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Biochemical and Biophysical Research Communications 308 (2003) 23–28
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Neuregulin-1 suppresses muscarinic receptor expression and acetylcholine-activated muscarinic K^+ channels in cardiac myocytes

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Received 24 June 2003

Abstract

The neuregulin-1 family of growth factors regulates nicotinic acetylcholine receptor synthesis in skeletal muscle, but its role in cardiac myogenesis remains unclear. Here, we investigate the involvement of neuregulins in the development of cardiac cholinergic responsiveness. Treatment of chick cardiac myocytes with neuregulin-1 inhibited mRNA expression of the M4 muscarinic receptor, but not the M2 receptor. In addition, mRNA levels of GIRK1 were reduced in myocytes by treatment with neuregulin-1. Activation of cholinergic receptors in cultured chick atrial myocytes by carbachol produced an outward potassium current ($I_{K(ACh)}$), which was attenuated by 24–48-h pre-treatment with neuregulin-1. These data suggest that neuregulins can regulate cardiac parasympathetic tone and may be involved in the pathogenesis of cardiac arrhythmias and heart failure.
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Keywords: Acetylcholine; Acetylcholine receptor inducing activity; Arrhythmia; Glial growth factor; Heart failure; Heregulin; Ion channels; Neu differentiation factor; Receptors

Neuregulins are a family of growth factors that include acetylcholine receptor inducing activity (ARIA), glial growth factor (GGF), heregulin, and neu differentiation factor (NDF) [11,17,24,27]. Neuregulin transcripts have been detected in the endocardium of rodent [6,19,26] and chick hearts [12], while their receptors (erbB receptors) are present in the myocardium and in mesenchymal cells of the developing atrioventricular valves [15,21,26]. Neuregulins have been shown to play an essential role in cardiac development. Targeted mutations of neuregulin and its receptors in mice by homologous recombination were shown to result in embryonic lethality around embryonic day 10 (E10) and were associated with severe defects in ventricular morphogenesis in the developing heart [9,15,19,21,26]. Many possibilities exist that may explain the death of the embryos, including reduced contractility and perfusion resulting from the lack of ventricular trabeculae in the heart or changes in cardiac excitability due to alterations in ion channel expression in myocytes. However, a specific role for neuregulins in myocardial development has not been elucidated.

Neuregulins are expressed in motor neurons [6] and have been shown to increase nicotinic acetylcholine (ACh) receptors [11] and voltage-gated sodium channels [5] in skeletal muscle. It is plausible that neuregulins may play similar roles in cardiac myogenesis. The heart is innervated by afferent nerves of both the sympathetic and parasympathetic nervous systems. Neuregulins are expressed in the neurons of the sympathetic ganglia and dorsal motor nucleus of the vagus nerve [6], although it is presently unknown whether neuregulins are expressed in the cardiac autonomic ganglia. Cholinergic parasympathetic responsiveness in the heart is mediated through ACh release from the vagal inputs to the SA node [22]. The modulation of cardiac activity by ACh is mediated by M2 and M4-type muscarinic acetylcholine

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01319-6

receptors [13]. These receptors are coupled to G-proteins, which activate a hyperpolarizing inwardly rectifying potassium channel. The ACh-induced potassium current ($I_{K(ACh)}$) produces both negative inotropic and chronotropic effects on the heart [22,35]. $I_{K(ACh)}$ in the heart is carried by a heteromultimer of two inwardly rectifying potassium channel subunits GIRK1/Kir 3.1/KGA and GIRK4/Kir 3.4/CIR [34]. GIRK channels are activated directly by interaction with the βγ subunits of a pertussis toxin-sensitive G-protein [20].

In this study, we investigated whether neuregulins are involved in the regulation of functional cholinergic responsiveness in the heart. Specifically, we investigated whether neuregulins influence the expression of mRNA coding for M2 and M4 muscarinic receptors as well as the GIRK1 and GIRK4 subunits of the ACh-sensitive inward rectifying K^+ channel in cultured chick heart cells. We also examined whether neuregulins can regulate ACh-sensitive ionic currents in cardiac myocytes.

