

Interaction and Inhibitory Cross-Talk between Endothelin and ErbB Receptors in the Adult Heart^[S]

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ABSTRACT

Endothelin-1 (ET-1) regulates contractility and growth of the mammalian heart by binding endothelin receptor type A (ET_A) and endothelin receptor type B (ET_B) G-protein-coupled receptors. To identify growth signaling pathways associated with ET-1 receptors in adult myocardium, a combined immunoprecipitation/proteomic analysis was performed. Signaling proteins believed to function downstream of ET_A such as G_{αq}, phospholipase C-β1, protein kinase C (PKC) ε, and PKCδ were identified in immunoprecipitates of ET_A by matrix-assisted laser desorption ionization/time of flight mass spectrometry. Also prominent were the growth factor receptor tyrosine kinases erbB2 and erbB4 and their downstream growth signaling effectors phosphoinositide-3 kinase (PI3 kinase), Akt, Raf-1, mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinase (Erk). Western blot analysis confirmed coimmunoprecipitation of erbB2/4, PI3 kinase, and Akt with ET_A, and confocal microscopy revealed their colocalization in cardiac transverse tubules (T-tubules). The erbB4

receptor ligand neuregulin-1β (NRG1β) promoted erbB2/4 tyrosine 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 1) prolonged exposure to ET-1; 2) degeneration of T-tubules; and 3) therapies targeted at erbB2 inhibition.

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 believed 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 (Prenzel et al., 1999; Shah and Catt, 2004; Waters et al., 2004).

In heart tissue, transactivation of EGFR (erbB1) by ET-1 has been shown to occur in neonatal rat cardiac myocytes (Asakura et al., 2002; Anderson et al., 2004). Much of our current understanding of cross-talk between GPCRs and RTKs is based on 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

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ABBREVIATIONS: ET-1, endothelin-1; ET_A, endothelin receptor type A; ET_B, endothelin receptor type B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptor; NRG, neuregulin; PMA, phorbol 12-myristate-13-acetate; PLC, phospholipase C; PI3 kinase, phosphoinositide-3 kinase; PKC, protein kinase C; RTK, receptor tyrosine kinase; T-tubule, transverse tubule; Bim, bis-indolylmaleimide; IP, immunoprecipitate; IB, immunoblot; Erk1/2, extracellular signal-regulated kinases 1 and 2; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance; MEK, mitogen-activated protein kinase kinase; NP-40, Nonidet P-40; PD98059, 2'-amino-3'-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; BQ788, *N*-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; BQ123, cyclo(p-Asp-Pro-D-Val-Leu-D-Trp).

identify proteins stably associated with ET receptors in adult myocytes after detergent solubilization and immunoprecipitation. The erbB2/4, PI3 kinase, and 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 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.

Materials and Methods

Materials. All reagents were obtained from Sigma Chemical 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 Biotechnology (Lake Placid, NY), Alexa 488- or 568-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA), and other primary and secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein G Sepharose 4 Fast Flow and *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). BQ123, BQ788, bis-indoylmaleimide, and phorbol 12-myristate-13-acetate (PMA) were from Sigma Chemical Co. U0126 and PD98059 were from Promega Corporation (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 described previously (Huang et al., 1996). Myocytes were maintained in 0.5 mM Ca²⁺ Ringer's solution (125 mM NaCl, 5 mM KCl, 2 mM NaH₂PO₄, 5 mM sodium pyruvate, 1.2 mM MgSO₄, 11 mM glucose, 0.5 mM CaCl₂, and 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 phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 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 phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitor cocktail, pH 7.4) on ice for 30 min and centrifuged at 10,000g for 10 min at 4°C. One milliliter of cell lysate (1 mg of protein/ml) was used for each immunoprecipitation. Cell lysates from lysis buffer A were incubated with ET_A antibody-conjugated *N*-hydroxysuccinimide-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, pH 2.5). 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 h 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, and 1% β-mercaptoethanol) by boiling for 3 min. Coimmunoprecipitated proteins were separated by SDS-PAGE. To control for nonspecific 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 (Supplemental Fig. S1).

In-Gel Digestion. A series of gel segments from top to near bottom of each lane were cut out of Coomassie-stained gels (Supplemental Fig. S1). Gel segments were destained by rinsing three times in 50% acetonitrile/25 mM NH₄HCO₃ and dried in a SpeedVac (Thermo Electron, Waltham, MA). Samples were reduced with 100 mM dithiothreitol/25 mM NH₄HCO₃ at 56°C and alkylated with 55 mM iodoacetamide/25 mM NH₄HCO₃ to modify cysteines. Samples were dried and subjected to "in-gel digestion" with Glu-C or trypsin for 24 h at 37°C in 30 μl of digestion solution. Peptides were extracted with 0.1% trifluoroacetic acid and then twice with 50% acetonitrile/5% trifluoroacetic acid solution. Extracts were pooled and dried in a SpeedVac.

