ARIA/HRG Regulates AChR ε Subunit Gene Expression at the Neuromuscular Synapse via Activation of Phosphatidylinositol 3-Kinase and Ras/MAPK Pathway

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Abstract. AChR-inducing activity (ARIA)/heregulin, a ligand for erbB receptor tyrosine kinases (RTKs), is likely to be one nerve-supplied signal that induces expression of acetylcholine receptor (AChR) genes at the developing neuromuscular junction. Since some RTKs act through Ras and phosphatidylinositol 3-kinase (PI3K), we investigated the role of these pathways in ARIA signaling. Expression of activated Ras or Raf mimicked ARIA-induction of AChR ε subunit genes in muscle cells; whereas dominant negative Ras or Raf blocked the effect of ARIA. ARIA rapidly activated erk1 and erk2 and inhibition of both erks also abolished the effect of ARIA. ARIA stimulated association of PI3K with erbB3, expression of an activated PI3K led to ARIA-independent AChR ε subunit expression, and inhibition of PI3K abolished the action of ARIA. Thus, synaptic induction of AChR genes requires activation of both Ras/MAPK and PI3K signal transduction pathways.

As the neuromuscular junction forms, signals from the motor neuron lead to accumulation of nicotinic acetylcholine receptors (AChR)[1] in the postsynaptic membrane (for review see Hall and Sanes, 1993). Both transcriptional and posttranslational mechanisms contribute to this synaptic specialization. First, nerve-supplied factors induce subsynaptic nuclei to increase synthesis of AChRs (for review see Chu et al., 1995*b*). Second, nerve-evoked electrical activity results in repression of AChR synthesis in extrasynaptic areas (Laufer and Changeux, 1989); and third, nerve-derived signals cause clustering of AChRs and other proteins on the muscle surface (for reviews see McMahan et al., 1992; Apel and Merlie, 1995; Bowe and Fallon, 1995). Recent progress has been made in elucidating the molecular details of all three transynaptic pathways. Here, we explore the signal transduction mechanisms involved in motor neuron–dependent induction of gene expression.

The hypothesis that a nerve-supplied factor selectively affects the transcriptional activity of subsynaptic nuclei arose from studies showing synaptic enrichment of AChR subunit mRNAs (Merlie and Sanes, 1985). Subsequently, reporter constructs linked to AChR genomic fragments were shown to be selectively expressed by underlying synaptic nuclei in transgenic mice (Klarsfeld et al., 1987; Sanes et al., 1991; Simon et al., 1992; Gundersen et al., 1993). These results supported a model in which the transcriptional potential of subsynaptic nuclei is controlled by a spatially restricted, nerve-supplied factor.

The leading candidate for this neuronal factor is Acetylcholine Receptor Inducing Activity (ARIA). Initially purified from chicken brain on the basis of its ability to stimulate AChR synthesis in aneural myotubes (Usdin and Fischbach, 1986), ARIA was shown to induce expression of AChR subunit genes (Harris et al., 1988; Martinou et al., 1991; Chu et al., 1995*a*; Jo et al., 1995). Sequence analysis of the ARIA gene (Falls et al., 1993) revealed that ARIA is encoded by the same gene that generates proteins initially isolated as ligands of the neu proto-oncogene (heregulin or NDF; Holmes et al., 1992; Wen et al., 1992) and as glial growth factor (Marchionni et al., 1993). Numerous alternatively spliced messages from the ARIA gene have been detected (for reviews see Ben-Baruch and Yarden, 1994; Corfas et al., 1995). In situ hybridization studies indicate that before innervation, ARIA is present in embryonic motor neurons lying in the ventral horn of the spinal cord (Falls et al., 1993; Marchionni et al., 1993). In the adult, immunoreactive ARIA protein can be detected at

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; AcRas, activated ras; ARIA, acetylcholine receptor inducing activity; ε-CAT, ε-chloramphenicol acetyltransferase; erbB, EGF receptor–related; GAP, guanine nucleotide-activating protein; HRG, heregulin; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RTK, erbB receptor tyrosine kinase.

synaptic sites in vivo (Chu et al., 1995*a*; Jo et al., 1995; Sandrock et al., 1995). Functionally, ARIA has been shown to induce specific expression of the AChR genes in tissue culture models (Martinou et al., 1991; Chu et al., 1995*a*; Jo et al., 1995).

ARIA/heregulin/GGF are ligands for EGF receptor–related (erbB) tyrosine kinases neu/erbB2/HER2 (Bargmann et al., 1986), erbB3/HER3 (Kraus et al., 1989), and erbB4/HER4(Plowman et al., 1990; 1993). All erbB receptor tyrosine kinases (RTKs) have been shown to interact with heregulin (HRG) in various cell types (for review see Carraway and Cantley, 1994; Carraway and Burden, 1995). In muscle, ARIA, erbB2, and erbB3 are colocalized and enriched at the neuromuscular end-plate (Altiok et al., 1995; Jo et al., 1995; Moscoso et al., 1995; Zhu et al., 1995) and ARIA has been shown to cause phosphorylation of erbB receptor complexes in muscle cells (Altiok et al., 1995; Jo et al., 1995). Thus, erbB kinases are likely to be physiologically relevant ARIA receptors at the neuromuscular synapse.

The actions of a number of RTKs, including the receptors for EGF, PDGF, and insulin have been shown to involve recruitment of SH2-domain–containing proteins (for review see Schlessinger, 1994) which activate the GDP/GTP-binding protein p21Ras, implicated as a key intermediate relaying signals between RTKs and a number of effectors (McCormick, 1994; Burgering and Bos, 1995). A major downstream target of Ras is the serine/threonine protein kinase Raf-1 (Daum et al., 1994; McCormick, 1994), which in turn controls the mitogen-activated protein kinase (MAPK) cascade that controls gene expression and protein synthesis by phosphorylation of transcription factors and other kinases (for review see Marshall, 1994; Robbins et al., 1994). Growth factors and neurotransmitters activate the Ras cascade (Ras/Raf/MEK/MAPK) in a number of cell types (Blenis, 1993).

It has been predicted that tyrosine–phosphorylated erbB2 and erbB3 may interact with a number of SH2-domain–containing proteins including phospholipase C -γ, guanine nucleotide-activating protein (GAP), the Ras adapter Grb2, SHC, the p85 subunit of PI3K, and others (Carraway and Cantley, 1994). Heregulin activates the MAPK cascade in proliferating mammary epithelial cells overexpressing erbB2 (Marte et al., 1995). Moreover, activation of erbB2 by point mutation (Ben-Levy et al., 1994) or by monoclonal antibodies directed against the extracellular domain of erbB2 (Marte et al., 1995), increases the activities of the MAP kinases, erk1 and erk2, in various cell lines. Thus, based on the results obtained in nonmuscle cells, transduction of the ARIA/HRG signal between muscle and nerve may involve activation of the Ras/MAPK pathway.

More recently, experimental evidence has linked activation of erbB3 to that of phosphatidylinositol 3-kinase (PI3K), a lipid kinase previously shown to mediate the mitogenic signal of the PDGF receptor (Cantley et al., 1991; Valius and Kazlauskas, 1993). PI3K products (PI(3,4)P and PI(3,4,5)P) have been implicated in regulation of mitogenesis, membrane ruffling, glucose uptake, and receptor sorting and downregulation in response to growth factors (for review see Kapeller and Cantley, 1994). ErbB3 is the only erbB kinase which contains six putative tyrosine phosphorylation sites (YXXM) in its cytoplasmic domain for specific association with PI3K. Association of PI3K with erbB3, but not with EGFR or erbB2, has been observed in EGF-treated human cancer cell lines (Soltoff et al., 1994). Heregulin-induced tyrosine phosphorylation and association of erb2 and erbB3 with PI3K has been reported in PC-12 cells (Gamett et al., 1995), in mouse fibroblasts transfected with a chimeric EGFR/erbB3 receptor (Fedi et al., 1994), and in fibroblasts overexpressing erbB2 and transfected with erbB3 (Carraway et al., 1995). Thus, recruitment of PI3K by an activated erbB3 may be another mechanism by which ARIA conveys its synaptic signal to alter transcription of specific genes in muscle.