Materials and methods

Heart cell cultures. Cultures of embryonic chick myocytes were prepared as previously described [12]. Briefly, hearts were removed aseptically from 14 day chick embryos, minced, and subjected to trypsin (0.01% in PBS) digestion at 37 °C. Dissociated cells were pooled, centrifuged, and resuspended in 10 ml of myocyte growth medium (MGM; DMEM with glutamax, 10% fetal bovine serum, 50 U penicillin, and 50 μg/ml streptomycin). Dissociated cells were preplated for 45–60 min in uncoated tissue culture treated dishes to reduce the number of fibroblasts. Unattached cells (myocytes) were removed and plated at a density of $2.5–3.5 \times 10^5$ cells/cm^2 in collagen coated plates for 2–6 days. Cultures were incubated at 37 °C in 10% CO_2.

Neuregulin-1 treatment and RT-PCR. Myocytes were treated with varying concentrations of recombinant neuregulin β1 EGF-like domain (EGF β1; residues 176–246 [17]; R&D Systems, Minneapolis, MN) for 24–48 h. Total RNA was isolated from myocytes using the RNAqueous RNA isolation system (Ambion). RNA (1 μg) was reverse transcribed using the Promega Reverse Transcription System. For detection of cardiac-specific gene transcripts by RT-PCR analysis (RT-PCR), PCR were performed in 50 μl of buffer containing 2 μl of the RT reaction product, 1× PCR buffer, 0.2 mM PCR nucleotide mix (Boehringer Mannheim), 1.25 U of Taq polymerase, and 25 nM of each sense and antisense primers for specific genes. PCR primers were designed using MacVector and based on sequences obtained from GenBank for chick GIRK1 [14], chick GIRK4 [31], and chick M2 [33] and M4 [32] muscarinic receptors. The primer nucleotide (nt) sequences used were: GIRK1, forward (nt 155–177), reverse (nt 431–411); GIRK4, forward (nt 4–27), reverse (nt 338–315); M2, forward (nt 712–734), reverse (nt 1146–1126); and M4, forward (nt 1590–1611), reverse (nt 2012–1993). PCR with all primers were linear between 25 and 45 cycles, and therefore 35 cycles were used in all reactions. All reactions were run on the PTC-100 Thermal Controller (MJ Research, Watertown, MA) for the appropriate number of cycles: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. In addition, a separate reaction was performed in parallel for each sample, using primers specific for chick actin [18], a transcript that remained at a constant level in the cultured heart cells. PCR products were electrophoretically separated on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and illuminated with UV light. The ethidium bromide signals were quantitated and analyzed using Kodak Scientific Imaging software. Relative mRNA levels were expressed as a ratio of the intensity of the individuals PCR products to the intensity of the actin control.

RNA isolation and Northern blot. Total RNA was isolated from embryonic chick hearts by homogenization in Ultraspec (Biotecx Labs, Houston) or from culture cardiac myocytes by first rinsing with PBS, adding Ultraspec directly to the dish, and then scraping cells into the solution. Total RNA was prepared according to the manufacturer's instructions and stored at −70 °C until used. Northern blot analysis was performed as described previously [6] using 5 μg of RNA per lane. Oligonucleotide probes were prepared from E14 chick heart RNA by RT-PCR amplification using the primers listed above and was used for random priming labeling using Prime-it II (Stratagene, La Jolla, CA). Quantitation of transcripts from Northern blots was performed using a Molecular Dynamics (Sunnyvale, CA) phosphoimager.

