We detected expression of two Raf isoforms, c-Raf and A-Raf, in neonatal rat heart. Both isoforms phosphorylated, activated, and formed complexes with mitogen-activated protein kinase kinase 1 in vitro. However, these isoforms were differentially activated by hypertrophic stimuli such as peptide growth factors, endothelin-1 (ET1), or 12-O-tetradecanoylphorbol-13-acetate (TPA) that activate the mitogen-activated protein kinase cascade. Exposure of cultured ventricular myocytes to acidic fibroblast growth factor activated c-Raf but not A-Raf. In contrast, TPA produced a sustained activation of A-Raf and only transiently activated c-Raf. ET1 transiently activated both isoforms. TPA and ET1 were the most potent activators of c-Raf and A-Raf. Both utilized protein kinase C-dependent pathways, but stimulation by ET1 was also partially sensitive to pertussis toxin pretreatment. c-Raf was inhibited by activation of cAMP-dependent protein kinase although A-Raf was less affected. Fetal calf serum, phenylephrine, and carbachol were less potent activators of c-Raf and A-Raf. These results demonstrate that A-Raf and c-Raf are differentially regulated and that A-Raf may be an important mediator of mitogen-activated protein kinase cascade activation when cAMP is elevated.

The exposure of cells to mitogenic agents that act through protein tyrosine kinase receptors activates a cascade of protein kinases including c-Raf, mitogen-activated protein kinase kinase (MEK), and mitogen-activated protein kinases (MAPKS) (1, 2). This protein kinase cascade provides a link between growth factor stimulation and the transcriptional changes that occur in the cell nucleus (3, 4). More recently, the activation of receptors coupled to heterotrimeric G-proteins has also been shown to activate MEK, p42MAPK, and p44MAPK (5, 6). The upstream events in this signaling cascade may also involve c-Raf (7). Thus, activation of c-Raf may integrate signals from tyrosine kinase receptors and G-protein-coupled receptors.

c-Raf was originally identified as the cellular homolog of an oncogene of a murine sarcoma virus, but the regulation of c-Raf activity and its role in normal cellular functions have, until recently, remained unclear (8). c-Raf has been shown to interact directly with Ras.GTP (9–12). This interaction translocates c-Raf to the membrane (13, 14) and, coupled with other events that may include phosphorylation, activates c-Raf (15–17). Activated c-Raf directly phosphorylates MEK1 on two serine residues within a conserved regulatory region between the "DFG" and "A/S/PE" motifs (18). This identification of MEK1 as a physiological substrate allows assay of Raf activation (14, 19).

c-Raf may not be the only MEK activator in the eukaryotic cell. Recent studies have shown that c-Raf is not the major upstream activator of MAPKs in rat fibroblasts (20, 21), adipocytes (22), and PC12 cells (23, 24). c-Raf is a member of a family of related protein kinases that includes A-Raf and B-Raf (25–27). In contrast to the ubiquitous expression of c-Raf, the expression of A-Raf and B-Raf is restricted (28). Although the expression of truncated, constitutively active forms of A-Raf and B-Raf leads to activation of MAPK (29), the regulation of these isoforms has not been well-characterized. It is possible that different Raf isoforms may regulate different cellular events. Thus, B-Raf has been proposed as a major activator of MEK in nerve growth factor-stimulated PC12 cells (24) and brain extracts (30, 31).

The exposure of ventricular myocytes to growth promoting stimuli such as tumor-promoting phorbol esters, endothelin-1 (ET1), α₁-adrenergic agonists, peptide growth factors, or mechanical stretch activates a series of genetic changes that leads to cell hypertrophy rather than cell division (32, 33). Many of these agonists have also been shown to activate MEK and MAPK in these cells (34–37). Recent studies suggest that the MAPK cascade may play a role in the transcriptional changes associated with hypertrophy (38). In this study, we have examined the expression and activation of Raf isoforms in cultured neonatal rat ventricular myocytes. We show that ventricular myocytes express c-Raf and A-Raf and that hypertrophic stimuli differentially activate these two Raf isoforms.