In this study, we sought to determine whether the Ras/MAPK or PI3K pathways were involved in mediating the effects of ARIA on induction of the AChR subunit genes. For this purpose, we studied expression of lacZ and CAT reporter constructs containing the AChR ε subunit promoter in an ARIA-responsive muscle cell line, Sol 8. Differentiated Sol 8 myotubes increase AChR ε subunit expression in response to ARIA (Chu et al., 1995*a*) and express both erbB2 and erbB3 receptors (Moscoso et al., 1995); and therefore they represent a suitable model for investigation of the signaling pathways activated by ARIA. Results obtained with pharmacological inhibitors and constructs encoding dominant negative or activated elements of the Ras and PI3K signaling pathways indicate that ARIA-dependent induction of AChR ε subunit gene requires both pathways. These results provide the first demonstration that these signaling proteins can regulate expression of synapse-specific genes in response to the ARIA synaptic signal.

Materials and Methods

Reagents

Recombinant heregulin (HRGβ1 residues 177-244) was provided by M.X. Sliwkowski of Genentech (San Francisco, CA). PD 098059, the selective MEK inhibitor was provided by A. Saltiel of Parke-Davis/Warner-Lambert Pharmaceuticals (Ann Arbor, MI). The PI3K inhibitor, LY294002, was purchased from BiomolResearch (Plymouth Mtg., PA). Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and used according to the manufacturer's specifications. Radiolabeled PIP marker for PI3K chromatography was obtained from H. Hope and prepared as described (Hope and Pike, 1994). Unless otherwise specified, all other chemicals were purchased from Sigma Pharmaceuticals (St. Louis, MO).

Tissue Culture and Transfections

Sol 8 cells, a fusion-competent mouse muscle cell line (Mulle et al., 1988) were maintained and transfected as described in Chu et al. (1995*a*). In experiments where no signaling construct was used, the pSKII+ plasmid was cotransfected as filler DNA. In experiments where CAT constructs were used, RSV-lacZ was cotransfected to adjust for variations in transfection efficiency (Chu et al., 1995*a*). Cells were transfected on day 1, switched to differentiation media (2% horse serum) on day 2, and stimulated with ARIA/recombinant HRG (3 nM) on day 3 for the times specified in each experiment. In experiments involving inhibition of MEK, cells were pretreated for 30 min with 50 μM PD 098059 (dissolved in DMSO to stock concentration of 50 mM) or DMSO vehicle before the addition of ARIA. In experiments involving PI3K inhibition, cells were pretreated for 30 min with 20 μM LY294002 (dissolved in DMSO to a stock concentration of 8 mM) or DMSO vehicle before the addition of ARIA. Transfected cells were harvested for β-galactosidase histochemistry or CAT assays on day 5. Transfected cells were harvested on day 4 in experiments involving use of

pharmacological inhibitors. ARIA-dependent MAPK activation was measured in untransfected cells harvested on day 6 after plating.

Plasmid Constructs

Constructs referred to as ε-CAT and εnlacZ are ε150CAT and ε3500nlacZ, respectively, and have been described previously (Sanes et al., 1991; Sunyer and Merlie, 1993). The ε150CAT contains the 150-bp promoter sequence fused to the CAT gene. The ε3500nlacZ construct contains the entire 3.5-kb of the AChR ε subunit gene promoter fused to nlacZ. The construct ActinCAT contains a 245-bp EcoRI–HindIII fragment of promoter sequence from the human cardiac α-actin gene (Miwa and Kedes, 1987) fused to the CAT gene. The construct RSV-lacZ described previously (Pronin and Gautam, 1992) was cotransfected with CAT constructs to normalize for transfection efficiency in Sol 8 cells.

All signaling constructs were subcloned into a pSK-based parent vector driven by the 850-bp fragment of the AChR α-subunit promoter to direct myotube-specific expression (Klarsfeld et al., 1987; Prody and Merlie, 1991) of the signaling constructs and avoid expression in myoblasts that might interfere with proliferation and subsequent myotube fusion. The parent vector also contains the AChR α-subunit polyadenylation signal downstream of the inserted cDNAs for efficient transcript processing in muscle cell lines. The activated Ras construct, referred to as AcRas, contains the HaRas (RasVal12) cDNA (clone HB-11) and was obtained from Amer. Type Culture Collection (Rockville, MD). The dominant-negative Ras construct, referred to as DnRas contains the cDNA originally described as N17Ras (Feig and Cooper, 1988). The dominant-negative Raf (DnRaf), the negative control for dominant-negative Raf (ΔDnRaf), and the activated Raf (AcRaf) constructs contain the cDNAs described previously as RafC4, RafC4pm17, and RafBXB, respectively (Bruder et al., 1992). The activated PI3K (AcPI3K) and negative control PI3K (ΔAcPI3K) constructs contain the cDNAs originally described as p110* and p110*Δkin, respectively (Hu et al., 1995) and were obtained from Anke Klippel at Chiron Corporation (Emeryville, CA).

β-Galactosidase Assay

Transfected cells were rinsed twice with PBS and fixed in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS for 5 min. Cells were stained using X-GAL histochemistry procedures described previously (Sanes et al., 1986). Enzymatic quantitation of β-galactosidase activity was performed using ONPG as the substrate with minor modifications of the protocol previously described (Chu et al., 1995*a*). Cells were rinsed twice with PBS and harvested in 1× Reporter Lysis Buffer (Promega, Madison, WI) for 15 min and subjected to centrifugation at 12,000 rpm for 5 min. Cell lysates were incubated in 0.1 M sodium phosphate (pH 7.0) containing 0.64 μg/ml ONPG, 10 mM $MgCl_2$, and 450 mM β-mercaptoethanol until reaction was visibly yellow. Absorbance was measured at OD420.

CAT Assays

CAT assays were performed according to the method of Gorman (1985) as modified by Chu et al. (1995*a*). β-Galactosidase activity (from the cotransfected RSV-lacZ plasmid) was used to adjust for variations in transfection efficiency. Extracts were assayed for CAT activity per β-galactosidase OD unit which was then expressed as Relative CAT activity. CAT assay reaction buffer consisted of 0.25 M Tris-HCl (pH 8.0) with 0.2 μg/ml bovine serum albumin as carrier. Samples were subjected to thin layer chromatography in buffer containing 95% chloroform/5% methanol (vol/vol) and reaction products were quantified using phosphorimager analyses (Molecular Dynamics, Sunnyvale, CA).

Immune Complex Kinase Assays

Cells were harvested for MAP kinase activity assays as described by Sevetson et al. (1993). Antibodies against erk1 (X-837) and erk2 were kindly provided by Melanie Cobb and John Lawrence, respectively, and used in immunoprecipitations as described (Sevetson et al., 1993). The immunoprecipitated MAP kinase activity and unprecipitated material were assayed using [γ-^{32}P]ATP and myelin basic protein (MBP) as substrates as described by Boulton and Cobb (1991) with modifications of Sevetson et al. (1993).

In-gel MAPK Assays

Cell lysates were prepared as described above for immune complex kinase assays. Myelin basic protein was used as a substrate with 25 μg total protein of each cell lysate as described (Samuels et al., 1993). Radioactivity was visualized by phosphorimager analyses.

PI3K Activity Assays

Cells were lysed in PI3K lysis buffer as described by Newberry and Pike (1995). Approximately 400 μg of lysate protein and 45 μl of anti-phosphotyrosine (4G10) antibody (Upstate Biotechnology, Inc.), anti-erbB2 (sc-284, Santa Cruz Biotechnology, Santa Cruz, CA), anti-erbB3 (sc-285, Santa Cruz) antibody, or nonimmune rabbit IgG were added to the samples and incubated overnight at 4°C while rotating end over end. Immune complexes were isolated using 25 μl of Immuno-Precipitin (BRL) beads and washed twice with PBS + 1% Triton X-100, twice with 0.5 mM LiCl, 10 mM Tris, pH 7.5, and twice with 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA. PI3K activity was measured essentially as described by Auger et al. (1990). PI 3-P was identified based on its migration relative to that of a radiolabeled PI marker prepared as described by Hope and Pike (1994). Radioactivity was visualized by phosphorimager scanning.