Mass Spectrometry. Extracted peptides were desalted by use of C₁₈ ZipTips (Millipore, Framingham, MA) as described in the manufacturer's instructions and eluted directly onto the MALDI-TOF plate. One microliter 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 Reflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Ballerica, MA) internally calibrated using the autoproteolytic fragments of trypsin or Glu-C. Peaks within the 800 to 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 nonspecifically 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; and no post-translational modifications.

Western Blotting. Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Millipore). Nonspecific sites were blocked by bovine lacto transfer optimizer [150 mM NaCl, 20 mM Tris, pH 7.4, 0.05% (v/v) Tween 20, and 5% powdered milk] for 1 h at room temperature, and Western analysis was carried out with enhanced chemiluminescence detection (GE Healthcare) as described previously (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 reprobing with the antibody for erbB itself. Blots illustrated in the figures are representative of a single experiment, whereas bar graphs represent the summarized results of three to five separate experiments in which the ratio of blot intensities to loading controls is shown.

Immunofluorescence. Isolated cardiac myocytes were skinned with 100 μg/ml saponin in relaxing solution (100 mM KCl, 1 mM MgCl₂, 2 mM EGTA, 4.5 mM ATP, and 10 mM imidazole, pH 7.0) and then washed and blocked by 2% bovine serum albumin in relaxing solution. Skinned myocytes were incubated with primary antibody overnight at 4°C. After 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 laser-scanning confocal microscope (MRC 1024; Bio-Rad Laboratories, Hercules, CA) equipped with an argon/krypton laser controlled by 24-bit LaserSharp software.

Twitch Measurements. Isolated myocytes were resuspended in 1 mM Ca²⁺ Ringer's solution. Cell twitches were initiated by electric field stimulation with a SD9 stimulator (Grass Instruments, Quincy, MA) in a modified PH1 chamber (Warner Instruments, Hamden, CT) mounted on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan). 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, Lawrenceville, NJ).

Statistical Analysis. Data are expressed as mean \pm 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.

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 nonionic detergent (1% NP-40), and then ET_A receptors were immunoprecipitated with a monoclonal antibody. Coimmunoprecipitated proteins were resolved on a one-dimensional SDS-PAGE gel (Supplemental Fig. S1). In each lane, a series of eight gel pieces was cut out without a gap (details in Supplemental Fig. S1), 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 semiquantitative nature of proteomic analyses. Proteomic results from all immunoprecipitations of ET_A are 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 two groups for convenience. One group of candidate proteins was the known ET_A signaling molecules such as Gα_q (9 of 18 IPs, 11 unique peptides, 32% sequence coverage), phospholipase C-β₁ (10 of 18 IPs, 20 unique peptides, 24% sequence coverage), and PKCδ (5 of 18 IPs, 17 unique peptides, 28% sequence coverage) (Table 1). Association of these proteins in ET_A complexes was further supported by reciprocal coimmunoprecipitation using antibodies

to candidate proteins including Gα_q, PLC-β₁, and PKCδ (Supplemental Fig. S2).

A second group of protein hits included growth-related signaling proteins such as Akt-1 (12 of 18 IPs, 17 unique peptides, 41% sequence coverage), Akt-2 (8 of 18 IPs, 11 unique peptides, 29% sequence coverage), and PI3 kinase (3 of 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 coimmunoprecipitation of ET_A with Akt-1 and PI3 kinase (Fig. 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 (data not shown), despite the prevailing view that Akt is a cytosolic protein in unstimulated cells. Moreover, immunofluorescence analysis showed that both Akt-1 and Akt-2 partially colocalized with ET_A in cardiac T-tubules (Fig. 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 colocalized with ET_A in T-tubules (Fig. 1B).

By tradition, 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 (Fig. 1A), and both of these RTKs colocalized with ET_A in T-tubules (Fig. 1B). Taken together, the proteomic analysis, Western blots, and confocal immunofluorescence 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 phos-

TABLE 1

Summary of proteomic analysis of ET_A immunoprecipitates

Data are from a total of nine independent experiments on myocytes isolated from nine separate hearts. Four independent experiments (four hearts) were performed using 1% NP-40 (lysis buffer A) resulting in eight IPs: four IPs from vehicle-treated myocytes, and four IPs from ET-1-treated myocytes (Supplemental Figure S1). Another five independent experiments (five 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.

