Go protein subunit Go\(\alpha\) and the secretory process of the natriuretic peptide hormones ANF and BNP

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Abstract

Expression of the G protein subunit Go\(\alpha\) has been shown to be prominent in the atria of the rat heart and to be significantly associated with atrial natriuretic factor (ANF)-containing atrial-specific secretory granules by immunocytochemistry. In addition, differential expression profile analysis using oligonucleotide arrays has shown that the Go\(\alpha\) isoform 1 (Go\(\alpha\)1) is 2.3-fold more abundant in the atria than it is in the ventricles. In the present report, we show protein–protein interaction between Go\(\alpha\) and ANF by yeast two-hybrid and by immunoprecipitation. A cardiac conditional Go\(\alpha\) knockout model developed for the present study showed a 90% decrease in Go\(\alpha\) expression and decreased atrial expression and ANF and brain natriuretic peptides (BNP) content. Expression of chromogranin A, a specific atrial granule core constituent, was not affected. Morphometric assessment of atrial tissue showed a very significant decrease in atrial-specific granule density as well as granule core electron density. Atrial electrical activity was not affected. The results obtained are compatible with the suggestion that Go\(\alpha\) plays a role in ANF sorting during intracellular vectorial transport and with the presence of a mechanism that preserves the molar relationship between cellular ANF and BNP stores in the face of the decreased production of these hormones.

Key Words

- endocrine heart
- ANF
- G proteins
- hormone storage
- hormone secretion

Introduction

At least three types of secretory mechanisms for the natriuretic peptides (NP) atrial natriuretic factor (ANF, ANP) and the brain natriuretic peptides (BNP) operate in atrial cardiomyocytes, and they include constitutive, regulated, and constitutive-like secretions (Ogawa et al. 1999). The stimulated secretion of NP involves G protein-associated mechanisms. Neuroendocrine agonists, such as endothelin-1 (ET-1), angiotensin II, and catecholamines, are known to utilize receptors coupled to effectors by Gq proteins. On the other hand, ANF secretion promoted through atrial muscle stretch (stretch-secretion coupling) (Kuroski de Bold & de Bold 1991) utilizes a mechanism that is inhibited by pertussis toxin (PTX), a specific inhibitor of the Gi/o family of G proteins (Bensimon et al. 2004, McGrath & de Bold 2005). The stimulation of ANF secretion by ET-1 has been shown to proceed unimpaired in the atria from
PTX-treated rats, which shows that the Gq/11 and Gi/o pathways of signaling during stimulated ANF secretion are functionally independent and may function in parallel (Bensimon et al. 2004). This dual stimulatory pathway would be expected to be operational in hemodynamic overload conditions, such as chronic congestive heart failure, in which neuroendocrine activation coexists with increased intracardiac pressures that lead to increased atrial muscle stretch. Both agonist-mediated and stretch-mediated secretion of NP from atrial cardiomyocytes cause the release of newly transported peptides in preference over the older NP pool, as has been revealed by double-label pulse-chase studies (Mangat & de Bold 1993, Ogawa et al. 1999).

There is scant information regarding the details of G protein participation in agonist-promoted NP secretion. Expression of the G protein subunit Goα is prominent in the atria and is significantly associated with ANF-containing secretory granules, as has been shown by immunocytochemistry, and it is virtually absent in ventricular cardiomyocytes (Wolf et al. 1998, Bensimon et al. 2004). Differential expression profile analysis between rat atria and ventricles using oligonucleotide arrays revealed that the G protein Goα isoform 1 (Goα1) is 2.3-fold more abundantly expressed in the atria than in the ventricles (McGrath & de Bold 2009).

In the present report, we further explore the association between Goα and atrial secretory function by assessing Goα protein–protein interactions in atrial muscle using yeast two-hybrid screening and the biochemistry and morphology of atrial cardiomyocyte phenotype in transgenic mice carrying a conditional cardiac Goα knockout. In the latter, we studied the expression of secretory granule components, including ANF, BNP, and chromogranin A (ChgA), as well as ANF processing and the effect of the knockout on ultrastructure and on cardiac electrophysiological activity.

