Activation of the mitogen-activated protein kinase cascade by pertussis toxin-sensitive and -insensitive pathways in cultured ventricular cardiomyocytes

Marie A. BOGOYEYITCH,* Angela CLERK and Peter H. SUGDEN

Department of Cardiac Medicine, National Heart and Lung Institute (University of London), Dovehouse Street, London SW3 6LY, U.K.

The involvement of pertussis toxin (PTX)-sensitive and -insensitive pathways in the activation of the mitogen-activated protein kinase (MAPK) cascade was examined in ventricular cardiomyocytes cultured from neonatal rats. A number of agonists that activate heterotrimeric G-protein-coupled receptors stimulated MAPK activity after exposure for 5 min. These included foetal calf serum (FCS), endothelin-1 (these two being the most effective of the agonists examined), phenylephrine, endothelin-3, lysophosphatidic acid, carbachol, isoprenaline and angiotensin II. Activation of MAPK and MAPK kinase (MEK) by carbachol returned to control levels within 30–60 min, whereas activation by FCS was more sustained. FPLC on Mono Q showed that carbachol and FCS activated two peaks of MEK and two peaks of MAPK (p42^{MAPK} and p44^{MAPK}). Pretreatment

of cells with PTX for 24 h inhibited the activation of MAPK by carbachol, FCS and lysophosphatidic acid, but not that by endothelin-1, phenylephrine or isoprenaline. Involvement of G-proteins in the activation of the cardiac MAPK cascade was demonstrated by the sustained (PTX-insensitive) activation of MAPK (and MEK) after exposure of cells to AlF₄⁻. AlF₄⁻ activated PtdIns hydrolysis, as did endothelin-1, endothelin-3, phenylephrine and FCS. In contrast, the effect of lysophosphatidic acid on PtdIns hydrolysis was small and carbachol was without significant effect even after prolonged exposure. We conclude that PTX-sensitive (i.e. G_i/G_o-linked) and PTX-insensitive (i.e. G_q/G_s-linked) pathways of MAPK activation exist in neonatal ventricular myocytes. FCS may stimulate the MAPK cascade through both pathways.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) were originally identified as a family of protein Ser/Thr kinases activated in response to growth factors that signal through protein tyrosine kinase receptors [1]. This activation is the result of the phosphorylation of the Tyr- and Thr- residues within a conserved Thr-Gly-Tyr motif catalysed by MAPK kinase [or MEK, for MAP or extracellular signal-related kinase (ERK) kinase] which is, in turn, activated by phosphorylation by protein kinases such as Raf (for reviews, see [2–5]). The intracellular signalling events initiated by some G-protein-coupled receptors have also been shown to activate the MAPK pathway in a number of cells [6–8]. In many cases, this activation is not as potent as that caused by the growth factor tyrosine kinase receptors [9,10]. The upstream signalling events in the G-protein-activated pathway may also involve the activation of Raf or other protein kinases such as MEK kinase [9,11].

The ventricular cardiomyocyte responds to cardioactive growth-promoting stimuli such as endothelin-1 (ET-1) and α -adrenergic agonists by an increase in cell size (hypertrophy) rather than cell division. Development of the hypertrophic phenotype involves dramatic changes at the level of gene expression [12,13]. Exposure of cardiomyocytes to either ET-1 or α -adrenergic agonists, agents that both act via G-protein-coupled receptors, also stimulates phospholipase C β (PLC β)-mediated hydrolysis of membrane PtdIns pools, leading to activation of protein kinase C (PKC) [14,15]. This implies the operation of a G_q-dependent pathway. Both agents also inhibit adenylate cyclase activated in response to agonists such as isoprenaline, and this inhibition is sensitive to pertussis toxin (PTX) [16–21].

This implies operation of a G_i-linked pathway. ET-1 and α -adrenergic agonists stimulate MAPK activities in ventricular cardiomyocytes [8,22,23], although it is not clear whether this is G_q- or G_i-linked. We have suggested that the G-protein/MAPK pathway may be important in the mediation of the hypertrophic response by ET-1, α -adrenergic agonists and other interventions [8,22]. Here we have extended our earlier studies to examine G_i-linked pathways of MAPK activation in the heart.

EXPERIMENTAL

Materials

Radiochemicals, enhanced chemiluminescence (ECL) reagents, autoradiography film and pre-stained molecular mass standard proteins were from Amersham International, Amersham, Bucks., U.K. All agonists, PTX, tissue culture media, proteinase inhibitors and bovine myelin basic protein (MBP) were from Sigma, Poole, Dorset, U.K. Heat-inactivated foetal calf serum (FCS) was from Sera Lab., Crawley Down, West Sussex, U.K. Collagenase (Type 2; Worthington) was from Lorne Laboratories, Twyford, Berks., U.K. Other tissue culture products were from Life Technologies, Paisley, Renfrewshire, U.K. Centricon ultrafiltration units were from Amicon, Stonehouse, Gloucestershire, U.K. Recombinant murine p42^{MAPK} was expressed as a glutathione transferase (GST) fusion protein in *Escherichia coli* [24]. This clone and polyclonal antisera recognizing MAPK or MEK were gifts from Professor C. J. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London, U.K.).

