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Transcriptional Activation of the Glucose Transporter GLUT1 in Ventricular Cardiac Myocytes by Hypertrophic Agonists*

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Myocardial hypertrophy is associated with increased basal glucose metabolism. Basal glucose transport into cardiac myocytes is mediated by the GLUT1 isoform of glucose transporters, whereas the GLUT4 isoform is responsible for regulatable glucose transport. Treatment of neonatal cardiac myocytes with the hypertrophic agonist 12-*O*-tetradecanoylphorbol-13-acetate or phenylephrine increased expression of *Glut1* mRNA relative to *Glut4* mRNA. To study the transcriptional regulation of GLUT1 expression, myocytes were transfected with luciferase reporter constructs under the control of the *Glut1* promoter. Stimulation of the cells with 12-*O*-tetradecanoylphorbol-13-acetate or phenylephrine induced transcription from the *Glut1* promoter, which was inhibited by cotransfection with the mitogen-activated protein kinase phosphatases CL100 and MKP-3. Cotransfection of the myocytes with constitutively active versions of Ras and MEK1 or an estrogen-inducible version of Raf1 also stimulated transcription from the *Glut1* promoter. Hypertrophic induction of the *Glut1* promoter was also partially sensitive to inhibition of the phosphatidylinositol 3-kinase pathway and was strongly inhibited by cotransfection with dominant-negative Ras. Thus, Ras activation and pathways downstream of Ras mediate induction of the *Glut1* promoter during myocardial hypertrophy.

Myocardial cells can use a wide variety of substrates for energy production, including free fatty acids, glucose, lactate, and ketone bodies. Substrate selection by cardiac myocytes is developmentally regulated. During the perinatal period, substrate metabolism shifts from predominant non-oxidative glucose utilization to predominant fatty acid oxidation (1). This shift is associated with a shift in the expression of several regulatory proteins involved in glucose and fatty acid metabolism (2–7), including GLUT (glucose transport) proteins. Specifically, the ubiquitous glucose transporter GLUT1 is replaced by the insulin-regulated, muscle- and fat-specific isoform GLUT4 (2, 3, 6).

Myocardial hypertrophy is a pathological condition triggered by excess workload or cellular stress and is characterized by a number of phenotypic changes, including activation of immediate/early, fetal, and contractile protein genes, *e.g.* c-*fos*, atrial natriuretic factor, and myosin light chain-2. In addition, the hypertrophied heart exhibits a pattern of substrate metabolism similar to that of the fetal/neonatal heart with increased glycolytic flux and reduced fatty acid oxidation (8–10). Such alterations of the metabolic behavior could be explained by a resumption of the fetal expression pattern of proteins involved in glucose and fatty acid metabolism. Two preliminary reports have described increased expression of the *Glut1* isoform mRNA in myocardial hypertrophy induced in adult rats by pressure overload (11) or a large infarct of the left ventricle (12).

In response to treatment with a variety of different agonists, primary cultures of ventricular myocytes isolated from neonatal rat hearts display many of the features associated with hypertrophy *in vivo* and provide a useful model to study this nonproliferative growth response (13). We have used this model to investigate the regulation of GLUT1 expression in cardiac myocytes. Cardiac myocytes were transfected with a reporter construct encoding luciferase under the control of the *Glut1* promoter. The expression of this reporter in response to the hypertrophic agonists 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[1] and phenylephrine was assessed, and the signaling pathways responsible for increased expression of GLUT1 were investigated.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary ventricular myocytes were isolated from 1-day-old rats by collagenase digestion and maintained in Dulbecco's modified Eagle's medium/medium 199 (4:1) supplemented with penicillin and streptomycin (maintenance medium) as described previously (14). For transfection experiments, cells were plated at a density of 2.5×10^5/3.5-cm dish. For RT-PCR experiments or ERK activation assay, 2×10^6 cells were plated in 6-cm dishes. For activated Ras assays, 2×10^6 cells were plated in 10-cm dishes.

Cardiac fibroblasts cultures were prepared by two passages of the cells adherent to the culture dish during the pre-plating procedure. Cells were maintained in maintenance medium supplemented with 10% fetal calf serum. For transfection experiments, cells were plated at a density of 2.5×10^5/3.5-cm dish and grown for 24 h in maintenance medium with FCS before transfection. For activated Ras assays, 2×10^6 cells were plated in 10-cm dishes and grown in maintenance medium with FCS to subconfluence.

Cell Morphology—Cells for morphological analysis were plated on glass coverslips coated with gelatin and laminin. Cells were treated for 48 h and then fixed and stained with fluorescein isothiocyanate-conjugated phalloidin to show filamentous actin.

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; RSV, Rous sarcoma virus; MAP, mitogen-activated protein; MKP, MAP kinase phosphatase; MEK, MAP kinase/ERK kinase; RBD, Ras-binding domain; GST, glutathione *S*-transferase; PE, phenylephrine; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; JNK, c-Jun N-terminal kinase.