Materials and methods

Experimental animals

All of the experiments involving animals were approved by the Animal Care Committee of the University of Ottawa and were carried out according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Conditional Goα knockout

Goα-floxed mice with loxP sites flanking exons 5 and 6, which are common to both isoforms Goα1 and Goα2 (also Goz1 and Goz2) were generously provided by Dr Lutz Birnbaumer. NIEHS mice, strain 129SvEv, were used. The process for disrupting of this gene has been previously described (Chamero et al. 2011). α-myosin-heavy chain (α-MHC)-Cre mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), strain name B6.FVB-Tg(Myh6-cre)2182Mds/J, strain no. 011038. Expression of Cre, which is driven by the α-MHC (cardiac-specific) promoter, induces more than 90% recombination in cardiac muscle cells when two loxp sites are present.

Male mice that were obtained after a standard breeding scheme for F2 generation were used. Controls were C57BL/6 x129SvEv males.

Cre/lox generation

Standard Cre/lox tissue-specific breeding protocol was followed. Briefly, breeding a homozygous Goα-floxed mouse to a hemizygous α-MHC-Cre mouse produces 50% offspring that are heterozygous for the floxed allele that has been recombined in the presence of Cre to delete this allele in the heart (cGoα+/-). Breeding a homozygous Goα-floxed mouse to a hemizygous α-MHC-Cre produces 25% offspring that are homozygous for the floxed alleles that have both been recombined in the presence of Cre to delete both alleles in the heart (cGoα−−).

All of the genotypes were produced in Mendelian frequencies. Table 1 shows all of the genotypes obtained from the Cre/lox mating scheme and their respective transgenic titles and genotypes. The homozygous floxed mouse Goα flx/flx was used as the control mouse (Zhao et al. 2010).

Genotyping and primers

At 21 days of age, a 0.1 cm tail clipping was taken from each mouse. DNA extraction was performed using a REDExtract-N-Amp Tissue PCR Kit (XNAT, Sigma–Aldrich), and subsequent PCR amplification was performed using a REDExtract-N-Amp PCR Mix (XNAT, Sigma–Aldrich) in an Eppendorf Mastercycler Thermal Cycler (Eppendorf, Mississauga, Ontario, Canada). PCR parameters for genotyping the Goα-floxed alleles, the Cre alleles, and control alleles are listed in Tables 2 and 3. Oligonucleotide sequences for genotyping are listed in Table 2. Amplicons were run on a 1% agarose gel using a 1 kb ladder (G571A, Promega) for calibration purposes, except for Cre products, which were run on a 1.5% agarose gel using a 50 bp ladder to calibrate the migrations of the
amplicons (10488, Invitrogen). Primers were commercially synthesized by Operon (www.operon.com/).

Chromogranin A knockout mice
The generation of the Chga knockout mice has been previously reported (Hendy et al. 2006). Controls were Balb/c. Only male knockout and control mice were used.

Yeast two-hybrid
The methodology followed for yeast two-hybrid screening has been previously published (Ogawa et al. 2009). Briefly, poly (A)+ RNA was isolated from rat atria using the Absolutely mRNA Purification Kit (Stratagene, Agilent Technologies, La Jolla, CA, USA). An atrial tissue library was constructed using a HybriZAP 2.1 XR Library Construction Kit and a HybriZAP 2.1 XR cDNA Synthesis Kit (Stratagene). cDNA inserts were cloned into the HybriZAP 2.1 vector, and the HybriZAP 2.1 library was converted to the pAD-GAL-4-2.1 library (target protein) by mass excision, as described in the manufacturer’s instructions for the HybriZAP 2.1 XR Library Construction Kit and the HybriZAP 2.1 XR cDNA Synthesis Kit. DNA encoding amino acids 1–354 of the activated Q205L mutant of rat Goα1 (kindly provided by Brad Denker, Harvard Medical School, Cambridge, MA, USA) was cloned into the pBD-GAL4 Cam phagemid ‘bait’ vector (Stratagene) and cotransformed with w20!106 atrial cDNA clones contained within the pAD-GAL-4-2.1 ‘prey’ vector using yeast strain YRG-2 (Stratagene). Plasmid recovered from 92 positive clones that grew on triple dropout (-Trp/-Leu/-His) plates were sequenced. The yeast transformation was performed by the S. C. Easy Comp Transformation Kit (Invitrogen) according to the manufacturer’s instructions.

Plasma collection
Trunk blood was collected in 1.5 ml tubes that had previously been rinsed with 15% EDTA and centrifuged at 2000 g for 20 min at 4 °C, and the plasma was stored at −80 °C. The plasma samples were acidified with 100 μl 0.1 M HCl/ml plasma and were passed three times through Sep-Pak C18 cartridges (Millipore-Waters Corp., Milford, MA, USA) pre-wetted with 5 ml 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA), followed by 20 ml of 0.1% TFA. After processing, the cartridges that had received the plasmas were then eluted with 3 ml 80% ACN in 0.1% TFA. Samples were then freeze-dried and reconstituted in 220 μl of RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.01% sodium azide, 0.1% Triton X-100, and 0.1% heat-treated BSA).

