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Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity

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Binding of heregulin (HRG) to its receptor, ErbB3, results in a dimerization with ErbB2/neu and activation of their intrinsic tyrosine kinases, initiating a cascade of events resulting in the stimulation of acetylcholine receptor (AChR) genes in muscle. Here we have examined the signalling downstream of the HRG receptor. We show that phosphatidylinositol 3′-kinase (PI3K) and SHC bind to the HRG-activated ErbB3 in myotubes. Subsequently, p70S6 kinase ($p70^{S6k}$), and MAP kinase ERK2 and thereby $p90^{rsk}$ are activated. However, inhibition of PI3K and $p70^{S6k}$ by wortmannin and rapamycin, respectively, failed to antagonize AChR α-subunit gene expression stimulated by HRG, despite the fact that the activities of the kinases were inhibited. In contrast, these inhibitors elevated AChR α-subunit mRNA levels, by themselves, independently of muscle electrical activity. On the other hand, the 17mer antisense oligonucleotide, EAS1, caused a specific depletion of ERK2 and eliminated the ability of HRG to stimulate AChR α-subunit gene expression. These results indicate that HRG stimulates expression of AChR genes via ERK2 activation, and provide a physiological example of neurotrophic factor-associated repression of AChR genes by stimulation of $p70^{S6k}$ activity which may contribute to the expression of adult type AChR genes at the neuromuscular junction.

Keywords: acetylcholine receptor/heregulin/membrane depolarization/mitogen-activated protein kinase/p70S6 kinase

Introduction

Heregulin (HRG) is a recently identified epidermal growth factor (EGF)-like growth factor which is homologous to neu differentiatiation factor, glial growth factor and acetylcholine receptor (AChR)-inducing activity (ARIA) (Holmes *et al.*, 1992; Falls *et al.*, 1993; Marchionni *et al.*, 1993). Members of the HRG family have been reported to be mitogenic for a variety of cell types, such as Schwann cells, fibroblasts (Marchionni *et al.*, 1993; Carraway *et al.*, 1995) and some human mammary carcinoma cell lines (Alimandi *et al.*, 1995). By contrast, HRG causes differentiation in some other breast cancer cell lines (Peles *et al.*, 1992). Recent evidence suggests that the most likely explanation for these differences in cellular responses to the HRGs might result from differences in the signalling pathways stimulated by HRG due to the involvement of different receptor dimers in different cells. ErbB3, ErbB4 and ErbB2/neu are members of the EGF receptor (EGFR) family with intrinsic tyrosine kinase activities that mediate the proliferation and differentiation of a variety of cells (Kraus *et al.*, 1989; Peles *et al.*, 1992; Plowman *et al.*, 1993). We and others have shown recently that ErbB3 is the receptor for HRGs in muscle and various other cells, with binding of HRG leading to tyrosine phosphorylation of both itself and ErbB2/neu via heterodimerization of the two receptors (Sliwkowski *et al.*, 1994; Alimandi *et al.*, 1995; Altiok *et al.*, 1995; Marikovsky *et al.*, 1995). Activation of receptor tyrosine kinases creates binding sites for intracellular signalling proteins containing src homology 2 (SH2) domains, including those with enzymatic activity such as phospholipase C-γ and phosphatidylinositol 3′-kinase (PI3K), and those with no enzymatic activity including *shc* gene products (SHC) and growth factor receptor-bound protein 2 (GRB2) (reviewed by Pawson, 1995). Those SH2-containing proteins which do not possess enzymatic activity act as adaptor molecules, linking other functional elements with the activated receptors. An example of this is the p85 protein which interacts with the p110 subunit of PI3K which possesses the enzymatic activity (reviewed by Kapeller and Cantley, 1994). Among the EGF family of receptors, only ErbB3 has the ability to couple to PI3K (Fedi *et al.*, 1994; Soltoff *et al.*, 1994).

Another example of an SH2-containing adaptor molecule, SHC, is phosphorylated by and associates with activated receptor tyrosine kinases such as EGFR, ErbB2/neu (Segatto *et al.*, 1993) and ErbB3 (Prigent and Gullick, 1994). Following phosphorylation, SHC interacts with the GRB2 and $p21^{ras}$ (Ras) signalling pathway (Rozakis-Adcock *et al.*, 1992). Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), constitute a family of protein serine/threonine (Ser/Thr) kinases that are stimulated by a variety of growth factors signalling via tyrosine kinase receptors that activate Ras. MAPKs regulate gene expression by transcription factor phosphorylation involved in a range of cellular processes (reviewed by Davis, 1993; Avruch *et al.*, 1994).

HRG stimulates AChR gene expression via activation of tyrosine phosphorylation of ErbB3 and ErbB2/neu in muscle cells (Altiok *et al.*, 1995; Jo *et al.*, 1995). ErbB2/neu and ErbB3 co-localize and accumulate densely at the neuromuscular junction, suggesting their involvement in synapse-specific expression of AChR genes by HRG (Altiok *et al.*, 1995; Moscoso *et al.*, 1995; Zhu *et al.*, 1995). The purpose of this study was to investigate further

the signal transduction pathway involved in the stimulation of AChR gene expression by HRG in muscle, and to examine whether an interplay exists between the HRG-stimulated pathway and the Ser/Thr kinase pathway stimulated by muscle electrical activity, known to repress AChR genes in the extrajunctional regions of the muscle fibre (reviewed by Duclert and Changeux, 1995).