ERK Activation Assay—Cells were pretreated with or without PD98059 for 30 min and then treated with either TPA or phenylephrine for 10 min. Cells were then harvested in 500 μl of Laemmli buffer, and 50 μl of cell lysate were submitted to SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane. Total ERKs and doubly phosphorylated (Thr^{202}/Tyr^{204}) ERKs were detected by Western blotting using the K-23 rabbit polyclonal antibody (Santa Cruz Biotechnology) and the E10 monoclonal antibody (New England Biolabs Inc.), respectively.

Plasmids—The plasmid containing 1.4 kilobase pairs of the mouse *Glut1* promoter along with enhancer 1 (0.6 kilobase pair) and enhancer 2 (1.3 kilobase pair) was a kind gift from Dr. Takashi Murakami (15). The *Glut1* promoter along with enhancers 1 and 2 was subcloned into the pGL3basic multicloning site (Promega) to generate the luciferase reporter plasmid pLuc-GT1/E1/E2. The luciferase reporter was cotransfected with a Rous sarcoma virus (RSV)-β-galactosidase reporter plasmid (provided by Michael Kapiloff). The estrogen-regulated Raf-1 expression vector (pCEP4-ΔRaf-1:ER) expresses the kinase domain of Raf1 fused to the steroid-binding domain of the human estrogen receptor (16). The expression plasmids for the Ras mutants V12Ras (constitutively active) and A15Ras (dominant-negative) were constructed by subcloning a 650-base pair cDNA into an elongation factor 1α-driven plasmid. The expression plasmid for the MAP kinase phosphatase MKP-3 was constructed by cloning a 1.4-kilobase pair cDNA that was amplified from a rat cardiac cDNA library into an elongation factor 1α-driven plasmid. CL100 in the expression vector pSG5 was provided by Steve Keyse. The cytomegalovirus-driven ΔN3/S218E/S222D MEK1 construct (17) was provided by Nathalie Ahn. The expression plasmid pGEX-RBD, encoding the Ras-binding domain (RBD) of c-Raf-1 fused to GST (GST-RBD) (18), was donated by Stephen J. Taylor.

Gene Expression Assays—Transient transfections of myocytes were performed using the calcium phosphate precipitation method as described previously (14, 19, 20), using the amounts of plasmid DNA indicated in the figure legends. Transfection of fibroblasts was achieved using the LipofectAMINE Plus reagent (Life Technologies, Inc.) following the supplier's instruction. Luciferase and β-galactosidase assays were performed with reagents from Promega or Tropix Inc., respectively, as described by the manufacturer.

Expression of Endogenous Glucose Transporters—To evaluate the relative expression of the endogenous glucose transporter genes *Glut1* and *Glut4* by RT-PCR, we took advantage of regions of structural similarity and differences between the two isoforms (21). Untreated cells or cells stimulated with 1 μM TPA or 100 μM phenylephrine for 48 h were harvested in 750 μl of TRIZOL reagent (Life Technologies, Inc.), and total RNA was isolated following the manufacturer's instructions. Total RNA was used for reverse transcription and subsequent polymerase chain reaction using the Titan One Tube RT-PCR system from Boehringer Mannheim. Primers capable of amplifying both *Glut1* and *Glut4* cDNAs such that their respective products could be resolved on the basis of a 12-base pair size difference were used (21). PCR products were labeled by adding 0.05 μCi/μl [α-^{32}P]dCTP to the reaction mixture and subsequently resolved by electrophoresis on 15% polyacrylamide gels in 1× Tris borate/EDTA buffer. The gels were dried and exposed to storage phosphor screens. Band intensity was determined using a Molecular Dynamics PhosphorImager with ImageQuant software.

Activated Ras Detection Assay—Detection of Ras-GTP in cells extract was performed as described (18). Briefly, cell lysates were incubated with GST-RBD pre-bound to glutathione-Sepharose (Amersham Pharmacia Biotech) for 30 min at 4 °C. Bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer, resolved on 15% polyacrylamide gels, and subjected to Western blotting. Blots were probed using a rabbit anti-Ha-Ras polyclonal antibody (Santa Cruz sc-520).

RESULTS

TPA and Phenylephrine Induce Hypertrophy of Cardiac Myocytes—Fig. 1 shows phalloidin staining of rat neonatal ventricular myocytes treated with either TPA or phenylephrine. As described previously (13, 22–25), cells treated with either agonist for 48 h showed a dramatic increase in size, together with increased organization of myofibrils, two hallmarks of hypertrophy of ventricular myocytes.

Hypertrophic Agonists Stimulate the Expression of Endogenous GLUT1—The effect of the hypertrophic agonists TPA and PE on the relative expression of the glucose transporter isoforms *Glut1* and *Glut4* is shown in Fig. 2. Untreated myocytes had a *Glut1*/*Glut4* expression ratio close to 1 (1.2 ± 0.2), typical for neonatal cardiac myocytes (2, 3, 26). Following 48 h of treatment with either TPA or PE, the ratio of *Glut1*/*Glut4* mRNAs was markedly increased, to 5.1 ± 1.4 and 3.7 ± 1.7, respectively. This increase was mainly achieved by overexpression of *Glut1* and to a minor extent by a decrease in *Glut4* expression.