Protein	Molecular Weight	IPs	Tryptic Peptides	Glu-C Peptides	Coverage (Amino Acids)	Coverage %
Gα _q	41,470	9/18	9	2	113 ^a	32 ^a
PLC-β ₁	138,346	10/18	10	10	281 ^a	24 ^a
PKC-δ	77,521	5/18 ^b	17	0	190 ^a	28 ^a
PKC-ε	83,479	4/18 ^b	11	0	138 ^a	18
ET _A	48,245	1/18	2	0	33	8
ET _B	49,455	2/18	2	0	31	7
Akt-1	55,736	12/18	11	6	196 ^a	41 ^a
Akt-2	55,544	8/18	7	4	141 ^a	29 ^a
PI3K110β	122,609	3/18 ^b	11	4	195 ^a	18
PI3K85β	81,329	3/18 ^b	5	0	67	9
erbB4	146,960	3/18	4	4	115 ^a	9
erbB2	138,834	4/18	1	5	81	7
Erk-2	41,276	8/18	8	8	205 ^a	57 ^a
Mek-2	44,282	6/18	7	2	119 ^a	30 ^a
Raf-1	72,929	5/18	7	2	116 ^a	18

^a 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.

^b 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.

phorylation was monitored with an antiphosphotyrosine antibody. The classic ligand for erbB4, NRG1 β , stimulated tyrosine phosphorylation of erbB2 and erbB4 in adult myocytes in a concentration-dependent manner (Fig. 2A). NRG1 α 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 (Fig. 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 NRG1 β but instead inhibited it (Fig. 2C). To establish the ET receptor subtype 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 autophos-

phorylation was mediated by the ET_A subtype (Fig. 3). BQ123 and BQ788 themselves did not increase or inhibit NRG1 β -induced erbB receptor phosphorylation (Supplemental Fig. S3).

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 NRG1 β -induced erbB receptor autophosphorylation (Fig. 4), but Bim itself did not affect NRG1 β -induced erbB receptor phosphorylation (Supplemental Fig. S3). Furthermore, pretreatment with a direct PKC activator, PMA, mimicked the ET-1 response by strongly attenuating NRG1 β -induced erbB receptor autophosphorylation (Fig. 4). PKC may directly target erbB2/4 receptors or alternatively may function through an intermediary kinase