Western Blot Analyses

In experiments where confirmation of quantitative immunoprecipitation of erbB tyrosine kinases with anti-erbB2 or anti-erbB3 antibodies was performed, aliquots of immune complexes obtained as described for PI3K activity assays were solubilized in 5× Laemmli SDS sample buffer. Samples were boiled for 5 min and after centrifugation, a 30-μg protein was loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto PVDF (Immobilon, Millipore, Bedford, MA) membranes and immunoblotted with anti-erbB2 or anti-erbB3 antibody (Santa Cruz Biotechnology, sc-284 and sc-285, respectively, each diluted 1:1,000) or nonimmune IgG. After incubation with a goat anti–rabbit alkaline phosphatase-conjugated secondary antibody diluted 1:1,000 (Boehringer Mannheim, Indianapolis, IN), blots were subject to the enhanced chemiluminescent (ECL) detection method (Dupont, Boston, MA).

In experiments where the ARIA-dependent activation of erk1 and erk2 was measured, samples were harvested directly in 2× Laemmli SDS sample buffer, diluted appropriately, boiled, sonicated, and centrifuged before loading onto 10% SDS-polyacrylamide gels. After electrophoresis and electroblotting onto PVDF membranes, immunoblot analysis was performed using an anti-phosphotyrosine MAP kinase antibody diluted 1:1,000 (New England Biolabs, Beverly, MA) according to manufacturer's specifications.

Results

Requirement for Ras and Raf in ARIA-induced AChR ε Subunit Gene Expression

Since previous evidence suggests that ARIA receptors are erbB kinases, and erbB RTKs are known to activate the Ras pathway, experiments were performed to determine whether specific induction of the AChR ε subunit gene by ARIA requires Ras. Sol 8 cells transfected with εnlacZ, a construct bearing the full-length promoter of the AChR ε gene fused to the reporter lacZ, exhibited little expression of the AChR ε subunit reporter construct (Fig. 1 *a*). However, when transfected cells were treated with ARIA, εnlacZ expression was observed in nuclei within many myotubes (Fig. 1 *a'*). To investigate the role of Ras in ARIA-dependent gene expression, we placed various Ras constructs under the control of a myotube-specific promoter to achieve postmitotic expression of these signaling constructs and minimize their interference with early myogenesis (see Materials and Methods). First, we asked if coexpression of an activated ras (AcRas) had an effect on εnlacZ expression. AcRas is a RasVal12 mutant trapped in the active form (GTP-bound) due to its insensitivity to GTPase-activating proteins (Ellis et al., 1981). Cotransfection of AcRas with εnlacZ resulted in reporter gene activa-

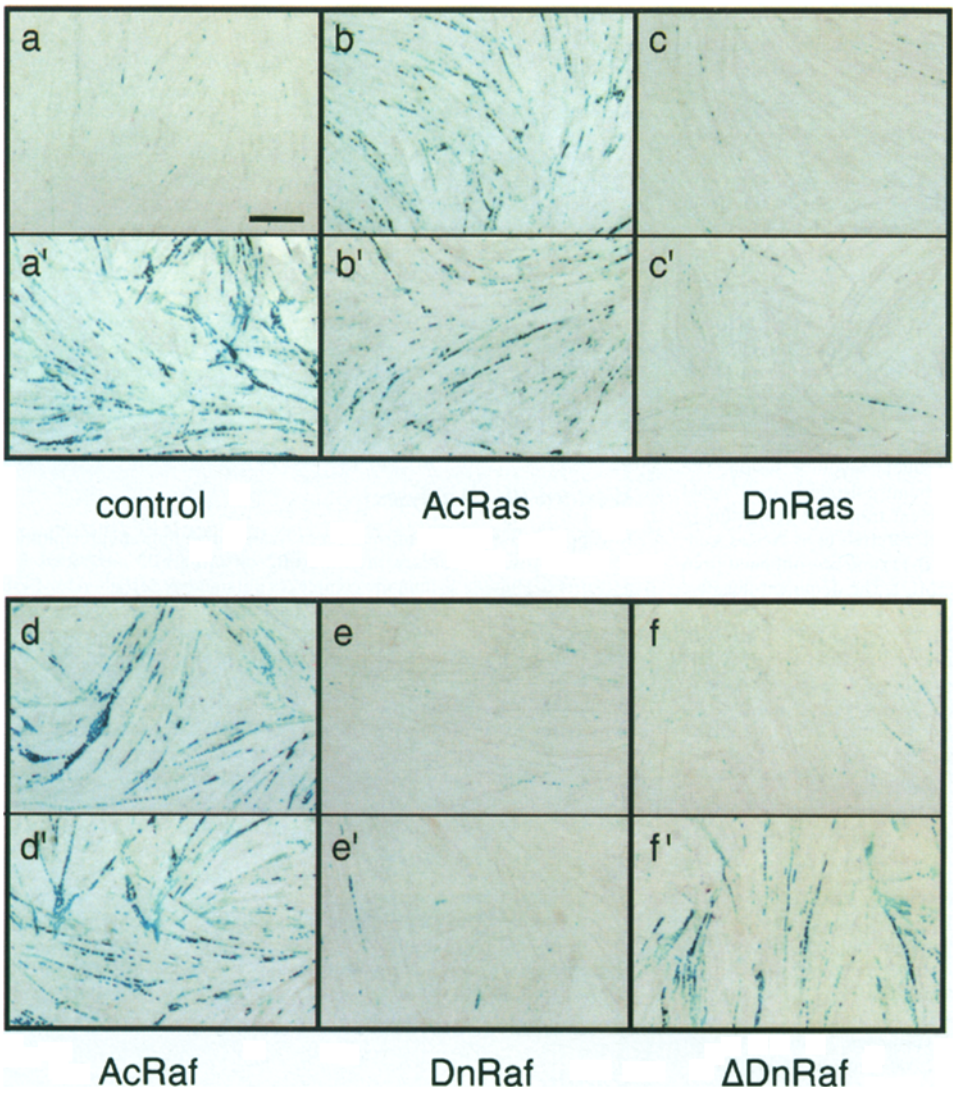

Figure 1. Activation of ϵnlacZ reporter by ARIA in Sol 8 myotubes and effects of various Ras and Raf constructs. Constructs were transiently transfected into Sol 8 myotubes. Cells transfected with ϵnlacZ plus (*a*) no signaling construct but pSKII+ plasmid used as filler DNA; (*b*) AcRas; (*c*) DnRas; (*d*) AcRaf; (*e*) DnRaf; and (*f*) ΔDnRaf. At 24 h after the induction of differentiation, ARIA (3 nM HRG) was added to the wells (*a'–f'*). Cells were stained for β-galactosidase 2 d later. Each plasmid set was transfected in duplicate; the data shown are representative for results obtained in four to five independent transfections. Bar, 300 μm.

tion in the absence or presence of ARIA (Fig. 1, *b* and *b'*). Thus, activated Ras can bypass the requirement for ARIA.

Next, we determined whether Ras was necessary for the response to ARIA. Sol 8 cells were transfected with ϵnlacZ and a dominant negative inhibitory (RasN17) mutant of Ras (DnRas). This mutant forms an inactive complex with Ras guanine nucleotide dissociation stimulators (i.e., Sos) and prevents activation of endogenous Ras proteins (Feig and Cooper, 1988). DnRas blocked the ARIA-induced AChR ϵ subunit expression (Fig. 1, *c* and *c'*), again suggesting that functional Ras is required for ARIA signaling.

