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Interaction and Inhibitory Cross-Talk between Endothelin and ErbB Receptors in the Adult Heart

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Abbreviations
Endothelin-1 (ET-1), ET_A (endothelin receptor type A), ET_B (endothelin receptor type B), EGF (epidermal growth factor), EGFR (epidermal growth factor receptor), Gαq (G protein α subunit q), G-protein coupled receptor (GPCR), neuregulin (NRG), PMA (phorbol 12-myristate-13-acetate), phospholipase C (PLC), phosphoinositide-3 kinase (PI3-kinase), protein kinase C (PKC), receptor tyrosine kinase (RTK), transverse tubule (T-tubule), *bis*-indoylmaleimide (Bim), immunoprecipitate (IP), immunoblot (IB), immunoglobulin (IgG), extracellular regulated kinases (Erk1/2), matrix assisted laser desorption ionization-time of flight (MALDI-TOF), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

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Abstract. Endothelin-1 (ET-1) regulates contractility and growth of the mammalian heart by binding ET_A and ET_B G-protein coupled receptors (GPCRs). To identify growth signaling pathways associated with ET-1 receptors in adult myocardium, a combined immunoprecipitation/proteomic analysis was performed. Signaling proteins thought to function downstream of ET_A such as Gαq, PLC-β1, PKCε and PKCδ were identified in immunoprecipitates of ET_A by MALDI-TOF mass spectrometry. Also prominent were the growth factor receptor tyrosine kinases erbB2 and erbB4, as well as their downstream growth signaling effectors PI3-kinase, Akt, Raf-1, MEK and Erk. Western blot analysis confirmed co-immunoprecipitation of erbB2/4, PI3-kinase, and Akt with ET_A, and confocal microscopy revealed their co-localization in cardiac transverse tubules (T-tubules). The erbB4 receptor ligand neuregulin-1β (NRG1β) promoted erbB2/4 tryosine phosphorylation and Akt serine phosphorylation in ventricular myocytes, whereas treatment with ET-1 did not. This observation argues against ET-1 growth signaling occurring via erbB2/4 transactivation in adult myocardium. ET-1 did however stimulate Erk1/2 phosphorylation and substantially blunted several NRG1β mediated actions including erbB2/4 phosphorylation, serine phosphorylation of Akt, and negative inotropy. This inhibitory cross-talk between ET_A and erbB2/4-Akt pathways was mimicked by a phorbol ester and blocked by pharmacological inhibition of PKC or MEK/Erk. The proteomic analysis and subsequent investigation of receptor cross-talk indicate that growth signaling between ET_A and erbB pathways is fundamentally different in adult versus neonatal cardiac myocytes. The results may be relevant to cardiomyopathies associated with i) prolonged exposure to ET-1, ii) degeneration of T-tubules, and iii) therapies targeted at erbB2 inhibition.

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INTRODUCTION

Endothelin-1 (ET-1) is a powerful growth factor in the developing heart that may also play a central role in triggering hypertrophy of the adult heart in response to mechanical stress (Goraca, 2002). ET-1 functions through G-protein coupled receptors (GPCRs) designated ET_A and ET_B (Sugden, 2003). The growth promoting effects of GPCR activation via ET-1 and angiotensin II are thought to be due in large part to transactivation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptors (EGFR) (Daub et al., 1996; Smith et al., 2004). The phenomenon of transactivation involves ligand binding and activation of a GPCR which promotes subsequent activation of an RTK via extracellular mobilization of matrix-bound growth factor, intracellular cross-talk, or both (Waters et al., 2004; Shah and Catt, 2004; Prenzel et al., 1999).

In heart tissue, transactivation of EGFR (erbB1) by ET-1 has been shown to occur in neonatal rat cardiac myocytes (Anderson et al., 2004; Asakura et al., 2002). Much of our current understanding of cross-talk between GPCRs and RTKs is based upon work in embryonic/neonatal tissues or immortalized cell lines. Very little is known about cross-talk between these receptor classes in terminally differentiated tissues such as adult ventricular myocytes. To address this gap in understanding, we performed a proteomic analysis to identify proteins stably associated with ET receptors in adult myocytes following detergent solubilization and immunoprecipitation. The erbB2/4, PI3kinase, Akt signaling system was found to be closely associated with the ET_A signaling system under these conditions. To address whether these proteins interact under more physiological conditions, we used isolated adult ventricular myocytes to examine

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subcellular localization of these signaling components and to investigate possible cross-talk between ET_A and erbB2/4 signaling pathways. The results reveal heretofore unexpected inhibitory cross-talk between ET_A and erbB2/4 in adult myocytes that differs from neonatal myocytes, and that could have relevance in cardiac pathology.

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MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma Chemincal Co. (St. Louis, MO) unless noted otherwise. Collagenase was from Worthington (Lakewood, NJ). Complete protease inhibitor cocktail, trypsin and Glu-C were from Roche (Mannheim, Germany). ET_A monoclonal antibody was from Transduction Laboratories (Lexington, KY), phosphotyrosine-specific antibody was from Upstate (Lake Placid, NY), Alexa 488 or 568-conjugated secondary antibodies were from Molecular Probes (Eugene, OR), and other primary and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein G Sepharose^{TM} 4 Fast Flow and NHS (N-hydroxysuccinimide)-activated Sepharose 4 Fast Flow were obtained from AmershamBioscience (Uppsala, Sweden). BQ123, BQ788, *bis*-indoylmaleimide and PMA were from Sigma Chemical Co (St Louis, MO). U0126 and PD98059 were from Promega Corp (Madison, WI).

Isolation of adult rat cardiac myocytes. Animal handling practices used in this study have been reviewed by and received approval from the Animal Care Committee of the University of Wisconsin. Ventricular cardiac myocytes were isolated from adult male Sprague-Dawley rats by enzymatic digestion with collagenase and hyaluronidase, as previously described (Huang et al., 1996). Myocytes were maintained in 0.5 mM Ca^{2+} Ringer's solution (125 mM NaCl, 5 mM KCl, 2 mM NaH_2PO_4, 5 mM sodium pyruvate, 1.2 mM $MgSO_4$, 11 mM glucose, 0.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4).

Immunoprecipitation. Cardiac myocytes from a single rat heart were divided into two equal aliquots, and half were treated with 10 nM ET-1 and half with vehicle at 37 °C for 10 min. Myocytes untreated or treated with ET-1 were lysed by either lysis buffer A (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1

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mM sodium orthovanadate, 1 mM sodium floride, protease inhibitor cocktail, pH 7.4) or lysis buffer B (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium floride, protease inhibitor cocktail, pH 7.4) on ice for 30 min, and centrifuged at 10,000 x *g* for 10 min at 4 °C. One ml of cell lysate (1 mg protein/ml) was used for each immunoprecipitation. Cell lysates from lysis buffer A were incubated with ET_A antibody-conjugated NHS-activated Sepharose beads that were prepared by manufacturer's instruction overnight at 4 °C. Beads were washed four times with lysis buffer A, and immunoprecipitates were eluted by elution buffer (50 mM glycine/HCl, pH2.5). Cell lysates from lysis buffer B were incubated with indicated antibodies overnight at 4 °C, and Protein G Sepharose™ 4 Fast Flow beads were added and incubated for 3 hr at 4 °C. Beads were washed four times with lysis buffer B, and immunoprecipitates were eluted by sample buffer (51 mM Tris-HCl, 4% SDS, 4 M urea, 5% glycerol, 0.001% bromophenol blue, 1% β-mercaptoethanol) by boiling for 3 min. Co-immunoprecipitated proteins were separated by SDS-PAGE. To control for non-specific binding to IgG-beads and other sources of spurious mass spectrometry peaks, the same immunoprecipitation procedure was performed with mouse IgG control, or with ET_A antibody without adding myocyte lysates (online supplement Figure 1S).

In-gel digestion. A series of gel segments from top to near bottom of each lane were cut out of Coomassie stained gels (see online supplement, Figure 1S). Gel segments were destained by rinsing three times in 50% acetonitrile/25 mM NH_4HCO_3, and dried in a SpeedVac. Samples were reduced with 100 mM DTT (dithiothreitol)/25 mM NH_4HCO_3 at 56 °C, and alkylated with 55 mM iodoacetamide/25 mM NH_4HCO_3 to

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modify cysteines. Samples were dried and subjected to 'in-gel digestion' with Glu-C or trypsin for 24 hr at 37 °C in 30 µl of digestion solution. Peptides were extracted with 0.1% trifluoroacetic acid (TFA**)**, and then twice with 50% acetonitrile/5% TFA solution. Extracts were pooled and dried in a SpeedVac.