PTX was dissolved in 50 mM Na₂HPO₄/NaH₂PO₄ (pH 7.2). Carbachol, ET-1 and angiotensin II were dissolved in distilled

Abbreviations used: AVP, [Arg⁸]vasopressin; ECL, enhanced chemiluminescence; ERK, extracellular signal-related protein kinase; ET, endothelin; FCS, foetal calf serum; GST, glutathione S-transferase; LPA, 1-oleoyl-lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK (or ERK) kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin.

* To whom correspondence should be addressed.

deionized water. 1-Oleoyl-lysophosphatidic acid (LPA) was dissolved in 30 % (v/v) ethanol; [Arg⁸]Vasopressin (AVP) was dissolved in dimethyl sulphoxide; and solutions of isoprenaline and phenylephrine were freshly prepared in 100 μ M L-ascorbic acid. AlF_4^- was freshly prepared by mixing 333 mM NaF and 1 mM AlCl_3 (9:1, v/v). Stock solutions of agonists were diluted in serum-free medium at 37 °C and used immediately. Preliminary experiments established that carry-over of solvent did not affect the results.

Ventricular cardiomyocyte cultures

Cardiac myocytes were dissociated from the ventricles of hearts from 1–2-day-old rats using 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 and 0.8 mM MgSO_4 (pH 7.35) [25]. 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) FCS and 100 units/ml penicillin and streptomycin. Cells were preplated (30 min) on uncoated culture dishes to remove non-myocytes, then either 4×10^6 myocytes were plated per 60 mm gelatin-precoated dish for preparation for samples for FPLC, or 1×10^6 myocytes were plated per 35 mm gelatin-precoated dish for all other assays. After 18 h, myocytes were confluent and spontaneously beating. Serum was withdrawn for 24 h before the cells were further treated. PTX was added when indicated to a final concentration of 150 ng/ml in the serum-free medium 24 h before exposure of myocytes to agonists.

Myocytes were exposed to agonists in serum-free medium at 37 °C, then washed with cold Ca^{2+} / Mg^{2+} -free Dulbecco's phosphate-buffered saline and scraped into cold lysis buffer [20 mM β -glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM sodium vanadate, 10 mM benzamidine, 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 supernatant fractions were retained for assay of MAPK or MEK activity, or for FPLC.

Assay of MAPK and MEK

MAPK was assayed by two methods. First, in the 'direct' assay, supernatant samples were incubated with MBP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described earlier [22]. The reaction was terminated by spotting 40 μ l of the reaction mixture on to P81 paper and washing in 75 mM H_3PO_4 (1 $\times$ 5 min; 3 $\times$ 30 min). Phosphate incorporation was measured by liquid scintillation counting. In these assays, 1 unit of MAPK catalyses the incorporation of 1 pmol of phosphate into MBP/min. All direct assays were performed on at least four independent preparations of myocytes. Secondly, in the 'in-gel' assay, supernatant samples (50 μ l) were denatured at 100 °C for 5 min with 25 μ l of SDS/PAGE sample buffer [26]. Samples were electrophoresed in SDS/10 % polyacrylamide gels formed in the presence of 0.5 mg/ml MBP. p42^{MAPK} , p44^{MAPK} and additional MBP kinase activities were detected by incubation of these gels with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and autoradiography as described earlier [8]. This assay was used to confirm that p42^{MAPK} and p44^{MAPK} activities changed in parallel to activities measured by the 'direct' assay.

MEK activity was assayed by a two-step assay using the activation of exogenous recombinant GST- p42^{MAPK} [8]. Supernatant samples were diluted 20-fold in buffer A (50 mM Tris/HCl, 0.1 mM EGTA, 0.1 mM Na_3VO_4 , pH 7.5) containing 0.1 % (w/v) BSA and 0.1 % (v/v) mercaptoethanol. After samples (10 μ l) had been mixed with 5 μ l of GST- p42^{MAPK} in buffer A containing 0.04 % (v/v) Brij 35, 0.1 % (v/v) mercaptoethanol

and 0.5 μ M okadaic acid, the activation of GST- p42^{MAPK} was initiated by the addition of 5 μ l of 80 mM magnesium acetate and 0.8 mM unlabelled ATP. After 15 min at 30 °C, the activity of GST- p42^{MAPK} was assayed by the addition of 25 μ l of 0.8 mg/ml MBP in buffer A containing 2.25 μ M cyclic AMP-dependent protein kinase inhibitory peptide (PKI), 2.25 μ M okadaic acid, and 5 μ l of 40 mM magnesium acetate/0.4 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.5 μ Ci per assay). After a further 15 min at 30 °C, the reaction was terminated by spotting 40 μ l of the reaction mixture on to P81 paper, which was washed with 75 mM H_3PO_4 as described for the MAPK assay above. To correct for endogenous MAPK activity in samples, results of control assays carried out in the absence of GST- p42^{MAPK} were subtracted. The auto-phosphorylation and autoactivation of GST- p42^{MAPK} occurring in the incubation of GST- p42^{MAPK} with ATP in these assays in the absence of cell lysates was also subtracted. One unit of MEK is the amount which, in 1 min of the preincubation in the presence of unlabelled ATP, specifically catalyses the activation of exogenous GST- p42^{MAPK} sufficient to transfer 1 pmol of phosphate into MBP/min in the subsequent MAPK assay.