Results

Association of ErbB3 with PI3K and SHC by HRG stimulation

To investigate the signalling pathway downstream of HRG-activated ErbB3, we examined binding patterns of signal transducing proteins to stimulated receptors in primary chick myotubes. ErbB3 possesses six putative tyrosine phosphorylation sites in its carboxy-terminal tail region that fit the YXXM motif known to mediate association with the SH2 domains of PI3K (Songyang *et al.*, 1994). Other members of the ErbB family do not contain this phosphorylation motif, suggesting that ErbB3 may be unique in its ability to associate directly with PI3K (Fedi *et al.*, 1994; Soltoff *et al.*, 1994). To test the involvement of PI3K in HRG signalling, immunoprecipitates prepared from HRG-stimulated and unstimulated cells using an antibody specifically reacting with the p85 subunit of PI3K were analysed for the association of ErbB3 by immunoblotting with anti-ErbB3 antibody. As shown in Figure 1A, p85 was co-immunoprecipitated with the HRG-activated ErbB3 receptor. p85 was also detected in the anti-ErbB3 immunoprecipitates from HRG-stimulated cells (not shown). The importance of tyrosine phosphorylation of ErbB3 in this event was shown by detection of ErbB3 in anti-p85 immunoprecipitates from HRG-stimulated cells, and to a greatly reduced extent from unstimulated cells, with anti-phosphotyrosine antibody (Figure 1A). However, p85 itself was not detected with anti-phosphotyrosine antibody, suggesting that it is not tyrosine phosphorylated to a significant extent after association with ErbB3.

The other element of the established SH2 signalling pathway is SHC, which is the major physiological substrate for a number of both receptor and non-receptor tyrosine kinases that have important roles in cellular growth and differentiation (Rozakis-Adcock *et al.*, 1992). To evaluate the association of SHC with activated ErbB3, SHC was immunoprecipitated with a specific antibody from HRG-stimulated and unstimulated cells and analysed by immunoblotting with anti-ErbB3 and anti-phosphotyrosine antibodies. Although the three forms of SHC (p66, p52 and p46) were detected in chick myotubes (not shown), only p52 was found associated with activated ErbB3 (Figure 1A). In contrast to the p85 subunit of PI3K, the p52 form of SHC was detected in the anti-phosphotyrosine immunoprecipitates from HRG-stimulated cells with anti-SHC antibody (Figure 1A), suggesting the tyrosine phosphorylation of p52 SHC by HRG stimulation. The tyrosine phosphorylation of the p52 form of SHC, stimulated by HRG, was also detected by the increase of ^{33}P incorporation into the anti-SHC immunoprecipitates from ^{33}P-labelled cells by autoradiography (not shown).

A number of reports have shown that GRB2 links the activated EGFR to the nucleotide exchange factor of Ras,

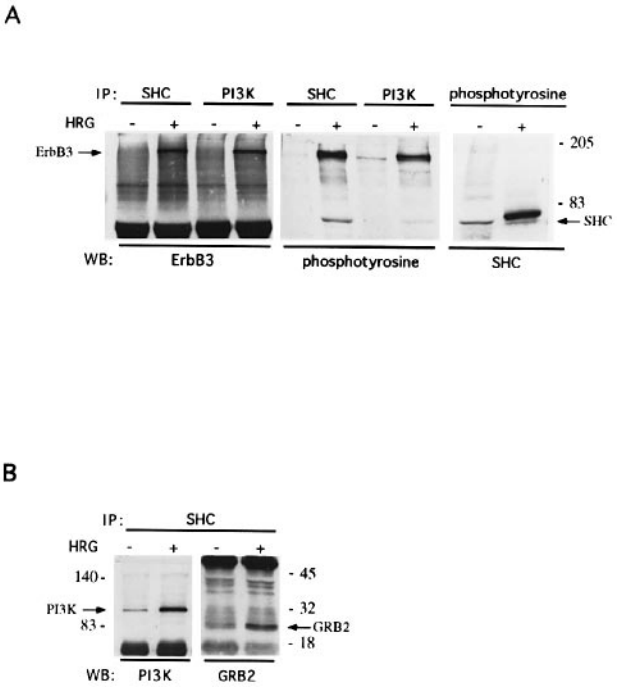

Fig. 1. HRG-induced association of the p85 subunit of PI3K and the p52 form of SHC with tyrosine-phosphorylated ErbB3. (**A**) At day 5 after plating, myotubes were stimulated with HRG (10 nM) (+) or vehicle (–) for 5 min. Cell lysates were prepared and immunoprecipitated (IP) with anti-SHC, anti-PI3K or anti-phosphotyrosine antibodies as indicated. Proteins present in the immune complexes were separated by 10% SDS–PAGE and blotted (WB) with anti-ErbB3, anti-phosphotyrosine or anti-SHC antibodies as indicated. (**B**) The anti-SHC precipitates (IP) were assayed for the presence of PI3K and GRB2 by 10 and 12% SDS–PAGE, respectively, and by blotting (WB) with anti-PI3K and anti-GRB2 antibodies. Molecular weight markers are shown on the left or right. IgG heavy chains are seen at the bottom or top of the blots.

the product of the *Drosophila Son of sevenless* gene (Sos), thereby localizing it close to the membrane (Buday and Downward, 1993; Chardin *et al.*, 1993). In chick myotubes, we could not detect tyrosine phosphorylation of GRB2 by HRG stimulation, but GRB2 was immunoprecipitated with anti-SHC antibody from HRG-stimulated cells (Figure 1B). This suggests that SHC associates with and is phosphorylated by activated ErbB3, resulting in its binding to the GRB2–Sos complex in myotubes, as has been described in other systems. In addition to GRB2, the p85 subunit of PI3K was also precipitated from HRG-stimulated cells with anti-SHC antibody (Figure 1B), which may further imply that both SHC and p85 are associated with ErbB3, and are thereby co-immunoprecipitated under non-denaturating conditions.

HRG activates MAP kinases and p90^{rsk}

To characterize the signal transduction pathway triggered by HRG to enhance AChR gene expression, we examined the contribution of the MAPKs, since HRG promoted association of SHC with ErbB3 in myotubes (Figure 1). MAPKs are a family of Ser/Thr kinases which are activated by both tyrosine and Ser/Thr phosphorylation after treatment of cells with mitogens and growth factors (reviewed by Davis, 1993; Avruch *et al.*, 1994). To determine directly whether MAPKs were activated by HRG treatment of myotubes, we assayed *in vivo* ^{33}P incorporation into