Mass spectrometry. Extracted peptides were desalted by use of C_{18} ZipTips (Millipore, Framingham, MA) as described in the manufacturer's instructions, and eluted directly onto the MALDI-TOF plate. One µl of the matrix solution (α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) was applied on top of peptides and allowed to air dry. Spectra were obtained by use of a Bruker Reflex II MALDI-TOF mass spectrometer (Ballerica, MA), internally calibrated using the autoproteolytic fragments of trypsin or Glu-C. Peaks within the 800-3000 molecular weight range were chosen for database searches. Mass peaks of control lanes (mouse IgG controls and ET_A antibody control without myocyte lysates) were subtracted from peak lists of ET_A immunoprecipitates to remove peptide masses from non-specifically bound proteins. For protein identification, peptide mass searches were performed using the MS-Fit program (http://prospector.ucsf.edu). SwissProt database was used with the following criteria: species: rat; maximum number of missed cleavages: 2; minimum number of peptides required to match: 3; mass tolerance: 50 ppm; no post-translational modifications.

Western blotting. Proteins separated by SDS-PAGE were transferred to PVDF membranes (Millipore, Bedford, MA). Nonspecific sites were blocked by Blotto (150 mM NaCl, 20 mM Tris, pH 7.4, 0.05% (v/v) Tween 20, 5% powdered milk) for 1 h at room temperature, and Western analysis was carried out with enhanced chemiluminescence detection (AmershamBioscience, Buckinghamshire, England) as

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described (Huang et al., 1997). Loading controls were performed for each blot either by probing for another cardiac protein in parallel, or by stripping the phospho-blot and re-probing with the antibody for erbB itself. Blots illustrated in Figures are representative of a single experiment, whereas bar graphs represent the summarized results of 3-5 separate experiments in which blot intensities were ratioed to loading controls.

Immunofluorescence. Isolated cardiac myocytes were skinned with 100 μg/ml saponin in relaxing solution (100 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 4.5 mM ATP, 10 mM imidazole, pH 7.0), then washed and blocked by 2% bovine serum albumin in relaxing solution. Skinned myocytes were incubated with primary antibody overnight at 4 °C. Following extensive washing with 2% bovine serum albumin in relaxing solution, myocytes were incubated with Alexa 488-conjugated anti-mouse IgG or Alexa 568-conjugated anti-rabbit IgG secondary antibodies (diluted 1:200) for 1 h at room temperature. After extensive washing, images were acquired with a Bio-Rad MRC 1024 laser-scanning confocal microscope equipped with an argon/krypton laser controlled by 24-bit LaserSharp software.

Twitch measurements. Isolated myocytes were resuspended in 1 mM Ca^{2+} Ringer's solution. Cell twitches were initiated by electric field stimulation with a SD9 stimulator (Grass Instrument) in a modified PH1 chamber (Warner Instrument) mounted on a Nikon Diaphot inverted microscope. The stimulation protocol was 0.5 Hz, 10 ms duration, and 50 V at room temperature. Individual myocytes were monitored with a model VED 104 video edge detector (Crescent Electronics, Sandy, UT) and cell shortening was recorded using Felix software (Photon Technology International).

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Statistical analysis. Data are expressed as mean ± S.E.M. and analyzed using an unpaired student's *t*-test, and a one-way ANOVA (where appropriate). Values of $P < 0.05$ were considered to be significant for both statistical tests.

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RESULTS

To identify signaling proteins associated with ET receptors in adult myocardium, a proteomic analysis was performed on isolated ventricular myocytes from adult rat heart. Cell membranes were solubilized in a non-ionic detergent (1% NP-40) and then ET_A receptors were immunoprecipitated with a monoclonal antibody. Co-immunoprecipitated proteins were resolved on a one-dimensional SDS-PAGE gel (on-line supplement Figure 1S). In each lane, a series of 8 gel pieces was cut out without a gap (details in on-line supplement), and proteins were identified in each gel piece by peptide mass fingerprinting using MALDI-TOF mass spectrometry. Similar results were obtained with a stronger detergent mixture (1% NP-40/0.25% deoxycholate) arguing against detergent dependent artifacts. The protein composition of immunoprecipitates assessed by MALDI-TOF analysis was not detectably altered by pretreatment of myocytes with ET-1, possibly reflecting the semi-quantitative nature of proteomic analyses. Proteomic results from all immunoprecitations of ET_A were combined and summarized in Table 1. Among the candidate proteins identified, we undertook further analysis of the strongest and most reproducible protein hits, which were separated into 2 groups for convenience. One group of candidate proteins was the known ET_A signaling molecules such as $G\alpha q$ (9 of 18 IPs, 11 unique peptides, 32% sequence coverage), phospholipase C-β_1 (10/18 IPs, 20 unique peptides, 24% sequence coverage), and PKCδ (5/18 IPs, 17 unique peptides, 28% sequence coverage) (Table 1). Association of these proteins in ET_A complexes was further supported by reciprocal co-immunoprecipitation using antibodies to candidate proteins including $G\alpha q$, PLC-β1, and PKCδ (on-line supplement, Figure 2S).

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A second group of protein hits included growth-related signaling proteins such as Akt-1 (12/18 IPs, 17 unique peptides, 41% sequence coverage), Akt-2 (8/18 IPs, 11 unique peptides, 29% sequence coverage) and PI3-kinase (3/18, 15 unique peptides, 18% sequence coverage) (Table 1). These proteins were not known to be functionally associated with ET_A in adult myocardium, but Western analysis confirmed co-immunoprecipitation of ET_A with Akt-1 and PI3-kinase (Figure 1A). Akt attracted our attention because it was the strongest hit in the proteomic analysis (i.e. most frequently detected protein and displayed the highest sequence coverage (Table 1)). Western blotting revealed that approximately half of the immunoreactivity for Akt-1/Akt-2 was in the membrane fraction of homogenates of adult rat ventricular myocytes (not shown), despite the prevailing view that Akt is a cytosolic protein in unstimulated cells. Moreover, immunofluorescence analysis showed that both Akt-1/Akt-2 partially co-localized with ET_A in cardiac T-tubules (Figure 1B), consistent with a pool of these molecules forming a macromolecular complex in a common subcellular compartment. A portion of the PI3-kinase immunoreactivity was also co-localized with ET_A in T-tubules (Figure 1B).

Traditionally, PI3-kinase and Akt are activated by growth factor RTKs, so we mined the proteomic data for evidence of such proteins. Two of the most prominent were erbB2 and erbB4 (Table 1), albeit less prominent than some of the intracellular signaling molecules. Reciprocal immunoprecipitation of erbB2 or erbB4 brought down ET_A (Figure 1A), and both of these RTKs co-localized with ET_A in T-tubules (Figure 1B). Taken together, the proteomic analysis, Western blots and confocal immunofluorescence

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strongly indicated that ET_A was physically associated with erbB2/4 receptors within cardiac T-tubules possibly in a macromolecular complex.

To examine possible receptor cross-talk between ET_A and erbB receptors at the molecular level, receptor tyrosine phosphorylation was monitored with an anti-phosphotyrosine antibody. The classical ligand for erbB4, NRG1β, stimulated tyrosine phosphorylation of erbB2 and erbB4 in adult myocytes in a concentration dependent manner (Figure 2A). NRG1α had less effect as expected. However, somewhat unexpectedly ET-1 at physiological and supraphysiological doses (10 and 100 nM) did not stimulate erbB tyrosine phosphorylation (Figure 2B). Therefore, the widely recognized phenomenon of transactivation of RTKs resulting from activation of GPCRs was not observed in these freshly isolated adult rat ventricular myocytes. Pretreatment of myocytes with 10 nM ET-1 also did not potentiate the subsequent response to NRG1β, but instead inhibited it (Figure 2C). To establish which ET receptor subtype was responsible, antagonists of ET_A and ET_B receptors (BQ123 and BQ788, respectively) were used and showed that the inhibitory effect of ET-1 on erbB receptor autophosphorylation was mediated by the ET_A subtype (Figure 3). BQ123 and BQ788 themselves did not increase or inhibit NRG1β-induced erbB receptor phosphorylation (on-line supplement, Figure 3S).