[^{3}H]QNB binding. [^{3}H]QNB Binding was carried out as previously described [29]. Briefly, binding experiments were performed on myocytes maintained in culture and treated with neuregulin-1 as described above. The plating medium was removed and the cells were rinsed twice with Earle's balanced salt solution containing 1 mg/ml bovine serum albumin (BSS/BSA). The cultures were incubated in BSS/BSA containing 0.1–0.2 nM of [^{3}H]QNB (39–40 Ci/mmol, Amersham). Nonspecific binding was defined as the amount of [^{3}H]QNB bound in the presence of 10 μM atropine. After 90 min at 37 °C, the cells were washed three times with ice-cold BSS/BSA and then dissolved in 1 M NaOH containing 0.5 mg/ml sodium deoxycholate. Lysates were transferred to vials containing scintillant and vigorously vortexed. Radioactivity was measured by liquid scintillation counting. Each point was determined in triplicate.

Recording of ACh-activated whole-cell currents ($I_{K(ACh)}$). For electrophysiological experiments, atrial myocytes were plated at a density of 5×10^3/cm^2 in collagen-coated 35 mm dishes and treated as before. Single cardiomyocytes were selected for patch-clamp recordings using the whole-cell variation of the patch-clamp technique [16]. The cells were held voltage-clamped using an Axopatch 200-A amplifier (Axon Instruments, Foster City, CA). Cells were placed in recording chamber and perfused continuously with extracellular solution at a rate of 1 ml/min. Patch pipettes (3–8 MΩ resistance) were prepared with borosilicate glass (VWR, Cat. # 53432-921). External solutions were exchanged with a rapid perfusion. $I_{K(ACh)}$ was activated by extracellular application of 100 μM carbachol, an esterase-insensitive ACh receptor agonist, for 2 s using a U-tube [1] and recorded at a holding potential of 0 mV. Data were stored using pClamp data acquisition programs. Membrane capacitance was determined online using the acquisition software program. For voltage-clamp recordings, the solutions used contained the following (in milliMolar) electrode solution: KCl 140, $MgCl_2$ 1, NaATP 5, NaGTP 0.2, EGTA 5, and Hepes 10, pH 7.4. L-15 medium was used as the external bath solution. All experiments were carried out at 22–23 °C. The current density was calculated as the peak current amplitude divided by the cell membrane capacitance to exclude the effect of different cell size.

Results

Downregulation of muscarinic receptor expression in chick heart cells by neuregulins

Cholinergic responsiveness in the heart is mediated by muscarinic acetylcholine receptors. Since the M2 and M4 muscarinic receptors are the only muscarinic receptors capable of activating $I_{K(ACh)}$ [13], we investigated whether the expression of these receptors was regulated by neuregulins using RT-PCR. Embryonic day 14 (E14) chick heart cell cultures were treated with 1 nM of the