FPLC and immunoblotting of fractions

Soluble fractions of myocyte extracts were applied to a Mono Q HR5/5 column (Pharmacia-LKB) equilibrated with buffer B [50 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 0.1 % (v/v) mercaptoethanol, 5 % (v/v) glycerol, 0.05 % (w/v) Brij 35, 0.3 mM sodium vanadate, 1 mM benzamidine and 4 μ g/ml leupeptin (pH 7.3)] at a flow rate of 1 ml/min [8]. After a 5 ml isocratic wash, proteins were eluted with a linear gradient of NaCl (0–0.3 M) in buffer B. Fractions (0.5 ml) were assayed for MAPK and MEK. For immunoblotting, fractions containing MAPK or MEK activity were concentrated (Centricon 10 units) and proteins separated by SDS/PAGE on 10 % gels. Proteins were transferred to nitrocellulose which was probed with antibodies to MAPK or MEK and finally developed using the ECL method [8].

Prelabelling and agonist-stimulating hydrolysis of PtdIns pools

Cells were incubated with serum-free medium (2 ml) containing 5 μ Ci of carrier-free $[\text{H}]\text{inositol}$ for 24 h before exposure to agonists in serum-free medium containing 10 mM LiCl. The incubation was terminated by removal of the medium and addition of 0.5 ml of 0.8 M HClO_4 . Each plate was scraped and washed with a further 0.5 ml of 0.8 M HClO_4 . Precipitated protein was removed by centrifugation and the total $[\text{H}]\text{inositol}$ phosphates in the neutralized supernatant fractions were determined [27].

Statistical methods

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.

RESULTS

Activation of MAPK by G-protein-coupled receptors

Activity of MAPK in the soluble fractions of cultured neonatal rat ventricular cardiomyocytes can be reliably assayed by the direct phosphorylation of MBP in the presence of inhibitors of cyclic AMP-dependent and Ca^{2+} -dependent protein kinases [8,22]. MAPK was activated in myocytes after 5 min of exposure (at which MAPK activities were maximal) to a variety of agonists that signal through heterotrimeric G-protein-dependent

Table 1 Activation of MAPK by agonists that act through G-protein-coupled receptors

Ventricular cardiomyocytes were exposed to isoprenaline, phenylephrine (PE), ET-1, ET-3, sarafotoxin 6c (STX 6c), carbachol (CB), LPA, angiotensin II (All), AVP or FCS for 5 min. MAPK activity in soluble extracts was assayed either in the 'direct assay' or in the 'in-gel' assay as described in the Experimental section. For the 'in-gel' assay, the bands corresponding to p42^{MAPK} and p44^{MAPK} were quantified by scanning laser densitometry of the autoradiograms. For both assays, results ($n = 4-10$ for 'direct' assay and $n = 3$ for 'in-gel' assay) are expressed relative to activities in control cells. Statistical significance: ^a $P < 0.10$, ^b $P < 0.05$, ^c $P < 0.01$ and ^d $P < 0.001$ versus control cells. N.D. = not determined.

Agonist	MBP kinase activity (% of control)			
	'Direct' assay	'In-gel' assay		
		p42 ^{MAPK} + p44 ^{MAPK}	p42 ^{MAPK}	p44 ^{MAPK}
Isoprenaline (50 μ M)	160 $\pm$ 10 ^d	300 $\pm$ 90 ^a	430 $\pm$ 40 ^b	240 $\pm$ 110 ^a
PE (50 μ M)	260 $\pm$ 30 ^d	650 $\pm$ 240 ^a	820 $\pm$ 100 ^b	690 $\pm$ 250 ^a
ET-1 (100 nM)	370 $\pm$ 40 ^d	1210 $\pm$ 320 ^b	1360 $\pm$ 120 ^c	1280 $\pm$ 410 ^b
ET-3 (100 nM)	220 $\pm$ 20 ^c	N.D.	N.D.	N.D.
STX 6c (100 nM)	130 $\pm$ 20	N.D.	N.D.	N.D.
CB (100 μ M)	180 $\pm$ 20 ^d	420 $\pm$ 220 ^b	520 $\pm$ 150 ^a	410 $\pm$ 240
LPA (100 μ M)	210 $\pm$ 10 ^d	560 $\pm$ 130 ^b	670 $\pm$ 90 ^c	530 $\pm$ 100 ^b
All (1 μ M)	150 $\pm$ 10 ^c	300 $\pm$ 60 ^b	330 $\pm$ 80 ^b	270 $\pm$ 40 ^b
AVP (1 μ M)	130 $\pm$ 10	N.D.	N.D.	N.D.
FCS (20%, v/v)	460 $\pm$ 40 ^d	1020 $\pm$ 290 ^b	1110 $\pm$ 110 ^c	1060 $\pm$ 420 ^a

receptors (Table 1). As we have shown previously [22], both ET-1 and the α -adrenergic agonist, phenylephrine, increased MAPK activity. Phenylephrine increased MAPK activity to 260 % of the control value. The β -adrenergic agonist isoprenaline, although significantly activating MAPK, was much less effective. ET-1 stimulated MAPK activity to 370 % of the control value. ET-3 activated MAPK to 200–250 %, but the ET_B receptor-selective agonist sarafotoxin 6c was without significant effect, suggesting the involvement of the ET_A receptor in the signalling pathways for ETs. Carbachol, LPA and angiotensin II were less effective activators of MAPK and stimulated activities to 150–250 % of control. AVP was without significant effect. The most potent activator of MAPK was FCS, which activated MAPK 4–5-fold. Thus FCS has a similar potency to phorbol 12-myristate 13-acetate (PMA) [22] with respect to activation of MAPK in this cell type.