A common downstream effector of ET_A is PKC. To investigate the involvement of PKC in the inhibitory effects of ET_A activation, the PKC inhibitor, *bis*-indolymaleimide (Bim) was used. Pretreatment of myocytes with Bim prevented the inhibitory effect of ET-1 on NRG1β-induced erbB receptor autophosphorylation (Figure 4), but Bim itself did not affect NRG1β-induced erbB receptor phosphorylation (on-line

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supplement, Figure 3S). Furthermore, pretreatment with a direct PKC activator, phorbol 12-myristate 13-acetate (PMA), mimicked the ET-1 response by strongly attenuating NRG1β-induced erbB receptor autophosphorylation (Figure 4). PKC may directly target erbB2/4 receptors or alternatively may function through an intermediary kinase cascade. To begin to address this question, we examined the involvement of the Raf-1/MEK/Erk cascade which was also found to immunoprecpitate with ET_A receptors in the proteomic analysis (Table 1). First, we showed that ET-1 promoted Erk1/2 phosphorylation in adult myocytes, and then established effective blocking concentrations for two commercial MEK antagonists, U0126 and PD98059, in our system (Figure 5A). At these concentrations, we found that each MEK antagonist attenuated the inhibitory cross-talk between ET-1 stimulation and erbB2/4 tyrosine phosphorylation, consistent with involvement of a PKC/Raf-1/MEK/Erk1/2 axis in this phenomenon. The MEK inhibitors alone had little or no effect on erbB2/4 tyrosine phosphorylation (on-line supplement, Figure 3S).

An analogous series of experiments was carried out with Akt-1/Akt-2 serine phosphorylation serving as the downstream effector of RTK stimulation. NRG1β promoted robust phosphorylation of Akt, but ET-1 treatment did not even when evaluated over a range of concentrations and incubation times (Figure 6A). However, as observed with erbB2/4 tyrosine phosphorylation, ET-1 pretreatment inhibited NRG1β-mediated Akt phosphorylation (Figure 5B). Thus, evidence for molecular cross-talk between ET_A and erbB2/4-Akt was obtained in the adult myocardium, but the nature of cross-talk was unexpected. ET-1 operating through ET_A receptors inhibited NRG1β signaling through

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erbB2/4 at both an early step (receptor autophosphorylation) and a subsequent step (Akt phosphorylation).

Finally, cross-talk between ET-1 and NRG1β was examined at the level of physiological function by evaluating inotropic responses in electrically-paced myocytes. NRG1β promoted a statistically significant ~20% decrease in twitch amplitude that developed within 5-10 minutes, and was often transient returning toward control twitch levels after 15 min (on-line supplement Figure 4S). Pretreatment with 10 nM ET-1 blocked this early onset negative inotropic effect (Figure 7), again consistent with its effects on erbB2/4 tyrosine phosphorylation and Akt serine phosphorylation.

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DISCUSSION

In this study, we investigated the components of ET-1 signaling complexes in adult cardiac myocytes by immunoprecipitating ET_A and identifying co-immunoprecipitated proteins by mass spectrometry. A number of proteins were identified that were previously reported to be functionally associated with ET_A such as Gαq, PLC-β, PKCε or PKCδ. Evidence was also obtained for the presence of a MAP kinase module, Raf-1/MEK/Erk. Unexpectedly, erbB2/4 and their downstream signaling molecules such as PI3-kinase and Akt were also present in ET_A co-immunoprecipitates. Further analysis indicated that the ET_A and erbB2/4 signaling pathways underwent cross-talk in intact myocytes suggesting that their close association may be physiologically relevant. This study presents the first evidence (to our knowledge) for co-localization and cross-talk between a GPCR and RTK in adult cardiac myocytes. EGF receptor (erbB1) which may be expressed in cardiac myocytes was not co-immunoprecipitated with ET_A possibly because of reduced expression compared to erbB2/4 (Iwamoto et al., 2003). This investigation focused on possible cross-talk between ET_A and erbB2/4 in adult cardiac myocytes because these were consistently co-immunoprecipitated under the conditions employed.

The nature of the cross-communication from ET_A to erbB2/4 was found to be inhibitory which was unexpected for several reasons. First, both ET-1 and NRG are thought to promote cardiac growth during development and during enlargement of diseased ventricles (i.e. cardiac hypertrophy) (Shah and Catt, 2004; Kedzierski and Yanagisawa, 2001; Negro et al., 2004). Second, studies in a variety of cell types including neonatal cardiac myocytes have revealed a common form of cross-talk between

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GPCRs and RTKs known as transactivation (Waters et al., 2004). With transactivation, stimulation of a GPCR promotes activation of a nearby RTK including its downstream signaling pathways which typically include PI3-kinase and Akt (Daub et al., 1996; Smith et al., 2004). Here we report that adult ventricular myocytes do not show transactivation of erbB2/4 RTKs when stimulated with the GPCR agonist ET-1, but instead show a profound inhibition of erbB2/4 signaling by ET-1.

Most members of the RTK family function by forming homo- and heterodimers upon ligand binding. This triggers subsequent autophosphorylation on tyrosine residues of the receptors followed by recruitment of signaling molecules such as Grb/SOS and PI3-kinase/Akt to the plasma membrane (Yarden and Sliwkowski, 2001). This appears to hold true for erbB2/4 in adult ventricular myocytes where activation by NRG1β stimulated tyrosine autophosphorylation and downstream activation of Akt. Interestingly, NRG1β also promoted a negative inotropic response in adult ventricular myocytes, as observed previously in papillary muscles (Lemmens et al., 2004). Elucidating precise intracellular mechanisms underlying this negative inotropic response will require further investigation.

Inhibitory cross-talk between ET_A and erbB2/4 receptors was detected at three different levels of neuregulin signaling including an early proximal step of erbB2/4 autophosphorylation, a downstream phosphorylation of Akt, and the integrated physiological response of negative inotropy. The inhibitory response was also found to be mediated by the ET_A subtype, which is known to couple strongly to Gαq-mediated PLC-β_1/PKC signaling in this cell type (Sugden, 2003). Our pharmacological analysis indicated that PKC and MEK/Erk kinases also play a role in mediating inhibitory cross-

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talk between ET_A and erbB2/4 in adult ventricular myocytes. Cross-talk between GPCRs and RTKs has been described in a wide variety of tissues, but interactions of the nature described here between ET_A and erbB2/4 have not been reported in any tissue. In studies of neonatal myocytes, ET-1 was shown to transactivate EGF receptors (Anderson et al., 2004; Asakura et al., 2002), whereas another study in the same system showed inhibition of EGF receptor-mediated Akt phosphorylation upon activation of Gαq (Sabri et al., 2002). In renal mesangial cells, Grewal *et al.* found that GPCRs desensitize and down-regulate EGF receptors (Grewal et al., 2001). Accumulating evidence also shows that ET-1 promotes insulin resistance in adipocytes and vascular smooth muscle via PKC-dependent inactivation of insulin receptor signaling (Jiang et al., 1999). A similar inhibitory cross-communication has been described involving angiotensin II/AT_1 receptor activation which inhibits insulin receptor function in a variety of tissues including adult myocardium (Velloso et al., 2006). This cross-talk appears to involve PKC and Erk mediated inhibition at multiple points in the insulin signaling cascade. Previous findings and the present study emphasize that activation of GPCRs do not always result in transactivation of RTKs, but can also lead to inhibitory modulation of RTKs.

The physiological role of this inhibitory cross-talk remains to be established. In this study, ET-1 inhibited the negative inotropic effects of NRG1β, suggesting a role in potentiation of ET-1's positive contractile effect on the heart. Interestingly, in the present study no evidence for cross-talk in the opposite direction (erbB2/4 to ET_A) was obtained, for instance, at the level of inotropic responses to ET-1 and NRG1β (on-line supplement, Figure 5S). Reciprocal cross-talk of this type (RTK to GPCR as opposed to GPCR to

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RTK) may be less well-developed as also found for communication between angiotensin II/AT_1 and insulin receptors (Velloso et al., 2006).