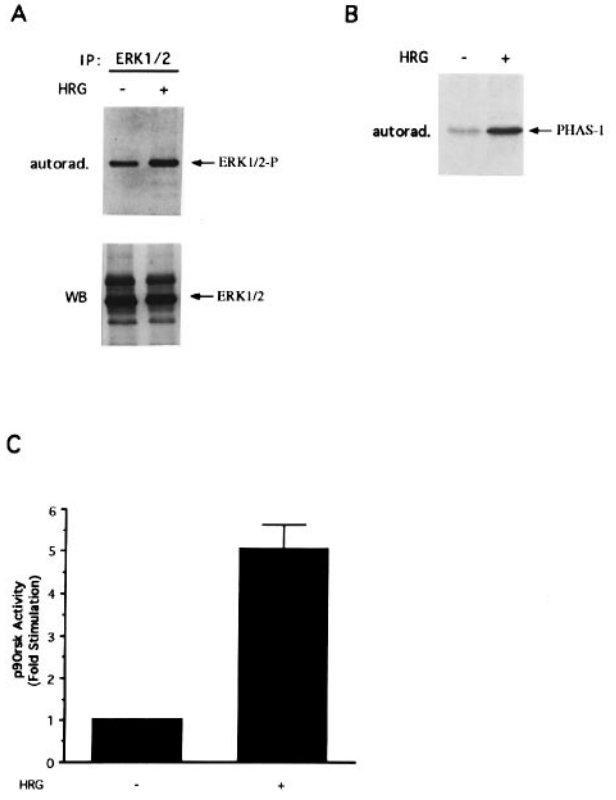

Fig. 2. HRG activates ERK1 and ERK2 (ERK1/2) and p90^{rsk} in myotubes. (**A**) At day 5 after plating, cells were biosynthetically labelled with [^{33}P]orthophosphoric acid for 3 h and either left untreated (–) or treated with HRG (10 nM) (+) for 10 min. The proteins in cell lysates were immunoprecipitated (IP) with anti-ERK1/2 and resolved by 10% SDS–PAGE, transferred to nitrocellulose and autoradiographed.The phosphorylated proteins are shown as ERK1/2-P in the autoradiographs. The nitrocellulose was then probed (WB) with anti-ERK1/2. IgG heavy chains are seen above the p44/42ERK1/2 bands in WB. (**B**) *In vitro* MAP kinase assay using PHAS-1 as an exogenous substrate in lysates of cells untreated (–) or treated with HRG (10 nM) (+) for 10 min, and (**C**) *in vitro* immune complex kinase assay using S6 peptide as an exogenous substrate in p90^{rsk} immunoprecipitates from cells untreated (–) or treated with HRG (10 nM) (+) for 10 min were carried out as described in Materials and methods. The results were converted to the ratio against the unstimulated value for the cells, and are the means of three separate experiments (means ± SD).

MAPKs which were immunoprecipitated from ^{33}P-labelled cells with an antibody which recognizes both ERK1 and ERK2 (ERK1/2). The amount of ^{33}P incorporated into the ERK1/2 was enhanced rapidly by HRG treatment, with a maximal increase at 10 min (Figure 2A, top panel). The same membrane was probed with the same antibody to ensure that equal amounts of ERK1/2 were immunoprecipitated from untreated and treated cells (Figure 2A, bottom panel). The activation of MAPKs by HRG was also examined in an *in vitro* kinase assay (Figure 2B). The treatment of myotubes with HRG increased the phosphorylation of the exogenous substrate PHAS-1 by MAPKs, with maximal stimulation in 5 min, which remained elevated during 60 min. This was consistent with the translocation of MAPKs to the nucleus within 45 min after HRG stimulation in immunocytochemical staining of myotubes (data not shown).

The ERK2 form of MAPKs phosphorylates and activates Ser/Thr kinase, the 90 kDa ribosomal S6 kinase (p90^{rsk}), which translocates to the nucleus where it phosphorylates numerous transcription factors (Rivera *et al.*, 1993; Ginty *et al.*, 1994). Since ERK2 is the major MAPK form that we detected in chick myotubes, we investigated the involvement of p90^{rsk} in the HRG signalling pathway. HRG stimulated p90^{rsk} activity by 5-fold in an *in vitro* immune complex kinase assay (Figure 2C).

HRG activates p70S6 kinase, which is inhibited by wortmannin and rapamycin

After having demonstrated the HRG induced association of the p85 subunit of PI3K with tyrosine-phosphorylated ErbB3 (Figure 1), we further investigated the role of downstream signalling elements of this pathway. p70S6 kinase (p70^{S6k}) is the major target of PI3K in several systems and it is activated by multisite serine phosphorylation (Chung *et al.*, 1994). In fact, we detected a retarded migration of the highly phosphorylated forms of p70^{S6k} immunoprecipitated from ^{33}P-labelled cells by HRG treatment on SDS–PAGE and subsequent immunoblotting with anti-p70^{S6k} antibody (Figure 3A, bottom panel). Autoradiography of the same membrane also showed an enhanced ^{33}P incorporation into p70^{S6k} by HRG stimulation (Figure 3A, top panel). Incubation of the cells with the specific inhibitors of PI3K, wortmannin (Ui *et al.*, 1995), and p70^{S6k}, rapamycin (Chung *et al.*, 1992), 1 h prior to HRG stimulation for 10 min, eliminated HRG-induced activation of p70^{S6k}. p70^{S6k} was also activated by addition of horse serum and embryonic extract (Figure 3A), and only in the absence of these factors was the activation of the kinase by HRG detectable.

The effect of HRG on the kinase activity of p70^{S6k} was also determined by using S6 peptide as an exogenous substrate in an *in vitro* kinase assay of immunoprecipitated p70^{S6k} from myotubes. Similarly to the enhancement of ^{33}P content of the kinase *in situ* as shown in Figure 3A, HRG treatment led to an ~8-fold increase in S6 peptide phosphorylation, which was abolished by both wortmannin and rapamycin treatments (Figure 3B). It is noteworthy that the stimulatory effect of HRG on ERK1/2 and p90^{rsk} activities was not altered by wortmannin or rapamycin treatment (not shown), thus excluding the cross-talk between the Ras/MAPK and PI3K/p70^{S6k} pathways.