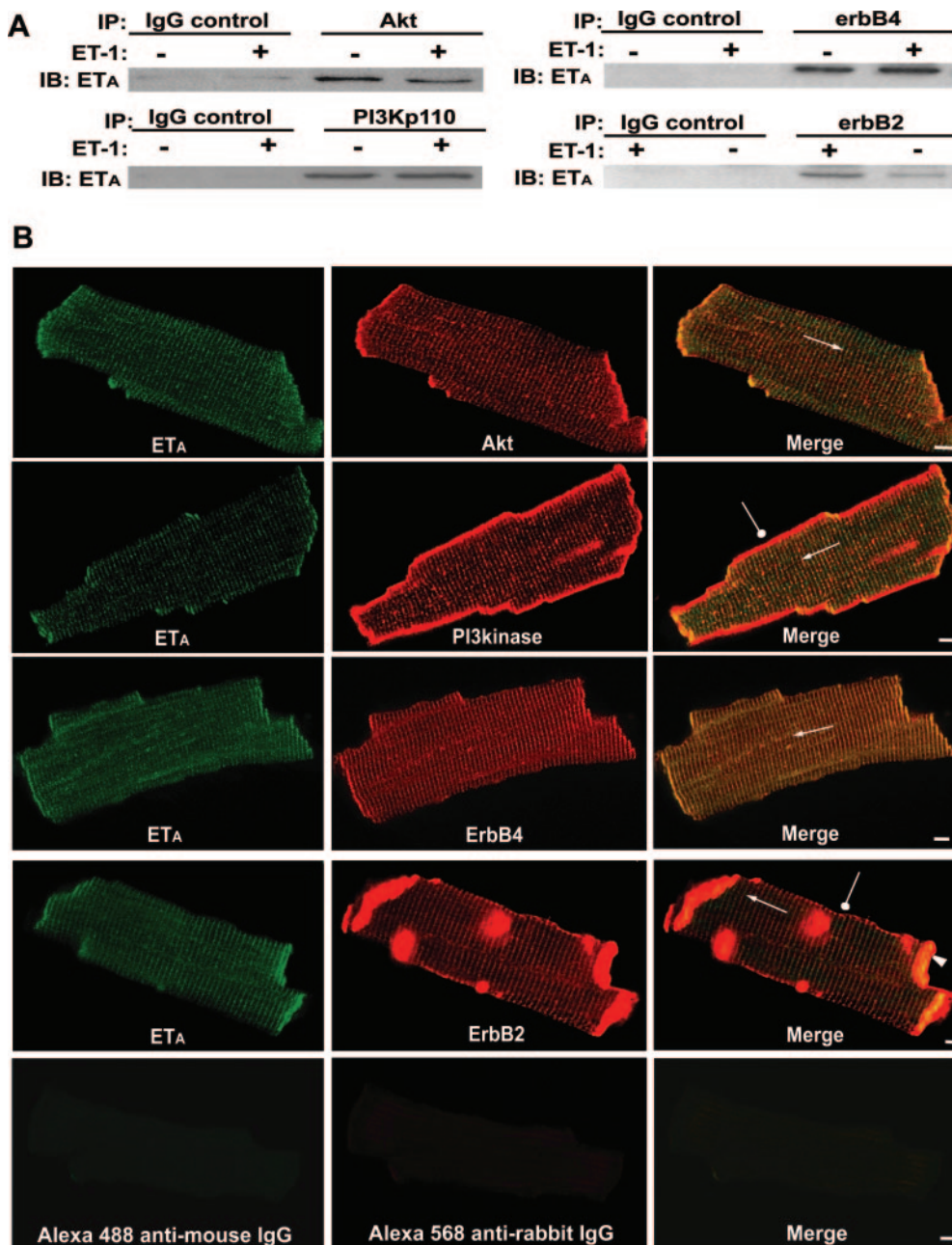


Fig. 1. Complex formation and colocalization of ET_A with Akt, PI3 kinase, and erbB2/4. **A**, isolated adult myocytes were solubilized in lysis buffer A, and proteins were immunoprecipitated (IP) by the antibodies indicated. Immunoprecipitates were blotted (IB) with an ET_A antibody. Lack of effects 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 under *Materials and Methods*). Arrows mark T-tubules, blunt arrows mark surface sarcolemma, and arrowheads mark intercalated discs. Scale bar, 10 μ m.

cascade. To begin to address this question, we examined the involvement of the Raf-1/MEK/Erk cascade, which was also found to immunoprecipitate 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 commer-

cial MEK antagonists, U0126 and PD98059, in our system (Fig. 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