ET_A localizes with its downstream signaling molecules such as PLCβ and PKCε at cardiac T-tubules (Robu et al., 2003), and it is now clear that erbB2/4 and associated signaling proteins are also enriched within the cardiac T-tubules in adult myocytes. Interestingly, the T-tubule compartment is absent in neonatal myocytes and may account for the fundamentally different type of GPCR-RTK cross-talk, i.e. transactivation, observed in those cells. Also of interest is the observation that cellular remodeling in the failing heart can result in degeneration of the cardiac T-tubule compartment (Balijepalli et al., 2003; Brette and Orchard, 2003). ET-1 and ET_A are increased in congestive heart failure, but the positive inotropic effects of ET-1 are attenuated (Pieske et al., 1999) or completely reversed (Thomas et al., 1996; MacCarthy et al., 2000) suggesting that intact T-tubule structure is important for normal physiological function of ET-1 in adult myocardium. The observation that erbB2/4 expression levels are reduced in failing heart (Rohrbach et al., 2005) may also be a direct result of their localization in this labile membrane compartment. The nature of cross-talk between ET_A and erbB receptors in failing hearts remains to be investigated.

Targeting the physical and functional interplay between ET-1 and erbB2/4 signaling systems may open new avenues for understanding and treating maladaptive remodeling of the human heart. Indeed, cross-talk between ET_A and erbB receptors may have particular significance in the setting of heart disease as erbB2/4 activation is thought to confer strong cardioprotective and anti-apoptotic signals in adult myocardium (Grazette et al., 2004; Zhao et al., 1998). Accordingly, chronic ET-1 stimulation may

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interfere with normal pro-survival signaling through erbB2/4 receptors, thereby shifting the balance toward cardiomyopathy. Such a chronic state could contribute to the transition from compensated to decompensated hypertrophy and failure, consistent with findings that ET_A antagonists improve survival in experimental models of heart failure (Sakai et al., 1996). In other studies, targeted activation of a G-protein that couples to ET_A, namely Gαq, initially induced cardiac hypertrophy, whereas chronic activation led to apoptosis and reduced basal and EGF-stimulated Akt activation (Adams et al., 1998; Sabri et al., 2002). All of these studies point to the importance of maintaining a critical balance between Gαq and Akt signaling.

Herceptin monoclonal antibodies targeted to erbB2 receptors show enormous promise in treatment of breast tumors, but cardiomyopathy is often a prominent side effect (Chien, 2006). Inhibitory erbB2 antibodies have been shown to cause mitochondrial-dependent apoptosis in ventricular tissues (Grazette et al., 2004), and erbB2 inhibition by conditional gene knockout in mice triggers myocardial apoptosis resulting in a severe dilated cardiomyopathy (Negro et al., 2004). In light of the inhibitory cross-talk between ET_A and erbB2 reported here, we speculate that breast cancer patients undergoing herceptin therapy might benefit from endothelin receptor anatagonists to minimize disruption of the balance of these autocrine/paracrine inputs in the heart.

In conclusion, we have used a combination of semi-quantitative approaches including immunoprecipitation/proteomics, immunoblotting, and subcellular localization by confocal microscopy to provide evidence for interactions and co-localization of ET_A and errB2/4 signaling systems in adult ventricular myocytes. A pharmacological analysis

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of cross-talk between these signaling systems further suggested close functional interactions. On this basis, we propose that 1) cardiac T-tubules contain macromolecular complexes with ET_A and erbB2/4 closely physically and functionally associated, 2) ET_A does not promote growth of the adult heart by erbB2/4 transactivation but more likely via a Raf-1/MEK/Erk1/2 signaling axis, and 3) altered cross-communication between these receptor classes may contribute to changes in neurohumoral and growth factor signaling observed in human cardiomyopathies, particularly those in which the T-tubule compartment is compromised.

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Figure Legends

Figure 1. Complex formation and co-localization of ET_A with Akt, PI3-kinase and erbB2/4. A. Isolated adult myocytes were solubilized in lysis buffer A, and proteins were immunoprecipitated (IP) by the antibodies indicated. Immunoprecipitates were blotted (IB) with an ET_A antibody. Lack of effects of preincubation of myocytes with 10 nM ET-1 (+) versus vehicle (-) for 10 min at 37 °C is also illustrated. B. Confocal images of cardiac myocytes immunostained by the antibodies indicated (details in Methods). Arrows mark T-tubules, blunt arrows mark surface sarcolemma, and arrowheads mark intercalated discs. Scale bar = 10 μm.

Figure 2. Attenuation of NRG1β-induced erbB2/4 receptor tyrosine phosphorylation by ET-1. A. Myocytes were incubated with NRG1β in a concentration dependent manner for 10 min at 37 °C. Cells were solubilized, subjected to immunoprecipitation (IP) and blotted (IB) with indicated antibodies. B. Myocytes were incubated with ET-1, NRG1α or NRG1β for 10 min at 37 °C, then analyzed as in A. C. Myocytes were pretreated with ET-1 for 10 min followed by NRG 1β for 10 min at 37 °C, then analyzed as in A. *$p < 0.05$ (n=4) by *t*-test, and one-way ANOVA confirmed non-identity of means of NRG treated samples. For Figures 2-6, blots are representative of a single experiment; bar graphs represent combined results of 3-5 separate experiments in which blot intensities are ratioed to loading controls.

Figure 3. Pharmacological analysis of the inhibitory effect of ET-1 on erbB2/4 tyrosine phosphorylation. Myocytes were incubated with ET_A antagonist BQ123 (2 μM) or ET_B

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antagonist BQ788 (2 μM) for 5 min followed by ET-1 for 10 min, and then treated with NRG1β for 10 min at 37 °C. Receptors were immunoprecipitated from myocyte extracts solubilized in buffer A, and immunoblotted with indicated antibodies. *$p < 0.05$ (n=3) by *t*-test, and one-way ANOVA confirmed non-identity of means of NRG treated samples.

Figure 4. Involvement of PKC. Isolated myocytes were pre-incubated with a PKC inhibitor (Bim, 1 μM) or vehicle for 10 min followed by 10 nM ET-1 or 1 μM PMA for 10 min, and treated with 10 ng/ml NRG 1β for 10 min at 37°C. ErbB4 (left panel) and erbB2 (right panel) receptors were immunoprecipitated from myocyte extracts solubilized in buffer A, and blotted with indicated antibodies. *$p < 0.05$ ($n \geq 4$) by *t*-test, and one-way ANOVA confirmed non-identity of means of NRG treated samples.

Figure 5. Involvement of MEK/Erk. A. Western blot with a phospho-Erk1/2 antibody before and after 10 nM ET-1 for 10 min, and the effects of two MEK antagonists, U=10 μM U0126 and PD=50 μM PD98059. α-Actinin was used as a loading control. B. Effects of MEK antagonist pretreatment on inhibitory cross-talk using standard doses of ET-1 and NRG1β. erbB4 was used as a loading control. *$p < 0.05$ (n=4) by *t*-test, and one-way ANOVA confirmed non-identity of means of NRG treated samples.

Figure 6. Attenuation of NRG 1β-induced Akt phosphorylation by ET-1. A. Myocytes were treated with increasing doses of NRG 1β or ET-1 for 10 min at 37°C, and Akt activation was measured by Western blotting with a phospho-Akt specific antibody. Incubation time in 10 nM ET-1 was also varied between 3 and 60 min. B. Myocytes

were treated with ET-1 for 10 min followed by NRG1β for 10 min at 37°C, and Akt activation was measured. Phospho-Akt signals were normalized to cardiac troponin I (cTnI) used as a loading control. *$p < 0.05$ (n=4) by *t*-test. One-way ANOVA revealed no statistical difference for means of all ET-1 treated samples.

Figure 7. Attenuation of NRG1β-induced negative inotropic response by ET-1. Myocytes were stimulated electrically, and cell shortening was measured 5 min after addition of NRG 1β as described in Methods. For ET-1 pretreated cells, NRG 1β was added after the ET-1 positive inotropic response stabilized (15-20 min; on-line supplement Figure 3S). Control is the time point when NRG1β was added. *$p < 0.05$ ($n \geq 5$) by *t*-test, and one-way ANOVA confirmed non-identity of mean values.