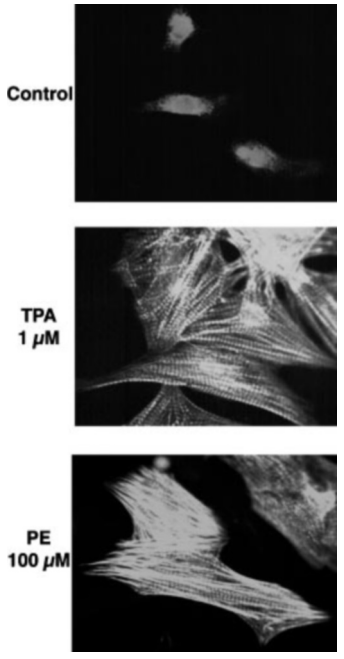

FIG. 1. **TPA and phenylephrine induce hypertrophy of rat neonatal ventricular myocytes.** Cells were plated onto glass coverslips coated with gelatin and laminin and left untreated or treated with TPA or PE for 48 h. Cells were then stained with fluorescein isothiocyanate-labeled phalloidin to show filamentous actin.

Hypertrophic Agonists Induce Transcription from the Glut1 Promoter—To determine whether the increased *Glut1* mRNA level in response to hypertrophic stimuli was caused by increased transcription from the *Glut1* promoter, we performed transient transfection experiments with the pLuc-GT1/E1/E2 construct. As shown in Fig. 3*A*, both TPA and PE stimulated transcription from the GT1/E1/E2 promoter construct in cardiac myocytes. TPA also induced transcription in cardiac fibroblasts, albeit to a lesser extent, whereas PE did not affect transcription in these cells. Fig. 3*B* shows the time course of induction of the *Glut1* promoter in cardiac myocytes. Induction by TPA was already detectable after 6 h of treatment and reached a plateau by 12 h, whereas induction by phenylephrine was slower, being detectable after 12 h and reaching a maximum by 48 h only. Therefore, an incubation time of 48 h was selected for subsequent experiments.

MAP Kinase Pathways Transduce the Hypertrophic Signal to the Glut1 Promoter—Stimulation of the MAP kinase pathways plays an important role in the development of hypertrophy of myocardial cells. We therefore assessed the involvement of MAP kinase pathways in the overexpression of *Glut1*. The MEK1 inhibitor PD98059 partially inhibited activation of ERK1 and ERK2 in response to either TPA or phenylephrine (Fig. 4*A*). Treatment with PD98059 did not affect base-line expression of either the *Glut1* or *Glut4* endogenous gene. However, treatment with PD98059 markedly reduced expression of the *Glut1* gene induced by either TPA or phenylephrine, without affecting expression of the *Glut4* gene (Fig. 4, *B* and *C*). We then assessed the involvement of MAP kinase pathways in the induction of the *Glut1* promoter by TPA and phenylephrine.

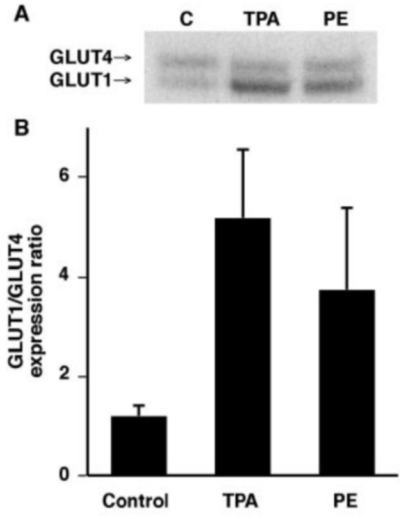

FIG. 2. **TPA and phenylephrine stimulate expression of the *Glut1* gene.** *A*, RT-PCR products obtained in a representative experiment. Myocytes (2×10^6 in 6-cm dishes) were untreated (control (*C*)) or treated with TPA or PE for 48 h. RT-PCR was performed with 1.25 μg of total RNA. *B*, PhosphorImager quantitation of RT-PCR experiments. Results are expressed as the *Glut1*/*Glut4* expression ratio and are means ± S.E. of six separate experiments.