RNA extraction and RT
Atrial appendages and the apical portions of the ventricles were immersed in 1 and 3 ml respectively of TRIzol Reagent (Invitrogen) immediately after removal and were stored at

Table 1  Genotypes obtained from F1 and F2 Cre/lox generation breeding scheme

<table>
<thead>
<tr>
<th>Goα genotype*</th>
<th>Transgenic title</th>
<th>Full genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (conditional) Goα→/→</td>
<td>Homozygous conditional knockout</td>
<td>αMHCcre; Goα flox/flx</td>
</tr>
<tr>
<td>cGoα+/→</td>
<td>Heterozygous conditional knockout</td>
<td>αMHCcre; Goα flox/wt</td>
</tr>
<tr>
<td>cGoα+/+</td>
<td>Homozygous floxed control</td>
<td>Wt; Goα flox/flx</td>
</tr>
<tr>
<td></td>
<td>Heterozygous floxed</td>
<td>Wt; Goα flox/wt</td>
</tr>
<tr>
<td></td>
<td>Hemizygous Cre</td>
<td>Wt; Goα Wt/wt</td>
</tr>
</tbody>
</table>

*Only the first and third genotypes were compared in the present report.

Table 2  Primers used for genotyping

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goα-floxed</td>
<td>AAGAATAGAAGCTAGGACCTGGAGG</td>
<td>GCAAGACAAGTGAAACAGATGCCTGAGAATCC</td>
</tr>
<tr>
<td>α-MHC-Cre</td>
<td>ATGACAAGACACAGATCCCTCTATCTCC</td>
<td>CTACCATCTCGTCTCCATATGGGC</td>
</tr>
<tr>
<td>TCR-α control</td>
<td>CAAATGTTGCTTGTCTGTTG</td>
<td>GTCAGTGGAGTCAGAGCATT</td>
</tr>
<tr>
<td>ANF WT</td>
<td>CTGTCACCAACAGATCTGAGT</td>
<td>CTGGTGACGCTATGGCCTAC</td>
</tr>
<tr>
<td>ANF mutant</td>
<td>CTGTCACCAACAGATCTGAGT</td>
<td>CTTCTTCAGCCCTTGCTG</td>
</tr>
<tr>
<td>ChgA WT</td>
<td>GATGAAATGTCGCTTGAAGTGCATCTCCGA</td>
<td>CTTGGAGAGCCAGGCTTGAAGTGCCTTTCA</td>
</tr>
<tr>
<td>ChgA mutant</td>
<td>ACAACAGCAATCGGCTGCT</td>
<td>CCATGGGTACAGCACAGGAT</td>
</tr>
</tbody>
</table>

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Further processing was done by homogenizing using a Polytron (PT10-35, Kinematica, Inc., Bohemia, NY, USA) at 70% power for 15–20 s and completing total RNA extraction according to the manufacturer’s instructions. The quality of the RNA extracted was assessed using a 2100 Bioanalyzer (Agilent Technologies) with an RNA 6000 Nano Kit (Agilent Technologies). cDNA was synthesized by a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) following the manufacturer’s instructions using 1 μg of total RNA.

### Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using LightCycler 480 SYBR Green I Master Mix and analyzed using the LightCycler 480 SW 1.5 quantification software (Roche Applied Science) according to the manufacturer’s instructions.

Primer nucleotide sequences were as published (Niu et al. 2005) or obtained from the literature and validated using Primer3 Input version 0.4.0 (http://frodo.wi.mit.edu). Primer nucleotide sequences are listed in Table 3. Glucose-6-phosphate dehydrogenase (G6PD) was used as the reference gene, and concentration ratios were normalized to the calibrator and corrected using primer efficiency. Analyses of each gene of interest were performed in triplicate. A standard curve was constructed in triplicate for each primer pair.

### Western blot

Freshly obtained tissues were placed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 1% Nonidet P-40, and 0.5% sodium deoxycholate) and 1:100 dilution of protease inhibitor cocktail (P8340, Sigma) and stored at −80 °C. The tissues were then homogenized using a Polytron at 70% power for 15–20 s, and the homogenate was centrifuged at 9300 g for 10 min at 4 °C. Protein quantification in the supernatant was determined using a Pierce BCA Protein Assay Kit (Pierce, Thermo Scientific, Ottawa, Ontario, Canada).