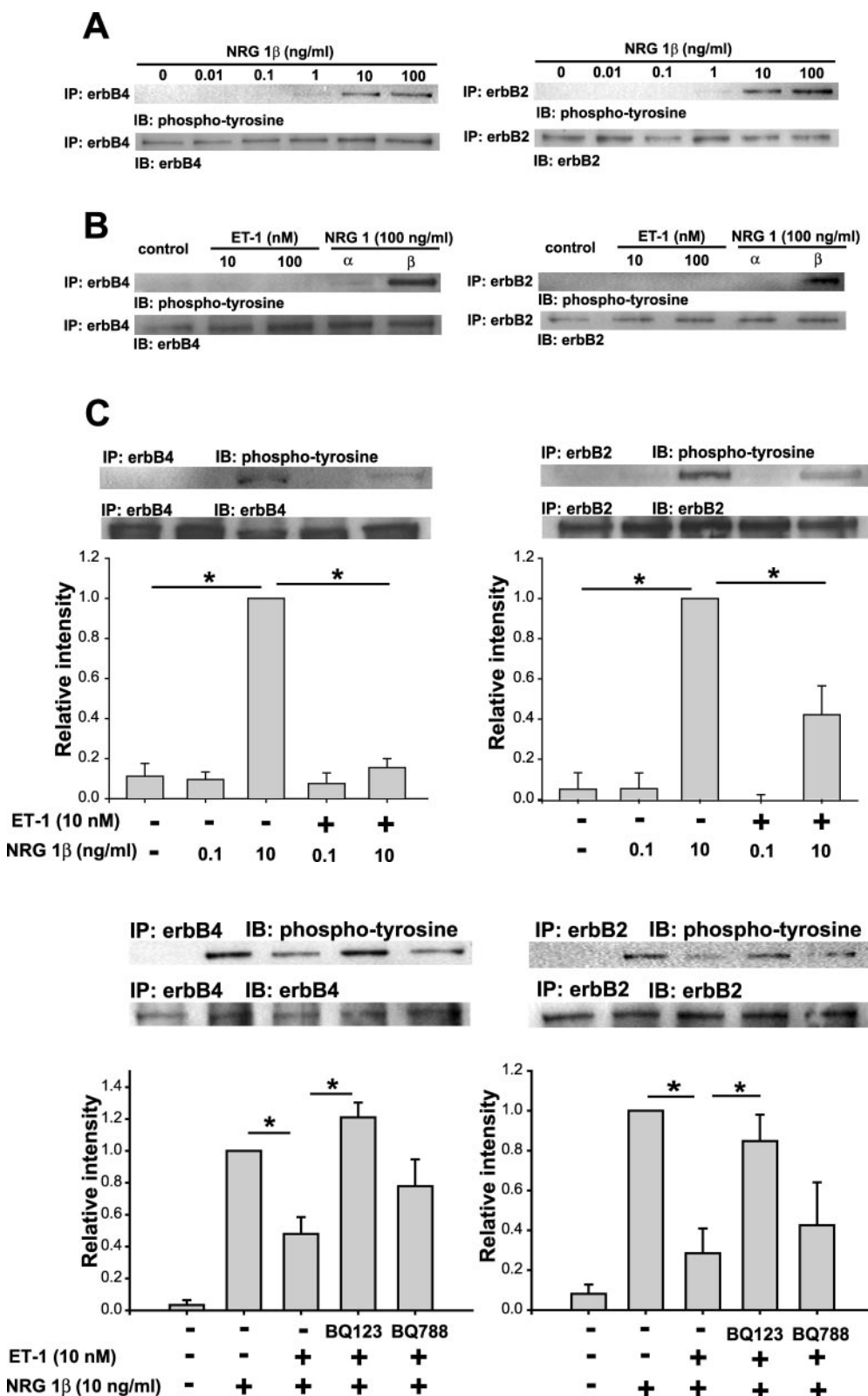


Fig. 2. Attenuation of NRG1 β -induced erbB2/4 receptor tyrosine phosphorylation by ET-1. A, myocytes were incubated with NRG1 β in a concentration-dependent manner for 10 min at 37°C. Cells were solubilized, subjected to immunoprecipitation (IP), and blotted (IB) with indicated antibodies. B, myocytes were incubated with ET-1, NRG1 α , or NRG1 β for 10 min at 37°C and then analyzed as in A. C, myocytes were pretreated with ET-1 for 10 min followed by NRG1 β for 10 min at 37°C and then analyzed as in A. *, $p < 0.05$ ($n = 4$) by t test, and one-way ANOVA confirmed nonidentity of means of NRG-treated samples. For Figs. 2 through 6, blots are representative of a single experiment; bar graphs represent the combined results of three to five separate experiments in which the ratio of blot intensities to loading controls is shown.

Fig. 3. Pharmacological analysis of the inhibitory effect of ET-1 on erbB2/4 tyrosine phosphorylation. Myocytes were incubated with ET_A antagonist BQ123 (2 μ M) or ET_B antagonist BQ788 (2 μ M) for 5 min followed by ET-1 for 10 min and then treated with NRG1 β 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 nonidentity of means of NRG-treated samples.

effect on erbB2/4 tyrosine phosphorylation (Supplemental Fig. S3).

An analogous series of experiments was carried out with Akt-1/Akt-2 serine phosphorylation serving as the downstream effector of RTK stimulation. NRG1 β promoted robust phosphorylation of Akt, but ET-1 treatment did not, even when evaluated over a range of concentrations and incubation times (Fig. 6A). However, as observed with erbB2/4 tyrosine phosphorylation, ET-1 pretreatment inhibited NRG1 β -mediated Akt phosphorylation (Fig. 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 NRG1 β signaling through erbB2/4 at both an early step (receptor autophosphorylation) and a subsequent step (Akt phosphorylation).

Finally, cross-talk between ET-1 and NRG1 β was examined at the level of physiological function by evaluating inotropic responses in electrically paced myocytes. NRG1 β promoted a statistically significant ~20% decrease in twitch amplitude that developed within 5 to 10 min and was often transient, returning toward control twitch levels after 15 min (Supplemental Fig. S4). Pretreatment with 10 nM ET-1 blocked this early onset negative inotropic effect (Fig. 7), again consistent with its inhibitory effects on erbB2/4 tyrosine phosphorylation and Akt serine phosphorylation.