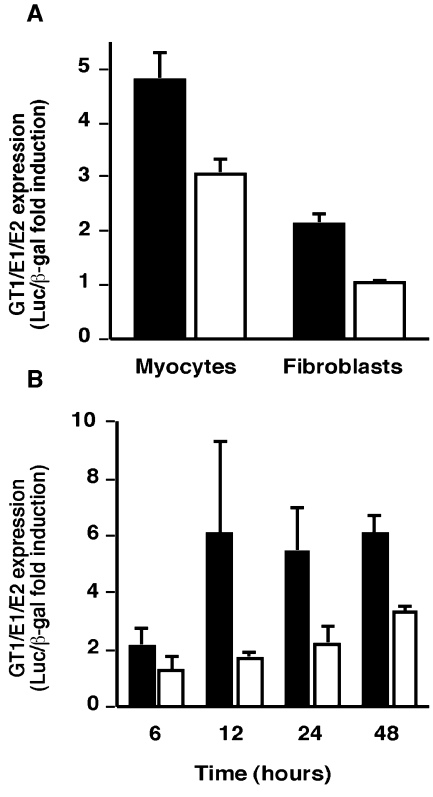

FIG. 3. **TPA and phenylephrine increase transcription from the *Glut1* promoter.** *A*, cardiac myocytes and fibroblasts were transfected with 1 μg of pLuc-GT1/E1/E2 and 0.7 μg of RSV-β-galactosidase (β-*gal*) and treated with 1 μM TPA (*black bars*) or 100 μM PE (*white bars*) for 48 h (myocytes) or 24 h (fibroblasts). Results are expressed as means ± S.E. of at least three experiments, each performed in triplicate. *B*, myocytes were transfected with 1 μg of pLuc-GT1/E1/E2 and 0.7 μg of RSV-β-galactosidase and treated with 1 μM TPA (*black bars*) or 100 μM PE (*white bars*) for 6–48 h. Results are expressed as means ± S.E. of at least three experiments, each performed in triplicate.

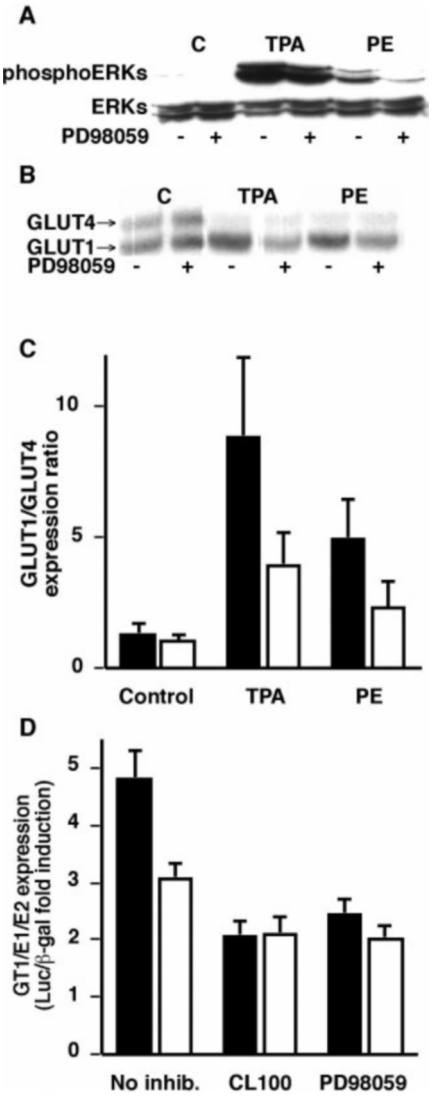

FIG. 4. **MAP kinase inhibitors block induction of the *Glut1* promoter.** *A*, the MEK inhibitor PD98059 reduces ERK activation by TPA or PE. Myocytes (2×10^6 in 6-cm dishes) were untreated (control (*C*)) or treated with TPA or PE in the presence or absence of 20 μM PD98059 for 10 min. Western blotting using an anti-doubly phosphorylated ERK antibody or an anti-ERK antibody was performed. *B*, RT-PCR products obtained in a representative experiment. Myocytes (2×10^6 in 6-cm dishes) were untreated or treated with TPA or PE in the presence or absence of 20 μM PD98059 for 48 h. RT-PCR was performed with 1.25 μg of total RNA. *C*, PhosphorImager quantitation of RT-PCR experiments. Cells were untreated or treated with TPA or PE in the presence (*white bars*) or absence (*black bars*) of 20 μM PD98059 for 48 h. Results are expressed as means ± S.E. of two to three experiments. *D*, myocytes were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase (β-*gal*), and 2 μg of either CL100 or empty vector plasmid. Cells were then stimulated with 1 μM TPA (*black bars*) or 100 μM PE (*white bars*) for 48 h in the presence or absence of 20 μM PD98059. Results are expressed as means ± S.E. of at least three experiments, each performed in triplicate.

Cotransfection of the cells with the broad specificity MAP kinase phosphatase CL100 significantly reduced induction of the *Glut1* promoter by either TPA or phenylephrine (Fig. 4*D*). PD98059 also inhibited the response to both hypertrophic agonists, confirming participation of the ERK pathway. In contrast, the p38 inhibitor SB203580 did not affect induction of the *Glut1* promoter by TPA or phenylephrine (data not shown).

To further confirm involvement of the ERK pathway in *Glut1* promoter induction, cells were cotransfected with increasing amounts of a plasmid expressing the ERK-specific phosphatase MKP-3 (Fig. 5) (27, 28). As expected, MKP-3 inhibited induction of the *Glut1* promoter by both TPA and phenylephrine.