Inhibition of p70^{S6k} does not block the activation of AChR α-subunit gene expression by HRG, but increases basal gene expression

We have shown previously that HRG treatment strongly increases AChR α-subunit mRNA levels in chick myotubes (Altiok *et al.*, 1995). Here we show that although HRG activated p70^{S6k}, inhibition of the kinase by rapamycin or by the PI3K inhibitor wortmannin did not block the ability of HRG to increase the expression of the α-subunit gene (Figure 4). In contrast, these inhibitors enhanced the basal levels of α-subunit mRNA. The ability of rapamycin to increase α-subunit mRNA levels was higher than that of wortmannin (Figure 4) although, as shown in Figure 3, both inhibitors blocked the HRG-stimulated p70^{S6k} activity with the same efficiency. These findings suggest that other factors may also activate p70^{S6k}, and its basal and possibly HRG-stimulated activity represses AChR α-subunit gene expression in myotubes.

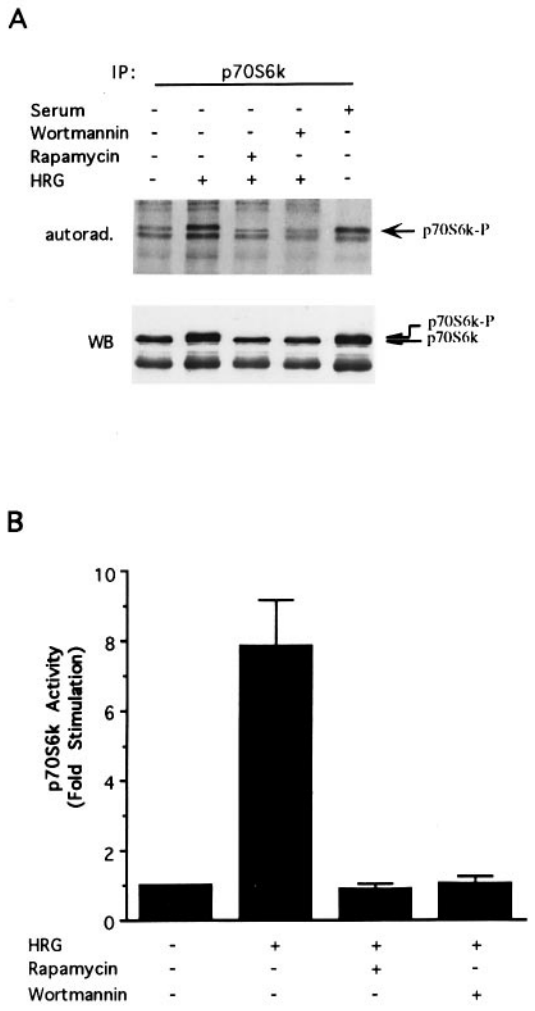

Fig. 3. HRG-activated $p70^{S6k}$ is inhibited by wortmannin and rapamycin. (**A**) At day 5 after plating, myotubes were incubated with wortmannin (0.1 μM), rapamycin (0.1 μM) or vehicle as indicated for 1 h, then labelled with [^{33}P]orthophosphoric acid for 3 h and either left untreated or treated with HRG (10 nM) or horse serum (4%) as indicated for 10 min. $p70^{S6k}$ was immunoprecipitated (IP) from the lysates and resolved by 10% SDS–PAGE, transferred to nitrocellulose and autoradiographed. The same membrane was probed (WB) with anti-$p70^{S6k}$. The slower migrating phosphorylated kinase is shown as p70S6k-P. IgG heavy chains are seen below the $p70^{S6k}$ bands in WB. (**B**) Cells were treated as described above, and *in vitro* immune complex kinase assays using S6 peptide as an exogenous substrate in $p70^{S6k}$ immunoprecipitates were performed as described in Figure 2C. –, absence; +, presence of drug.

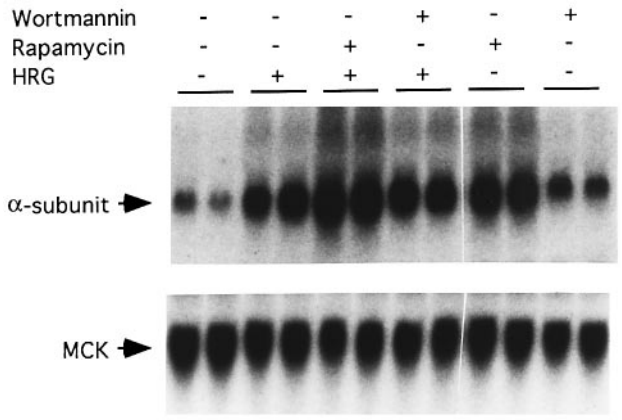

Fig. 4. Effect of wortmannin and rapamycin on basal and HRG-stimulated AChR α-subunit mRNA levels. Autoradiograms of Northern blot hybridization analysis of mRNA in chick myotubes treated for 30 min with wortmannin (0.1 μM) or rapamycin (0.1 μM) before addition of HRG (3 nM) as indicated. Cells were treated between days 4 and 7 after plating, and total RNA was extracted. AChR α-subunit and muscle creatine phosphokinase (MCK) mRNAs were analysed using their cRNA probes.The same blot was used for hybridization with both probes. The arrows show the position of AChR α-subunit and MCK mature mRNAs. –, absence; +, presence of drug.

It is important to note that fused and differentiated myotubes were treated in horse serum- and embryonic extract-deficient medium for the kinase assays as shown in Figure 3, whereas treatments for analysis of the mRNAs had been done in media containing these factors. This suggests that the observed enhancing effects of wortmannin and rapamycin on basal α-subunit mRNA levels are due to the elimination of a repression of α-subunit gene expression by these factors. Rapamycin and wortmannin treatments did not induce any morphological change in myotubes. None of these treatments modulated the levels of MCK (muscle creatine kinase) mRNA in these assays, showing the specificity of the treatments for α-subunit.