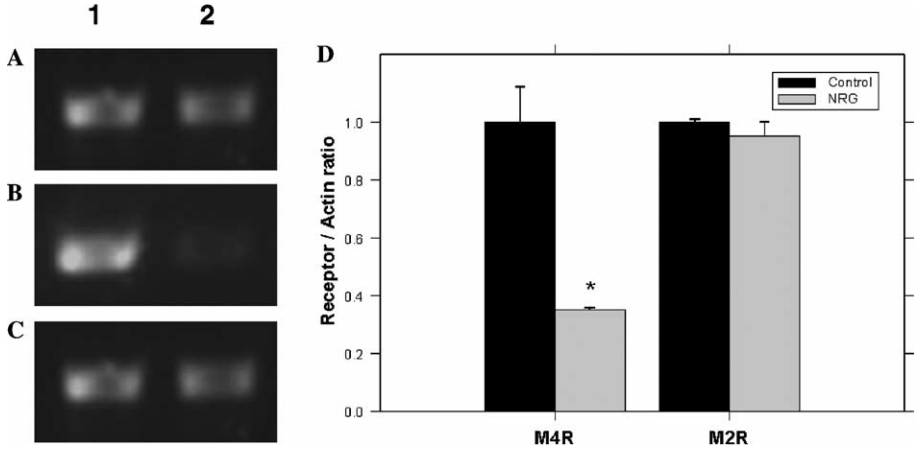

Fig. 1. Downregulation of muscarinic receptor mRNA expression by EGFβ1. M2R (A) and M4R (B) mRNA expression was examined in control and EGFβ1-treated E14 chick heart cells. Actin mRNA expression (C) was used to verify equal amounts of cDNA that were used in each reaction. After 24–48-h treatment of cells with medium containing 1 nM EGFβ1 or control medium, total RNA was collected and reverse transcribed into cDNA. M2R and M4R cDNAs were amplified by PCR and fractionated on a 1.5% agarose gel. Signal intensity was determined by densitometry. The graph (D) shows the average percentage of change ± SEM in M2R ($n = 5$) and M4R ($n = 3$) mRNA levels from EGFβ1-treated heart cells compared to control values after normalization to actin (*denotes $p < 0.05$).

EGF-like domain of the β1 isoform of neuregulin-1 (EGFβ1) or control medium for 24 h. Equal amounts of total RNA were prepared, transcribed into cDNA, and analyzed for the presence of mRNAs encoding M2 (M2R) and M4 (M4R) muscarinic receptors. Primers for M2R and M4R generated single bands of 435 and 423 bp, respectively (Figs. 1A and B). Treatment of heart cell cultures with EGFβ1 for 24 h resulted in a 85.3 ± 15.2% reduction of M4 muscarinic receptor (M4R) mRNA expression (Fig. 1B). However, only a slight reduction of M2 receptor (M2R) mRNA expression was seen after EGFβ1 treatment (Fig. 1A). Actin mRNA levels remained constant throughout the treatment. (Fig. 1C). Muscarinic receptor protein levels, determined by [^{3}H]QNB binding, were slightly reduced in response to EGFβ1 treatment (data not shown). This is consistent with the observation that the M2 receptor is the predominant form of muscarinic receptor found in chick heart, while M4 receptors comprised a small proportion of the total population of muscarinic receptors [25].

Neuregulins regulate GIRK1 expression in chick heart cells

To determine whether the effect of neuregulins on $I_{K(ACh)}$ was related to alterations in GIRK expression, we treated E14 chick heart cell cultures with EGFβ1 for 24 h and analyzed GIRK1 and GIRK4 mRNA expression using RT-PCR. Amplification products of 277 bp (GIRK1) and 335 bp (GIRK4) were observed for each pair of primers (Figs. 2A and B). Treatment of cells with EGFβ1 resulted in a suppression of GIRK1 mRNA expression (Figs. 2A and C). GIRK1 mRNA was reduced by 51.6 ± 10.6%. EGFβ1 did not alter the expression of GIRK4 in the cell cultures (Fig. 2B). No decrease in GIRK1 expression was noted when cells were treated with the α isoform of the