We next reasoned that if the ERK pathway was important for induction of the *Glut1* promoter, then introduction into cells

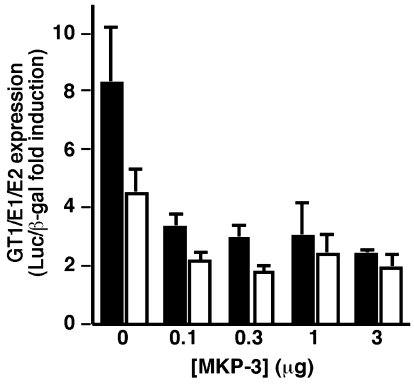

FIG. 5. **ERK activation is required for induction of the *Glut1* gene.** Myocytes were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase (*β-gal*), and increasing amounts of MKP-3, balanced with empty vector. Cells were then stimulated with 1 μM TPA (*black bars*) or 100 μM PE (*white bars*) for 48 h. Results are expressed as means ± S.E. of four experiments, each performed in triplicate.

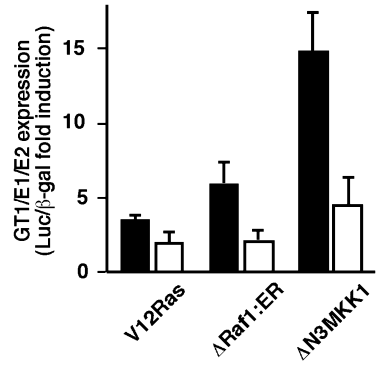

FIG. 6. **Constitutively active mutants of the ERK pathway induce the *Glut1* promoter.** Myocytes were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase (*β-gal*), and 2 μg of V12Ras or empty vector, 2 μg of estrogen-inducible ΔRaf-1:ER, or 1 μg of ΔN3/S218E/S222D MEK1 or wild-type MEK1. ΔRaf-1:ER-transfected cells were then treated with either 0.8 mM estradiol or the ethanol vehicle. Cells were also cotransfected with 2 μg of CL100 (*white bars*) or empty vector (*black bars*). Results are expressed as means ± S.E. of three to four experiments, each performed in triplicate.

of constitutively active versions of the proteins of the Ras/Raf/MEK/ERK cascade should mimic the effect of TPA and phenylephrine and induce the promoter. The results shown in Fig. 6 demonstrate that expression of a constitutively active mutant of Ras (V12Ras), an estrogen-inducible version of Raf-1 (ΔRaf-1:ER), or a constitutively active mutant of MEK1 (ΔN3/S218E/S222D MEK1) resulted in increased expression from the *Glut1* promoter. Furthermore, expression induced by all of these agonists was inhibited by cotransfection with the broad specificity MAP kinase phosphatase CL100.

Ras Activity Is Required for the Hypertrophic Response—Ras activation is required for phenylephrine-induced hypertrophy and is sufficient to induce both morphological and genetic markers of hypertrophy (19, 29, 30). We therefore tested whether Ras was also required for the *Glut1* response to TPA and phenylephrine. Fig. 7*A* shows that cotransfection of cardiac myocytes with increasing amounts of the dominant-negative Ras mutant A15Ras (31) strongly inhibited induction of the *Glut1* promoter by both TPA and phenylephrine. This result suggests that Ras activation is required for transduction of the signal elicited by both TPA and phenylephrine in cardiac myocytes. In contrast, expression of A15Ras in cardiac fibroblasts did not affect the *Glut1* promoter induction in response to TPA (Fig. 7*B*), whereas it markedly blunted induction of the *Glut1* promoter by serum (Fig. 7*C*). These results suggest that Ras does not participate in signal transduction activated by TPA in cardiac fibroblasts, although it does participate in induction by TPA in cardiac myocytes.

TPA Activates Ras in Myocytes, but Not in Fibroblasts—To further investigate the difference between cardiac myocytes and fibroblasts regarding the requirement for Ras for TPA-induced GLUT1 expression, we performed Ras-GTP loading assays in both cell types. As shown in Fig. 8, treatment of myocytes with 1 μM TPA or 10% FCS induced GTP loading of Ras. In contrast, TPA was unable to elicit activation of Ras in fibroblasts, although the cells responded to FCS stimulation. Therefore, TPA can induce Ras in muscle cells, but not in fibroblasts, thus explaining the Ras requirement only in muscle cells.

Involvement of the Phosphatidylinositol 3-Kinase Pathway in Glut1 Induction—In addition to the Ras/Raf/MEK/ERK pathway, GTP loading of Ras can trigger activation of other signaling pathways, including phosphatidylinositol 3-kinase (PI3K) (32). We therefore investigated whether activation of the PI3K pathway was required for induction of the *Glut1* promoter by hypertrophic agonists. As shown in Fig. 9, the selective PI3K inhibitor LY294002 (33) reduced base-line expression of both the *Glut1* and *Glut4* endogenous genes, but did not affect the *Glut1*/*Glut4* expression ratio in non-hypertrophic cells. However, treatment with LY294002 reduced the increase in the *Glut1*/*Glut4* ratio observed upon treatment with TPA or phenylephrine. In addition, LY294002 slightly, but significantly inhibited induction of the *Glut1* promoter by both TPA and phenylephrine (Fig. 9*C*). LY294002 did not inhibit induction of the *Glut1* promoter by V12Ras. These results suggest that PI3K activation contributes to the induction of the *Glut1* promoter in response to TPA and phenylephrine and that it acts upstream of Ras.

