Activation of Ras by phorbol esters in cardiac myocytes

Role of guanine nucleotide exchange factor

Christophe Montessuit[a], Andrew Thorburn[a,b,*]

[a] *Department of Oncological Sciences, Huntsman Cancer Institute, 2000 E North Campus Drive, University of Utah, Salt Lake City, UT 84112-5550, USA*

[b] *Program in Human Molecular Biology and Genetics, Departments of Oncological Sciences, Human Genetics and Internal Medicine, Huntsman Cancer Institute, 2000 E North Campus Drive, University of Utah, Salt Lake City, UT 84112-5550, USA*

Received 20 August 1999

Abstract The relationship between protein kinase C (PKC) activation and Ras function was investigated in cardiac cells. Ras function was required for ERK activation by phorbol esters in cardiac myocytes, but not in cardiac fibroblasts. Accordingly, treatment with phorbol esters resulted in GTP loading of Ras in cardiac myocytes, but not fibroblasts. Ras activation by phorbol esters was abolished by a PKC specific inhibitor, but was insensitive to tyrosine kinase inhibitors. Ras activation was mediated by stimulation of guanine nucleotide exchange. These results suggest the existence of a novel pathway for Ras activation, specific to cardiac myocytes, with implications for myocardial hypertrophy.

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Key words: Cardiac myocyte; Ras; Protein kinase C; Phorbol ester; Myocardial hypertrophy

1. Introduction

The small G-protein Ras plays a central role in signaling pathways leading to hypertrophy of cardiac myocytes [1–4]. Among the pathways that are activated downstream of Ras, the ERK1/2 MAP kinase pathway is one of the best understood. The immediate downstream effectors of Ras in this pathway are the Raf kinases A-Raf, B-Raf and Raf-1. Raf kinases can also be activated by phosphorylation by protein kinase C (PKC). Although activation of Raf by PKC was shown to be independent of Ras [5] in COS cells and NIH3T3 fibroblasts, experiments performed in PC12 pheochromocytoma cells indicated that the activity of Ras was required for Raf activation by PKC in this cell type [6,7]. In addition, in COS cells, activation of PKC by phorbol esters resulted in activation of Ras [8]. However, expression of dominant-negative Ras did not inhibit Raf activation by PKC in these cells. Thus, the relationship between Ras, Raf and PKCs appears to be cell-type specific.

In a recent study we observed activation of the glucose transporter *glut1* promoter by phorbol esters in two different types of primary cardiac cells, i.e. myocytes and fibroblasts [9]. The two cell types exhibited a major difference in that activation of the *glut1* promoter could be inhibited by dominant-negative Ras in cardiac myocytes, but not in cardiac fibroblasts. Moreover, we found that phorbol esters elicited GTP loading of Ras in cardiac myocytes exclusively. In the present study we further investigated the mechanisms by which phorbol esters activate Ras in primary cardiac myocytes.

2. Materials and methods

2.1. Cell culture

Primary ventricular myocytes were isolated from 1 day old rats by collagenase digestion and maintained in Dulbecco's modified Eagle's medium (DMEM)/medium 199 (4/1) supplemented with penicillin and streptomycin (maintenance medium, MM) as previously described [10]. Cytosine arabinoside was added at a final concentration of 10 μM to prevent growth of contaminating fibroblasts (typically less than 5% of the cells). For ERK activation experiments, cells were plated at a density of 2.5×10^5 per 3.5 cm dish. For activated Ras assay and determination of guanine nucleotide exchange factor (GEF) activity, 2×10^6 cells were plated in 6 cm dishes.

Cardiac fibroblast cultures were prepared by two passages of the cells adherent to the culture dish during the preplating procedure. Cells were maintained in MM supplemented with 10% fetal calf serum. Confluent 3.5 cm dishes were used for ERK1/2 activation assays and confluent 10 cm dishes were used for activated Ras assay.

2.2. ERK activation assay

Cells in 3.5 cm dishes were pretreated with or without lovastatin for 16 h, then treated with either tetradecanoyl phorbol acetate (TPA) or its vehicle for 5 min. Cells were then harvested in 200 μl RIPA buffer and 90 μg of proteins were subjected to SDS-PAGE and blotted onto PVDF. Total ERKs and doubly phosphorylated (Thr-202/Tyr-204) ERKs were detected by Western blotting using the K-23 rabbit polyclonal antibody (Santa Cruz) and the E10 monoclonal antibody (New England Biolabs), respectively. Signals were quantitated using the Lumi-Imager system with the LumiAnalyst software (Boehringer Mannheim).