Discussion

In this study, we investigated the components of ET-1 signaling complexes in adult cardiac myocytes by immunoprecipitating ET_A and identifying coimmunoprecipitated proteins by mass spectrometry. A number of proteins were identified that were reported previously to be functionally associated with ET_A such as G α_q , PLC- β , PKC ϵ , or PKC δ . Evidence was also obtained for the presence of a mitogen-activated protein kinase module, Raf-1/MEK/Erk. It was unexpected that erbB2/4 and their downstream signaling molecules such as PI3 kinase and Akt were also present in ET_A coimmunoprecipitates. 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 colocalization and cross-talk between a GPCR and RTK in adult cardiac myocytes. EGFR (erbB1), which may be expressed in cardiac myocytes, was not coimmunoprecipitated with ET_A possibly because of reduced expression compared with 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 coimmunoprecipitated under the conditions used.

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 believed to promote cardiac growth during development and during enlargement of diseased ventricles (i.e., cardiac hypertrophy) (Kedzierski and Yanagisawa, 2001; Negro et al., 2004; Shah and Catt, 2004). Second, studies in a variety of cell types, including neonatal cardiac myocytes have revealed a common form of cross-talk between 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 seems to hold true for erbB2/4 in adult ventricular myocytes, in which activation by NRG1 β stimulated tyrosine autophosphorylation and downstream activation of Akt. It is interesting that NRG1 β also promoted a negative inotropic response in adult ventricular myocytes, as observed previously in papillary muscles (Lemmens et al., 2004). Elucidating precise intracellular mecha-

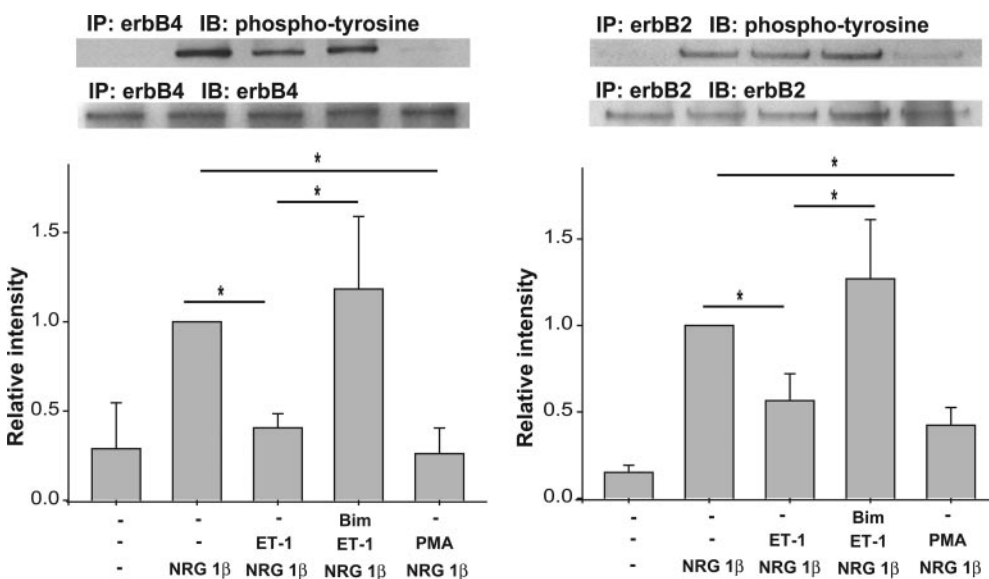


Fig. 4. Involvement of PKC. Isolated myocytes were preincubated with a PKC inhibitor (Bim, 1 μ M) or vehicle for 10 min followed by 10 nM ET-1 or 1 μ M PMA for 10 min and treated with 10 ng/ml NRG1 β for 10 min at 37°C. ErbB4 (left) and erbB2 (right) receptors were immunoprecipitated from myocyte extracts solubilized in buffer A and blotted with indicated antibodies. *, $p < 0.05$ ($n \geq 4$) by t test, and one-way ANOVA confirmed nonidentity of means of NRG-treated samples.

nisms 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 G α_q -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-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 re-

ported in any tissue. In studies of neonatal myocytes, ET-1 was shown to transactivate EGF receptors (Asakura et al., 2002; Anderson et al., 2004), whereas another study in the same system showed inhibition of EGF receptor-mediated Akt phosphorylation upon activation of G α_q (Sabri et al., 2002). In renal mesangial cells, Grewal et al. (2001) found that GPCRs desensitize and down-regulate EGF receptors. 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/AT₁ receptor activation, which inhibits insulin receptor function in a variety of tissues, including adult myocardium (Velloso et al., 2006). This cross-talk seems 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.