DISCUSSION

Myocardial hypertrophy is characterized by expression of immediate/early, fetal, and contractile genes. *In vivo*, hypertrophic hearts have a pattern of substrate metabolism resembling that observed in fetal hearts, with increased reliance on glycolysis for energy production and reduced oxidation of fatty acids (8–10). In this study, we observed that hypertrophy of rat neonatal ventricular myocytes is associated with increased expression of the glucose transporter *Glut1* isoform mRNA. GLUT1 is the principal isoform expressed in the fetal heart, and its expression is down-regulated following birth in normal myocardium (2, 3, 6), concomitantly with the shift from glycolytic to oxidative metabolism (1).

Our results suggest that regulation of *Glut1* expression during hypertrophy is primarily achieved at the transcriptional level. Transient transfection experiments with a luciferase reporter under the control of the mouse *Glut1* promoter indicated that treatment of myocytes with hypertrophic agonists resulted in increased transcription from the *Glut1* promoter occurring between 6 and 48 h following addition of the agonist. The two agonists we used (TPA and phenylephrine) activated the promoter with different kinetics, with TPA acting more rapidly and efficiently than phenylephrine. This is probably related to TPA being a better activator of the ERK mitogen-activated protein kinases compared with PE (Fig. 3*A*). The mode of action of these agonists is different. Phenylephrine is an α_1-adrenergic agonist whose receptor is coupled to a $G\alpha_q$-containing heterotrimeric G protein. Activation of G_q-containing heterotrimeric protein in cardiac myocytes leads to Ras activation via the tyrosine kinase/Shc/Grb2/Sos pathway (34). Ras activation, in turn, can trigger a variety of downstream signaling pathways, including Raf and phosphatidylinositol 3-kinase (for a review, see Ref. 35). TPA belongs to the phorbol ester family, a

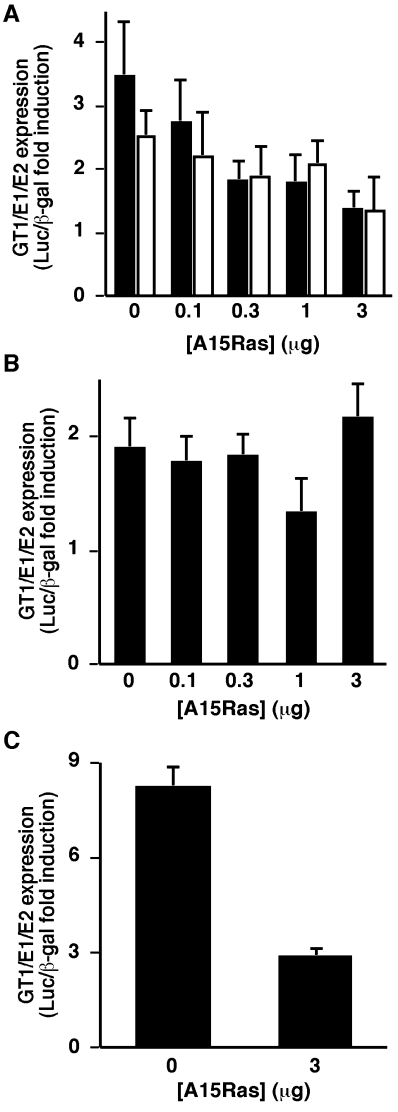

FIG. 7. **Ras activity is required for induction of the *Glut1* promoter in myocytes.** *A*, myocytes were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase (*β-gal*), and increasing amounts of A15Ras, balanced with empty vector. Cells were then stimulated with 1 μM TPA (*black bars*) or 100 μM PE (*white bars*) for 48 h. Results are expressed as means ± S.E. of four experiments, each performed in triplicate. *B*, fibroblasts were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase, and increasing amounts of A15Ras, balanced with empty vector. Cells were then stimulated with 1 μM TPA for 24 h. Results are expressed as means ± S.E. of four experiments, each performed in triplicate. *C*, fibroblasts were transfected with 1 μg of pLuc-GT1/E1/E2, 0.7 μg of RSV-β-galactosidase, and 3 μg of either A15Ras or empty vector. Cells were then stimulated with 10% FCS for 24 h.