To confirm that these agonists stimulated both p42^{MAPK} and p44^{MAPK}, soluble fractions of cardiomyocytes were assayed by the 'in-gel' kinase method [8,22,23]. In this assay, renaturable MBP kinases are detected in MBP-containing gels. Upon 5 min of exposure of the cardiomyocytes to a variety of agonists that activate G-protein-coupled receptors, only the kinases of 42 kDa and 44 kDa, i.e. p42^{MAPK} and p44^{MAPK}, were activated. We performed quantification by laser scanning densitometry to compare the 'direct' assay with the 'in-gel' assay. First, data for the 42 and 44 kDa kinases were combined (p42^{MAPK} + p44^{MAPK}) to compare directly the two assays (Table 1). The order of potency of activation assessed by the two assays was similar, with FCS and ET-1 being the most potent activators. Phenylephrine, LPA, carbachol and angiotensin II were less effective. In all cases the observed stimulation of activity was approx. 2–3-fold greater using the 'in-gel' compared with the 'direct' assay. This would suggest that high levels of MBP phosphorylation in the control cells may limit the stimulation observed in the 'direct' assay. Analysis of the p42^{MAPK} and p44^{MAPK} bands showed that both isoforms were activated by all agonists (Table 1).

Activation of MAPK and MEK by carbachol and FCS

The time courses of activation of MEK and MAPK after exposure to carbachol revealed that both enzymes were rapidly

Figure 1 Time courses for the activation of MAPK and MEK by carbachol

Ventricular cardiomyocytes were exposed to 100 μ M carbachol for the times indicated. In (a), soluble extracts were assayed directly for MAPK (■) or MEK (●) activity as described in the Experimental section. Results of a typical experiment are shown, similar results being obtained on three separate occasions. In (b), soluble extracts were assayed by the 'in-gel' MBP kinase method as described in the Experimental section. The numbers to the right of the Figure are the molecular masses (kDa) of the marker proteins. The lower and the upper bands migrating ahead of the 46 kDa marker protein are p42^{MAPK} and p44^{MAPK} respectively.

activated (Figure 1a). To allow direct comparison of the time courses and the extent of maximal activation achieved with each agonist, the activities of MAPK and MEK are presented in the same graphs but using different axes. The maximum activation of MAPK and MEK was achieved at 5 min and fell to control

Figure 2 Time courses for the activation of MAPK and MEK by FCS

Ventricular cardiomyocytes were exposed to 20% (v/v) FCS for the times indicated. In (a), soluble extracts were assayed directly for MAPK (■) or MEK (●) activity as described in the Experimental section. Results of a typical experiment are shown, similar results being obtained on three separate occasions. In (b), soluble extracts were assayed by the 'in-gel' MBP kinase method as described in the Experimental section. The numbers to the right of the Figure are the molecular masses (kDa) of the marker proteins. The lower and the upper bands migrating ahead of the 46 kDa marker protein are $p42^{\text{MAPK}}$ and $p44^{\text{MAPK}}$ respectively.

values within 30 min. The 'in-gel' MBP kinase assay confirmed the rapid changes in $p42^{\text{MAPK}}$ and $p44^{\text{MAPK}}$ activities (Figure 1b).

Similarly, MEK and MAPK activities were rapidly elevated by exposing myocytes to 20% (v/v) FCS (Figure 2a). However, MEK and MAPK activities remained elevated for at least 30 min (Figure 2a). 'In-gel' MBP kinase assays confirmed the rapid and relatively sustained activation of $p42^{\text{MAPK}}$ (5-fold at 30 min) and $p44^{\text{MAPK}}$ (12-fold at 30 min) (Figure 2b). The activation of $p42^{\text{MAPK}}$ and $p44^{\text{MAPK}}$ was decreased after a 60 min exposure to FCS (Figure 2b), and it may be that the sustained activation of kinase activity at 60 min observed in the 'direct' assay is a consequence of other protein kinases activated by prolonged exposure to FCS. These protein kinases are not detected in the 'in-gel' assay. Activation of MAPK could be observed at FCS concentrations as low as 1% (v/v) (results not shown), suggesting the presence of a potent MAPK activating agent(s). LPA may be an important growth-promoting agonist in serum [28]. However, the time course and extent of MEK or MAPK activation by LPA resembled that of carbachol rather than FCS (results not shown).

In all 'in-gel' MBP kinase assays with crude extracts, other MBP kinases with molecular masses of 160 and 68 kDa were detected (see Figures 2b and 3b). However, their activities did not consistently change during the exposure of cells to carbachol, FCS or LPA. The origin of these activities has been discussed previously [8].

FPLC of MEK and MAPK

MEK and MAPK activities in soluble fractions of carbachol- or FCS-treated cells were examined by FPLC on Mono Q (Figure

Figure 3 FPLC of MAPK and MEK in myocytes exposed to carbachol or FCS

Ventricular cardiomyocytes were exposed to (a) 100 μM carbachol or (b) 20% (v/v) FCS for 5 min. Soluble extracts were applied to a Mono Q HR5/5 column and proteins were eluted with a linear gradient of NaCl (0–0.3 M; straight dotted line). Fractions (0.5 ml) were collected and assayed for MEK (solid line: ●, + agonist; ○, control) and MAPK (dashed line: ■, + agonist; □, control). The two major peaks of MEK are labelled KK1 and KK2 and the two major peaks of MAPK are labelled K1 and K2.