For electrophoretic separation, the samples were prepared by adding 7 μg of atrial protein to 3X Blue loading dye (7722, Cell Signaling Technology, Danvers, MA, USA) and 10% dithiothreitol (DTT) and boiling for 2 min at 95 °C. A 30 μl sample was loaded into a 10% MiniPROTEAN TGX precast gel (Bio-Rad Laboratories, Inc.) and run at 200 V for 45 min, along with a biotinylated ladder (Precision-Plus Protein Western C, Bio-Rad Laboratories, Inc.) and mouse brain extract (sc-2253, Santa Cruz Biotechnology, Inc.). The gel was transferred to a 0.45 μm PVDF membrane (Millipore-Waters Corp.) at 100 V for 1 h and blocked for 1 h in a buffer containing 5% fat-free milk in Tris-buffered saline, 0.1% Tween-20 (TBST). The membranes were then rinsed three times with TBST and incubated overnight at 4 °C with antibodies diluted in blocking buffer (Goα 1:2000 (polyclonal anti-Goα, 551, MBL Int., Woburn, MA, USA), α-tubulin 1:400 (sc-8035, Santa Cruz Biotechnology, Inc.)). This Goα antibody cross-reacts with rat and human Goα, but it does not bind Gαi2 or Gαi3. Membranes were rinsed three times with TBST and incubated for 1 h at room temperature with secondary antibodies conjugated to HRP diluted in blocking buffer (goat anti-rabbit 1:5000 (sc-2004, Santa Cruz Biotechnology, Inc.)).

### Table 3 Oligonucleotide primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td>CAGCTCTTGAGGACCAAGGG</td>
<td>AGACCCAGGCAAGTTCAGA</td>
</tr>
<tr>
<td>Goα</td>
<td>TGCACGAGTCTCTCATGCTCT</td>
<td>AGATGTCGAAGGTGACCTCT</td>
</tr>
<tr>
<td>ANF</td>
<td>GCCGGTAAAGATGAGGTCA</td>
<td>GGGCTCAAATCTCGTCACT</td>
</tr>
<tr>
<td>G6PD</td>
<td>CCAGCTCTCAAGACACCTCA</td>
<td>AATAGCCACGACCCTCAGA</td>
</tr>
</tbody>
</table>

### Table 4 Identification of molecules associated with Goα1 (Q205L) by yeast two-hybrid

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate conductance calcium-activated potassium channel</td>
<td>AF190458</td>
<td>95</td>
</tr>
<tr>
<td>Natriuretic peptide precursor type A (eight clones)</td>
<td>NM_012612</td>
<td>99</td>
</tr>
<tr>
<td>Ankyrin repeat domain 1</td>
<td>BC072699</td>
<td>98</td>
</tr>
<tr>
<td>Myosin, heavy polypeptide 6, cardiac muscle</td>
<td>NM_017239</td>
<td>94</td>
</tr>
<tr>
<td>Ankyrin repeat domain 1 (cardiac muscle)</td>
<td>NM_013220</td>
<td>95</td>
</tr>
<tr>
<td>Actin-binding Rho-activating protein</td>
<td>NM_175844</td>
<td>97</td>
</tr>
<tr>
<td>Clusterin</td>
<td>BC061534</td>
<td>99</td>
</tr>
<tr>
<td>Ubiquitin A-52 residue ribosomal protein fusion</td>
<td>NM_031687</td>
<td>99</td>
</tr>
</tbody>
</table>
Cruz Biotechnology, Inc.), goat anti-mouse 1:5000 (1858413, Pierce, Thermo Scientific), and Precision Protein StrepTactin 1:5000 (161-0380, Bio-Rad Laboratories, Inc.). Membranes were rinsed three times with TBST and were visualized using the FluroChem Alpha Ease ECL imaging system (Alpha Innotech Corp., San Leandro, CA, USA) following enhancement with Luminata Forte chemiluminescence (Millipore-Waters Corp.).

Electron microscopy

Tissues were finely minced in saline and fixed in Karnovsky’s fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4)) overnight; they were then washed three times in washing solution (0.1 M Sorensen’s phosphate buffer (pH 7.4), 10% sucrose, 1% calcium chloride) and post-fixed in 2% osmium tetroxide in phosphate buffer. The tissues were then dehydrated in a graded series of alcohols and embedded in Spurr resin. Tissue sections mounted in copper grids were stained with lead citrate and uranyl acetate and then viewed in a JEM-1230 electron microscope. Granule counting was carried out in randomly chosen fields at 3000× magnification.