To investigate further the role of Ras and provide quantitation of the effects described above, we repeated the experiments using a construct bearing a fragment of the ϵ promoter fused to the bacterial reporter chloramphenicol acetyltransferase (ϵ-CAT). In cells transfected with ϵ-CAT alone, a 5–6-fold induction by ARIA of ϵ-driven CAT expression was observed (Fig. 2 *A*), consistent with previous findings (Chu et al., 1995*a*). Cotransfection of AcRas with ϵ-CAT caused a 6–7-fold increase in ϵ-driven CAT expression that was not increased further by ARIA. In contrast, cotransfection of DnRas with ϵ-CAT did not significantly change basal CAT expression, but DnRas abolished the ARIA-induced ϵ-CAT expression (Fig. 2 *A*). Transfection cells with a control construct bearing a fragment of the cardiac α-actin promoter fused to CAT (ActinCAT) resulted in a marked increase in CAT activity which was not changed by ARIA or either Ras construct. These results indicate that direct activation of Ras is sufficient to mimic ARIA as well as necessary for transduction of the ARIA signal.

Since Ras signaling by RTKs has been shown to be directly linked to activation of Raf, we next investigated whether Raf was involved in mediating the ARIA signal to increase AChR ϵ subunit expression. Cotransfection experiments were performed with activated and dominant negative Raf constructs using methods described above for Ras. Activated Raf (AcRaf) has constitutively active kinase activity because it lacks a cysteine-rich regulatory domain (Bruder et al., 1992). Cotransfection of ϵnlacZ with activated Raf (AcRaf) resulted in significant activation of ϵnlacZ expression in nuclei within many myotubes in the absence of ARIA (Fig. 1 *d'*). In contrast, cotransfection of a dominant negative Raf (DnRaf) which lacks the Raf kinase domain abolished the ARIA-induced ϵnlacZ

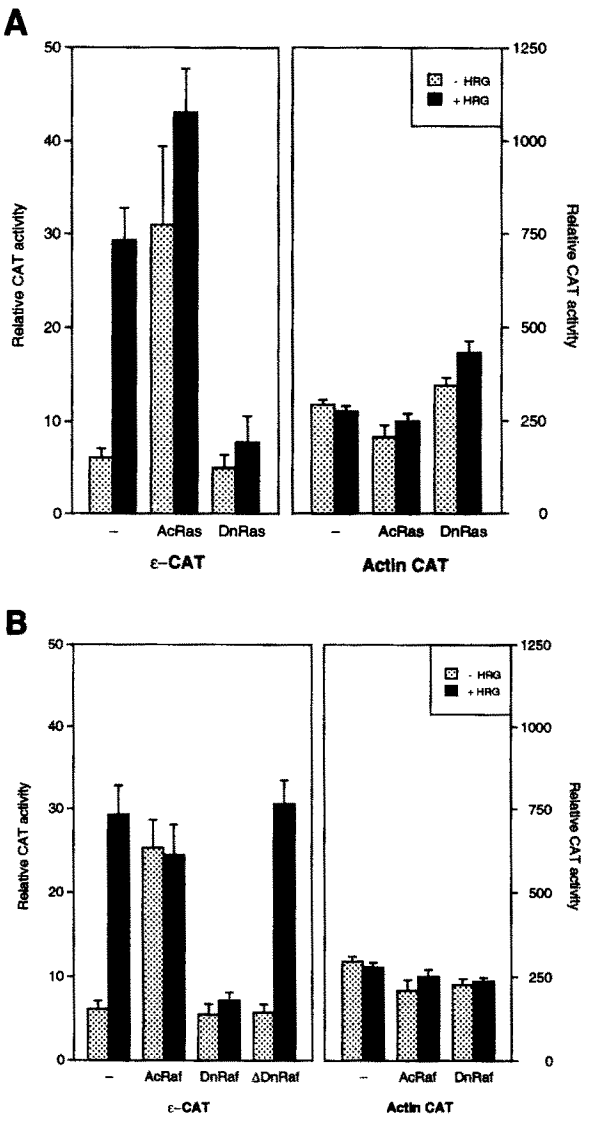

Figure 2. Induction of ε-CAT expression by ARIA in Sol 8 transient transfection and effect of Ras and Raf constructs. (*A*) Ras or (*B*) Raf constructs were transiently cotransfected with ε-CAT into Sol 8 myotubes. At 24 h after the induction of differentiation, ARIA (3 nM HRG) was added to the wells. Cells were harvested for analysis 2 d later. A cotransfected plasmid encoding β-galactosidase activity was used to normalize for variations in transfection efficiency among constructs. ActinCAT was used to demonstrate the specificity of ARIA induction of ε-CAT expression and showed no sensitivity to signaling constructs. Each plasmid set was transfected in duplicate; the data shown represent average values from five to six independent transfections. Error bars indicate SEM. See text for description of plasmids.

expression (Fig. 1, *e* and *e'*), whereas cotransfection with its negative control (ΔDnRaf) did not affect the ARIA-specific induction of εnlacZ expression (Fig. 1, *f* and *f'*) and gave the same results as εnlacZ alone (Fig. 1, *a* and *a'*). Quantitation of these effects using ε-CAT confirmed that expression of activated Raf results in ARIA-independent ε-CAT expression (Fig. 2 *B*) and that expression of dominant negative Raf (DnRaf) abolishes the ARIA-dependent increase. Cotransfection of Raf constructs had no effect on ActinCAT expression or its ARIA-responsiveness. Thus, activation of a Raf-mediated pathway can substitute for ARIA-receptor activation and is necessary for ARIA-induced AChR ε subunit expression.

Requirement for MAPK in ARIA-induced AChR ε Subunit Gene Expression

Although Raf has been demonstrated to activate multiple substrates, its most common target is the MAPK kinase or MEK. Therefore, we examined the role of MEK and its known downstream effectors, the MAP kinases, erk1 and erk2, in mediating the ARIA signal by use of a selective inhibitor of MEK activation, PD 098059. Incubation of cells with PD 098059 blocks the activation of MEK and the downstream MAPKs, erk1 and erk2 (Dudley et al., 1995; Pang et al., 1995). Moreover, PD 098059 does not inhibit the MAPK homologues JNK (jun kinase) and P38, PI3K, other protein serine/threonine kinases including Raf, cAMP-dependent protein kinase and protein kinase C, or the tyrosine kinase activities of v-*src*, EGFR, insulin receptor, or PDGFR (Dudley et al., 1995). More importantly, in Sol 8 cells, PD 098059 did not inhibit PI3K activity (see below). Thus, we reasoned that inhibitory effects of PD 098059 on AChR ε subunit expression would likely be a result of specific inhibition of MEK. Sol 8 cells transfected with εnlacZ or ε-CAT were pretreated with PD 098059 for 30 min before treatment with ARIA. 24 h later, cultures were either fixed and subjected to β-galactosidase histochemistry or harvested for CAT assays. MEK inhibitor did not affect basal εnlacZ or ε-CAT expression but specifically abolished the ARIA-induced increase in AChR ε subunit expression (Fig. 3, *A* and *B*). As expected, Sol 8 myotubes pretreated with MEK inhibitor 30 min before stimulation with ARIA displayed levels of erk1 and erk2 phosphorylation indistinguishable from that in unstimulated cells (Fig. 4 *B*).

To further test the involvement of MAPKs, we evaluated the temporal characteristics of MAPK activation in response to ARIA stimulation by using an antibody specific for activated (phosphorylated) forms of the MAPKs erk1 (p44) and erk2 (p42). Stimulation with ARIA resulted in significant increases in the activated forms of erk1 and erk2 (Fig. 4 *A*). Similarly, we observed a 2–4-fold increase in endogenous erk1 and erk2 activity within minutes of ARIA stimulation which remained elevated for at least 1 h, when MAPK activity was measured with an immune complex assay (data not shown). Thus, the rapid and prolonged activation of erk1 and erk2 in ARIA-treated Sol 8 cells and the inhibitory action of PD 098059 on both ARIA-induced MAPK activation and AChR ε subunit expression, are consistent with the necessary role of the MAPK pathway in transduction of the ARIA signal.