MAP kinase antisense oligonucleotide EAS1 depletes MAP kinase ERK2 and prevents the HRG-stimulated increase in AChR α-subunit gene expression

To test directly in a physiological system whether a MAP kinase mediates the signalling pathway by which HRG stimulates AChR α-subunit gene expression, we used an oligonucleotide-based antisense strategy for depleting MAP kinases. The phosphorothioate 17mer oligonucleotides with the sequence 5′-GCC GCC GCC GCC GCC AT-3′, referred to as antisense EAS1, and 5′-CGC GCG CTC GCG CAC CC-3′ (scrambled) as a control were used as described (Sale *et al.*, 1995). The antisense target region on MAP kinases ERK1 and ERK2 is unique to these kinases when compared with cDNA sequences from all other known proteins (Charest *et al.*, 1993). The first six amino acid residues (MAAAAA) originating with the putative initiating methionyl residue of human ERK1 are identical to the N-terminal sequences of the MAPK isoforms from mice, rats and *Xenopus* species (Charest *et al.*, 1993). Exposure of chick myoblasts to 5 μM antisense oligonucleotide EAS1 caused a depletion of the major MAPK isoform, ERK2, expressed in these cells over 3 days as determined by immunoblotting (Figure 5A, top panel). Incubation of the cells with 5 μM scrambled oligonucleotide did not affect the expression of ERK1 and ERK2 (Figure 5A, top panel), indicating that antisense EAS1 was acting specifically in depleting ERK2. This was supported further by the lack of effect of antisense EAS1 on expression of other signalling proteins involved in HRG action, such as $p70^{S6k}$ (Figure 5A, bottom panel) and ErbB3 (not shown).

The depletion of ERK2 by EAS1 treatment in myotubes as described above caused a nearly total inhibition of α-subunit mRNA expression stimulated by HRG (Figure 5B). Treatment with scrambled oligonucleotide had no effect on the expression of α-subunit mRNA stimulated by HRG (Figure 5B), indicating that the oligonucleotide-

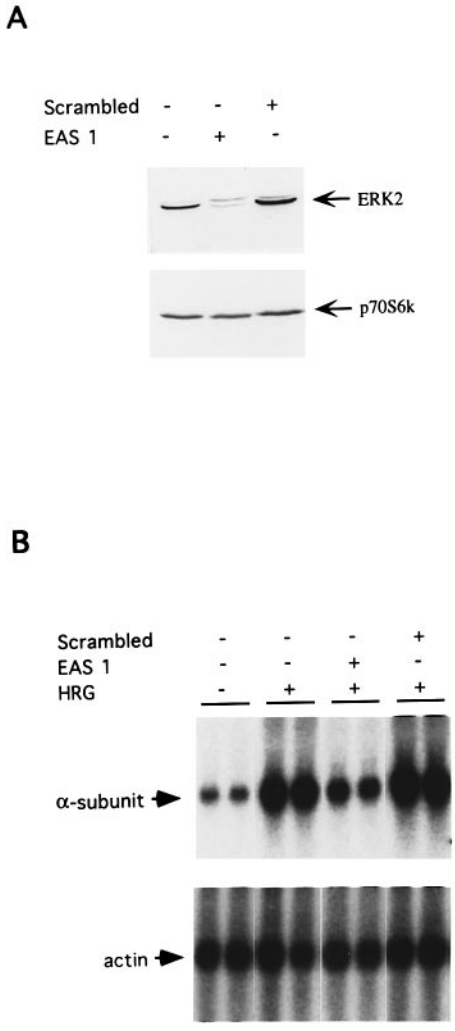

Fig. 5. Antisense EAS1 inhibited expression of ERK2 and HRG-stimulated AChR α-subunit mRNA. At day 1 after plating, myoblasts were exposed to antisense EAS1 (5 μM) or scrambled (5 μM) oligonucleotides as indicated, for 3 days as described in Materials and methods. (**A**) At day 4, lysates from myotubes were separated by 10% SDS–PAGE and blotted with anti-ERK1/2 and as an internal control with anti-$p70^{S6k}$ antibodies. (**B**) Myotubes were treated with HRG (3 nM) between days 4 and 5 and total RNA was extracted. AChR α-subunit and actin mRNAs were analysed using their cRNA probes. The same blot was used for hybridization with both probes. The arrows show the position of the AChR α-subunit and sarcomeric α-actin mature mRNAs. –, absence; +, presence of treatment.

mediated inhibition of the HRG effect was specific for antisense EAS1 and was not mediated by the control scrambled oligonucleotide. Treatment of chick myoblasts with EAS1 did not affect the ability of myoblasts to fuse and differentiate into myotubes. However, basal expression of the MCK gene, which is regulated by differentiation, was also slightly decreased by EAS1 treatment. Therefore, we measured the expression of actin mRNA in the same blots as an internal control. Actin mRNA levels were not affected by EAS treatment (Figure 5B). In EAS1-treated cells, HRG-induced $p90^{rsk}$ and MAPK activities were decreased by 70% in *in vitro* kinase assays (data not shown).

$p70^{S6k}$-mediated inhibition of AChR gene expression is independent of electrical activity-mediated repression of the AChR gene

In myotube cultures, the spontaneous or stimulated electrical activity of the myotubes represses AChR biosynthesis, whereas treatment of myotubes with the Na^+ channel blocker tetrodotoxin (TTX) or with Ca^{2+} channel blockers increases α-subunit mRNA levels by blocking Ca^{2+} entry from L-type channels leading to activation of a Ser/Thr kinase (Klarsfeld *et al.*, 1989; Laufer *et al.*, 1991; Huang *et al.*, 1994) which has not yet been identified. Since inhibition of Ser/Thr kinase $p70^{S6k}$ by rapamycin strongly increased AChR α-subunit mRNA levels in spontaneously contracting myotubes, we tested whether the activity of this kinase is regulated by electrical activity. However, blocking the spontaneous activity of myotubes by TTX treatment did not alter the basal or HRG-stimulated *in vivo* phosphorylation and *in vitro* kinase activity of the enzyme (not shown). Similarly, basal and HRG-stimulated phosphorylation of ERK1/2 was unaffected by TTX treatment (not shown). These findings indicate that HRG and electrical activity trigger different kinase cascades to repress AChR gene expression.