EXPERIMENTAL PROCEDURES

Materials—Sprague-Dawley rats were bred in the National Heart and Lung Institute. [γ-32P]ATP was from DuPont NEN. ET1, 12-O-tetradecanoylphorbol-13-acetate (TPA), 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), pertussis toxin, Medium 199 (M199), Dulbecco’s modified Eagle’s medium (DME), n-octyl β-D-glucopyranoside, protease inhibitors, Protein A-Sepharose, bovine myelin basic protein (MBP), Kemp, and other biochemicals were purchased from Sigma unless stated otherwise. Culture dishes (Primaria) were from Falcon and heat-inactivated fetal calf serum (FCS) was from Sera-Lab. Other tissue culture products were from Life Technologies, Ltd. Glutathione-Sepharose was from Pharmacia and Ni2+-NTA-agarose was from Qiagen. Recombinant human acidic fibroblast growth factor (aFGF) was provided by Dr. P.
Dimension: 612.0 x 792.0

Cells/mm² on gelatin-precoated 60-mm dishes (41). After culture for 18 protein by the method of Bradford (39) were from Bio-Rad.

**Cummins (Department of Physiology, University of Birmingham, Birmingham, United Kingdom). Affinity-purified antibodies raised against C-terminal peptide sequences of c-Raf (CTLTTSRPLPVF), A-Raf (CLLSAARLVP), and B-Raf (ASPKPTPQAGGYGAFPHV), and the corresponding peptides were from Santa Cruz Biotechnology Inc. Enchanced chemiluminescence (ECL) immuno blotting detection reagents and ECL film were from Amersham. The prestained molecular mass standard proteins and reagents to assay protein by the method of Bradford (39) were from Bio-Rad.**

**Expression of Recombinant Protein Kinases—Recombinant p42MAPK and MEK1 were expressed as glutathione S-transferase (GST) fusion proteins (18, 40) and were purified by glutathione-Sepharose affinity chromatography. Further purification of the His6-tagged GST MEK1 was achieved by Ni²⁺-NTA agarose chromatography (18). For the assay of MEK phosphorylation by the immunoprecipitated protein kinases, recombinant MEK1 was modified by site-directed mutagenesis to produce GST-MEK1(R1/30finaldilution) were added to samples of the supernatants.

**3** (Lys97

**MEK1 in which a Lys-residue essential for kinase activity is mutated to produce GST-MEK1(R97/A291/A385), a catalytically inactive form of recombinant MEK1 was modified by site-directed mutagenesis to produce GST-MEK1(G202/E204) for use in these assays.**

**Activation of MEK by Immunoprecipitated c-Raf and A-Raf—**

**Protein Serine/threonine Kinase Activity—**

**Phosphorylation of MEK1 by Immunoprecipitated c-Raf and A-Raf—**

**Activation of A-Raf and c-Raf in Ventricular Myocytes**

**Oocytes—**

**Immunoblot Analysis—All samples were boiled in SDS-PAGE sample buffer for 5 min. Proteins were separated by SDS-PAGE (10% polyacrylamide gels), then transferred to nitrocellulose. The membrane was blocked with a mixture of 10% non-fat milk in PBS containing 0.05% Tween-20 for 1 h and then incubated with primary antibody at 4°C overnight. The membranes were washed three times in 75 mM phosphoric acid. Reactions in which antibodies were omitted were included as controls.**

**Results—Data presented are expressed as means ± S.E. for the number of independent experiments (n) indicated or as examples of representative experiments, these being performed on at least three independent occasions.**
Expression of Raf Isoforms in Ventricular Tissue—Because agonists that cause hypertrophy of the ventricular myocyte activate the MAPK cascade (34, 37), we investigated the expression and regulation of activators of MEK in the heart. Immunoprecipitation of Raf isoforms with affinity-purified antibodies followed by immunoblotting demonstrated the expression of c-Raf (Fig. 1A, lane 1) and A-Raf (Fig. 1B, lane 3) in the ventricles of neonatal rat hearts. Molecular masses (kilodaltons) of standard proteins are shown to the left of each panel. The specific bands for c-Raf, A-Raf, and B-Raf are indicated by the arrows to the right of each panel. Experiments were carried out on three separate occasions with similar results.