2.3. Activated Ras assay

The expression plasmid pGEX-RBD, encoding the Ras binding domain of cRaf-1 fused to GST (GST-RBD) [11], was kindly donated by Stephen J. Taylor. Detection of Ras-GTP in cell extracts was performed as described [11]. Briefly, cell lysates were incubated with GST-RBD pre-bound to glutathione-Sepharose (Pharmacia) for 30 min at 4°C. Bound proteins were eluted with SDS-PAGE sample buffer, resolved on 12% polyacrylamide gels and subjected to Western blotting. Blots were probed using an anti-H-Ras rabbit polyclonal antibody (Santa Cruz sc-520). Aliquots (10%) of total cell lysate were also resolved for quantitation of total Ras and determination of ERK1/2 phosphorylation.

2.4. Determination of guanine exchange factor activity

GEF activity in myocytes and fibroblasts was determined as previously described [12]. Briefly, cells were scraped in ice-cold permeabilization buffer containing TPA, bFGF or their vehicle, pelleted and permeabilized at 37°C with 0.1% digitonin in the presence of [α-^{32}P]GTP. Incubation was stopped after 2 or 5 min with ice-cold lysis buffer and Ras was immunoprecipitated from cell lysates with 1 μg anti-Ras antibody. Non-specific (time 0) [α-^{32}P]GTP binding was de-

*Corresponding author. Fax: (1) (801) 585 0900.
E-mail: andrew.thorburn@hci.utah.edu

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PII: S0014-5793(99)01223-5

termined by adding lysis buffer before [α-^{32}P]GTP and was subtracted. Guanine nucleotides were then eluted from immunoprecipitated Ras and radioactivity of the eluate counted by liquid scintillation.

3. Results

3.1. Ras requirement for ERK activation in cardiac cells

In a previous study we found that Ras activity was required for activation of the GLUT1 promoter by the PKC activator TPA [9]. In cardiac fibroblasts, however, TPA could activate the GLUT1 promoter independently of Ras. To extend these observations, we used the hydroxymethylglutaryl CoA inhibitor lovastatin to inhibit Ras [13]. Inhibition of Ras farnesylation and geranylgeranylation by lovastatin led to a dose-dependent reduction of ERK1/2 activation by TPA in cardiac myocytes, while lovastatin was without effect in cardiac fibroblasts (Fig. 1A). To confirm that lovastatin actually inhibited Ras function in fibroblasts, we showed that activation of ERK1/2 by bFGF was abolished by pretreatment with lovastatin in cardiac myocytes as well as fibroblasts (Fig. 1B). Fig. 1C shows quantitation of ERK1/2 activation in response to TPA and bFGF in cardiac myocytes and fibroblasts. Pretreatment of the cells with 30 μM lovastatin markedly reduced activation of ERK1/2 by bFGF in both cell types. In addition, lovastatin reduced ERK1/2 activation by TPA in cardiac myocytes, but was without effect on TPA-activated ERK1/2 in cardiac fibroblasts. The residual ERK1/2 activation observed in lovastatin-pretreated myocytes stimulated with TPA can probably be attributed to contaminating fibroblasts, since in these cells ERK activation by TPA was more prominent than in myocytes (39-fold vs. 23-fold).