Requirement for PI3K in ARIA-induced AChR ε Subunit Gene Expression

Activation of an erbB2/erbB3 receptor complex by ARIA may recruit not only the Ras pathway but also the PI3K pathway (see Introduction). We asked whether there is detectable activation of PI3K in our system, and if such activation is necessary and sufficient for eliciting the ARIA-induced increase in AChR ε subunit gene expression. Using a phosphorylation assay which detects PI to PI(3)P

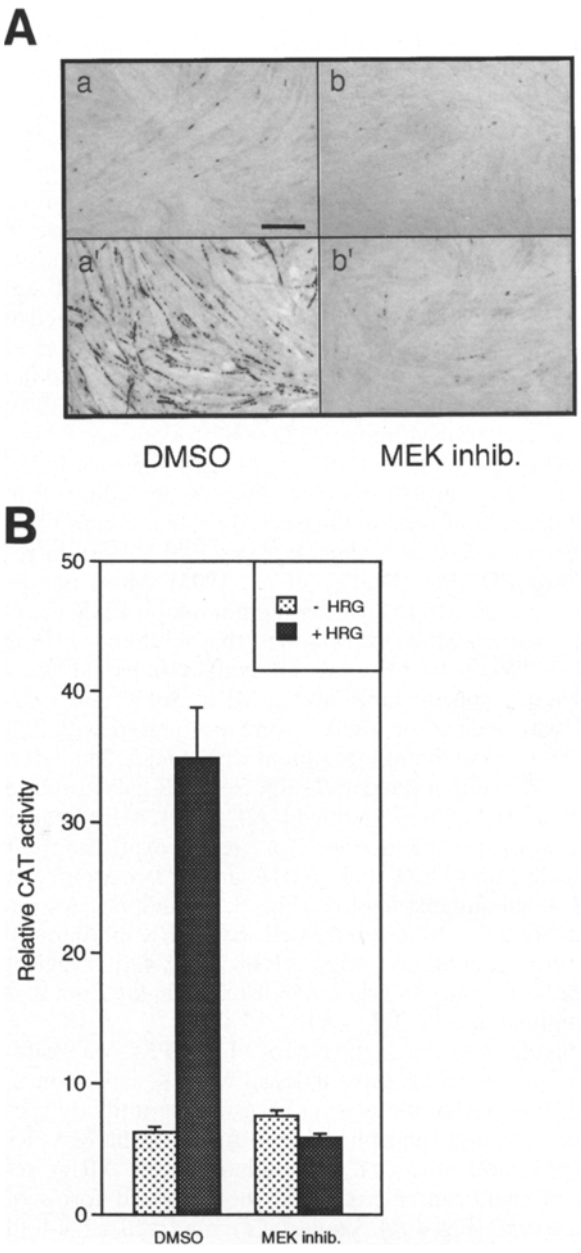

Figure 3. Inhibition of ARIA-induced expression of ϵnlacZ and ϵ-CAT by the MEK inhibitor PD 098059. (*A*) ϵnlacZ or (*B*) ϵ-CAT constructs were transiently transfected into Sol 8 myotubes. At 24 h after the induction of differentiation, a DMSO vehicle (*a* and *a'*) or the MEK inhibitor PD 098059, 50 μM (*b* and *b'*) was added to the wells 30 min before addition of ARIA (3 nM HRG) (*a'* and *b'*). One day later, cultures were either fixed or harvested for analysis. Each condition was performed in duplicate. The data shown in *A* are representative of results obtained in three to four independent transfections; Bar, 300 μm. In *B*, the data shown represent average values from four to five independent transfections. Error bars indicate SEM. See text for description of plasmids and inhibitor.

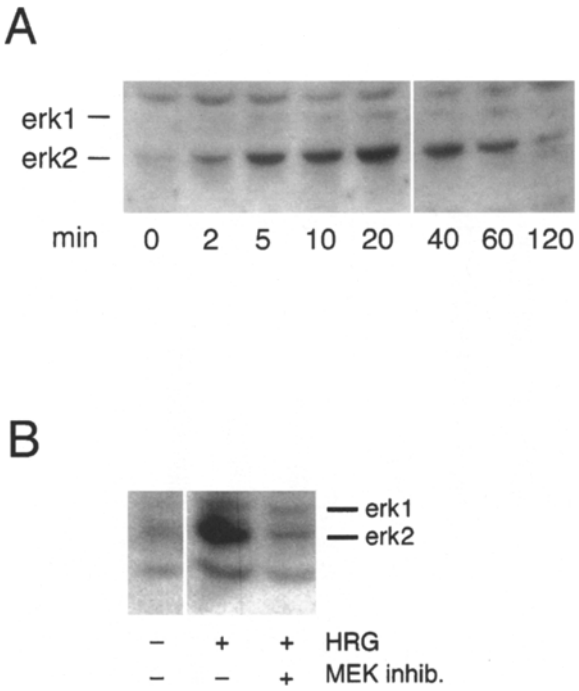

Figure 4. ARIA-dependent MAPK ativation and inhibition by the MEK inhibitor PD 098059. (*A*) Western blot analysis of ARIA-induced MAPK activation in Sol 8 cells. 15 μg of Sol 8 myotube protein in SDS Laemmli sample buffer were subject to electrophoresis on 10% polyacrylamide gels, blotted, and probed with antibodies specific for tyrosine/threonine phosphorylated MAPKs, erk1 and erk2 (NEB). MAPK activity, as indicated by phosphorylation, increased within minutes of stimulation with ARIA, and remained elevated for as long as 1 h. Data shown represent data obtained from three independent experiments. (*B*) In-gel MAPK assay. Extracts (25 μg protein) prepared from Sol 8 cells treated with MEK inhibitor (PD 098059, 50 μM) and ARIA (3 nM HRG), as indicated, were used as a source of MAPK (erk) activity in an in-gel kinase assay with myelin basic protein (MBP) and [γ-^{32}P]ATP as substrates. Radioactivity was visualized by phosphorimager analysis. ARIA-dependent activation of erk1 and erk2 was inhibited by PD 098059. Data shown represent data obtained from three to four independent experiments. See text for description of inhibitor.

conversion, we measured PI3K activity in extracts from ARIA-treated and untreated Sol 8 myotubes. Cells were stimulated with ARIA for specified periods of time and harvested in PI3K lysis buffer. Since activated PI3K associates with tyrosine-phosphorylated proteins, cell lysates were immunoprecipitated by using anti-phosphotyrosine antibody (4G10) to enrich lysates with PI3K protein before PI3K assays (Newberry and Pike, 1995). We observed a rapid and transient 10–12-fold increase in PI3K activity in extracts of ARIA-treated Sol 8 myotubes (Fig. 5 *A*). Thus, an activated (tyrosine phosphorylated) ARIA receptor may recruit PI3K to mediate the ARIA signal.