RESULTS

Expression of Raf Isoforms in Ventricular Tissue. ET1 (Fig. 2, A, and 3' A) activates c-Raf and A-Raf in cultured ventricular myocytes. ET1 activates c-Raf and A-Raf in ventricular myocytes.
Activation of A-Raf and c-Raf in Ventricular Myocytes

by its ability to activate GST-MEK1 in a coupled assay in vitro (Fig. 3A, closed symbols). Peak activation occurred at 1.5–3 min and had decreased to control levels within 10–30 min (8 ± 3% of peak activity after 30 min exposure, n = 5). There was no detectable activity in c-Raf or A-Raf immunoprecipitated from serum-starved ventricular myocytes exposed to serum-free medium for 1–30 min (results not shown). We confirmed that amounts of c-Raf immunoprecipitated from the extracts of cells used in the time course did not vary greatly (Fig. 3B, upper panel). Activity of immunoprecipitated c-Raf was also assayed by its phosphorylation of a reconstituent mutant MEK1, GST-MEK1(R97/A291/A385). The Lys → Arg mutation prevents autophosphorylation and the Thr291 → Ala and Thr385 → Ala mutations prevent the "retrophosphorylation" of MEK by endogenous MAPK (49) which may be greater than that achieved by c-Raf (50). This assay confirmed the rapid (within 1 min) and transient activation of immunoprecipitated c-Raf (Fig. 3B, lower panel). A reaction in the absence of immunoprecipitated kinases (Fig. 3, B and C, lanes B) confirmed that there was no autophosphorylation of GST-MEK1(R97/A291/A385).

We compared the time courses for activation of c-Raf and A-Raf in ET1-stimulated cells. ET1 activated A-Raf measured using the coupled assay (Fig. 3A, open symbols). A-Raf activities were maximal at 3–5 min but were lower than those for c-Raf (49 ± 4% of maximal c-Raf activities, n = 3). A-Raf activity decreased to control levels within 30 min of exposure (21 ± 8% of peak A-Raf activities, n = 3). We confirmed that the amounts of A-Raf immunoprecipitated from these cells did not vary (Fig. 3C, upper panel). The assay of A-Raf by its phosphorylation of GST-MEK1(R97/A291/A385) confirmed that MEK1 is phosphorylated by immunoprecipitated A-Raf (Fig. 3C, lower panel).

TPA Differentially Activates c-Raf and A-Raf—In the ventricular myocyte, TPA activates classical and novel isoforms of PKC (51, 52) and causes sustained activation of MEK2 and MAPK (37). Activation of c-Raf (as measured by the coupled assay) by TPA (1 μM) was rapid and maximal at 3–5 min (Fig. 4A, closed symbols). Activity then fell within 30 min of exposure (32 ± 5% of maximal response, n = 4). This was confirmed in

2 M. A. Bogoyevitch, A. Clerk, and P. H. Sugden, unpublished results.
more sustained (69 ± 4 of c-Raf activity at 3 min, subsequent assays, were always performed (lane B). The position of the mutant GST-MEK1 is indicated by the arrow at the right of the panel. Experiments on c-Raf activation were carried out on four separate occasions, and these results were compared with A-Raf activation carried out on two of those four occasions.

the phosphorylation assay (Fig. 4B). Transient activation of c-Raf in the presence of sustained MEK and MAPK activation has been reported (14).

A-Raf was also activated as shown by the coupled assay (Fig. 4A, open symbols) or by the direct phosphorylation assay (Fig. 4C). A-Raf was activated to a lesser extent than c-Raf (75 ± 8% of c-Raf activity at 3 min, n = 4) and was slower than c-Raf activation (Fig. 4A, open symbols). Activation of A-Raf was more sustained (69 ± 3% of maximal response at 30 min exposure, n = 4) than the activation of c-Raf so that the activity of A-Raf exceeded that of c-Raf after a 30-min exposure (178 ± 48% of c-Raf activity at 30 min, n = 4).

aFGF activates c-Raf, but Not A-Raf—aFGF concentrations (25 ng/ml) which maximally activate MAPK and MEK (37) activated c-Raf within 1–3 min (Fig. 5A). The activation of c-Raf was confirmed by the direct phosphorylation of GST-MEK1(R97/A291/A385) (Fig. 5B). A-Raf activation by aFGF was undetectable at any time point examined (Fig. 5A).