In order to investigate further the relationship between the Ser/Thr kinase involved in electrical activity-linked repression of the AChR gene and $p70^{S6k}$ activity, we used okadaic acid, a specific inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), which has been shown to block the differentiation of muscle cells by extinguishing expression of myogenic factors, myoD1 and myogenin (Kim *et al.*, 1992). Okadaic acid is a tumour promoter and has no direct effect on the protein kinases tested to date (Cohen *et al.*, 1990). However, the inhibition of protein phosphatases potentiates the activity of protein kinases constitutively present in the cell and results in enhanced phosphorylation of their substrates. Okadaic acid, at 2.5 nM concentration which inhibits only PP2A, blocked the increase of AChR protein and α-subunit mRNA levels by TTX (Figure 6A and B). The $p70^{S6k}$ inhibitor rapamycin was still able to increase surface AChR and α-subunit mRNA levels in the presence of okadaic acid; however, it did not eliminate its inhibitory effect while the broad spectrum kinase inhibitor staurosporine completely reversed this inhibition (Figure 6A and B). Consistent with this finding, okadaic acid or staurosporine did not alter the *in vivo* phosphorylation or activity of $p70^{S6k}$ in *in vitro* kinase assays (not shown). In addition, TTX and rapamycin additively increased the number of surface AChRs (Figure 6A). These results indicate that $p70^{S6k}$ and the Ser/Thr kinase activated by electrical activity inhibit α-subunit gene expression via different mechanisms in muscle (Figure 7).

Discussion

Here we have demonstrated that HRG-stimulated tyrosine phosphorylation of ErbB3 led to recruitment of the p52 form of SHC and PI3K and thereby activated the MAPKs, ERK1/2 and $p70^{S6k}$ in chick myotubes. ErbB3, the major receptor of HRGs in a variety of cell types, appears to cooperate with ErbB2/neu by simultaneous recruitment of complementary signal transduction pathways. Chick myotubes express three forms of SHC protein; however,

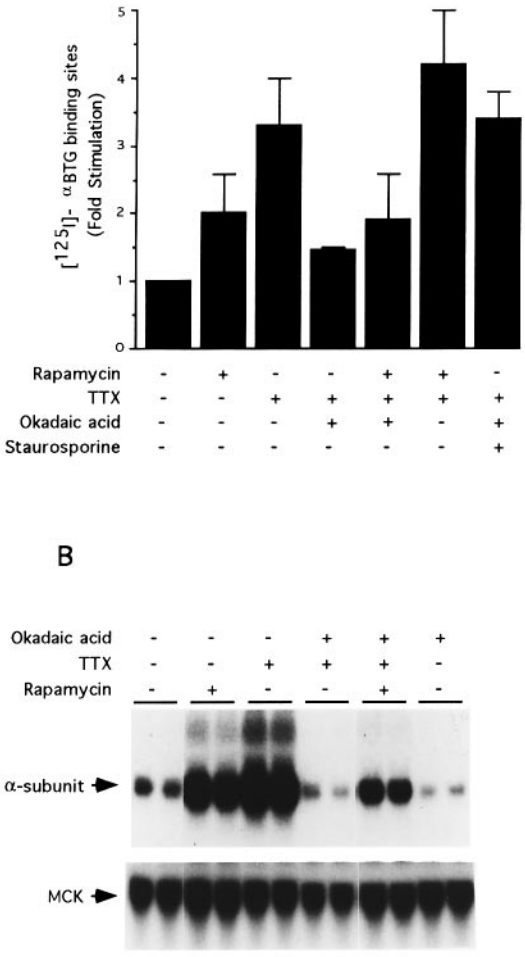

Fig. 6. Rapamycin increases (**A**) surface ^{125}I-labelled α-BTG (bungarotoxin) binding sites and (**B**) α-subunit mRNA levels independently of muscle electrical activity. At day 5 after plating, myotubes were treated with rapamycin (0.1 μM) and staurosporine (2.5 nM) 30 min before addition of tetradotoxin (TTX, 1 μM) and okadaic acid (2.5 nM) as indicated, then further incubated for 2 days. Surface ^{125}I-labelled α-BTG binding sites were quantitated as described in Materials and methods. The results are expressed as fold increase over the untreated value of cells, and are the means of six separate experiments (means ± SD). AChR α-subunit and MCK mRNAs were analysed as described in Figure 4. The arrows show the position of AChR α-subunit and MCK mature mRNAs. –, absence; +, presence of drug.

only the p52 form of SHC was significantly tyrosine phosphorylated by activated HRG receptors. While the p85 subunit of PI3K only binds to ErbB3, SHC may bind to both ErbB3 and ErbB2/neu in a cooperative manner since both receptors have SHC binding sites in their carboxy-terminal domains (Segatto *et al.*, 1993; Prigent *et al.*, 1994). Multiple transducing signals converge downstream of SHC phosphorylation at the MAPKs (reviewed by Davis, 1993; Avruch *et al.*, 1994). We show here that specific depletion of ERK2 by the antisense oligonucleotide EAS1 completely blocked the HRG-stimulated AChR α-subunit gene expression in chick myotubes. The oligonucleotide sequence of ERK1/2 in the antisense EAS1 probe target region is conserved in mouse, rat and human (Sale *et al.*, 1995), indicating that antisense EAS1 should be generally applicable in depleting ERK1/2 from cells of a wide range of species. In fact, in chick myotube cultures, EAS1 efficiently depleted ERK2 which is the major MAPK isoform in these cells.

One of the signalling proteins whose recruitment is increased by the ErbB3 and ErbB2/neu heterodimer is PI3K in mammary carcinoma cells and fibroblasts transfected with both receptors (Alimandi *et al.*, 1995; Carraway *et al.*, 1995; Marikovsky *et al.*, 1995). It has been postulated that PI3K is an upstream mediator of p70^{S6k} in some cells since the specific PI3K inhibitor wortmannin causes a parallel inhibition of both kinases in several cell types (Chung *et al.*, 1994). In our study, although wortmannin (Ui *et al.*, 1995) and the specific p70^{S6k} inhibitor, rapamycin (Chung *et al.*, 1992), both inhibited the activation of p70^{S6k}, they did not block the ability of HRG to stimulate AChR α-subunit gene expression. By contrast, both inhibitors increased the steady-state levels of AChR α-subunit mRNA in myotube cultures. The implications of these findings are: (i) even though the PI3K/p70^{S6k} pathway is activated by HRG, the stimulatory effect of HRG on AChR gene expression is not mediated by these kinases; and (ii) the endogenous activities of PI3K and, more markedly, p70^{S6k} have tonic inhibition on the AChR α-subunit gene. It is possible that growth factors present in horse serum and embryonic extract may contribute to the basal activation of these kinases. However, rapamycin has a more profound effect on increasing basal α-subunit mRNA levels than wortmannin, suggesting that p70^{S6k} might also be activated via other pathways which are also blocked by rapamycin. Indeed, it has been reported that wortmannin and rapamycin block p70^{S6k} activation by inhibiting different inputs (for review, see Proud, 1996). For example, rapamycin antagonizes the G_1–S cell cycle transition or blocks translation initiation via both p70^{S6k}-dependent and -independent pathways (Chung *et al.*, 1994; Proud, 1996); however, these mechanisms are not likely to occur in these terminally differentiated myotubes where rapamycin selectively increased AChR synthesis and α-subunit mRNA levels.