3). Two peaks of MEK activity (KK1 and KK2) and two peaks of MAPK activity (K1 and K2) were observed. MEK peak KK1 was greater than KK2. Peaks of MEK and MAPK activity were lower in myocytes exposed to carbachol (Figure 3a) than in cells exposed to FCS (Figure 3b). The differences in the activities observed in the FPLC profiles are consistent with the lower activities of MEK and MAPK in carbachol-treated cells (Figure 1) compared with those treated with FCS (Figure 2). When fractions from FCS-treated cells were examined by immunoblotting, anti-MEK1 antiserum recognized a 50 kDa protein in concentrated peak fractions of MEK peaks KK1 and KK2. Antiserum that recognizes both $p42^{\text{MAPK}}$ and $p44^{\text{MAPK}}$ detected a 42 kDa protein in concentrated peak fractions from MAPK peak K1 and a 44 kDa protein in concentrated peak fractions from MAPK peak K2 (results not shown). Similar results have been previously observed after FPLC of myocytes exposed to ET-1 and acidic fibroblast growth factor, indicating that agonists that use different signalling pathways proximal to the cell membrane can activate the MAPK cascade in these cells [8,22].

Activation of MAPK by AlF_4^-

AlF_4^- has been used as a reagent to investigate the involvement of G-proteins in signalling events in intact cells. It activates G-proteins by forming a $\text{GDP} \cdot \text{AlF}_4^-$ complex at the GTP binding site on $\text{G}\alpha$, thereby mimicking the trigonal-bipyramidal tran-

Figure 4 Time courses for the activation of MAPK and MEK by AIF_4^-

Ventricular cardiomyocytes were exposed to AIF_4^- (30 mM NaF, 10 μM AlCl_3) for the times indicated. In (a), soluble extracts were assayed directly for MAPK (■) or MEK (●) activity as described in the Experimental section. Results of a typical experiment are shown, similar results being obtained on three separate occasions. In (b), soluble extracts were assayed by the 'in-gel' MBP kinase method as described in the Experimental section. The numbers to the right of the Figure are the molecular masses (kDa) of the marker proteins. The lower and the upper bands migrating ahead of the 46 kDa marker protein are p42^{MAPK} and p44^{MAPK} respectively.

sition state of GTP during the hydrolytic GTPase reaction [29]. MEK activity was elevated after a 5 min exposure of myocytes to AIF_4^- and continued to increase in the subsequent 10 min of exposure (Figure 4a). In contrast to carbachol (Figure 1), FCS (Figure 2) or LPA (results not shown), no significant activation of MAPK could be detected at 5 min ($84 \pm 21\%$ of control; $n = 3$; Figure 4a), and maximum activation ($299 \pm 29\%$ of control; $n = 6$; $P < 0.001$) was not observed until 15–30 min. The extent of maximal activation is only marginally less than that observed for FCS (see Table 2 and compare Figures 2 and 4). The activities of MAPK and MEK upon continued exposure to AIF_4^- remained elevated until at least 60 min (Figure 4a). The activation of p42^{MAPK} and p44^{MAPK} was confirmed by the 'in-gel' MBP kinase (Figure 4b). The activity of the 68 kDa MBP kinase also increased after prolonged exposure to AIF_4^- (Figure 4b).

Effects of PTX on activation of MAPK by agonists

To investigate the role of G-proteins in the activation of the MAPK pathway, ventricular myocytes were pre-incubated with PTX (150 ng/ml) in serum-free medium for 24 h prior to exposure to agonists. This concentration of PTX effectively ADP-ribosylates and inactivates G_i and G_o in cultured neonatal ventricular myocytes [25]. The activation of MAPK by carbachol, LPA and FCS is mediated by PTX-sensitive G-proteins (Table 2). Activation by carbachol and LPA was inhibited so that activities were no longer significantly different from the control activity (+PTX pretreatment) (Table 2). Although activation by FCS was also significantly decreased, activity remained at 230 %

Table 2 Sensitivity of agonist-stimulated MAPK activation to PTX pretreatment

Ventricular cardiomyocytes were preincubated for 24 h in serum-free medium without further addition (–PTX pretreatment) or in serum-free medium supplemented with 150 ng/ml PTX (+PTX pretreatment). Cells were then exposed to isoprenaline, phenylephrine (PE), ET-1, carbachol (CB), LPA, fluoroaluminate (AIF_4^-), PMA or FCS. Exposure time to the agonists was 5 min except for when the agonist was AIF_4^- (30 min). MAPK activity in soluble extracts was assayed directly by the phosphorylation of MBP as described in the Experimental section. Results ($n = 4-7$) are expressed relative to activity in control cells. Statistical significance: * $P < 0.05$ for agonist-stimulated activity (+PTX pretreatment) versus agonist-stimulated activity (–PTX pretreatment). † $P < 0.05$ for agonist-stimulated activity (+PTX pretreatment) versus control activity (+PTX pretreatment).