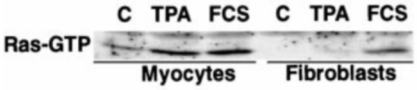

FIG. 8. **Stimulation of Ras-GTP loading by TPA in myocytes.** Cells (2×10^6 in 10-cm dishes) were serum-starved for 24 h and then stimulated for 1 h with either 1 μM TPA or 10% FCS. Cells were lysed, and GTP-bound Ras was affinity-precipitated using GST-RBD and detected by Western blotting. *C*, control.

class of compounds able to directly stimulate the classical and novel protein kinase C (PKC) isoforms. Stimulation of PKC activity can cause activation of Raf and MAP kinases independently of Ras (36). Thus, the modes of action of both agonists may converge onto Raf. In some cell types, TPA-induced activation of MAP kinases involves Ras (37–39). Our data indicate

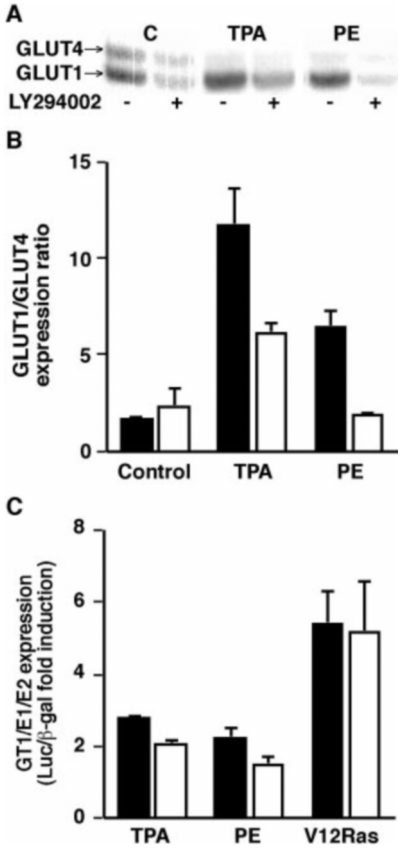

FIG. 9. **Inhibition of the phosphatidylinositol 3-kinase pathway reduces hypertrophic *Glut1* induction.** *A*, shown are RT-PCR products obtained in a representative experiment. Myocytes (2×10^6 in 6-cm dishes) were untreated (control (*C*)) or treated with TPA or PE in the presence or absence of 50 μM LY294002 for 48 h. RT-PCR was performed with 1.25 μg of total RNA. *B*, RT-PCR experiments were subjected to PhosphorImager quantitation. Cells were untreated or treated with TPA or PE in the presence (*white bars*) or absence (*black bars*) of 50 μM LY294002 for 48 h. Results are expressed as means ± S.E. of two experiments. *C*, myocytes were transfected with 1 μg of pLuc-GT1/E1/E2 and 0.7 μg of RSV-β-galactosidase and stimulated with 1 μM TPA or 100 μM PE for 48 h. Alternatively, cells were cotransfected with 2 μg of V12Ras. Cells were incubated in the presence (*white bars*) or absence (*black bars*) of the selective phosphatidylinositol 3-kinase inhibitor LY294002 (50 μM). Results are expressed as means ± S.E. of three to four experiments, each performed in triplicate.

that primary ventricular myocytes fall into this class (see below).

GLUT1 is a ubiquitous isoform of the glucose transporter, expressed at a significant level in virtually every tissue of the body. Therefore, it was a potential concern that the effect of TPA, a non-tissue-specific protein kinase C agonist, could be due to increased expression of the *Glut1* promoter in contaminating non-myocyte cardiac cells, mainly cardiac fibroblasts. We are confident, however, that this is not the case, for the following reasons. 1) Phenylephrine, which activates common signaling pathways with TPA, but through α_1-adrenergic receptor stimulation, induced a similar response from the *Glut1* promoter in myocyte culture. It failed, however, to induce the *Glut1* promoter in cultures of non-myocyte cardiac cells, consistent with the presence of the α_1-adrenergic receptor on myocytes only (40). 2) TPA-induced activation of the *Glut1* promoter was less in cultures of cardiac fibroblasts than in cultures of myocytes. The converse would be expected if the effect observed in myocytes was due to contaminating fibroblasts, which represent at most only 5% of the cells in myocyte cultures. 3) TPA-induced activation of the *Glut1* promoter was almost totally abolished by cotransfection with dominant-neg-

ative Ras in myocytes, as was phenylephrine-induced activation of *Glut1*; it was, however, unaffected in non-myocytes. This observation is corroborated by the finding that TPA treatment resulted in activation of Ras in myocytes, but not in fibroblasts (see below).