Comparison of the Stimulation of c-Raf and A-Raf by Agonists—We have identified TPA as a potent activator of MAPK in cultured ventricular myocytes (37). In this study, TPA was the most potent activator of c-Raf and A-Raf resulting in at least a 10-fold activation of both Raf isoforms (Fig. 4). Activation of c-Raf by ET1 achieved 90% of the level of TPA stimulation whereas activation by FCS and aFGF was 30–40% of the TPA stimulation (Fig. 6). Phenylephrine and carbachol were the poorest activators examined (Fig. 6). A similar order of potency was observed for A-Raf activation, with the exception that aFGF did not activate A-Raf (Fig. 6).

c-Raf and A-Raf Activation by ET1 or TPA Requires Classical and/or Novel PKCs—Prolonged treatment (24 h) with TPA down-regulates classical and novel isoforms of PKC in ventricular myocytes, and this decreases signaling of ET1 to MAPK by 62% (37). When activities of c-Raf were examined after prolonged TPA pretreatment, basal activities were enhanced (452 ± 54% of values in the absence of TPA pretreatment, n = 3) (Fig. 7A). Similar effects of TPA pretreatment have been reported (53). This complicated calculation of the inhibition in-

![Figure 5](image1.png)

**Fig. 5. aFGF activates c-Raf but not A-Raf.** Cultured ventricular myocytes were treated with 25 ng/ml aFGF at 37°C for the times indicated. A-Raf and c-Raf were then immunoprecipitated from the detergent-soluble cell lysates as described under "Experimental Procedures." A, activity of the immunoprecipitated c-Raf (●) or A-Raf (○) was measured in a coupled assay as MEK activating activity. B, the kinase activity of c-Raf was measured directly by the phosphorylation of GST-MEK1(R97/A291/A385). Blank reactions in which c-Raf antibody was incubated with Buffer A and Protein A-Sepharose, then used in the subsequent assays, were always performed (lane B). The position of the mutant GST-MEK1 is indicated by the arrow at the right of the panel. Experiments on c-Raf activation were carried out on four separate occasions, and these results were compared with A-Raf activation carried out on two of those four occasions.

![Figure 6](image2.png)

**Fig. 6. TPA and ET1 were the most potent activators of c-Raf and A-Raf identified in ventricular myocytes.** Cultured ventricular myocytes were treated with 1 µM TPA, 100 nM ET1, 50 µM phenylephrine (PE), 20% (v/v) FCS, 25 ng/ml aFGF (FGF), or 100 µM carbachol (CB) at 37°C for 3 min. Activity of c-Raf (solid bars) or A-Raf (open bars) immunoprecipitated from the detergent-soluble cell lysates was measured in a coupled assay as MEK activating activity as described under "Experimental Procedures." Results (means ± S.E.) are expressed as a percentage of the TPA response measured on each separate preparation of myocytes and are an average of 3–15 independent observations.

![Figure 7](image3.png)

**Fig. 7. Classical and novel PKC isoforms are involved in the activation of c-Raf and A-Raf.** Cultured ventricular myocytes were either incubated in serum-free medium (solid or open bars) or pre-treated with 1 µM TPA for 24 h (crosshatched or diagonally striped bars), then exposed to serum-free medium (CON), 100 nM ET1, 1 µM TPA, or 20% (v/v) FCS at 37°C for 3 min. Activity of c-Raf (panel A) or A-Raf (panel B) immunoprecipitated from the detergent-soluble cell lysates was measured in a coupled assay as MEK activating activity as described under "Experimental Procedures." The experiment was performed on three separate occasions with similar results.
duced by TPA pretreatment. However, the activity of c-Raf elicited by acute ET1 or TPA exposure of TPA-pretreated cells was similar to that of control TPA-pretreated cells (Fig. 7A). These results suggest that pretreatment with TPA down-regulates the subsequent activation of c-Raf by ET1 or TPA and implies participation of classical/novel PKCs. In contrast, FCS produced a small stimulation of c-Raf activity in TPA-pretreated cells (Fig. 7A). This suggests that FCS may activate c-Raf via pathways that are independent of classical/novel PKCs.