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Table 1. Summary of proteomic analysis of ET_A immunoprecipitates (IPs).[#]

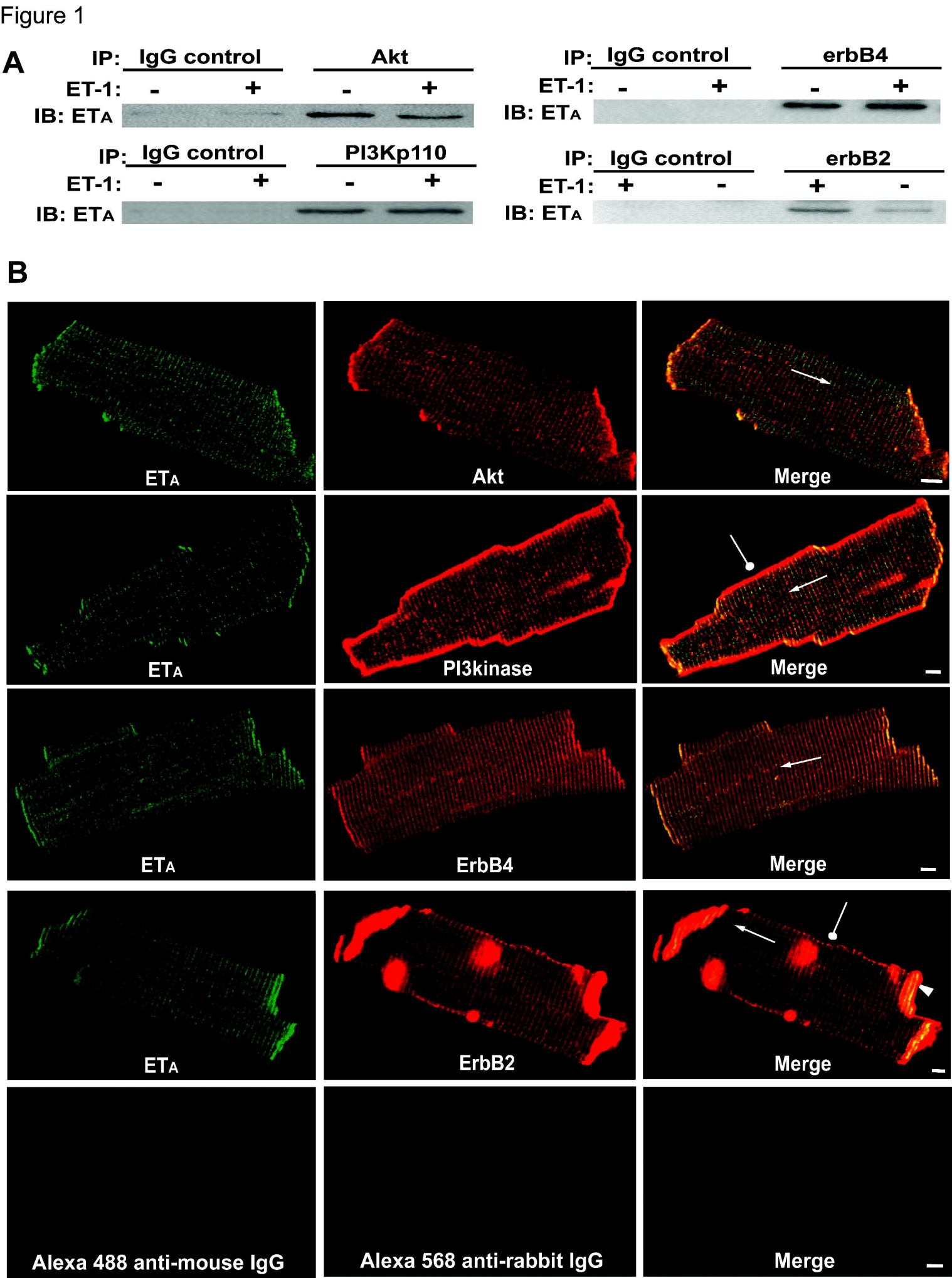
Protein	MW	IPs	Tryptic peptides	GluC peptides	Coverage (aa's)	Coverage (%)
Gαq	41470	9/18	9	2	113*	32*
PLC-β1	138346	10/18	10	10	281*	24*
PKC-δ	77521	5/18[@]	17	0	190*	28*
PKC-ε	83479	4/18[@]	11	0	138*	18
ET_A	48245	1/18	2	0	33	8
ET_B	49455	2/18	2	0	31	7
Akt-1	55736	12/18	11	6	196*	41*
Akt-2	55544	8/18	7	4	141*	29*
PI3K110β	122609	3/18[@]	11	4	195*	18
PI3K85β	81329	3/18[@]	5	0	67	9
erbB4	146960	3/18	4	4	115*	9
erbB2	138834	4/18	1	5	81	7
Erk-2	41276	8/18	8	8	205*	57*
Mek-2	44282	6/18	7	2	119*	30*
Raf-1	72929	5/18	7	2	116*	18

[#] Data are from a total of 9 independent experiments on myocytes isolated from 9 separate hearts. Four independent experiments (4 hearts) were performed using 1% NP-40 (lysis buffer A) resulting in 8 IPs, 4 IPs from vehicle treated myocytes and 4 IPs from ET-1 treated myocytes (online supplement Figure 1S). Another 5 independent experiments (5 hearts) were performed using 1% NP-40/0.25% deoxycholate (lysis buffer B) resulting in 10 IPs, 5 IPs from vehicle treated myocytes and 5 IPs from ET-1 treated myocytes. Each experiment was analyzed either by trypsin or Glu-C digestion. IPs were analyzed by SDS-PAGE, 'in gel' digestion and MALDI-TOF mass spectrometry. Proteins are grouped by functional relatedness.

* Significant hits were determined on the basis of sequence coverage by one of two criteria: 1) >110 amino acids covered (equivalent to ~12,000 Daltons) or 2) >20% of the protein's entire sequence covered.

[@] Detected only in experiments with the milder detergent 1% NP-40 (lysis buffer A), and accounts in part for the lower number of total IPs in which these proteins were detected.

Figure 1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Akt
ET-1: - + - +
IB: ETA
IP: IgG control
PI3Kp110
ET-1: - + - +
IB: ETA
IP: IgG control
erbB4
ET-1: - + - +
IB: ETA
IP: IgG control
erbB2
ET-1: + - + -
IB: ETA
B
ETA
Akt
Merge
ETA
PI3kinase
Merge
ETA
ErbB4
Merge
ETA
ErbB2
Merge
Alexa 488 anti-mouse IgG
Alexa 568 anti-rabbit IgG
Merge