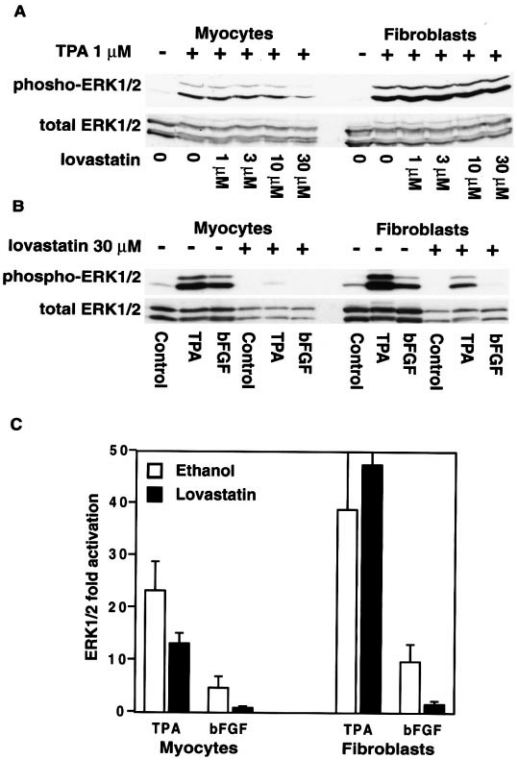


Fig. 1. Ras is required for ERK activation by TPA only in cardiac myocytes. A: Cardiac myocytes (2.5×10^5/3.5 cm dish) or cardiac fibroblasts (confluent in 3.5 cm dishes) were pretreated with varying concentrations of lovastatin for 16 h. Cells were then stimulated with TPA or its vehicle for 5 min, harvested in RIPA buffer and doubly phosphorylated ERK1/2 and total ERK1/2 were detected by Western Blot. B: Cardiac cells were pretreated with 30 μM lovastatin or its vehicle for 16 h before stimulation with TPA or bFGF for 10 min. Doubly phosphorylated ERK1/2 and total ERK1/2 were detected by Western blot. C: Quantitation of ERK1/2 activation. Cardiac cells were pretreated with 30 μM lovastatin (black bars) or its vehicle (white bars) for 16 h before stimulation with TPA or bFGF for 10 min. Doubly phosphorylated ERK1/2 and total ERK1/2 were quantitated using the LumiImager system and the phosphoERK/total ERK ratio was determined. This ratio was arbitrarily set to 1 in untreated cells. Results are mean ± S.E.M. of six (TPA) or three (bFGF) experiments.

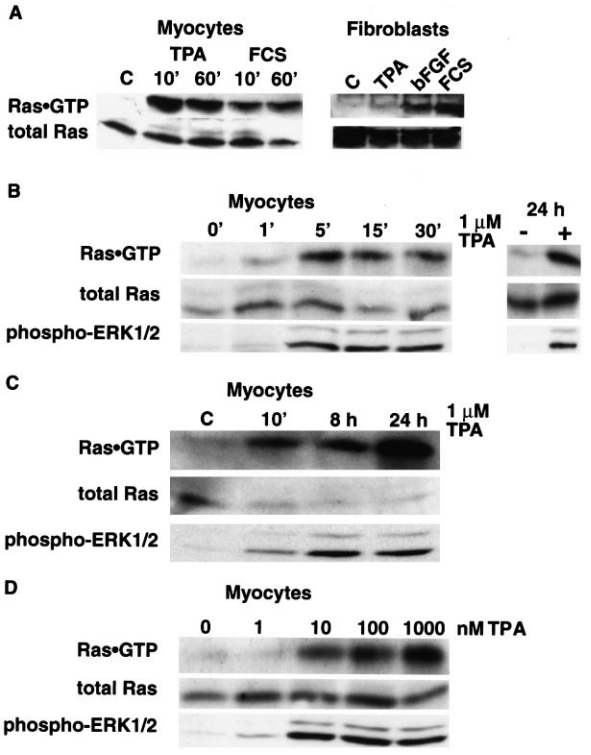


Fig. 2. TPA activates Ras in cardiac myocytes, not in cardiac fibroblasts. A: Cardiac myocytes (2×10^6/6 cm dish) or cardiac fibroblasts (confluent in 10 cm dish) were treated for 10 min with either TPA (1 μM), bFGF (25 ng/ml) or serum (FCS, 10%) as indicated. Cells were then lysed and Ras-GTP was affinity-precipitated using GST-RBD and detected by Western blotting. Aliquots of total cell lysate were run in parallel for detection of total Ras and doubly phosphorylated ERK1/2. B: Time course of Ras activation by TPA in cardiac myocytes. Cardiac myocytes were treated with 1 μM TPA for the indicated time periods before Ras-GTP was quantitated as described above. C: Reversal of Ras activation. Cardiac myocytes were treated with either 0.1% ethanol (C) or 1 μM TPA. TPA was removed (and replaced with 0.1% ethanol) after 10 min or 8 h or left on the cells for 24 h. Ras-GTP was detected after 24 h, regardless of the incubation time with TPA. D: Dose response of Ras activation by TPA. Cardiac myocytes were treated with the indicated concentrations of TPA for 10 min before Ras-GTP was quantitated as described above.

3.2. Activation of Ras by TPA in cardiac myocytes

To explain the requirement for Ras for ERK1/2 activation in response to TPA, we hypothesized that TPA was able to induce GTP loading of Ras in cardiac myocytes. As shown in Fig. 2A, TPA rapidly and potently activated Ras in cardiac myocytes, but was unable to do so in fibroblasts. To confirm that fibroblasts were responsive, we showed that bFGF and

FCS could activate Ras in these cells. In cardiac myocytes, Ras activation by TPA occurred very rapidly (about 1 min) and preceded activation of ERK1/2 (Fig. 2B). Both Ras GTP loading and ERK1/2 dual phosphorylation were sustained for at least 24 h in cardiac myocytes. Interestingly, Ras and ERK1/2 activation persisted for 24 h even when TPA was removed after 10 min or 8 h (Fig. 2C). Surprisingly, long-term treatment of cardiac myocytes with TPA, with or without removal, also resulted in down-regulation of total Ras.