In contrast to c-Raf, basal A-Raf activity was not enhanced by TPA pretreatment (Fig. 7B). ET1, TPA, and FCS activation of A-Raf were all decreased by TPA pretreatment, although ET1 stimulation of A-Raf (solid bars) or A-Raf (open bars) immunoprecipitated from the detergent-soluble cell lysates was measured in a coupled assay as MEK activating activity as described under "Experimental Procedures." The experiment was performed on three separate occasions with similar results.

Activation of c-Raf and A-Raf by FCS or ET1 Is Sensitive to Pertussis Toxin—The activation of MAPK by ET1 is largely insensitive to pertussis toxin (41). Pertussis toxin pretreatment (150 ng/ml) decreased the ET1-stimulation of c-Raf or A-Raf activities by 71 ± 5% (n = 4) or 60 ± 2% (n = 4), respectively (Fig. 8, A and B). Activation of c-Raf or A-Raf by FCS was also decreased by 68 ± 2% (n = 3) and 93 ± 4% (n = 3), respectively (Fig. 8, A and B). In contrast, activation of c-Raf or A-Raf by TPA was decreased by only 19 ± 6% (n = 3) or 27 ± 8% (n = 3), respectively (Fig. 8, A and B). These results suggest that ET1 and FCS use pertussis toxin-sensitive G-proteins in the activation of A-Raf and c-Raf.

Activation of c-Raf by ET1 Is Inhibited by Activation of PKA—Although activation of PKA can inhibit the MAPK signaling pathway in some cells (54), activation of MAPK by ET1 (100 nM) in the ventricular myocyte is largely insensitive to inhibition by simultaneous PKA activation.3 We investigated whether PKA could inhibit the activation of c-Raf and A-Raf isoforms upon exposure of ventricular myocytes to ET1. Exposure of myocytes to CPT-cAMP (100 μM) increased the PKA activity ratio from 0.10 ± 0.04 (n = 3) to 0.78 ± 0.04 (n = 3) within 1 min. PKA activity was sustained at 3 min (PKA activity ratio, 0.89 ± 0.03, n = 3) and did not alter basal c-Raf or A-Raf activity (Fig. 9). Exposure to 100 μM CPT-cAMP decreased activation of c-Raf by ET1 by 89 ± 5% (n = 4) (Fig. 9). Activation of A-Raf was decreased by only 33 ± 14% (n = 4) (Fig. 9). c-Raf activity in cells exposed to ET1 alone was 210 ± 45% (n = 4) of the activity of A-Raf. After CPT-cAMP exposure, the activity of A-Raf in ET1-stimulated cells was 240 ± 9% (n = 4) of the activity of c-Raf. This suggests that A-Raf provides a signaling pathway to MAPK in the presence of activated PKA.

The Role of the MAPK Cascade in Cardiac Hypertrophy—The ventricular myocyte responds to growth-promoting stimuli by changes in gene expression and an increase in cell size (hypertrophy) in the absence of cell division (32). We have proposed that activation of the MAPK pathway may be involved (34, 37). Direct support for this hypothesis has come from studies in which transfection of ventricular myocytes with Ras(V12) (55) and constitutively activated forms of MEK4 produce changes in gene expression qualitatively similar to those observed after treatment with hypertrophic agonists (32). Furthermore, Ras (55) and MAPK (38, 57) appear to be necessary for phenylephrine-stimulated changes in gene expression.