Activation of the MAP kinase pathways is involved in the process of myocardial hypertrophy. Previous results have shown that Ras or Raf-1 activity is required for expression of the c-*fos*, atrial natriuretic factor, and myosin light chain-2 promoters in phenylephrine-induced hypertrophy (14, 19). In addition, active MAP kinase is required for induction of the c-*fos* and atrial natriuretic factor promoters by phenylephrine (41, 42), and expression of constitutively active MEK1 results in overexpression of hypertrophic genes (43). In this study, we found that activation of the ERK mitogen-activated protein kinase pathway is required for activation of the *Glut1* promoter during myocardial hypertrophy. PD98059, a specific inhibitor of the ERK kinase MEK1 (44), partially inhibited both overexpression of the endogenous *Glut1* mRNA and activation of the *Glut1* promoter in response to hypertrophic agonists. Inhibition was only partial, however, as was inhibition of ERK activation, possibly because MEK2, which is much less sensitive to PD98059 than MEK1, is the predominant ERK kinase in cardiac myocytes. Cotransfection of the cells with the MAP kinase phosphatases CL100 (45) and MKP-3 (27, 28) also significantly reduced induction of the *Glut1* promoter by TPA and phenylephrine. Because of the low transfection efficiency in primary myocytes, it was not possible to assess the effect of transfection with these molecules on expression of endogenous *Glut1* mRNA. Although MKP-1, the mouse homologue of CL100, was initially thought to be specific for the ERKs, we have previously shown that, in our cells, CL100 could inhibit MEK kinase-induced JNK activity as well as ERK activity (25). Therefore, to further restrict involvement of the MAP kinase pathways to the ERKs, we also used the MAP kinase phosphatase MKP-3, which is highly specific for ERK (28). Together, the results obtained with PD98059, CL100, and MKP-3 suggest that activation of the ERK pathway plays a major role in transducing hypertrophic signals to the *Glut1* promoter. Furthermore, transfection of cardiac myocytes with constitutively active or estrogen-inducible mutants of proteins of the Ras/Raf/MEK/ERK cascade also leads to increased expression from the *Glut1* promoter. Conversely, cotransfection of the cells with a dominant-negative version of Ras (A15Ras) strongly inhibits induction of the *Glut1* promoter by either TPA or phenylephrine, suggesting that activation of Ras is not only sufficient, but also necessary for induction of the *Glut1* promoter. The finding that A15Ras strongly inhibits TPA-induced activation was somewhat unexpected in view of the ability of active PKC to stimulate Raf independently of Ras in COS and NIH3T3 cells (36). However, Marais *et al.* (39) recently showed that TPA activation of ERK required active Ras in COS cells. Furthermore, TPA increased the amount of Ras-GTP in these cells. In our experiments, TPA increased Ras-GTP in myocytes, but not in fibroblasts, a finding consistent with inhibition of the TPA effect by A15Ras in myocytes only. These results therefore suggest that Ras-mediated Raf activation, rather than PKC-mediated Raf phosphorylation, is the main pathway leading to stimulation of ERK activity in myocytes treated with phorbol esters. The reason why TPA activates Ras in myocytes but not in fibroblasts remains unknown, but could be related to expression of different PKC isoforms.

We have also shown that PI3K activation is involved, at least in part, in the transduction of the signal from hypertrophic agonists to the *Glut1* promoter. The exact position of PI3K in these signal transduction pathways remains somewhat controversial. Although an initial report described PI3K as being a direct target of Ras (32), other studies have suggested that PI3K could act upstream of Ras (46, 47). The present study does not allow us to draw firm conclusions on this issue. However, the fact that the PI3K inhibitor LY294002 does not inhibit induction of *Glut1* by V12Ras suggests that PI3K does not act downstream of Ras in this model. Recently, two reports showed that PI3K activity was required for activation of some PKC isoforms, including the TPA-activated isoform nPKCδ (48, 49). This would place PI3K upstream of Ras in cardiac myocytes in which Ras is activated as a result of PKC activation.

In addition to the ERK pathway, phenylephrine has been shown to potently activate the JNK and p38 MAP kinase pathways (50, 51). Experiments using the specific p38 inhibitor SB203580 (52, 53) or cotransfection with the JNK inhibitory protein JIP (data not shown) have shown that the p38 pathway was not significantly involved and the JNK pathway contributed very little to induction of *Glut1* transcription during myocardial hypertrophy.

Although this study used pharmacological agonists to study the *in vitro* hypertrophic response of glucose transporters, it has implications for the understanding of glucose metabolism in pathophysiological situations. Physiological agonists that, like phenylephrine, signal through $G\alpha_q$-coupled receptors and activate ERKs, such as endothelin (54, 55) and angiotensin II (56), have been implicated in the pathogenesis of myocardial hypertrophy *in vivo*. In addition, a specific inhibition of $G\alpha_q$-dependent signaling blocks pressure overload-induced hypertrophy in transgenic mice (57). Other stimuli, such as oxidative stress (58) or hypoxia/reoxygenation (59), that lead to activation of the Ras/Raf/MEK/ERK pathway or ischemia/reperfusion leading to translocation and activation of PKC isoforms (60, 61) could also potentially lead to increased *Glut1* transcription. In fact, overexpression of *Glut1* has recently been reported in an *in vivo* model of ischemia/reperfusion (62), a situation in which PKC activation and oxidative stress take place.

In conclusion, our data indicate that *Glut1* expression in hypertrophied myocytes can be explained primarily through activation of the Ras/Raf/MEK/ERK pathway. Interestingly the signaling pathways that are used in different cell types in the heart to activate the ERK molecules are different.

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