NP extraction

Tissues were extracted in 3 ml of a solution containing 0.1 M HCl, 1.0 M acetic acid, and 1% NaCl using a Polytron at 70% power for 15–20 s. The homogenates were centrifuged at 10 000 g for 30 min at 4 °C, and the supernatants were processed using Sep-Pak C18 (Millipore-Waters Corp.) cartridges as described in the previous sections, separated into two aliquots, and freeze-dried. The freeze-dried samples were solubilized in either 0.1% TFA or RIA buffer for HPLC and RIA respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) analysis was carried out in a C18 column using a linear gradient elution profile from 15 to 85% of 80% ACN in 0.1% TFA at 1.5 ml/min with monitoring at 275 nm over a total period of 80 min. Fractions were collected every 2 min. One hundred microliters of an aqueous solution of 1 mg/ml BSA was added to each fraction before it was freeze-dried.

RIAs

RIAs for ANF and BNP were performed as previously described (Sarda et al. 1989, Bensimon et al. 2004).

Immunoprecipitation of Goα/ANF complexes

Immunoprecipitation of Goα/ANF complexes was carried out in atrial or ventricular homogenates that were obtained by homogenizing ~50 mg of tissue in 0.01 M phosphate buffer (pH 6.5) containing 1 µM GDP, I

### Table 5  
ECG parameters in mice. Values are averages of the same time point (1600–1900 h or 0400–0700 h) over 3 days. n = 3

<table>
<thead>
<tr>
<th>Phase/time</th>
<th>Genotype</th>
<th>RR-I (ms)</th>
<th>HR (bpm)</th>
<th>R-amp (mV)</th>
<th>P-amp (mV)</th>
<th>QRS-dur (ms)</th>
<th>PR-I (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest 1600–1900 h</td>
<td>Control</td>
<td>107.4864</td>
<td>569.1028</td>
<td>0.725167</td>
<td>0.087531</td>
<td>15.43949</td>
<td>34.4616</td>
</tr>
<tr>
<td>Rest 1600–1900 h</td>
<td>cGoα−/−</td>
<td>106.8187</td>
<td>572.3695</td>
<td>0.852373</td>
<td>0.076315</td>
<td>14.30988</td>
<td>33.22616</td>
</tr>
<tr>
<td>Awake 0400–0700 h</td>
<td>Control</td>
<td>107.071</td>
<td>576.5367</td>
<td>0.735165</td>
<td>0.083329</td>
<td>15.08417</td>
<td>33.66375</td>
</tr>
<tr>
<td>Awake 0400–0700 h</td>
<td>cGoα−/−</td>
<td>106.3489</td>
<td>576.0036</td>
<td>0.84694</td>
<td>0.079072</td>
<td>14.34907</td>
<td>33.42748</td>
</tr>
</tbody>
</table>

RR-I, interval between R waves; HR, heart rate; R-amp, amplitude of the R wave; P-amp, amplitude of the P wave; QRS-dur, duration of the QRS complex; PR-I, interval between the P wave and the R wave.
30 mM AlF₄⁻ (30 mM AlCl₃, 1.2 mM NaF), 6 mM MgCl₂, and 1% NaCl. Following centrifugation, the pellet was re-homogenized, and the supernatants were combined. After incubation overnight with 1 ml of undiluted ANF antibody, 20 ml of Protein A/G Plus Agarose (Santa Cruz Biotechnology, Inc.) were added and mixed by inversion for 1 h at 4°C. Following centrifugation at 400 g for 30–60 s, the resulting pellet was washed twice with 0.01 M phosphate buffer containing 1 mM GDP, 30 mM AlF₄⁻, 6 mM MgCl₂, and 1% NaCl. Electrophoretic separation of samples and western blotting was carried out as described above. Goα (polyclonal anti-G α, 551, MBL Int.) and ANF RyB8 antibody (an ANF-specific polyclonal antibody produced in our laboratory) were used at a concentration of 1:1333 and 1:2000 respectively. This Goα antibody cross-reacts with rat and human Goα, but it does not bind Gi2α and Gi3α.

**Electrocardiography**

ECG transmitters (ETA-F20, Data Science International, San Paul, MN, USA) were implanted in mice pretreated with s.c. buprenorphine (0.05 mg/kg) and anesthetized with isoflurane (1.5–2%). A 2–3 cm midline abdominal incision was made, and the telemetry unit was placed within the abdominal cavity. Telemetry data gathering began on post-surgery day 3 using the software supplied with the telemetry unit.