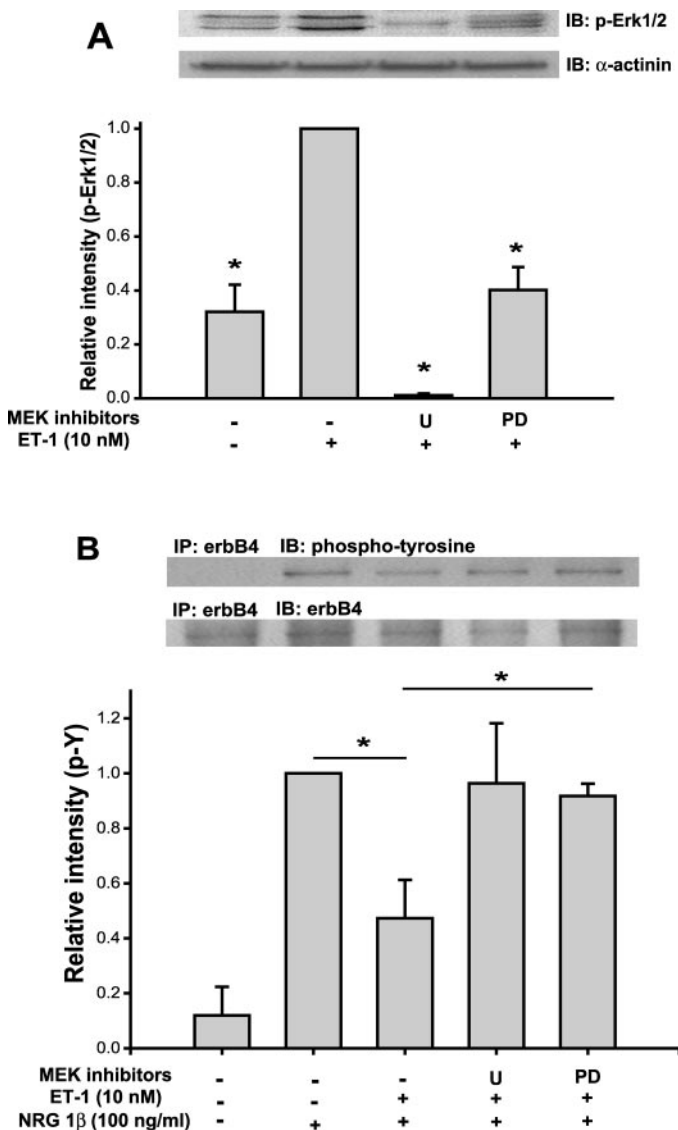


Fig. 5. Involvement of MEK/Erk. **A**, Western blot with a phospho-Erk1/2 antibody before and after 10 nM ET-1 for 10 min and the effects of two MEK antagonists, U = 10 μ M U0126, and PD = 50 μ M PD98059. α -Actinin was used as a loading control. **B**, effects of MEK antagonist pretreatment on inhibitory cross-talk using standard doses of ET-1 and NRG1 β . erbB4 was used as a loading control. *, $p < 0.05$ ($n = 4$) by t test, and one-way ANOVA confirmed nonidentity of means of NRG-treated samples.