Agonist	MBP kinase activity (% of control)	
	(–PTX pretreatment)	(+PTX pretreatment)
Control	100 ± 0	118 ± 10
Isoprenaline (50 μM)	162 ± 4	158 ± 11†
PE (50 μM)	255 ± 15	224 ± 15†
ET-1 (100 nM)	427 ± 10	383 ± 20†
CB (100 μM)	204 ± 29	145 ± 13*
LPA (100 μM)	197 ± 22	146 ± 14*
AIF_4^- (300 mM NaF, 0.1 mM AlCl_3)	302 ± 40	308 ± 43†
PMA (1 μM)	480 ± 15	561 ± 33†
FCS (20%, v/v)	411 ± 21	238 ± 29*†

Figure 5 Stimulation of $\text{Ptd}[^3\text{H}]\text{Ins}$ hydrolysis by agonists

Ventricular cardiomyocytes were exposed to 100 μM carbachol (○, CB), 50 μM phenylephrine (□, PE), 20% (v/v) FCS (△), 100 nM ET-1 (●), AIF_4^- (■, 30 mM NaF/10 μM AlCl_3), 100 μM LPA (▲) for the times indicated. △---△, Control in the absence of agonist (CON). $\text{Ptd}[^3\text{H}]\text{Ins}$ hydrolysis was measured as described in the Experimental section and is expressed relative to control cells at time zero ($n = 4-7$).

of the (–PTX pretreatment) control value and this was significantly greater than the control activity (+PTX pretreatment) (Table 2). This suggests that the response to FCS may be only partially mediated by PTX-sensitive G-proteins. Activation of MAPK by ET-1, adrenergic agonists or PMA was not significantly decreased by PTX pretreatment (Table 2), suggesting that the inhibitory effects of PTX on MAPK did not result from non-specific effects. Activation of MAPK by AIF_4^- was also not inhibited by PTX, suggesting the presence of other potent G_i -

independent activation pathways in the ventricular myocyte (Table 2). The reason that the activation of MAPK by AIF_4^- was not at least partially PTX-sensitive is not clear.

Activation of membrane PtdIns hydrolysis

To investigate further the signalling pathways utilized by agents that activate MAPK, we examined the activation of membrane PtdIns hydrolysis (Figure 5). ET-1 and phenylephrine were the most potent activators of PtdIns hydrolysis. This is in agreement with the potent activation of MAPK by these agonists (Table 1). ET-3 stimulated PtdIns hydrolysis to $302 \pm 32\%$ ($n = 3$, $P < 0.005$) of control at 10 min, whereas a 10 min exposure to sarafotoxin 6c was without significant effect ($126 \pm 12\%$; $n = 3$). Both ET-1-stimulated and ET-3-stimulated PtdIns hydrolysis were effectively inhibited by incubation with the ET_A receptor antagonist BQ123 ($10 \mu\text{M}$; results not shown).

AIF_4^- also stimulated PtdIns hydrolysis, but only after 20–30 min of exposure (Figure 5). This delay is similar to that observed for MAPK activation (Figure 3), suggesting that AIF_4^- may slowly activate G_q or a G_q -like G-protein that subsequently activates the MAPK pathway. LPA caused only small changes in PtdIns hydrolysis during prolonged incubations, whereas FCS was moderately effective (Figure 5). No significant activation of PtdIns hydrolysis by carbachol was observed (Figure 5).

DISCUSSION

We have proposed that the MAPK signalling pathway may contribute to the changes in gene expression that occur during the development of the hypertrophic phenotype in the ventricular cardiomyocyte [8]. Recent direct evidence now supports this hypothesis [30–33]. In the present study, we have examined whether the stimulation of a variety of G-protein-coupled receptors leads to activation of the MAPK pathway in the cardiomyocyte. We wished to elucidate the signalling pathways which link G-protein-coupled receptor activation to MAPK activation and to correlate these pathways of MAPK activation with the development of the hypertrophic phenotype. The present study had demonstrated that a variety of agonists that activate heterotrimeric G-protein-coupled receptors can activate MAPK, albeit to different extents (Table 1). Direct G-protein activation by AIF_4^- also activates MEK and MAPK (Figure 4 and Table 2). The most potent activators of MAPK identified in this study, i.e. ET-1, phenylephrine and FCS, have been described as potent activators of ventricular myocyte growth and/or hypertrophy ([25,34–36] and A. Clerk, M. B. Andersson, M. A. Bogoyevitch and P. H. Sugden, unpublished work).

The receptors for ET-1 and α -adrenergic agonists such as phenylephrine couple to at least two major classes of G-protein α -subunits, G_{12} and G_{13} . The activation of G_{12} has been classically characterized as leading to inhibition of agonist-stimulated adenylate cyclase activity [16–19]. The activation of G_{13} activates PLC β , which hydrolyses PtdIns to produce $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol [37,38]. The production of diacylglycerol subsequently activates the isotypes of PKC predominating in the cardiac myocyte, namely PKC- δ and PKC- ϵ [23,39]. In the present study, ET-1 and phenylephrine were potent stimulators of PtdIns hydrolysis (Figure 5), and this agrees with their activation of PKC [23]. FCS and fluoroluminale also stimulated PtdIns hydrolysis, but to lesser extents (Figure 5).

There is now substantial evidence for the involvement of G_q and the subsequent activation of PKC in the development of the

hypertrophic phenotype. Previous studies have demonstrated that the growth-promoting effects of phenylephrine in the cardiomyocyte could not be blocked by pretreatment with PTX, thereby indirectly implicating a role for G_q [25]. (Although ET-1 also promotes myocyte hypertrophy [34,35], we are not aware of reports of the sensitivity of this response to PTX.) More recently, ventricular cardiomyocytes transfected with either activated G_q or activated PKC isotypes have shown increased expression of marker genes of ventricular hypertrophy (β -myosin heavy chain [40,41], atrial natriuretic factor [42–44] and myosin light chain-2 [42]). Recently, the hypertrophic response to α -adrenergic agonists was demonstrated to be blocked by microinjection of neutralizing antibodies to G_q [43]. This implicates a role for G_q /PtdIns hydrolysis and PKC activation in the hypertrophic response to α -adrenergic stimulation. Presumably ET-1 activating via the ET_A receptor would utilize similar signalling pathways to produce hypertrophic effects on the ventricular cardiomyocyte.