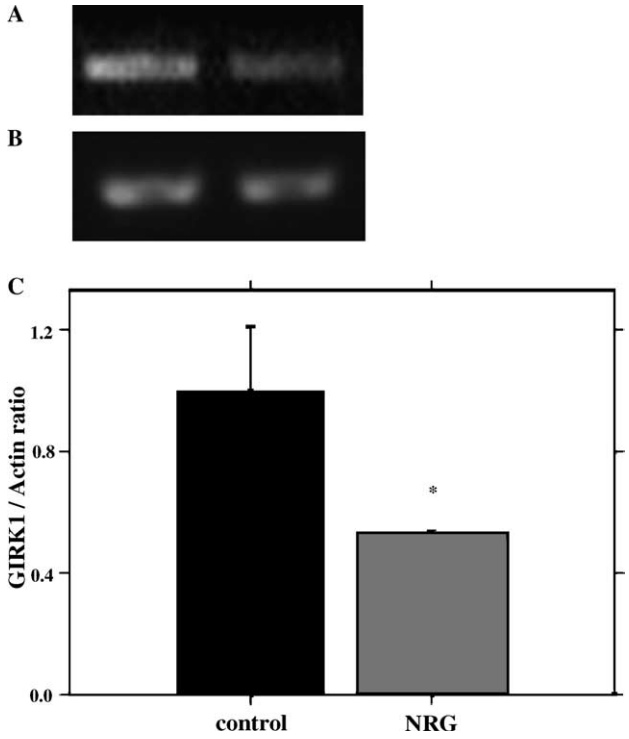

Fig. 2. EGFβ1 downregulates GIRK mRNA expression. GIRK1 mRNA expression was examined in control and EGFβ1-treated E14 chick heart cells. After 24–48-h treatment of cells with medium containing1 nM EGFβ1 or control medium, total RNA was collected and reverse transcribed into cDNA. GIRK1 (A) and GIRK4 (B) cDNAs were amplified by PCR and fractionated on a 1.5% agarose gel. The graph (C) shows the average percentage of change ± SEM in GIRK1 ($n = 5$) mRNA levels from EGFβ1-treated heart cells compared to control values after normalization to actin (* denotes $p < 0.05$).

neuregulin-1 EGF-like domain at concentrations ranging from 0.1 to 100 nM (data not shown).

Characterization of the neuregulin-induced downregulation of GIRK1

The time course for the neuregulin-stimulated downregulation of GIRK1 mRNA in E14 chick heart cells was also determined. Fig. 3A shows a Northern blot measuring the time course of NRG mRNA expression after the administration of EGFβ1 to E14 myocyte cultures. Cells were treated with EGFβ1 at the indicated time intervals. The level of GIRK1 mRNA was decreased to 25% of control within 3 h of EGF β1 treatment. The time course of neuregulin-induced GIRK1 downregulation in chick heart cells was also characterized by RT-PCR analysis (Fig. 3B). The expression of GIRK1 mRNA was reduced by EGF β1 within 1 h of treatment and remained suppressed for >24 h in the continued presence of EGFβ1. In control, untreated cultures, GIRK1 expression remained constant over this 24 h period.

Neuregulins suppress acetylcholine-activated potassium currents in chick heart cells

To investigate the role of neuregulins in the regulation of cardiac cholinergic responsiveness, we treated E14 chick atrial cells with EGFβ1 and determined the ability of cells to produce ionic currents in response to carbachol application. Isolated, spontaneously beating chick atrial myocytes were patch-clamped in whole-cell configuration when the holding potential was set at −70 mV. $I_{K(ACh)}$ was activated by the extracellular application of 100 μM carbachol and recorded at a holding potential of 0 mV. Fig. 4 demonstrates the characteristic currents seen in control and EGFβ1-treated cells following a two-second application of carbachol. Carbachol evoked an outward current ($I_{K(ACh)}$) in control atrial myocytes with an average current density of 3.03 ± 0.54 pA/pF ($n = 18$) (Figs. 4A and C). To determine whether neuregulins can regulate the responsiveness of atrial cells to carbachol, cells were pre-treated with 1 nM EGFβ1 for 24–48 h prior to electrophysiological studies. EGFβ1 treatment of atrial cells resulted in a 47.9% reduction in the ACh-induced current (Figs. 4B and C). Atrial cells treated with EGFβ1 produced current of 1.73 ± 0.31 pA/pF ($n = 18$) after carbachol application. The current density varied markedly among individual cells ranging from 0.6 to 9.6 pA/pF in control cells and from 0.5 to 4.6 pA/pF in