To determine if ARIA-induced specific association of PI3K with erbB kinases, lysates from ARIA-treated Sol 8 cells were immunoprecipitated with an anti-erbB2 or anti-erbB3 antibody and processed for either PI3K activity assay or immunoblot analysis. In unstimulated Sol 8 cells, no significant association of PI3K activity with erbB2 or erbB3 was detected. However, after ARIA stimulation, erbB3-associated PI3K activity increased dramatically (Fig. 5 *B*). In contrast, association of PI3K activity with erbB2 was not observed in extracts from ARIA-treated cells immunoprecipitated with anti-erbB2 antibody. West-

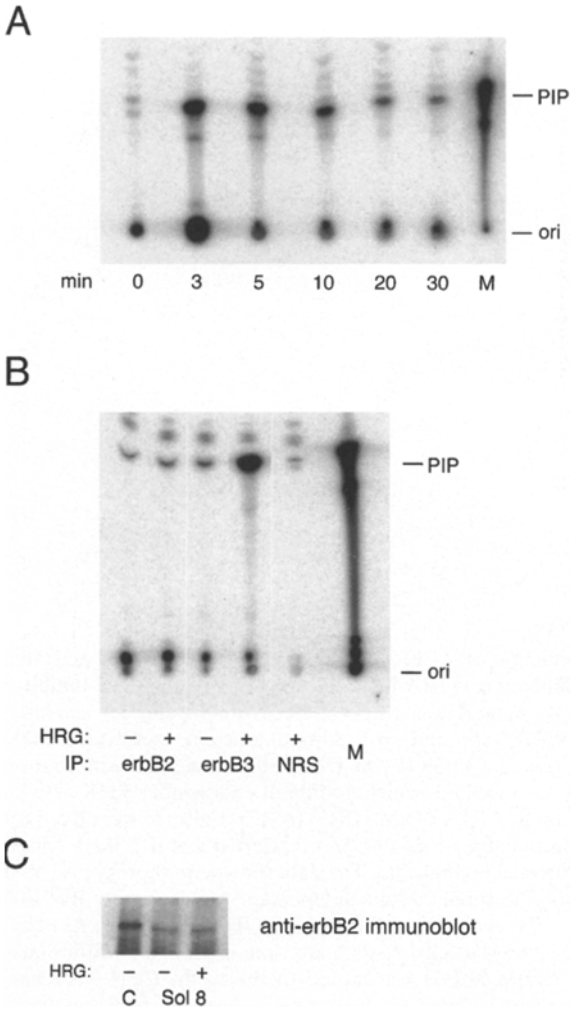

Figure 5. ARIA-dependent PI3K activity and association with ErbB3 in Sol 8 myotubes. (*A*) ARIA-dependent PI3K activity analyzed by thin layer chromatography. Cells were treated with ARIA (3 nM HRG) for the times indicated in minutes, lysed, immunoprecipitated with anti-phosphotyrosine antibody, and assayed for PI3K activity. The origin (*ori*) of the chromatogram and the position of PI 3-phosphate (*PIP*) are indicated. *M*, radiolabeled PIP marker. Sample variations at the origin can be ascribed to carry-over of highly radioactive aqueous (nonlipid) material during organic extractions (Auger et al., 1990). (*B*) ARIA-dependent association of PI3K activity with erbB3. Cells were treated with ARIA (3 nM HRG) for 3 min, lysed, immunoprecipitated with antibodies to erbB2 and erbB3, and assayed for PI3K activity. Lysates incubated with normal rabbit serum (*NRS*) were used as controls for nonspecific associations of PI3K in stimulated cells. *ori*, origin; *PIP*, PI 3-phosphate; *M*, radiolabeled PIP marker. (*C*) Western blot analysis was performed to confirm quantitative immunoprecipitation of erbB2 with anti-erbB2 antibody from unstimulated and ARIA-treated Sol 8 cells. Aliquots of immune complexes obtained as described for PI3K activity assays were solubilized in Laemmli SDS sample buffer and 30 μg protein subjected to 10% PAGE, blotted, and probed with antibodies specific for erbB2 or erbB3 (not shown). Immunoprecipitation of erbB2 from extracts of HRG-treated mammary carcinoma cells (MDA-MB-453) was used as a positive control, lane labeled as *C*.

ern blot analysis was performed to confirm quantitative immunoprecipitation of erbB2 (Fig. 5 *C*). We found that comparable levels of erbB2 were immunoprecipitated from extracts of Sol 8 cells and a breast carcinoma cell line overexpressing erbB2 (Culouscou et al., 1993). Thus, we conclude that ARIA stimulation leads to rapid recruitment of PI3K to the activated erbB3 receptor.

Given the ARIA-dependent activation of PI3K, we determined whether the activation of PI3K is sufficient to substitute for the ARIA-elicited increase in AChR ε expression. Cells were cotransfected with either εnlacZ or ε-CAT reporter constructs and a constitutively active PI3K (AcPI3K) (see Materials and Methods). Expression of the activated PI3K, but not a kinase-deficient PI3K construct (ΔAcPI3K), increased expression of εnlacZ (Fig. 6 *A*). Similarly, expression of AcPI3K but not ΔAcPI3K, increased expression of ε-CAT (Fig. 6 *B*). Thus, an activated PI3K turns on a pathway that mimics ARIA-receptor activation and thus, elicits increases in AChR ε subunit gene expression.

Since our results suggested that activation of a PI3K-dependent pathway was sufficient to substitute for ARIA receptor activation, we investigated whether PI3K activation was necessary as well as sufficient for AChR gene expression. LY294002, a potent and stable synthetic inhibitor of PI3K, has been shown to inhibit activation of PI3K in human neutrophils and smooth muscle cells. Moreover, it does not inhibit a number of ATP-requiring enzymes, including PI4K, EGF receptor tyrosine kinase, c-src, MAPKs, S6 kinase, diacylglycerol kinase, protein kinase C, cAMP-dependent protein kinase, and ATPase (Vlahos et al., 1994). To establish the selectivity of LY294002 for PI3K in Sol 8 cells, we confirmed that the compound did not inhibit ARIA-induced activation of MAPKs. Stimulation with ARIA (5 min) in cells pretreated for 30 min with LY294002 or DMSO did not inhibit the ARIA-dependent activation of both erk1 and erk2 MAPKs as measured by in-gel kinase assay (data not shown), indicating that LY294002 does not inhibit MAPK activity in our cell culture system. Therefore, any effects of LY294002 on ARIA-induction of ε gene expression could be reasonably attributed to specific inhibition of PI3K activity. Sol 8 cells were transfected with either εnlacZ or ε-CAT. Before stimulation with ARIA, cells were pretreated for 30 min with LY294002 or the DMSO vehicle and were assayed for lacZ or CAT 24–48 h later. LY294002 did not affect basal expression of εnlacZ; however, it completely abolished the ARIA-induced increase in AChR ε subunit expression as indicated by minimal β-galactosidase nuclear staining (Fig. 6 *A*). Thus, selective inhibition of PI3K prevents transduction of the ARIA signal and blocks activation of εnlacZ. Comparable results were obtained with LY294002 in ε-CAT–transfected cells (data not shown). Thus, we have demonstrated that (*a*) ARIA stimulation results in rapid association of PI3K with the erbB3 receptor, (*b*) that the presence of a constitutively active PI3K is sufficient to mimic the action of ARIA on AChR ε subunit gene expression, and (*c*) that selective inhibition of PI3K blocks the ARIA induction of AChR ε subunit gene expression. Together, these results suggest that activation of a PI3K-dependent pathway is a prerequisite for eliciting a complete ARIA-mediated increase in AChR ε subunit gene expression.

