ^b

by isoprenaline, we investigated the contribution of extracellular Ca^{2+} to the activation of MAPK by isoprenaline, noradrenaline or PMA. Activation of MAPK by isoprenaline was inhibited (80–85%) by two L-type Ca^{2+} -channel antagonists, nifedipine or verapamil. Depletion of extracellular Ca^{2+} by inclusion of 3 mM 1,2-bis(2-aminophenoxy)ethane-*NN'*-tetra-acetate (BAPTA) in the medium induced a similar degree of inhibition (Table 5). Thus, isoprenaline in the presence of these inhibitors of Ca^{2+} mobilization did not activate the MAPKs significantly above control levels. In contrast, nifedipine, verapamil, or BAPTA did not significantly affect activation by noradrenaline or PMA (Table 5), nor the activation of MAPKs by endothelin-1 or acidic fibroblast growth factor (results not shown). 'In gel' MBP phosphorylation assays confirmed that isoprenaline-induced activation of 42 and 44 kDa MBPKs was decreased by these treatments, but that the 107 and 65 kDa kinase activities were unaffected (results not shown). These results suggest the relative importance of extracellular Ca^{2+} in the activation of MAPK by isoprenaline.

DISCUSSION

The cardiac myocyte is a terminally differentiated cell that responds to a wide variety of growth-promoting stimuli by an increase in cell size rather than cell division. Recently a role for Ras and Raf in hypertrophy has been proposed [8,25]. MAPK activation is now recognized as a major downstream signalling event of Ras/Raf-dependent pathways. We have suggested that MAPK activation in the cardiac myocyte may be important in the mediation of the hypertrophic response to agents that use dissimilar signalling pathways proximal to the cell membrane [6]. Specifically, we have observed that both PKC-dependent (e.g. PMA) and PKC-independent (e.g. acidic fibroblast growth factor) pathways are involved in the activation of MAPK in these cells. Other responses (e.g. endothelin-1) appear to utilize both PKC-dependent and PKC-independent components in the signalling pathways leading to MAPK activation [5,6].

In the present study, we have shown that adrenergic receptor stimulation activates the MAPK cascade in the cardiac myocyte via at least two distinct signalling pathways. In the first pathway, noradrenaline activates α_1 -adrenergic receptors (Table 3) that stimulate a pathway including the hydrolysis of PtdIns. This pathway is partially sensitive to down-regulation of the PMA-sensitive PKC isotypes (PKC- α , - δ and - ϵ) (Table 2), but insensitive to inhibition of G_i by pertussis toxin [3]. In these respects this is similar to the response to endothelin-1 [3,6].

In the second pathway, isoprenaline acting via the β -adrenergic

receptor (Table 3) activates a pathway that does not appear to be sensitive to down-regulation of the PMA-sensitive isotypes of PKC (Table 2). Stimulation of MAPK by isoprenaline is also not sensitive to inhibition by pertussis toxin [3]. Although the slow activation of PtdIns hydrolysis elicited by isoprenaline (significant after 40 min) may prove to be important in the development of hypertrophy (Table 1), the time course of its activation appears to preclude it playing a role in MAPK activation (maximal at 5 min, Figure 1).

In many cell types the activation of the MAPK cascade may be antagonized by the simultaneous activation of cyclic AMP-dependent protein kinase [14,26–30]. This is a result of the direct phosphorylation of c-Raf by cyclic AMP-dependent protein kinase [31]. However, these effects of cyclic AMP may be cell-specific as it has also been shown that elevation of cyclic AMP may lead to activation (rather than inhibition) of MAPK in PC12 cells [32] or COS-7 cells [33]. This cyclic AMP-dependent mechanism is not utilized by isoprenaline in the myocyte because agents that activate cyclic AMP-dependent protein kinase (IBMX, CPT-cyclic AMP, glucagon, forskolin) in the absence of β -adrenergic receptor stimulation fail to activate MAPK (Table 4).

A role for Ca^{2+} rather than cyclic AMP has been established in the β -adrenergic regulation of myocardial gene expression [10]. Furthermore, mobilization of Ca^{2+} has been shown to activate MAPK [34–37]. Elevation of intracellular Ca^{2+} activated MAPK in the myocyte to a level comparable with that achieved by isoprenaline (Table 4). Indeed, the activation of MAPK by isoprenaline was dependent on extracellular Ca^{2+} (Table 5). This is similar to the recent findings that angiotensin II may also utilize a Ca^{2+} -dependent pathway to activate MAPK in the cultured ventricular myocyte [7].

The upstream activators of MEK in the isoprenaline-stimulated myocytes must be identified. It is not clear how isoprenaline can increase MEK and MAPK activity (Figures 4 and 5) in the presence of elevated cyclic AMP-dependent protein kinase activity. Elevation of cyclic AMP-dependent protein kinase activity by exposure of the ventricular myocyte to CPT-cyclic AMP or forskolin does not decrease endothelin-1-stimulated MAPK activation (M. A. Bogoyevitch and P. H. Sugden, unpublished work). However, CPT-cyclic AMP does significantly decrease endothelin-1-stimulated c-Raf activity without altering basal c-Raf activity [38]. Interestingly, activation of A-Raf in the myocyte is less sensitive to CPT-cyclic AMP [38]. Although this might suggest that A-Raf could provide a cyclic AMP-insensitive step in the MAPK cascade [38], isoprenaline fails to activate either c-Raf or A-Raf in the ventricular myocyte (M. A. Bogoyevitch and P. H. Sugden, unpublished work). The β -adrenergic pathway for MEK activation must utilize other activators of MEK. However, two well-characterized MEK activators, MEKK and B-Raf, are also sensitive to inhibition by cyclic AMP-dependent protein kinase activation [39–41]. Recent studies suggest that cyclic AMP-insensitive steps may bypass c-Raf, although the identity of these components remains elusive [42].

There is increasing evidence for a role of MAPK in the regulation of gene expression. MAPK is able to phosphorylate a number of transcription factors [43]. MAPK is also active in the nuclei of many cell types and may undergo translocation to the nucleus upon activation [44]. MAPK may therefore provide an important link between the β -adrenergic receptor and the cell nucleus of the cardiac myocyte.

From the results of studies on the activation of MAPK in the cultured neonatal cardiac myocytes, there appears to be a correlation between the strength and completeness of the hyper-

trophic response and the activation of the MAPK cascade. Thus, agonists such as endothelin-1, α -adrenergic agonists and PMA, which produce a powerful hypertrophic response [4], are also the most effective activators of the MAPK cascade [5,6]. In contrast, as we show here, agonists such as isoprenaline which produce a weaker hypertrophic response [9] also are weaker activators of the MAPK cascade. We propose that the cardiac myocyte may possess mechanisms downstream from MAPK that monitor its signal strength and this leads to the hierarchical nature of hypertrophic agonism.

This work was supported by grants from The Wellcome Trust, British Heart Foundation, and the Biotechnology and Biological Sciences Research Council.

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