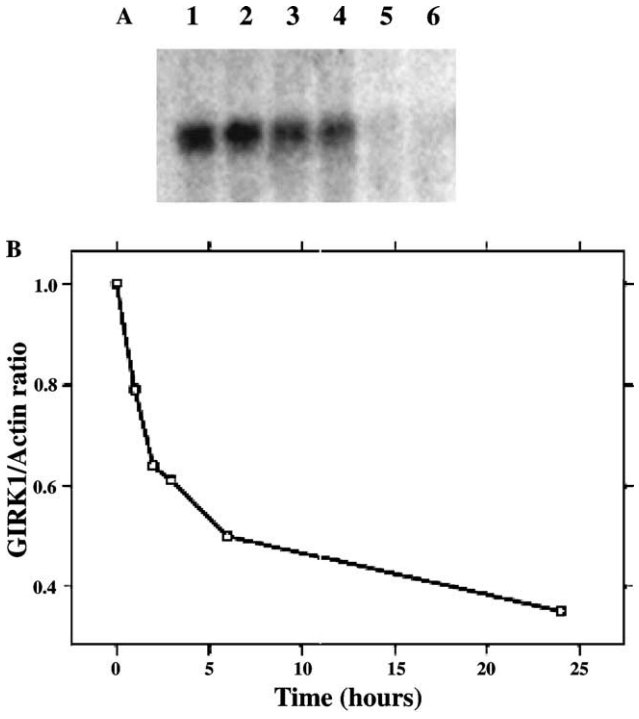

Fig. 3. Time course GIRK1 and M4R mRNA down-regulation by EGFβ1 in E14 chick heart cells. (A) Downregulation of GIRK1 mRNA (A) over time in E14 chick heart cells treated with 1 nM of EGFβ1. Lanes are: 0 h (1,2); 1 h (3,4); 3 h (5); and 6 h (6). (B) RT-PCR analysis of GIRK1 mRNA expression following treatment with 1 nM EGFβ1 ($n = 2$).

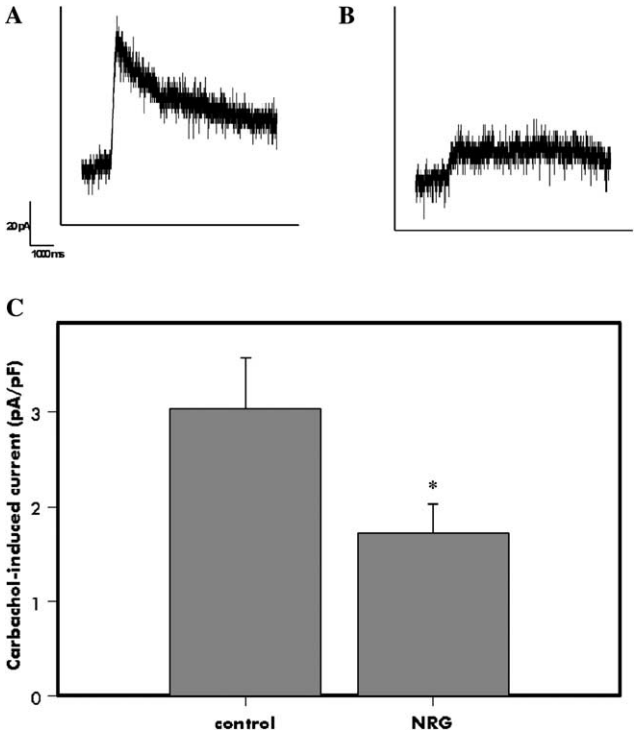

Fig. 4. Suppression of ACh-induced potassium currents ($I_{K(ACh)}$) by EGFβ1. E14 chick atrial cells were treated 24–48 h with 1 nM EGFβ1 or control medium. $I_{K(ACh)}$ was activated by extracellular application of 100 μM carbachol and recorded at a holding potential of 0 mV using the patch-clamp method. Representative traces of $I_{K(ACh)}$ from control (A) and EGFβ1-treated (B) atrial myocytes. (C) Graph shows average $I_{K(ACh)}$ from control ($n = 21$) and EGFβ1 ($n = 21$) treated atrial myocytes. Current density was calculated by dividing the peak amplitude of $I_{K(ACh)}$ divided by the cell membrane capacitance. Data are shown as means ± SEM (* denotes $p < 0.05$).

EGFβ1-treated cells. All cells in this study responded to carbachol treatment. No response was seen in myocytes when only extracellular solution was applied to the cells (not shown). No significant difference in membrane capacitance was noted between control and EGFβ1-treated cells.