Dose-response experiments showed that activation of Ras in cardiac myocytes was clearly apparent with 10 nM TPA and maximum activation was reached between 100 nM and 1 μM TPA (Fig. 2D). Activation of ERK1/2 by TPA followed a similar dose-response curve, suggesting that activation of ERK1/2 by TPA is actually mediated by Ras activation.

3.3. Ras activation by TPA is mediated by a PKC

Recently RasGRP, a guanine nucleotide release factor that activates Ras in response to phorbol esters, has been described and cloned from rat brain [14], thus providing an example of how phorbol esters can directly stimulate a Ras GEF. Such a mechanism of activation does not require PKC activity. To assess whether Ras activation by TPA was mediated by a PKC, we performed experiments with the selective PKC inhibitor chelerythrin [15]. As shown in Fig. 3, chelerythrin totally suppressed activation of Ras in response to TPA. In contrast, several broad-spectrum tyrosine kinase inhibitors (lavendustin A, genistein and herbimycin A) failed to inhibit activation of Ras by TPA. The tyrosine kinase inhibitors were, however, able to reduce bFGF-induced activation of ERK1/2. Consistently with Ras-GTP being unaffected by tyrosine kinase inhibition, the tyrosine kinase inhibitors did not reduce ERK activation induced by TPA, while the PKC inhibitor chelerythrin did.

3.4. TPA stimulates GRF activity in myocytes

The balance between GEF and GTPase activating protein (GAP) activities determines the type of nucleotide (GTP vs. GDP) bound to Ras. GEF activity in cardiac myocytes was markedly stimulated by treatment of the cells with TPA. In contrast, GEF activity in cardiac fibroblasts was only minimally increased by TPA treatment (data not shown); this minor increment is probably caused by a small number of contaminating myocytes in the fibroblast preparation.

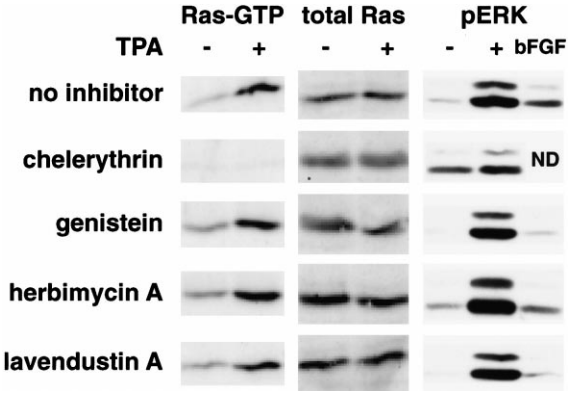


Fig. 3. Ras activation by TPA is PKC-mediated. Cardiac myocytes were pretreated for 30 min with either chelerythrin (5 μM), genistein (50 μM), herbimycin A (1 μM), lavendustin A (10 μM) or the vehicle (0.1% DMSO). The cells were then stimulated for 10′ with 1 μM TPA (+), 0.1% ethanol (−) or 25 ng/ml bFGF (bFGF). Cells were then lysed and Ras-GTP was affinity-precipitated using GST-RBD and detected by Western blotting. Doubly phosphorylated ERK1/2 (pERK) was determined by Western blot. ND: not done.

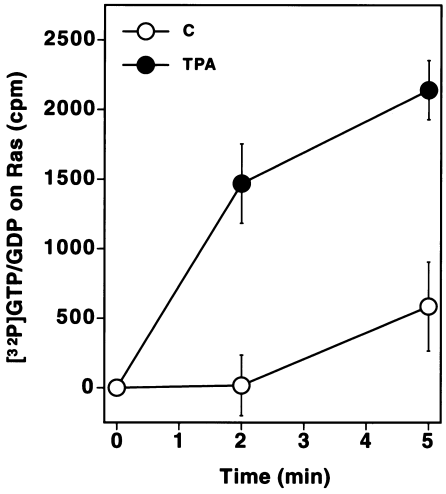


Fig. 4. TPA stimulates GEF activity in cardiac myocytes. Cardiac myocytes (2×10^6/6 cm dish) were scraped in permeabilization buffer containing TPA or its vehicle, then permeabilized in the presence of [α-^{32}P]GTP for various times. The radiolabel on Ras was then determined by immunoprecipitation. Results are shown as average ± S.E.M. of four experiments.