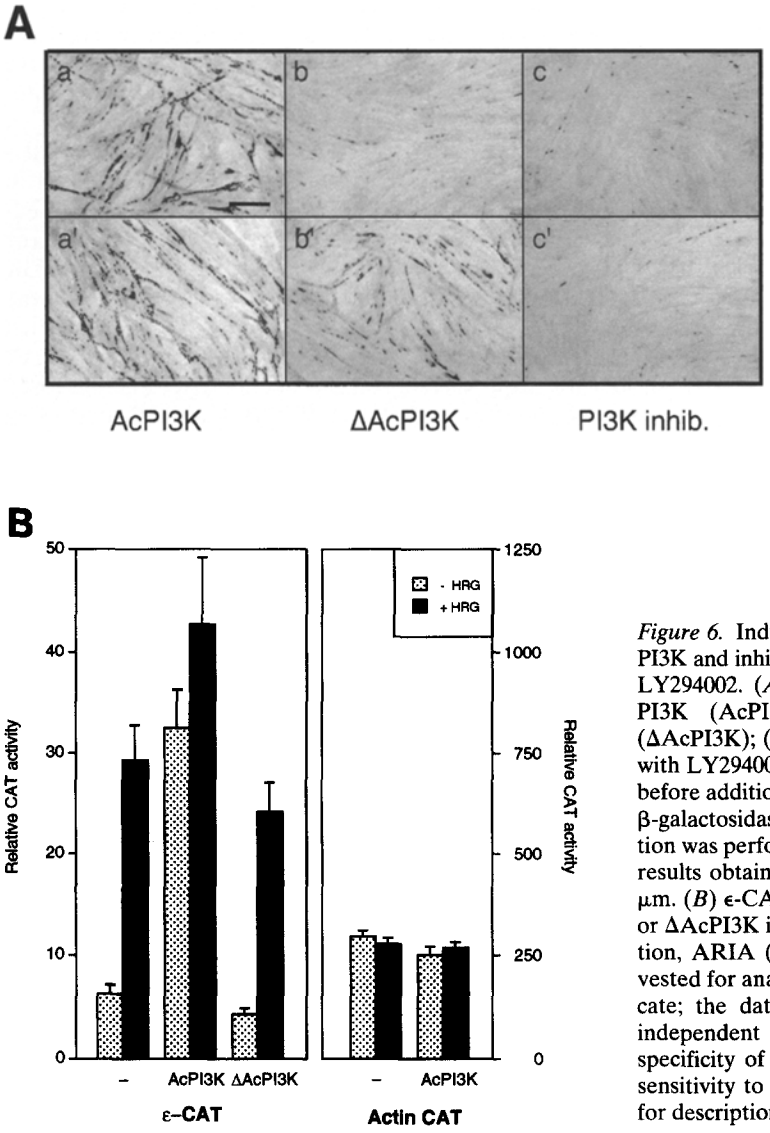

Figure 6. Induction of AChR ε expression in Sol 8 cells by activated PI3K and inhibition of ARIA-induced expression by the PI3K inhibitor LY294002. (*A*) εnlacZ was cotransfected with (*a* and *a'*) activated PI3K (AcPI3K); (*b* and *b'*) kinase-deficient activated PI3K (ΔAcPI3K); (*c* and *c'*) pSKII+ as DNA filler plus 30 min incubation with LY294002 in DMSO vehicle to inhibit endogenous PI3K activity before addition of ARIA (3 nM HRG) (*a'–c'*). Cultures were fixed for β-galactosidase analysis 2 d later or 1 d later (*d* and *d'*). Each condition was performed in duplicate. The data shown are representative of results obtained in three to four independent transfections. Bar, 300 μm. (*B*) ε-CAT was cotransfected with pSKII+ DNA filler, AcPI3K, or ΔAcPI3K into Sol 8 cells. At 24 h after the induction of differentiation, ARIA (3 nM HRG) was added to the wells. Cells were harvested for analysis 2 d later. Each plasmid set was transfected in duplicate; the data shown represent average values from four to five independent transfections. ActinCAT was used to demonstrate the specificity of ARIA induction of ε-CAT expression and showed no sensitivity to signaling constructs. Error bars indicate SEM. See text for description of plasmids.

Relationship between Ras/MAPK and PI3K in ARIA Signaling

Since our results suggest that ARIA signaling requires both a Ras-dependent and a PI3K-dependent component, we investigated how the two pathways interact to induce AChR ε subunit expression. We performed a series of cotransfection experiments to establish the epistatic relationship between the two in this system.

First, we tested the possibility that Ras/Raf/MAPK were downstream effectors of PI3K. We asked whether inhibition of the Ras/MAP kinase cascade could interfere with the ability of AcPI3K to activate the AChR ε subunit reporter construct. The ARIA-independent increase in ε-CAT expression stimulated by the AcPI3K construct alone is abolished when the cells are also expressing a dominant-negative Ras, and is significantly attenuated when the cells are expressing AcPI3K plus a dominant-negative Raf (Fig. 7 *A*). We next investigated if pharmacological inhibition of the MAPK pathway downstream of Ras could also block the inductive effect of AcPI3K on AChR ε subunit expression. Cells transfected with ε-CAT plus the activated PI3K (AcPI3K) were treated with the MEK inhibitor PD 098059 before stimulation with ARIA. We found that the ARIA-independent increase in ε-CAT expression induced by AcPI3K was completely abolished by the MEK inhibitor. Thus, ε-CAT expression in cells transfected with AcPI3K and treated with PD 098059 is indistinguishable from basal ε-CAT expression in unstimulated cells (Fig. 7 *A*). Thus, the MEK inhibitor blocked both the expression of AChR ε subunit by ARIA (Fig. 3) and by AcPI3K. These results may imply that AcPI3K mimics ARIA by activating a Ras/Raf/MAPK-mediated pathway essential for ARIA-induced AChR ε subunit expression and without these downstream effectors, activation of PI3K in this system is insufficient for full induction of the AChR ε subunit construct. Alternatively, these results may suggest that if the Ras- and PI3K-dependent components are distinct, the PI3K-activated component alone is not sufficient for full expression of the AChR ε subunit gene.

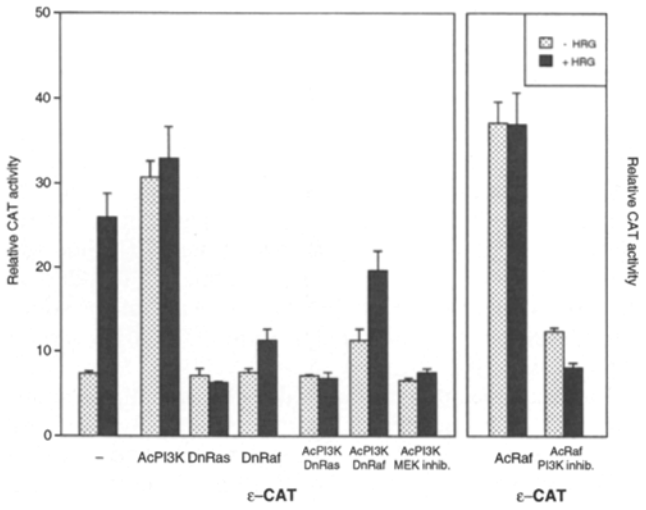

Figure 7. Interactions of PI3K, Ras, Raf and MEK activities in ARIA-dependent induction of ϵ-CAT expression in Sol 8 transient transfection. Cells were cotransfected with ϵ-CAT and signaling constructs as indicated and subject to inhibition of the MAPK pathway (with PD 098059) or inhibition of PI3K (with LY294002) before addition of 3 nM HRG where indicated. Cells were harvested for CAT analysis as described elsewhere. Each plasmid set was transfected in duplicate; the data shown represent average values from four independent transfections. Error bars indicate SEM. See text for description of plasmids and inhibitors.

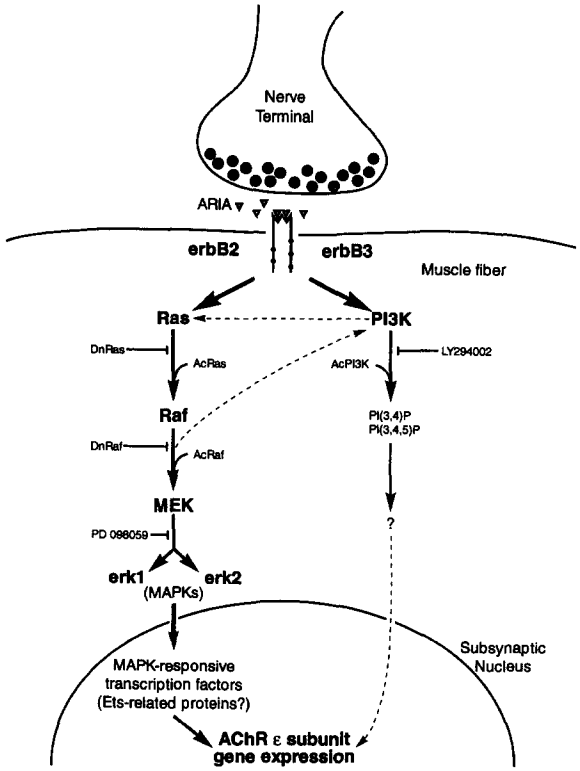

Figure 8. Schematic model of signaling pathways required for ARIA-regulated synapse-specific AChR ϵ gene expression at the neuromuscular junction. The nerve terminal provides the neurotrophic factor ARIA at the nerve end-plate. Probable receptors for ARIA include erbB2 and erbB3. ARIA-dependent dimerization and activation of these receptors leads to the association of various SH2 domain–containing signaling molecules (i.e., PI3K, Ras, etc.) with the tyrosine-phosphorylated RTKs. Transduction of the ARIA transynaptic signals to increase AChR ϵ subunit expression requires the interaction of a Ras/MAPK-dependent component and a PI3K-dependent component. Activation of either pathway is sufficient to mimic ARIA-induced gene expression. Inhibition of either pathway by dominant-negative constructs (DnRas, DnRaf) or pharmacological inhibitors (PD 098059 and LY294002) is sufficient to block ARIA-induced AChR ϵ expression via the other. Transduction of the transynaptic signals to the nucleus may lead to activation of regulatory molecules involved in transcription of AChR and other synapse-specific genes. Dashed arrows represent potential routes suggested by our data. See Discussion for detailed description of the model.