Other agonists with less well characterized (or sometimes controversial) effects on the development of the hypertrophic phenotype include isoprenaline, carbachol, LPA and angiotensin II [25,45–48]. These agonists activated MAPK to a lesser extent (Table 1). Activation of MEK and MAPK by carbachol or LPA was rapid and transient (Figure 1) and independent of hydrolysis of PtdIns (Figure 5). It is therefore unlikely that the effects of FCS that we observe are mediated solely by LPA. Carbachol also failed to activate PKC isotypes PKC- α , PKC- δ , PKC- ϵ or PKC- ζ after short-term exposure (A. Clerk and P. H. Sugden, unpublished work). Activation of MAPK by carbachol and LPA therefore appears to be independent of G_q /PtdIns hydrolysis and PKC activation.

Further examination of the effects of carbachol and LPA showed the activation of MAPK to be sensitive to pretreatment with PTX (Table 2). In addition, the effects of FCS were also partially sensitive to PTX, and this agrees with the finding that PTX may inhibit growth in some cells [49]. The sensitivity of MAPK activation of PTX implies coupling through G-proteins such as G_i or G_o to this pathway in the ventricular myocyte. Neonatal rat ventricles express G_i protein and mRNAs for G_{12} , G_{13} and G_o [50]. Previous reports have suggested that LPA, carbachol and thrombin may activate the MAPK cascade via G_{12} in cells such as fibroblasts [6,51–54], and the same may thus apply in the neonatal ventricular myocyte. However, the role of G_i in the growth of the cardiomyocyte has not been reported. Future studies must address the effects of agents such as LPA or carbachol on the development of the hypertrophic response.

In summary, this study demonstrates that a variety of G-protein-coupled agonists activate the MAPK pathway in cultured ventricular myocytes. G_{12} may mediate the response to carbachol or LPA and may partially mediate the response to FCS. An increase in the expression of G_{12} in the heart in the development of hypertension [55] (although controversial [56]) may therefore alter cyclic AMP production and contractility, and have direct consequences on the regulation of the MAPK signal transduction pathway in the heart. In addition, the levels of G_{12} and muscarinic receptors are developmentally regulated in the heart [50,57,58], and this may have implications for the activation of the MAPK cascade. A direct assessment of the relative roles of G_{12} and G_{13} in the signal transduction pathways in the ventricular cardiomyocyte will require the introduction of these proteins into this cell type.

This work was supported by grants from The Wellcome Trust and the British Heart Foundation.