Figure 2

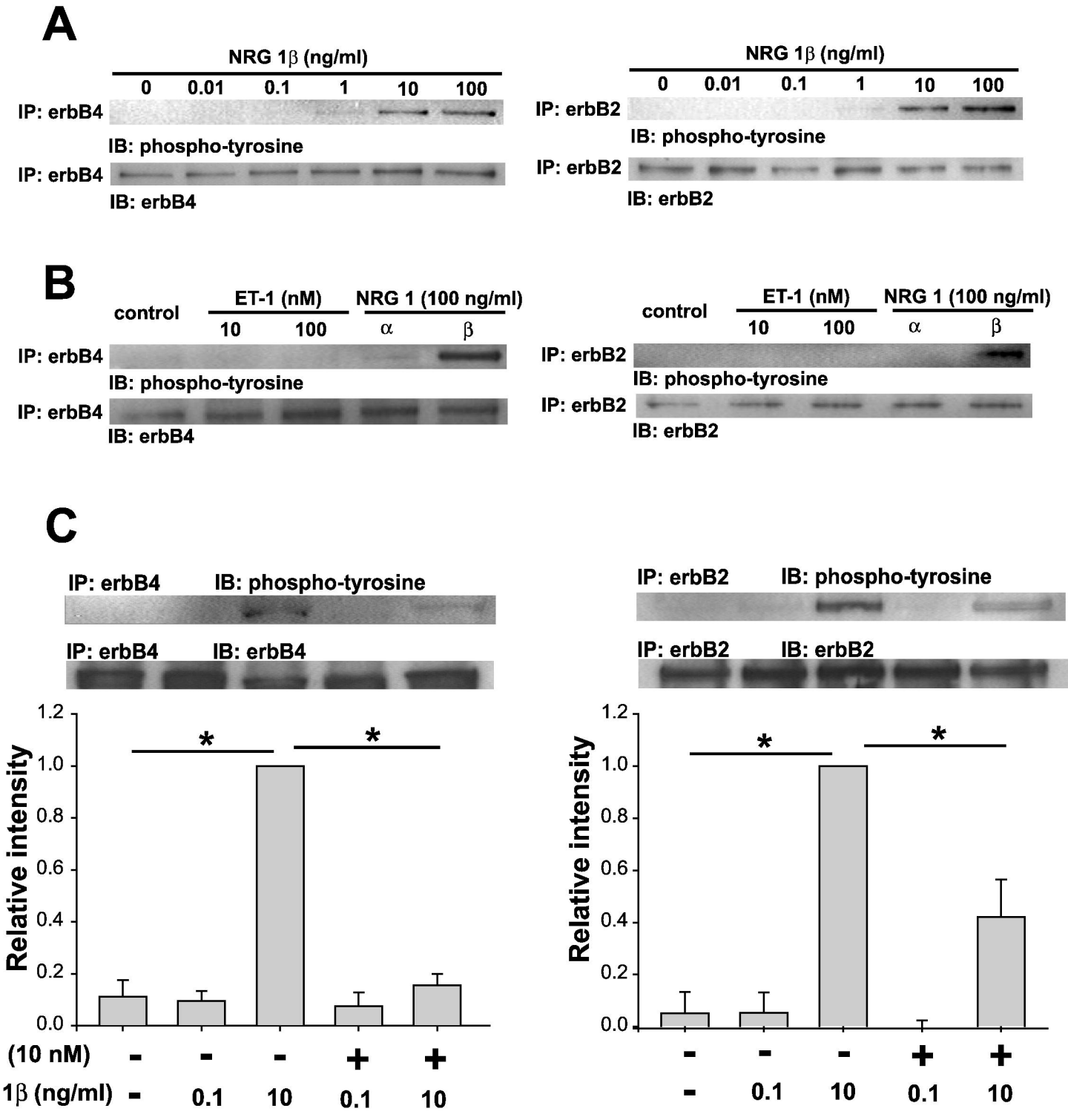

A
NRG 1β (ng/ml)
0 0.01 0.1 1 10 100
IP: erbB4
IB: phospho-tyrosine
IP: erbB4
IB: erbB4
NRG 1β (ng/ml)
0 0.01 0.1 1 10 100
IP: erbB2
IB: phospho-tyrosine
IP: erbB2
IB: erbB2
B
control
ET-1 (nM)
10 100
NRG 1 (100 ng/ml)
α β
IP: erbB4
IB: phospho-tyrosine
IP: erbB4
IB: erbB4
control
ET-1 (nM)
10 100
NRG 1 (100 ng/ml)
α β
IP: erbB2
IB: phospho-tyrosine
IP: erbB2
IB: erbB2
C
IP: erbB4 IB: phospho-tyrosine
IP: erbB4 IB: erbB4
IP: erbB2 IB: phospho-tyrosine
IP: erbB2 IB: erbB2
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
ET-1 (10 nM) - - - + +
NRG 1β (ng/ml) - 0.1 10 0.1 10
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
- - - + +
- 0.1 10 0.1 10

Figure 3

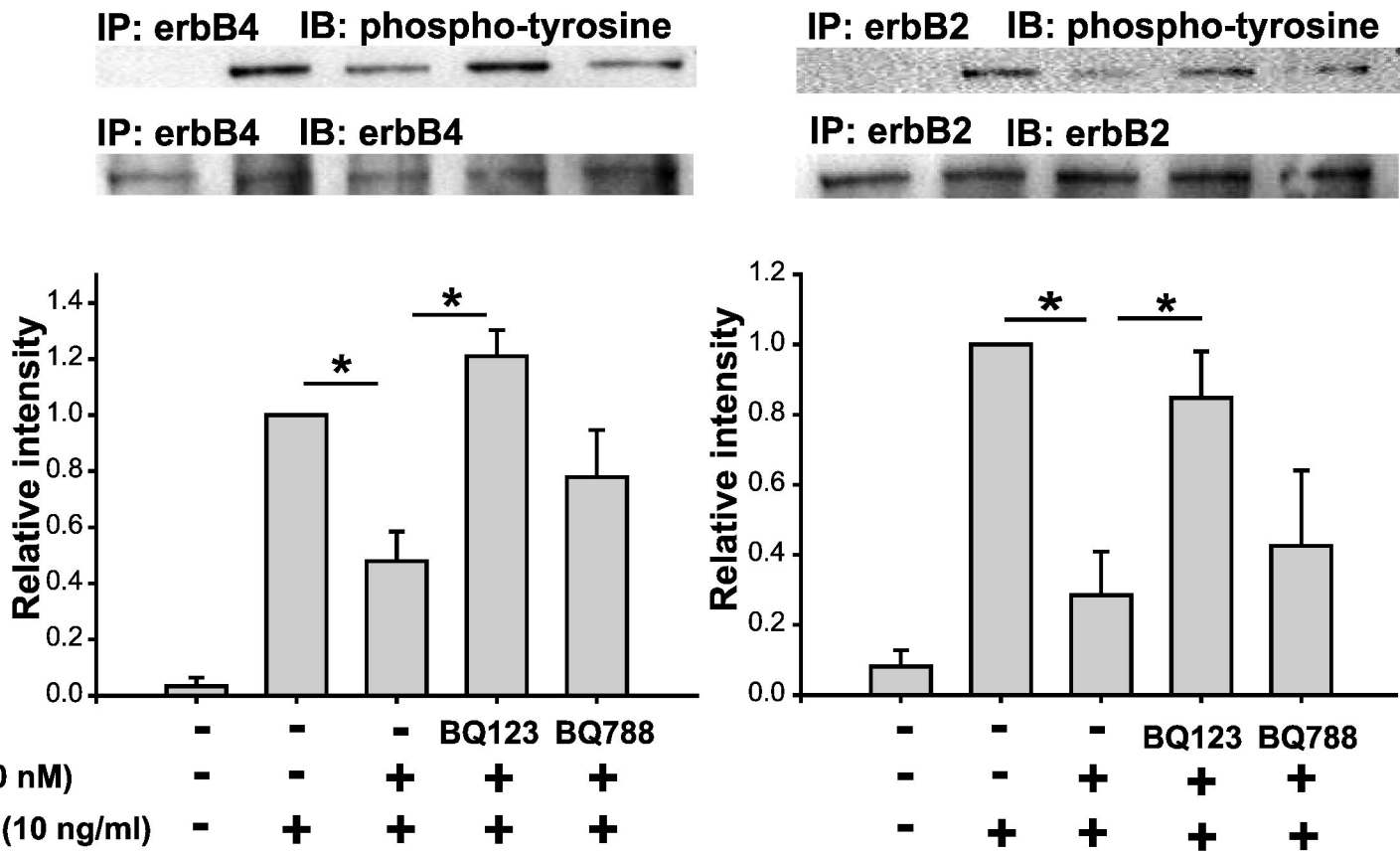
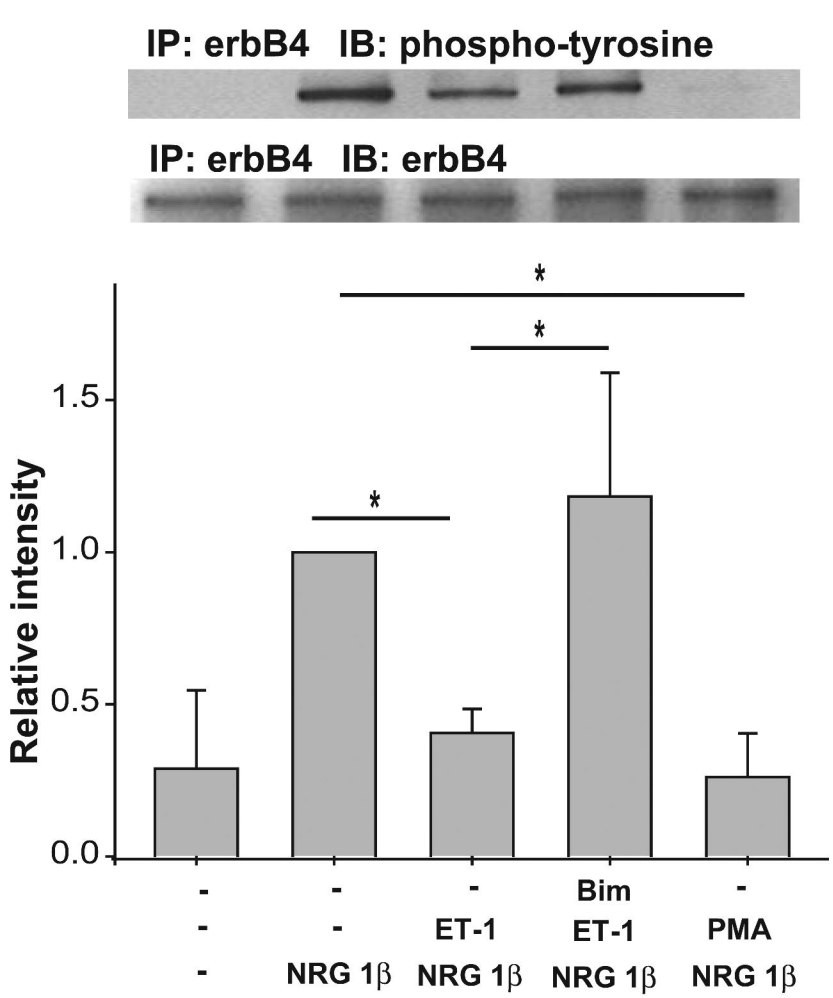