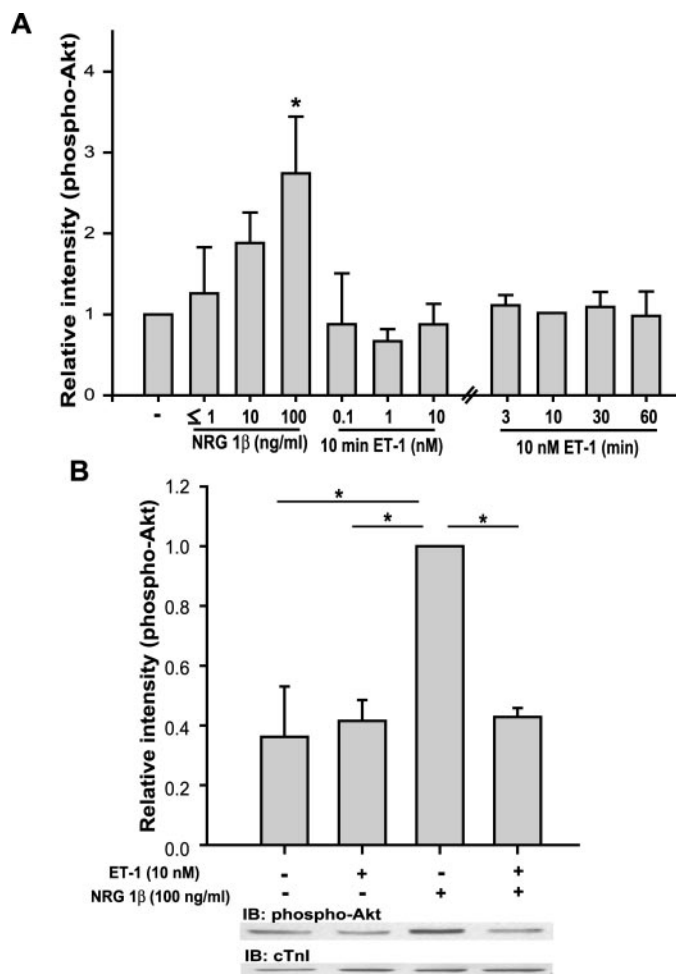


Fig. 6. Attenuation of NRG1 β -induced Akt phosphorylation by ET-1. **A**, myocytes were treated with increasing doses of NRG1 β or ET-1 for 10 min at 37°C, and Akt activation was measured by Western blotting with a phospho-Akt specific antibody. Incubation time in 10 nM ET-1 was also varied between 3 and 60 min. **B**, myocytes were treated with ET-1 for 10 min followed by NRG1 β 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.

The physiological role of this inhibitory cross-talk remains to be established. In this study, ET-1 inhibited the negative inotropic effects of NRG1 β , suggesting a role in potentiation of ET-1's positive contractile effect on the heart. It is interesting that 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 NRG1 β (Supplemental Fig. S5). Reciprocal cross-talk of this type (RTK to GPCR as opposed to GPCR to RTK) may be less well-developed as also found for communication between angiotensin II/AT₁ and insulin receptors (Velloso et al., 2006).

ET_A localizes with its downstream signaling molecules such as PLC β and PKC ϵ 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. It is interesting that 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 are reversed completely (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 because erbB2/4 activation is believed to confer strong cardioprotective and antiapoptotic signals in adult myocardium (Zhao et al., 1998; Grazette et al., 2004). Therefore, long-term ET-1 stimulation may interfere with normal prosurvival signaling through erbB2/4 receptors, thereby shifting the balance toward cardiomyopathy. Such a long-term 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 G α_q , initially induced cardiac hypertrophy, whereas long-term 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 G α_q 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 antagonists to minimize disruption of the balance of these autocrine/paracrine inputs in the heart.

In conclusion, we have used a combination of semiquantitative approaches including immunoprecipitation/proteomics, immunoblotting, and subcellular localization by confocal microscopy to provide evidence for interactions and colocalization of ET_A and erbB2/4 signaling systems in adult ventricular myocytes. A pharmacological analysis 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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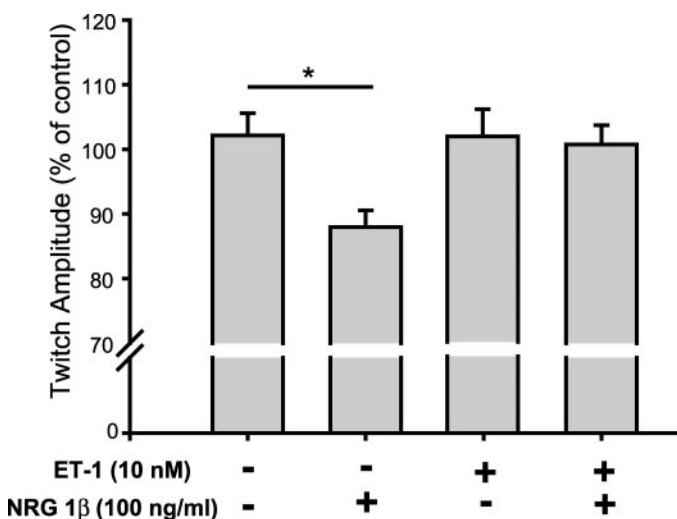


Fig. 7. Attenuation of NRG1 β -induced negative inotropic response by ET-1. Myocytes were stimulated electrically, and cell shortening was measured 5 min after the addition of NRG1 β as described under *Materials and Methods*. For ET-1-pretreated cells, NRG1 β was added after the ET-1-positive inotropic response stabilized (15–20 min; Supplemental Fig. S3). Control is the time point when NRG1 β was added. *, $p < 0.05$ ($n \geq 5$) by t test, and one-way ANOVA confirmed nonidentity of mean values.

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