**Statistical analysis**

Data are reported as means±S.E.M. Student’s t-tests were performed to determine statistical significance between groups. P≤0.05 was considered significant.

**Results**

**Yeast two-hybrid and co-immunoprecipitation**

To determine whether the previously observed microscopic co-localization of Goα and ANF could reflect protein–protein interaction at the cellular level, two-hybrid and co-immunoprecipitation studies were conducted. Screening of a rat atrial cDNA library with a mutationally active form of Goα1 (Q205L) identified several cDNA clones of interest, including eight clones that contained open reading frames corresponding to proANF. No clones containing BNP sequences were found, and only single clones were detected for the other species listed in Table 4.

Immunoprecipitation of atrial tissue homogenates using anti-ANF antibodies produced complexes that upon electrophoretic and western blot analysis yielded bands consistent with protein–protein interaction between Goα and ANF. Immunoprecipitates isolated from ventricular tissue homogenates did not reveal bands for either ANF or Goα (Fig. 1).

These results support the view that activated forms of Goα and ANF interact to form a complex in vivo (Table 5).

**Cardiac Goα knockout**

Given the association between Goα and ANF that was found by yeast two-hybrid and co-immunoprecipitation,
we examined the role of Goα in atrial cardiomyocyte phenotype using a conditional Goα-floxed mouse in which two loxP sites exist in both alleles flanking the fifth and sixth exons common to the two variants (Goα1 and Goα2). By expressing Cre, the loxP sites recombine to delete the fifth and sixth exons of Goα, which thereby produces a truncated mRNA sequence and deletes the C-terminal functional amino acids (Jiang et al. 1997, Chamero et al. 2011). The N-terminus of Goα lacks the functional binding domains to its signaling pathways with adenylyl-cyclase and phospholipase C as well as its binding to the Gβγ complex (Birnbaumer 2007, Zhao et al. 2010).

Through the standard breeding of Goα-floxed mice and α-MHC-Cre mice, heart-specific conditional Goα knockout mice were derived after two generations of mating. Continuous breeding of α-MHC-Cre mice to Goα-floxed mice produced six genotypes of mice (Table 1). No Cre toxicity was observed in these mice (Baba et al. 2005), because no two animals expressing α-MHC-Cre were mated.

Genotyping of tail, ventricular, or atrial tissue using Goα-floxed primers to distinguish between the WT, heterozygous, and homozygous Goα-floxed and Goα-deleted genotypes showed a 2142 bp PCR product that represented the Goα allele and contained two loxP sites in the Goα gene (Goα-floxed alleles), an 1868 bp PCR product that represented the Goα WT allele, and a 442 bp PCR product that represented the Goα allele after recombination by Cre recombinase, which had thereby produced a deleted allele and truncated band.

Genotyping of tail, ventricular, and atrial tissue using α-MHC-Cre primers to distinguish between the mice carrying the Cre enzyme and those without the Cre enzyme showed a 300 bp PCR product that represented the presence of Cre in the genome (one Cre allele) and a 200 bp PCR product that represented the internal control in a sample without the presence of Cre.

**Goα, NP, and ChgA mRNA and protein expression**

In the atria of cGoα−/− mice, Goα mRNA was reduced by 90% (P < 0.001) as compared to control mice (Fig. 2A). In line with these results, Goα protein expression was much less abundant in the atria of cGoα−/− mice as compared to control mice (Fig. 2B). ANF was knocked down by 23% in the atria of cGoα−/− mice as compared to controls (P < 0.05; Fig. 3). BNP had a more significant reduction in expression (63%) than ANF did (P < 0.01; Fig. 4).

ChgA knockout mice did not show any changes in either ANF or BNP transcript levels in the atria (data not shown).

The quantitation of peptide content by RIA in atrial tissue extracts of control and cGoα−/− mice showed that the atrial ANF content in cGoα−/− mice (16 489 pg/mg of tissue) was not statistically different from that in control mice (18 546 pg/mg of tissue) (Fig. 5). Tissue BNP content was significantly lower in the atria of cGoα−/− mice. Control mice had an average BNP concentration of 118 pg/mg of tissue, whereas the cGoα−/− mice had an average BNP concentration of 84 pg/mg of tissue (P < 0.05) (Fig. 6). ChgA knockout mice did not show any changes in either ANF or BNP peptide levels in the atria or plasma (data not shown).
HPLC analysis of ANF tissue stores

Molecular forms of intracellular ANF in atrial tissue extracts were examined by RP-HPLC to determine the ratio content of low molecular weight processed ANF (ANF$_{99-126}$) and high molecular weight proANF (ANF$_{1-126}$). RP-HPLC profiles showed the presence of both forms of ANF in each genotype, with similar ratios of proANF to processed ANF (Fig. 7A and B).