REFERENCES

- 1 Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morganbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) *Cell* **65**, 663–675
- 2 Roberts, T. M. (1992) *Nature (London)* **360**, 534–535
- 3 Crews, C. M. and Erikson, R. L. (1993) *Cell* **74**, 215–217
- 4 Blumer, K. J. and Johnson, G. L. (1994) *Trends Biochem. Sci.* **19**, 236–240
- 5 Marshall, C. J. (1994) *Curr. Opin. Genet. Dev.* **4**, 82–89
- 6 Kahan, C., Seuwen, K., Meloche, S. and Pouyssegur, J. (1992) *J. Biol. Chem.* **267**, 13369–13375
- 7 Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K. and Yokoyama, M. (1992) *Circ. Res.* **71**, 620–630
- 8 Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 1110–1119
- 9 Hilal-Dandan, R. R. and Johnson, G. L. (1993) *J. Biol. Chem.* **268**, 17896–17901
- 10 Vouret-Craviari, V., van Obberghen-Schilling, E., Scimeca, J.-C., van Obberghen, E. and Pouyssegur, J. (1993) *Biochem. J.* **299**, 209–214
- 11 Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. and Johnson, G. L. (1993) *Science* **260**, 315–319
- 12 Chien, K. R., Knowlton, K. U., Zhu, H. and Chien, S. (1991) *FASEB J.* **5**, 3037–3046
- 13 Chien, K. R., Zhu, H., Knowlton, K. U., Miller-Hance, W., Van Bilsen, M., O'Brien, T. X. and Evans, S. M. (1993) *Annu. Rev. Physiol.* **55**, 77–95
- 14 Rana, R. S. and Hokin, L. E. (1990) *Physiol. Rev.* **70**, 115–164
- 15 Stabel, S. and Parker, P. J. (1991) *Pharmacol. Ther.* **51**, 71–95
- 16 Buxton, I. L. O. and Brunton, L. L. (1985) *J. Biol. Chem.* **260**, 6733–6737
- 17 Buxton, I. L. O. and Brunton, L. L. (1986) *Am. J. Physiol.* **251**, H307–H313
- 18 Hilal-Dandan, R., Urasawa, K. and Brunton, L. L. (1992) *J. Biol. Chem.* **267**, 10620–10624
- 19 Hilal-Dandan, R., Merck, D. T., Lujan, J. P. and Brunton, L. L. (1994) *Mol. Pharmacol.* **45**, 1183–1190
- 20 James, A. F., Xie, L.-H., Fujitani, Y., Hayashi, S. and Horie, M. (1994) *Nature (London)* **370**, 297–300
- 21 Ono, K., Tsujimoto, G., Sakamoto, A., Eto, K., Masaki, T., Ozaki, Y. and Satake, M. (1994) *Nature (London)* **370**, 301–304
- 22 Bogoyevitch, M. A., Glennon, P. E. and Sugden, P. H. (1993) *FEBS Lett.* **317**, 271–275
- 23 Clerk, A., Bogoyevitch, M. A., Andersson, M. B. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 32848–32857
- 24 Stokoe, D., Campbell, D. G., Nakielnny, S., Hidaka, H., Leever, S. J., Marshall, C. and Cohen, P. (1992) *EMBO J.* **11**, 3985–3994
- 25 Iwaki, K., Sukhatme, V. P., Shubeita, H. E. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 13809–13817
- 26 Bogoyevitch, M. A., Parker, P. J. and Sugden, P. H. (1993) *Circ. Res.* **72**, 757–767
- 27 Sugden, P. H., Fuller, S. J., Mynett, J. R., Hatchett, R., Bogoyevitch, M. A. and Sugden, M. C. (1993) *Biochim. Biophys. Acta* **1175**, 327–332
- 28 Durieux, M. E. and Lynch, K. R. (1993) *Trends Pharmacol. Sci.* **14**, 249–254
- 29 Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. and Sprang, S. R. (1994) *Science* **265**, 1405–1412
- 30 Thorburn, J. and Thorburn, A. (1994) *Biochem. Biophys. Res. Commun.* **202**, 1586–1591
- 31 Thorburn, J., Frost, J. A. and Thorburn, A. (1994) *J. Cell Biol.* **126**, 1565–1572
- 32 Sugden, P. H., Gillespie-Brown, J., Fuller, S. J., Cowley, S., Ashworth, A. and Marshall, C. J. (1994) *Circulation* **90**, I-303 (Abstract)
- 33 Thorburn, J., McMahon, M. and Thorburn, A. (1994) *J. Biol. Chem.* **269**, 30580–30586
- 34 Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembofski, C. C., Brown, J. H. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 20555–20562
- 35 Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K. and Marumo, F. (1991) *Circ. Res.* **69**, 209–215
- 36 Knowlton, K. U., Michel, M. C., Itani, M., Shubeita, H. E., Ishihara, K., Heller Brown, J. and Chien, K. R. (1993) *J. Biol. Chem.* **268**, 15374–15380
- 37 Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- 38 Exton, J. H. (1994) *Annu. Rev. Physiol.* **56**, 349–369
- 39 Pucéat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L. L. and Brown, J. H. (1994) *J. Biol. Chem.* **269**, 16938–16944
- 40 Kariya, K.-I., Karns, L. R. and Simpson, P. C. (1991) *J. Biol. Chem.* **266**, 10023–10026
- 41 Kariya, K.-I., Karns, L. R. and Simpson, P. C. (1994) *J. Biol. Chem.* **269**, 3775–3782
- 42 Shubeita, H. E., Martinson, E. A., Van Bilsen, M., Chien, K. R. and Brown, J. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1305–1309
- 43 LaMorte, V. J., Thorburn, J., Absher, D., Spiegel, A., Brown, J. H., Chien, K. R., Feramisco, J. R. and Knowlton, K. U. (1994) *J. Biol. Chem.* **269**, 13490–13496
- 44 Decock, J. B., Gillespie-Brown, J., Parker, P. J., Sugden, P. H. and Fuller, S. J. (1994) *FEBS Lett.* **356**, 275–278
- 45 Dubus, I., Samuel, J.-L., Marmotte, F., Delcayre, C. and Rappaport, L. (1990) *Circ. Res.* **66**, 867–874
- 46 Bishopric, N. H. and Kedes, L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2132–2136
- 47 Baker, K. M. and Aceto, J. F. (1990) *Am. J. Physiol.* **259**, H610–H618
- 48 Sadoshima, J.-I. and Izumo, S. (1993) *Circ. Res.* **73**, 413–423
- 49 Zhang, L. M., Newman, W. H., Castresana, M. R. and Hildebrandt, J. D. (1994) *Endocrinology (Baltimore)* **134**, 1297–1304
- 50 Luetje, C. W., Tietje, K. M., Christian, J. L. and Nathanson, N. M. (1988) *J. Biol. Chem.* **263**, 13357–13365
- 51 Howe, L. R. and Marshall, C. J. (1993) *J. Biol. Chem.* **268**, 20717–20720
- 52 van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. and Moolenaar, W. H. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1257–1261
- 53 Hordijk, P. L., Verlaan, I., van Corven, E. J. and Moolenaar, W. H. (1994) *J. Biol. Chem.* **269**, 645–651
- 54 Winitz, S., Gupta, S. K., Qian, N.-X., Heasley, L. E., Nemenoff, R. A. and Johnson, G. L. (1994) *J. Biol. Chem.* **269**, 1889–1895
- 55 Anand-Srivastava, M. B. (1992) *Biochem. J.* **288**, 79–85
- 56 Michel, M. C., Brodde, O. E. and Insel, P. A. (1993) *J. Hypertens.* **11**, 355–363
- 57 McMahon, K. K. (1989) *J. Pharmacol. Exp. Ther.* **251**, 372–377
- 58 Kojima, M., Ishima, T., Taniguchi, N., Kimura, K., Sada, H. and Sperelakis, N. (1990) *Br. J. Pharmacol.* **99**, 334–339