Finally, we considered the possibility that the Ras and PI3K-dependent components may be distinct and may have to be active simultaneously for AChR ϵ subunit induction. Thus, we asked whether induction of AChR ϵ subunit expression by AcRaf also displayed dependence on activation of endogenous PI3K. Sol 8 cells were transfected with ϵ-CAT plus AcRaf and subsequently treated with LY294002 to inhibit endogenous PI3K activity. We found that the PI3K inhibitor blocks all expression of ϵ-CAT, both ARIA-induced and AcRaf-induced (Fig. 7 *B*). As expected, LY294002 also blocked AcRas-induced ϵ-CAT expression (data not shown). More importantly, high levels of MAPK activity in Sol 8 cells transfected with AcRaf and treated with LY294002 confirmed adequate expression of AcRaf and selectivity of the inhibitor for PI3K (data not shown). Therefore, the PI3K inhibitor blocked induction of AChR ϵ subunit by ARIA (Fig. 3 *A*) and by AcRaf. Thus, PI3K activity appears to be necessary for complete induction of AChR ϵ subunit expression by an activated Ras/MAPK cascade.

Discussion

We have investigated the signaling pathways by which ARIA exerts its inductive effects on gene expression at the neuromuscular junction. In the studies reported here, we have demonstrated (*a*) ARIA-independent induction of AChR ϵ subunit gene expression by direct activation of either PI3K or the Ras/MAPK pathway, (*b*) inhibition of ARIA-induced AChR ϵ subunit expression by interference with the MAPK cascade or PI3K activity, and (*c*) ARIA-dependent association of PI3K activity with erbB3 receptor.

Based on these results and previous studies, we propose a model for ARIA-induced synapse-specific gene expression (Fig. 8). ARIA is released by the motor-neuron in a spatially restricted manner into the synaptic cleft where it binds to and activates an erbB2/erbB3 heterodimeric complex at the motor-endplate (Altiok et al., 1995; Moscoso et al., 1995; Zhu et al., 1995). ARIA 1/2induced cross-phosphorylation of erbB3 by erbB2 might be expected to result in tyrosine phosphorylation of specific residues, some of which may represent docking sites for the p85 regulatory subunit of PI3K (YXXM) or for the Ras adapter Grb2 (YXNR) (Carraway and Cantley, 1994). Recruitment of PI3K and Ras by the activated ARIA-receptor

complex then initiates the two signal transduction pathways (Fig. 8) shown in our studies to be necessary for AChR ε subunit expression. The target transactivating factors responsible for direct induction of AChR ε subunit expression in subsynaptic nuclei are unknown at present. However, in light of recent reports of Ets-binding proteins as targets of Ras-mediated signaling in *C. elegans*, *Drosophila*, and other mammalian systems, these highly conserved transcription factors are likely to play an important role (Wasylyk et al., 1993; Conrad et al., 1994).

Cross-talk between various signaling pathways involving protein phosphorylation is an emerging theme in intracellular communication (for review see Bourne, 1995; Hunter, 1995). Our findings indicate that transduction of the ARIA neurotrophic signal for induction of AChR ε subunit gene expression at the neuromuscular synapse requires the concomitant activation of PI3K and a Ras-mediated pathway. Specifically, inhibition of either pathway with either dominant-negative constructs or pharmacological inhibitors was sufficient to block expression of AChR ε subunit by ARIA. Moreover, even when one pathway was artificially turned on with activated constructs, inhibition of the second pathway with either dominant-negative constructs or pharmacological inhibitors blocked the ability of the activated pathway to induce expression of AChR ε subunit reporter construct. Since the specificity of the drugs is critical to the proposal that both pathways are necessary for induction of AChR ε subunit expression, it is important to note that although the PI3K inhibitor (LY294002) blocked AcRaf induction of AChR gene expression, it did not inhibit ARIA-induced MAPK activation in Sol 8 cells. Likewise, although the MEK inhibitor (PD 098059) blocked AcPI3K induction of AChR gene expression, we confirmed that it did not do so by inhibiting endogenous PI3K activation. Thus, although it may be argued that the lack of induction of AChR ε subunit was the result of either drug blocking the activity of some other protein(s), it seems unlikely that another protein equally sensitive to both LY294002 and PD 098059 is involved in induction of AChR subunit gene expression. Therefore, a more likely explanation for these results is that each pathway depends on the other for complete activation, since both are necessary and neither alone is sufficient for induction of AChR ε subunit gene. In addition, results with activated constructs and epistatic experiments suggested that there is cross-talk between the Ras and PI3K pathways. It was recently reported that the p85 regulatory subunit of PI3K can bind directly to the Ras adapter Grb2 (Wang et al., 1995). Therefore, activation of one pathway may result in subsequent activation of the other, generating the necessary downstream signals for full induction of AChR ε subunit expression in subsynaptic nuclei.

Alternatively, ARIA may activate separate Ras-dependent and PI3K-dependent signaling components, neither of which is able to generate all the necessary downstream signals to increase AChR ε subunit expression when the other is inhibited. Thus, a basal level of activity from each ARIA-induced signaling pathway may be required in a permissive manner for full activation of gene expression by the other ARIA-induced pathway. PI3K may work partly through Ras in this and other systems but may also provide a Ras-independent signal which alone is insufficient to mediate the ARIA signal for induction of AChR ε subunit gene. It should be noted that some PI3K-mediated actions have been shown to be Ras-dependent while others have not. In PC12 cells, NGF induces differentiation and survival via recruitment of PI3K but not Ras or MAPKs (Yao and Cooper, 1995). In contrast, PI3K requires Ras for c-fos transcription in fibroblasts and oocyte maturation in Xenopus (Hu et al., 1995) as well as for mediating the PDGF receptor mitogenic signal (Valius and Kazlauskas, 1993). In our system, the apparent Ras-dependent component of PI3K activity may account for some, but not all, of its actions in activation of AChR ε subunit expression.

Future studies may focus on defining how the synaptic pathways activated by ARIA are coordinately regulated with pathways elicited by electrical activity known to repress AChR gene expression. Both a cAMP-dependent (Chahine et al., 1993) and a protein kinase C–dependent pathway (Klarsfeld et al., 1989; Huang et al., 1994) have been implicated in mediating this repression. PKC may antagonize the action of ARIA by inhibiting the formation of activated ARIA receptor complexes (Altiok et al., 1995) or by inactivating transcription factors in the myogenic pathway necessary for AChR gene expression (Laufer and Changeux, 1989; Li et al., 1992). These and other alternatives remain to be explored.

In summary, regulation of synapse-specific gene expression by ARIA via the PI3K and the Ras/MAPK pathway further expands the range of developmentally regulated cellular processes shown to be critically determined by these signaling pathways. Moreover, it raises the possibility that similar signaling mechanisms may be involved in the development and differentiation of central synapses. Future investigations of signal transduction mechanisms involved in the development and maintenance of the neuromuscular junction may be aided by the molecular tools designed for these studies.

This paper is dedicated to John P. Merlie, who died on May 27, 1995.

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