ANF peptide content in plasma

Plasma ANF concentration did not differ between cGo$_{a}$/K$_{m}$ mice and control mice (Fig. 8). BNP plasma level concentration was below assay sensitivity for normal and transgenic animals.

Microscopy

Figure 9A and B shows medium-power images of atrial cardiomyocytes by conventional transmission electron microscopy. The general morphological features of both transgenic and control atrial cardiomyocytes did not show obvious morphological differences except for an apparent loss of electron density in the atrial granule’s core in transgenic animals (Fig. 9B). However, even by simple inspection, it was obvious that cardiomyocytes of the Go$_{a}$/−/− mice contained fewer granules than the controls did. Quantification of the number of granules per square micron of tissue section from Go$_{a}$ conditional knockouts showed a near halving in the number of granules as compared to controls (Fig. 10).

Electrocardiography

In order to determine differences in the electrical conductance of cGo$_{a}$/−/− mice, telemetry recordings were obtained via an implantable telemetry unit. A 3-day continuous recording period was carried out in three mice per genotype during daytime hours (at rest) and during nighttime hours (while awake). The parameters analyzed in these mice included RR intervals, heart rate, R-wave amplitude, P-wave amplitude, QRS duration, P-wave duration, and PR interval. Both genotypes had similar recordings, and none of the aforementioned parameters was significantly different in the cGo$_{a}$/−/− mice as compared to the control mice (Table 3). An ECG tracing that represents four cardiac cycles is shown in Fig. 11.

Discussion

The present study was designed to determine the role of the Go protein $\alpha$ subunit Go$_{a}$ in determining atrial cardiomyocyte phenotype, given previous indications that it is involved with atrial secretory granules and with ANF secretion. Generally, heterotrimeric G proteins are associated with secretory granule biogenesis in other

Figure 6
Concentration of BNP peptide in atrial tissue. Values are means ± S.E.M.; n = 6; *P < 0.05.

Figure 7
RP-HPLC profiles of atrial tissue ANF in (A) control mice and (B) cGo$_{a}$/−/−.
endocrine organs (Barr et al. 1991, Burgoyne & Morgan 1993, Gasman et al. 1997). The Goα subunit has been implicated in various parts of this pathway (Zhao et al. 2010). Far greater amounts of Goα are found within the atria of the heart as compared to the ventricles (Eschenhagen et al. 1995). In addition, G proteins such as Go, are stretch-sensitive (Van Wagoner 1993), and they are involved in several secretory processes, including ANF secretion, together with K+ channels (Ogawa et al. 2009).

Both light and electron microscopy immunocytochemistry have shown that Goα co-localizes in atrial granules with ANF with a preferential distribution in the granule membrane (Wolf et al. 1998, Bensimon et al. 2004, Muth et al. 2004). The bulk of ventricular cardiomyocytes do not display Goα immunoreactivity.

An additional indication of Gi/o involvement in ANF secretion is the fact that PTX can completely abolish stretch-secretion coupling (Kuroski de Bold & de Bold 1991, Bensimon et al. 2004). Finally, a comparison of gene expression profiles using oligonucleotide arrays has shown a uniquely high expression of Goα1 in the atria (McGrath & de Bold 2009). The results in the present study, which were obtained by yeast two-hybrid and immunoprecipitation techniques further suggest an association between ANF and Goα.

ANF exists in the form of proANF within atrial secretory granules. The latter appears to play a master regulatory role in the formation of secretory granules, as is suggested by the fact that ANF knockout mice lack secretory granules (John et al. 1995, Dikeakos & Reudelhuber 2007). Given the association of ANF with Goα, it is reasonable to postulate a sorting role for membrane-bound Goα during granule maturation, a model that was proposed by Arvan & Halban (2004). This model, which was denominated ‘sorting by retention’, suggests that proteins within secretory vesicles (e.g., proANF) that arise from the TGN and are destined to be secreted by a regulatory mechanism are retained in secretory granules (e.g., by interaction with Goα), whereas other proteins are sorted into other compartments, such as lysosomes or endosomes.

In all, these previous studies do not provide a clear view of how Goα participates in the biogenesis of...