Discussion

The results of this study demonstrate that neuregulins decrease cholinergic receptor signaling in embryonic cardiac myocytes. To our knowledge, this is the first published report describing the regulation of ACh receptors or ion channel expression and function in the heart by neuregulins. Perhaps, the most intriguing observation in this study was the acute inhibition of GIRK1 expression. Inward rectifying K^+ channels are known to participate in setting the resting membrane potential and regulating heart rate in cardiac myocytes [34]. The intrinsic embryonic heart rate is developmentally regulated and precisely optimized for the cardiac output required at a specific embryonic stage [3,7]. Alterations in heart rate dramatically decrease cardiac output and may be rapidly fatal [7]. In early cardiogenesis, GIRK channels are not expressed in the heart although all other components of the parasympathetic signaling pathway are present, including muscarinic receptors and G-proteins [23]. Based on our findings, we propose that the absence of GIRK channels in the embryonic heart myocytes results from suppression by high levels of neuregulin-1 expression in the endocardium. Thus, with the absence of neuregulin signaling in neuregulin-1 and erbB knockout mice, GIRK channels should be anomalously expressed. Consistent with this hypothesis, preliminary data from our laboratory demonstrate that while no GIRK1 mRNA is seen in E10 wild-type mouse embryos, GIRK1 is expressed in littermates lacking the neuregulin-1 gene (data not shown).

Aberrant expression of GIRK channels in the developing heart could lead to a decrease in myocardial excitability and reduced cardiac output. In fact, recent studies in neuronal cultures demonstrated that overexpression of GIRK channel subunits by adenovirus gene transfer in hippocampal cells resulted in an inhibition of action potential firing by increasing the threshold current for firing [8]. The data herein suggest a role for neuregulins in the development of cardiac excitability and may offer an explanation for the embryonic lethality of the neuregulin-1 and erbB knockout mice.

In the adult heart, the PNS appears to protect against the ischemia-induced arrhythmia and myocardium injury [30]. Vagal stimulation has been shown to terminate tachyarrhythmias, while reduced vagal tone is directly correlated with increased incidence of ventricular fibrillation. During the initial stages of heart failure, heightened sympathetic activity and reduced parasympathetic responsiveness are involved in compensatory mechanisms to sustain cardiac output and systemic perfusion [4]. The results of this study suggest that neuregulins can regulate the balance between sympathetic and parasympathetic responsiveness and consequently may play a role in modulating cardiac contractility and in the pathogenesis of cardiac arrhythmias.

Several lines of evidence suggest that neuregulins and their receptors may be involved in the development of cardiac hypertrophy and heart failure. Neuregulin-erbB signaling was shown to promote survival and induce hypertrophic growth in cultured rat cardiac myocytes [2,36]. A recent in vivo study found that the expression of neuregulin-1 and its receptors erbB2 and erbB4 was unchanged at the stage of compensatory hypertrophy in an aortic stenosis rat model of LV pressure-overload hypertrophy, however at the stage of early heart failure, erbB2 and erbB4 mRNA and protein were severely reduced [28]. Additional observations in humans implicate neuregulin-1 signaling via erbB2 receptors in the development of heart failure. Clinical trials of Herceptin, a monoclonal antibody directed against the erbB2 receptor, in breast cancer patients demonstrated a 5 to 6-fold increase in the percentage of patients who developed severe class III or IV heart failure in direct response to Herceptin treatment [10].

The results of this study demonstrate that neuregulins suppress the expression of muscarinic receptors and GIRK channels in the chick heart. The suppression of GIRK and ACh receptor gene expression by neuregulins significantly affect excitability in cardiac myocytes and may subsequently regulate cardiac performance during early embryogenesis and in the adult. These observations taken together also suggest that regulation of gene expression by neuregulin-erbB signaling pathways in the heart is cardioprotection and is involved in the transition from compensatory hypertrophy to heart failure.

Acknowledgments

This work was supported in part by National Institutes of Health Grant NS18458 and a National Science Foundation Postdoctoral Fellowship (B.D.F.).

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