IP: erbB4 IB: phospho-tyrosine
IP: erbB4 IB: erbB4
IP: erbB2 IB: phospho-tyrosine
IP: erbB2 IB: erbB2
Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
- - - BQ123 BQ788
ET-1 (10 nM) - - + + +
NRG 1β (10 ng/ml) - + + + +
- - - BQ123 BQ788
- - + + +
- + + + +

Figure 4



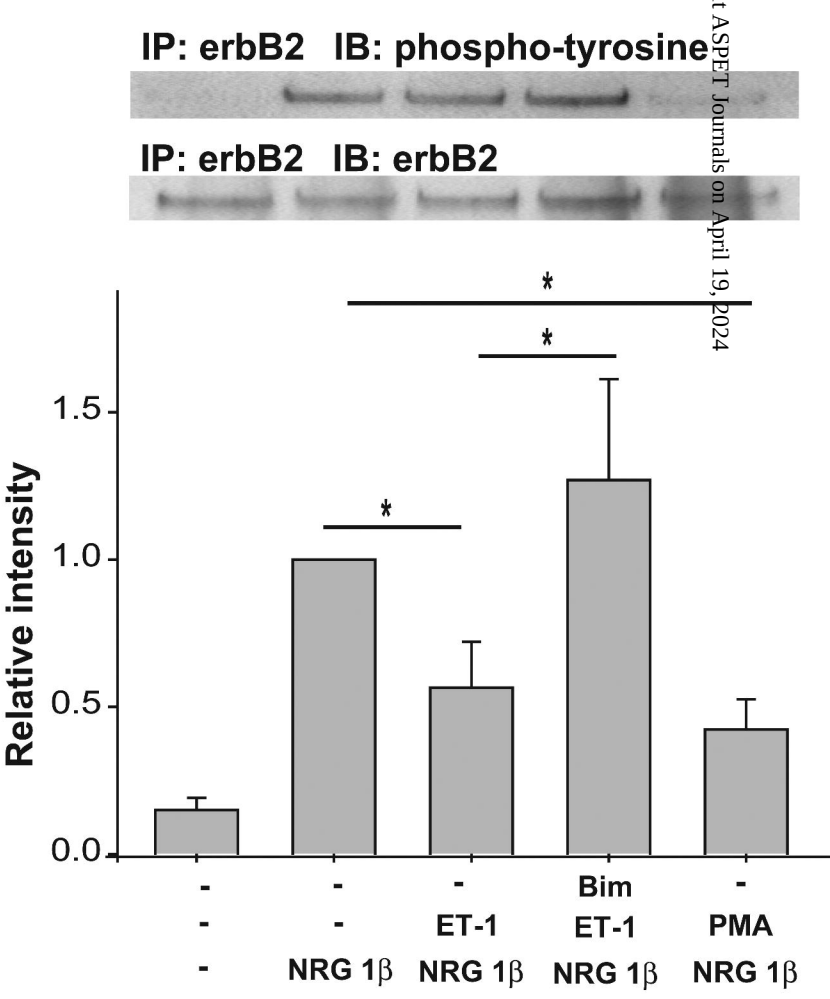
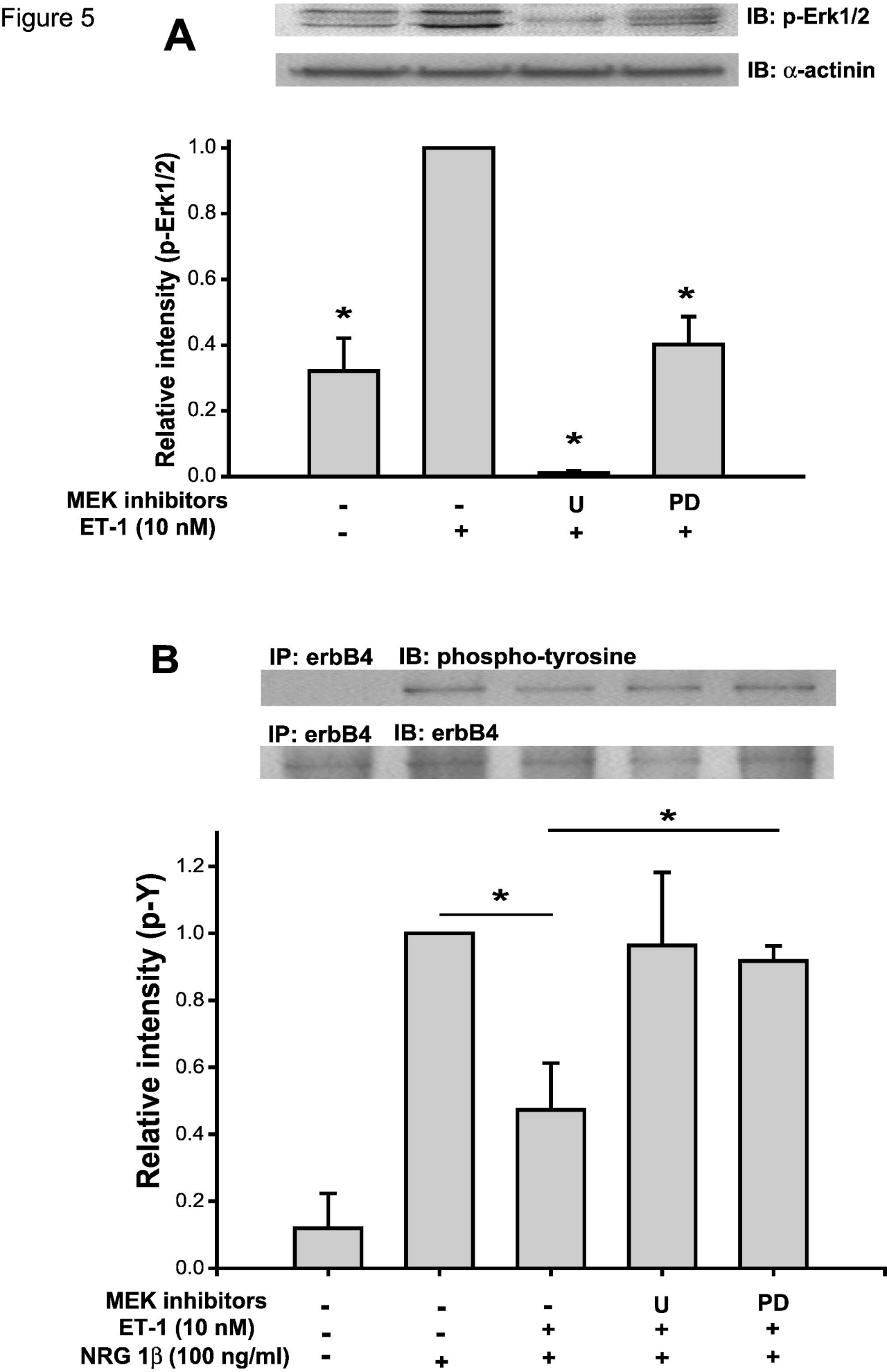


Figure 5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IB: α-actinin
Relative intensity (p-Erk1/2)
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0.8
0.6
0.4
0.2
0.0
*
*
*
MEK inhibitors
ET-1 (10 nM)
-
-
U
PD
-
+
+
+
B
IP: erbB4
IB: phospho-tyrosine
IP: erbB4
IB: erbB4
Relative intensity (p-Y)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
MEK inhibitors
ET-1 (10 nM)
NRG 1β (100 ng/ml)
-
-
-
U
PD
-
-
+
+
+
-
+
+
+
+