4. Discussion

The present study demonstrates that activation of the ERK1/2 MAP kinase by phorbol esters is brought about by different mechanisms in two distinct cardiac cell types in primary cultures. In cardiac myocytes, Ras is required for ERK1/2 activation by the phorbol ester TPA; in addition TPA induces GTP loading of Ras in this cell type. In contrast, in cardiac fibroblasts Ras activity is not required for activation of ERK1/2 by TPA and activation of PKC by TPA does not affect the activity of Ras.

Both the small G-protein Ras and proteins of the PKC family play major roles in the control of cell growth, differentiation and proliferation (see [15] for review). In cardiac myocytes Ras is a key element of the signaling pathways leading to hypertrophy [1–4]. PKCs have also been involved in the progression to hypertrophy in cardiac myocytes (reviewed in [16]). Signaling downstream of Ras and PKC is usually thought to converge onto activation of the kinases of the Raf family, since Raf is recruited and activated by active Ras on the one hand, and PKC can, at least in some cell types, phosphorylate and activate Raf independently of Ras activity [5].

In this study we have shown that phorbol esters activate Ras in cardiac myocytes, thus demonstrating an additional level of integration between signaling pathways involved in cardiac myocyte hypertrophy. The rapid (1 min) appearance of Ras-GTP in response to TPA treatment suggests that few steps take place between activation of the phorbol ester target, presumably a PKC, and activation of Ras. Based on such a rapid kinetics for Ras activation, paracrine or autocrine effects can be ruled out. The phorbol ester's direct target in this

setting is most likely a PKC for the following reasons: (1) Ras activation by TPA could be prevented by pretreatment with a specific PKC inhibitor, chelerythrin, and (2) the dose-response curve for activation of Ras by TPA is compatible with the EC_{50} reported for activation of members of the classic family of PKC, i.e. 25–130 nM depending on the isozyme [17].

Ras output is tightly regulated by cycling between an active GTP-bound conformation (Ras-GTP) and an inactive GDP-bound state (Ras-GDP). The balance between Ras-GTP and Ras-GDP is altered by modulation of the activity of GEFs and GAPs. GEFs trigger the release of the guanine nucleotide from Ras, thus enabling loading of GTP, which is much more abundant than GDP in the cell cytoplasm. On the other hand, GAPs enhance the intrinsic GTPase activity of Ras, thereby terminating Ras signaling. In the present study we demonstrated that activation of Ras in response to phorbol esters occurred by stimulation of GEF activity (Fig. 4). Activation of a GEF by phorbol esters has been previously demonstrated. The Ras-activating guanine nucleotide exchange protein Vav is known to be activated by phorbol esters [18]. Expression of Vav is, however, limited to hematopoietic cells. Recently, Ebinu and coworkers identified and cloned RasGRP, a Ras guanine nucleotide release protein with calcium and diacylglycerol binding motifs [14]. It was subsequently shown that activation of RasGRP by phorbol esters requires binding of these compounds to a C1 domain very similar to that found in PKC [19]. However, we do not believe that such a mechanism is operative in cardiac myocytes for the following reasons: (1) no expression of RasGRP could be shown in heart tissue [14] and (2) activation of Ras by TPA could be prevented by pretreatment with chelerythrin, a catalytic domain inhibitor specific for PKC [15].

Studies in pancreatic acinar cells have shown that activation of PKC by phorbol esters led to Ras-GEF activation through tyrosine phosphorylation of the adapter protein Shc [20,21]. However, in this study activation of Ras by TPA in cardiac myocytes was resistant to inhibitors of tyrosine kinases, therefore suggesting a different mechanism, which remains unknown. Also, the reason for the cardiac myocyte specificity of the effect remains to be investigated. Several hypotheses can be envisaged: (1) a PKC isoform that is present in myocytes but not in fibroblasts is able to somehow activate GEFs; (2) a myocyte-specific GEF is a substrate for PKCs or (3) a PKC substrate present in myocytes only mediates GEF activation.

In conclusion, this work demonstrates that in cardiac myocytes activation of PKC apparently results in activation of Ras GEF independently of tyrosine kinases. This information is of particular relevance for the understanding of the development of cardiac hypertrophy, which is often associated with stimuli that cause diacylglycerol production. This additional level of integration between signaling pathways provides a way by which PKC-activating agonists can stimulate signaling pathways downstream of Ras that are more usually activated in response to tyrosine kinases.

Acknowledgements: We thank Jacqueline Thorburn for the preparation of cell cultures and Stephen Taylor for providing the pGEX-RBD plasmid. This work was supported by Grants 823A-050451 from the Swiss National Science Foundation to C.M. and HL-50210 from NIH to A.T.

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