Recently it has been reported that HRG activates both MAPK and p70^{S6k} irrespective of whether it stimulates proliferation or differentiation of mammary epithelial cells (Marte *et al.*, 1995). In a very recent publication, it was reported that besides MAPKs, PI3K was also involved in the stimulatory effect of HRG on AChR ε-subunit gene expression in the Sol 8 mouse muscle cell line (Tansey *et al.*, 1996). However, our study demonstrates that the HRG-activated Ras/MAPK pathway is involved in the stimulation of AChR α-subunit gene expression, whereas activation of PI3K/p70^{S6k} inhibits AChR α-subunit gene expression in chick myotubes. The biological significance of the opposing effects of two parallel pathways stimulated by HRG is presently unknown. One possible interpretation of these observations is that p70^{S6k} activation by HRG may suppress fetal-type AChR gene expression in the synaptic region of myofibres independently of the evoked electrical activity. Concordant with our findings, local neurotrophic repression of gene transcripts encoding fetal AChRs at rat neuromuscular synapses has been reported recently (Kues *et al.*, 1995). Indeed, blocking the spontaneous electrical activity of myotubes by TTX treatment did not alter the activity of p70^{S6k}. In addition, the p70^{S6k} inhibitor rapamycin did not eliminate the repression of AChR α-subunit gene expression by okadaic acid, which

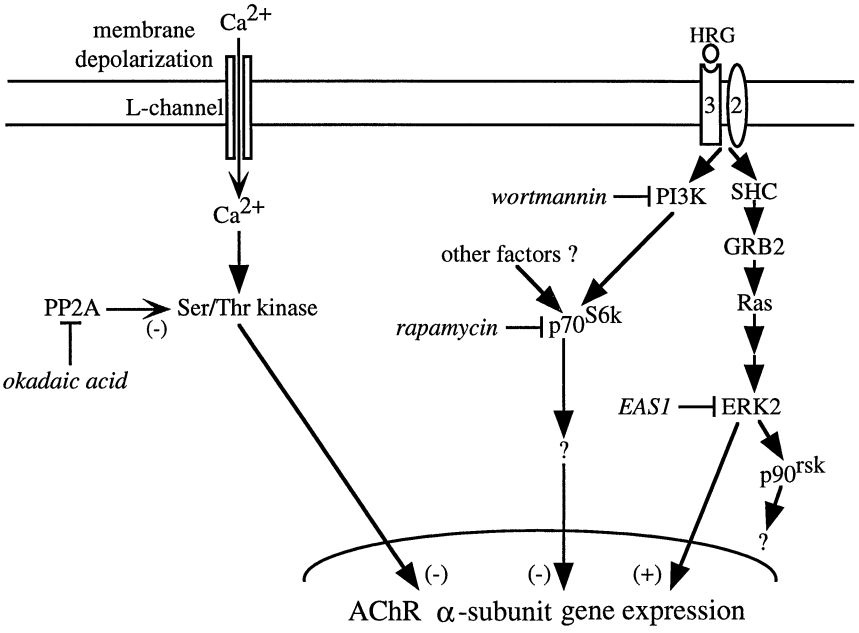

Fig. 7. Schematic representation of parallel kinase cascades triggered by HRG and membrane depolarization which regulate AChR α-subunit gene expression in chick myotubes. HRG binds to ErbB3 (3) and induces its dimerization with ErbB2/neu (2). Tyrosine phosphorylation of the two proteins recruits PI3K and SHC molecules, and thereby activates $p70^{S6k}$, and the MAPK, ERK2, respectively. While activation of $p70^{S6k}$ by HRG and possibly by other factors inhibits, activation of ERK2 stimulates AChR gene expression. MAPK antisense oligonucleotide EAS1 treatment abolishes AChR α-subunit gene expression. Wortmannin and rapamycin block the inhibition of AChR gene expression by $p70^{S6k}$ activation. In a parallel independent pathway, an increase in intracellular Ca^{2+} concentration caused by membrane depolarization activates a Ser/Thr kinase to repress AChR genes in myotube nuclei. This Ser/Thr kinase is susceptible to inhibition by protein phosphatase 2A (PP2A) which is blocked by okadaic acid.

potentiates the inhibitory effect of the Ser/Thr kinase activated by muscle electrical activity, suggesting that these two pathways are separate. We have demonstrated previously that phorbol ester treatment inhibited the HRG-dependent tyrosine phosphorylation of both ErbB3 and ErbB2/neu, suggesting that Ca^{2+}-dependent protein kinase Cs (PKCs) are able to interfere with the initial step of the stimulatory pathway triggered by HRG (Altiok *et al.*, 1995). Here we show that, even though a Ca^{2+}-dependent PKC isoform may be activated by muscle electrical activity to block ErbB2/neu, it is different from the Ser/Thr kinase which represses AChR genes in the myotube nuclei.

In conclusion, our results indicate that HRG stimulates AChR α-subunit mRNA expression via activation of ERK2, and suggest that ErbB3 may have an important role in the regulation of AChR gene expression through activation of the PI3K/$p70^{S6k}$ pathway, which consequently may induce sustained expression of adult type AChR genes in the synaptic nuclei of adult muscle.