Figure 6

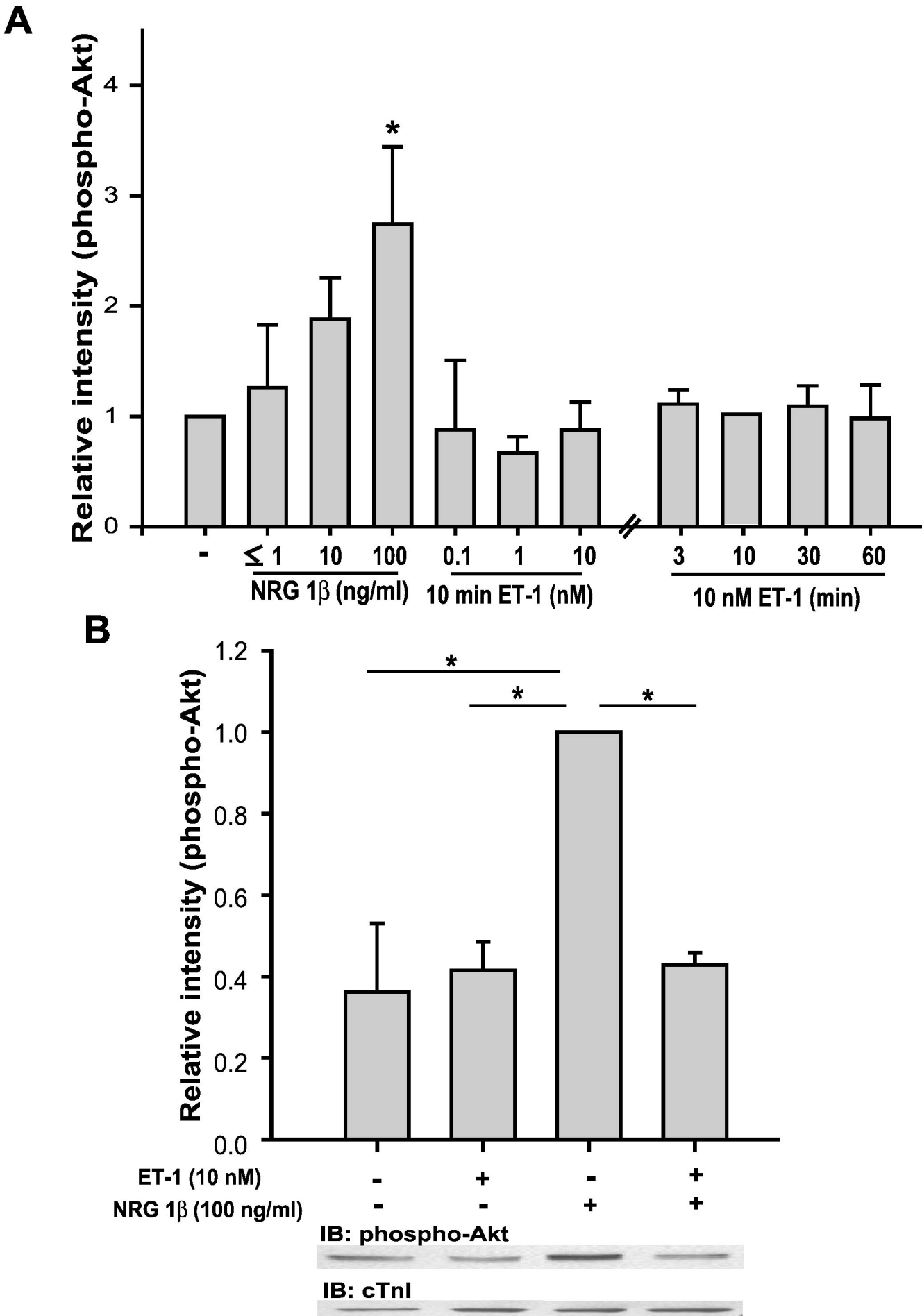
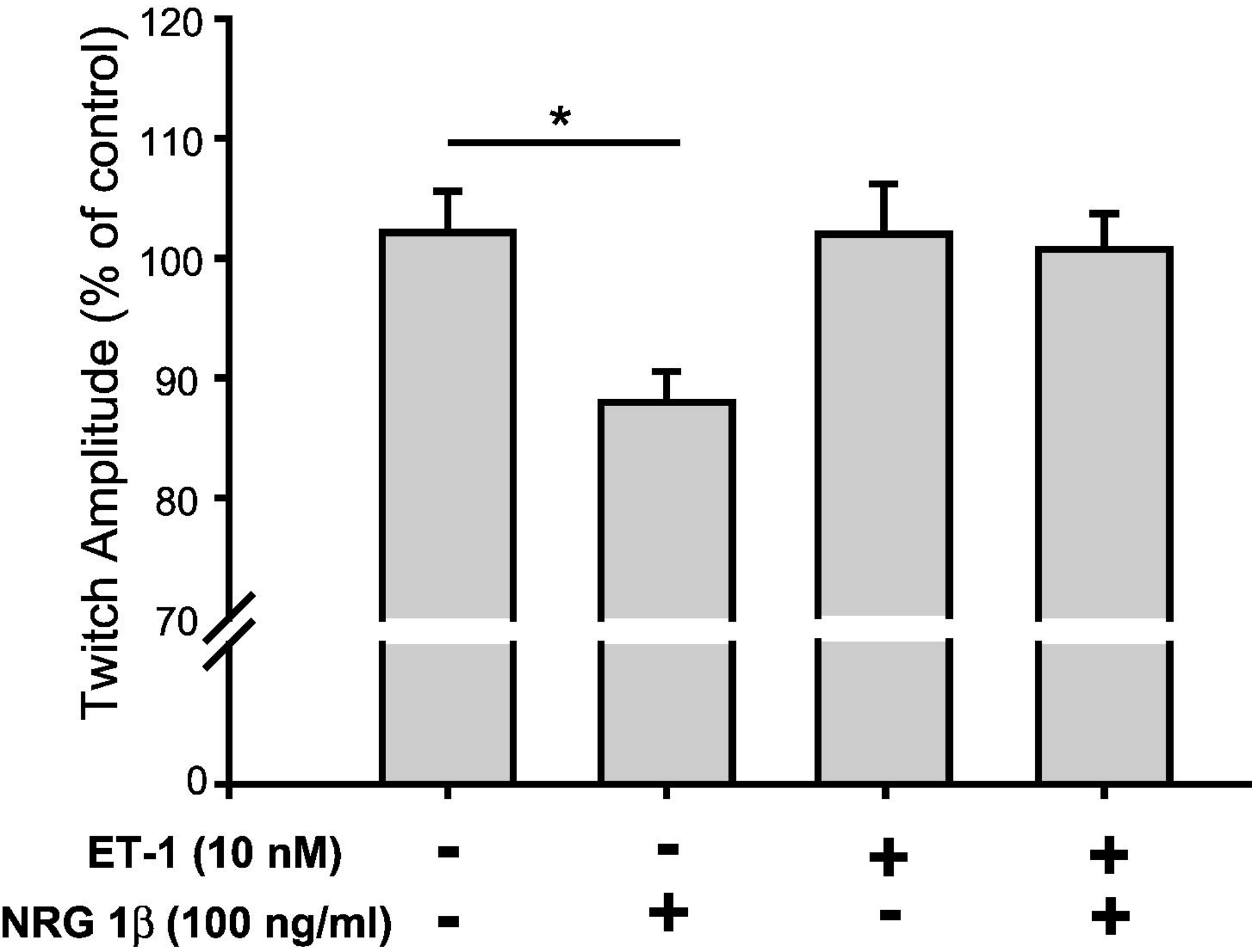

A
Relative intensity (phospho-Akt)
0
1
2
3
4
*
-
≤ 1
10
100
0.1
1
10
3
10
30
60
NRG 1β (ng/ml)
10 min ET-1 (nM)
10 nM ET-1 (min)
B
Relative intensity (phospho-Akt)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
*
*
ET-1 (10 nM)
NRG 1β (100 ng/ml)
IB: phospho-Akt
IB: cTnI

Figure 7


Twitch Amplitude (% of control)
120
110
100
90
80
70
0
*
ET-1 (10 nM)
NRG 1β (100 ng/ml)
-
-
+
+
-
+
-
+