Two protein kinase activators of MEK have been identified in somatic cells, namely Raf and MEKK (1, 58). Although both Raf and MEKK may act downstream of Ras (59, 60), recent evidence indicates that MEKK participates in the activation of Raf.34

**DISCUSSION**

3 M. A. Bogoyevitch, M. B. Andersson, J. Gillespie-Brown, A. Clerk, P. E. Glennon, S. J. Fuller, and P. H. Sugden, manuscript submitted for publication.

4 Gillespie-Brown, J., Fuller, S. J., Bogoyevitch, M. A., Cowley, S., and Sugden, P. H. (1995) J. Biol. Chem. 270, in press.
stress-activated protein kinase pathways (61–63). Either Raf or MEKK may be important in the regulation of the Ras-dependent hypertrophic responses previously observed (55), but recent studies have demonstrated changes in gene expression typical of the hypertrophic response after transfection with activated forms of c-Raf.

Differential Activation of Raf Isoforms—The activation of c-Raf has been identified as an important event linking the membrane-associated signaling events (such as activation of Ras) with the stimulation of a cascade of cytosolic Ser/Thr protein kinases (59, 65). Recent studies have addressed the differences in regulation of activation of c-Raf and B-Raf (23, 30, 31, 66). Notably B-Raf, not c-Raf, appears to be a major form of MEK activator identified in brain extracts (30, 31).

We have observed that exposure of the ventricular myocyte to aFGF activated c-Raf, but not A-Raf. The reason for this differential activation of Raf isoforms by aFGF is unclear and requires further investigation. The functional effectors of Raf in addition to Ras and Src remain to be identified (15–17). Although lipids may interact with the Cys-rich Zn²⁺-finger of the Raf molecule, diacylglycerol and phosphatidyserine do not appear to activate c-Raf in vitro (67). Recently, c-Raf activity has shown to be further enhanced by a lipid factor (68). It may be that the differential activation of A-Raf and c-Raf reflects different requirements for effectors (either species or concentrations). The interaction of c-Raf with other proteins has been suggested (14). Thus, 14–3–3 proteins interact with c-Raf (69), and this prevents the deactivation of c-Raf by phosphatases (70).

The activation of c-Raf by ET1 in ventricular myocytes was sensitive to inhibition by PKA. This is consistent with previous studies which demonstrated the inhibition of the MAPK pathway by PKA (71–75). Ser⁴³ of c-Raf is directly phosphorylated by PKA, and this decreases the affinity of interaction between Ras and c-Raf (71). This provides negative regulation of c-Raf activity and cross-talk between signal transduction cascades (54). The N-terminal regions of both B-Raf and A-Raf do not have a site analogous to the Caspase cleavage site (25, 26). However, B-Raf is also inhibited by PKA activation (23), and other sites of phosphorylation (such as in the kinase domain) may also contribute to the PKA-mediated inhibition of c-Raf (76) and possibly B-Raf. We found that A-Raf was less sensitive than c-Raf to inhibition by PKA. The activation of MAPK by ET1 is also relatively insensitive to inhibition by PKA in ventricular myocytes,² and A-Raf potentially provides a signaling pathway for MAPK activation that is not inhibited when CAMP concentrations are elevated.

A Role for PKC in the Regulation of c-Raf and A-Raf—The mechanism of activation of the MAPK signaling cascade by phorbol esters and G-protein-coupled receptors remains unclear (77). The elucidation of this pathway may be especially important in the ventricular myocyte where these agents produce the greatest hypertrophic responses (78–81) and the largest increases in MEK and MAPK activities (34, 37, 41, 82).

TPA was the most potent activator of both c-Raf and A-Raf in cultured ventricular myocytes. The activation of c-Raf was rapid and transient whereas activation of A-Raf was slower and sustained. Because the classical and novel isoforms of PKC are major targets of phorbol ester action in the eukaryotic cell (83), a role for PKC in the activation of both Raf isoforms is implied. In ventricular myocytes, activation of PKC-α, PKC-δ, and PKC-ε by TPA is rapid (<30 s for maximal response (52, 84)) and precedes activation of A-Raf or c-Raf. A number of studies have demonstrated activation of c-Raf by PKC (85–87), and PKC-α directly activates c-Raf by phosphorylation of Ser⁴⁹⁹ (87) which lies within a regulatory region between the “DFG” and “A(S)PE” motifs (88). A-Raf does not have a residue analogous to Ser⁴⁹⁹, and its direct regulation by PKC has not been examined.