Materials and methods

Cell culture

Myoblasts were obtained from the hindlimbs of 11-day-old chick embryos by mechanical dissociation and were seeded at a density of 2×10^4 cells/mm^2 in gelatin-coated, 100 mm plastic dishes and grown in medium containing 4% horse serum and 2% embryonic extract, as described previously (Klarsfeld *et al.*, 1989).

Chemicals

[^{33}P]Orthophosphoric acid, [γ-^{33}P]ATP and [^{32}P]UTP were from Amersham. The EGF domain of recombinant heregulin (rHRG-β$1_{177–244}$) (Holmes *et al.*, 1992) was provided by Dr Mark Sliwkowski (Genentech Inc., San Francisco, CA). FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan. GF 109203X was kindly provided by Dr Jorge Kirilovsky (Glaxo, France). Wortmannin, rapamycin and HA 1004 were from Biomol Research Lab, USA. Staurosporine was from Research Biochemicals, USA. Okadaic acid was from Upstate Biotechnology Inc., Lake Placid, NY. All the other chemicals were from Sigma Chemical Co., St Louis, MO.

Antibodies

Rabbit polyclonal antibodies against peptides corresponding to amino acids 1307–1323 of ErbB3 (sc-285), 305–327 of ERK1(sc-94), 485–502 of $p70^{S6k}$ (sc-230), 195–217 of GRB2 (sc-255) and 508–525 of $p90^{rsk}$ were from Santa Cruz Biotechnology, Inc., USA. Mouse monoclonal anti-phosphotyrosine antibody, 4G10, and rabbit polyclonal antibodies against the p85 subunit of PI3K (catalogue No. 06 195), amino acids 366–473 of SHC (catalogue No. 06-203) and amino acids 63–98 of ERK1 (06-183) were obtained from Upstate Biotechnology Inc. (UBI), Lake Placid, NY.

Oligonucleotide treatment of cells

Phosphorothioate oligonucleotides were synthesized and purified by Genset, Paris. At day 1 after plating, myoblasts (80% confluent in 60 mm dishes) were washed with minimal essential medium (MEM). Dilutions of oligonucleotide in 100 μl of MEM were pre-incubated at room temperature for 30 min with 100 μl of MEM containing 20 μg/ml lipofectamine (Gibco). This mixture was added to the cells together with a further 1.4 ml of MEM. Cells were incubated for 2 h at 37°C in the presence of 5% CO_2. Then the medium was removed and the incubation continued for the times indicated in the figure legends using 4 ml of growth medium containing the indicated oligonucleotide concentrations in the presence of 10% horse serum. The medium was replaced every 24 h with new medium containing the indicated oligonucleotide concentrations and other additions.

In vivo biosynthetic labelling

Myotubes were washed with serum and phosphate-free MEM (Sigma), then treated with inhibitors as described in the figure legends and incubated with 250 μCi/100 mm dish of [^{33}P]orthophosphoric acid (3000 Ci/mmol, Amersham) for 3 h in 4 ml of the same medium. Cell lysis and immunoprecipitation were carried out as described below.

Cell lysis and immunoprecipitation

After treatment with the drugs, as described in the figure legends, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin. Insoluble material was removed by centrifugation at 13 000 *g* for 20 min at 4°C. Proteins were precipitated with the antibodies, as indicated, and protein G plus protein A–agarose (Santa Cruz Biotechnology, Inc.). Immunoprecipitation with anti-ERK1 antibody (sc-94) was carried out under denaturating conditions. Immune complexes were washed four times with lysis buffer.

Western blot analysis

An equivalent volume of 2× SDS sample buffer was added to immune complexes and boiled for 5 min. The supernatants were subjected to electrophoresis on SDS–PAGE gels as indicated and transferred to nitrocellulose using a Bio-Rad apparatus. Membranes were blocked for 1 h at room temperature in PBS containing 3% non-fat dried milk. Protein blots were probed overnight at 4°C with primary antibodies. Blots were analysed by using goat anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase at a 1:3000 dilution in blocking solution for 1 h, followed by BCIP/NBT staining. The blots with labelled phosphoproteins were exposed to autoradiography and quantified by phosphoimager analysis.

Kinase assays

The immunoprecipitates of $p90^{rsk}$ and $p70^{S6k}$ from drug-treated and untreated cells in serum and embryonic extract-free medium were resuspended in kinase buffer (20 mM MOPS, pH 7.4, 5 mM EGTA, 25 mM $MgCl_2$, 1 mM sodium vanadate, 1 mM dithiothreitol). The immune complexes were then incubated for 10 min at 30°C with 40 μl of kinase buffer containing 100 μM ATP, 200 μCi/ml of [γ-^{33}P]ATP (>1000 Ci/mmol, Amersham), 4 μM PKC inhibitor peptide, 0.5 μM PKA inhibitor peptide, 5 μM Compound R24571 and S6 peptide (AKRRRLSSLRA) (UBI). The reactions were stopped by rapid centrifugation, and the aliquots were spotted on squares of P-81 paper, washed and counted as described (Ahn *et al.*, 1990). MAPK activity was assayed in cell extracts by using PHAS-1 as substrate (Stratagene, USA) in the presence of 4 μM PKC inhibitor peptide, 0.5 μM PKA inhibitor peptide and 5 μM Compound R24571.

Northern blot analysis

After treatment of chick myotube cultures in growth medium, RNA was purified. The average yield was 100 μg of total RNA per 100 mm dish. Northern blots were made with 15 μg of total RNA in each lane. Blots were hybridized with a riboprobe synthesized from a pGEM3 vector (Promega, Madison, WI) containing a 2.3 kb fragment of the AChR α-subunit cDNA which contained exons 2–6. [^{32}P]UTP (800 Ci/mmol, Amersham) was used as the label. The same blots were rehybridized with an MCK- or mouse actin-specific RNA probe, as described previously (Klarsfeld and Changeux, 1985; Laufer *et al.*, 1991).

Quantification of surface AChRs

Surface AChR levels were measured by ^{125}I-labelled α-bungarotoxin (α-BTG; ≈200 Ci/mmol, Amersham) binding to 35 mm myotube cultures as previously described (Klarsfeld *et al.*, 1989).

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