Both c-Raf and A-Raf have been identified to interact specifically with HRas after screening a randomly primed mouse embryo cDNA library in the yeast-two hybrid system (10). An alternative route of Raf activation may involve a PKC-dependent activation of Ras (e.g., activation of Ras guanine nucleotide exchange factors to promote formation of RasGTP) (89). Other sites for PKC-independent actions of phorbol esters cannot be ruled out (90, 91). Differences in the mechanism of activation of c-Raf and A-Raf by phorbol esters may help to explain the differences in their time courses of activation.

Exposure of ventricular myocytes to ET1, a potent hypotrophic agonist (80, 81), leads to rapid (within 30 s) stimulation of hydrolysis of membrane phosphatidylinositols (37),² equally rapid activation of PKC-δ and PKC-ε (92), and activation of MEK and MAPK (34, 37, 92). ET1 activated c-Raf and A-Raf to 70–90% of the levels achieved by TPA. Both Raf isoforms followed similar rapid and transient time courses of activation in response to ET1. Depletion of novel and classical PKC isoforms which inhibited the ET1-stimulated MAPK activation (37) also inhibited c-Raf and A-Raf activation. These findings are consistent with a hypothesis that activation of PKC leads to activation of both Raf isoforms. Therefore, there must be a major PKC-dependent pathway from the ET1 receptor to Raf activation in these cells. In addition, ET1 elicits tyrosine phosphorylation of Shc in astrocytes (93) which leads to its association with GRB2 (93) and provides a Ras-dependent mechanism for activation of Raf by endothelin.

PKC-independent Pathways of MAPK Activation—Recent studies on the regulation of the MAPK pathway by G-protein-coupled receptors have focused on regulation via Gαi (77). The introduction of activated forms of Gαi into Rat 1a fibroblasts stimulates MAPK (5, 94, 95) and βγ subunits may also activate the MAPK pathway (96–98). Inactivation of Gαi by preincubation of cells with pertussis toxin inhibits activation of the Ras/MAPK pathway by agents such as lysosphosphatidic acid, α₂-adrenergic agonists, and thrombin (47, 99, 100). In earlier studies, we identified carbachol and lysosphosphatidic acid as utilizing pertussis toxin-sensitive pathways of MAPK activation in the ventricular myocyte (41). In this study, carbachol was a poor activator of c-Raf or A-Raf. This result is consistent with the proposal that the agents that signal through the Gαi pathway may signal through activators of MEK other than Ras (5, 77). In the present study the activation of c-Raf or A-Raf by ET1 or FCS was substantially inhibited by pertussis toxin. This suggests that a Gαi pathway initiated by these agonists may signal to both isoforms of Raf. We cannot distinguish between a role for activated Gαi or βγ subunits.

Cross-talk between Gαi and Gαi-coupled pathways is implied from the findings that pretreatment with either TPA or pertussis toxin down-regulates activation of A-Raf. Thus, activation of PKC downstream from both the stimulation of phosphatidylinositol hydrolysis and Gαi is necessary for activation of A-Raf by ET1. Alternatively, activation of phosphatidylinositol hydrolysis by ET1 may be stimulated through Gαs as well as Gαq. The anomalous activation of c-Raf by TPA pretreatment prevents similar attempts at interpretation.

A potent Raf-independent pathway of MAPK activation is suggested by our studies with phenylephrine which activates MAPK and MEK (34, 57, 92). Here, we demonstrate that phenylephrine is a poor activator of both A-Raf and c-Raf. Future studies must identify which pathways of activation of MEK are utilized by agonists such as phenylephrine. It is equally important to clarify the different mechanisms of activation of A-Raf
Raf isoform to activation of the MAPK cascade.

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