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**Figure 8**
ANF concentration in plasma of control or cGoα−/− genotypes. Values are means ± S.E.M.; n=4 for control; n=5 for cGoα−/−.

**Figure 9**
Electron microscopy images of atrial cardiomyocytes. Atrial muscle cell in control mice (A) and cGoα−/− mice (B). SG, secretory granules; G, Golgi complex; N, nucleus; M, mitochondria; My, myofibrils. Magnification 5000 x.
ANF-containing granules. Therefore, we postulated that the suppression of Goα expression would contribute information in this respect. A complication in taking this approach is that the homologous recombinant knockout for the Gnao1 gene induces severe neurological complications and very early lethality (Jiang et al. 1997). For this reason, we generated a conditional, heart-specific knockout of Goα in mice according to published breeding protocols for Cre/lox mice (Niu et al. 2005). In the present study, heart-specific Goα knockout mice were produced in Mendelian frequencies without any severe motor or neurological symptoms nor any gross morphological features in the heart associated with this deletion. These mice appeared normal and lived into adulthood.

The yeast two-hybrid experiments and the immunoprecipitation studies carried out as part of the present investigation clearly show an association of Goα with ANF in a fashion that suggests that Goα plays a role in proANF sorting into storage vesicles, which is in accordance with the sorting by retention model proposed by Arvan & Halban (2004).

No protein–protein interaction was detected between BNP and Goα, but atrial stores of BNP nonetheless decreased. The finding that BNP decreased more significantly than ANF did may reflect the fact that the determination of ANF introduces a larger variance than the determination of BNP does because of the large dilutions required to perform the RIA. Nevertheless, the finding suggests that a mechanism exists to keep the molecular ratios of these two hormones constant within the storage granules. A second suggestion brought about by the present observations is that the association between proANF and Goα protects the former molecule but not BNP from proteolytic processing, which thus explains why the storage forms of NP in the atria consist of proANF and processed BNP. In addition, the fact that Chga expression was not affected in the knockouts suggests that Goα function is restricted to the NP.

We have previously reported on the relationship between ANF secretion, K+ channels, and Goα based on evidence obtained through oligonucleotide array analysis and yeast two-hybrid analysis (McGrath & de Bold 2009, Ogawa et al. 2009). Pharmacological interventions with drugs known to pharmacologically affect the function of specific K+ channels, including adenosine triphosphate-sensitive K+ channels, TWIK-related K+ channel-1 (TREK-1), and the Ca2+-activated intermediate conductance K+ channel (SK4) modified ANF secretion to various extents. SK4 was expressed specifically in the atria, and the yeast two-hybrid analysis specifically demonstrated an interaction of this channel with Goα. However, in the present investigation, we found no changes in cardiac electrophysiological activity in cGoα−/− animals.

The most notable phenotypic change that was observed in atrial cardiomyocytes of Goα−/− animals was a loss of electron density of the granule’s core and
a significant reduction in the number of granules. The conditional Goz deletion led to a 90% loss of Goz mRNA, so these mice had an average 10% genetic expression of Goz, which was reflected by the small but significant amount of Goz protein evident in the western blots. This remaining protein likely represents cells in which Cre-induced deletion may not have been completed on both chromosomes.

Whether a total deletion would have led to a complete disruption of the formation of granules cannot be determined from the present investigations. Together with the changes in phenotype revealed by electron microscopy, it was evident that both ANF and BNP atrial content decreased (albeit not significantly for ANF) in the transgenic animals. That stored ANF content would diminish alongside a decrease in its interacting partner, Goz, was not surprising, but the significant decrease in BNP was not expected, and it hints at a mechanism that preserves a predefined molar relationship of stored NP in both normal and transgenic hearts.

The present investigation was designed to determine cardiomyocyte secretory phenotype changes rather than changes in the contractile phenotype, cardiac function, or other parameters in the animal as a whole. Therefore, we did not attempt to determine if the knockout had whole-animal consequences (e.g., effects on blood pressure, cardiac remodeling, etc.). This point should be addressed in future investigations.

Goz regulates insulin granule dynamics by acting as a represor and inhibitor of vesicular docking in pancreatic β cells during normal physiological functions (Zhao et al. 2010). In pancreatic-specific conditional Goz knockout mice, 35–100% more insulin vesicles are docked at the plasma membrane as compared to control β cells (Zhao et al. 2010). Therefore, in the pancreatic β-cell model, Goz acts as a key regulator of granule docking by interacting at either the docking or the priming station of cells to prevent the oversecretion of insulin (Zhao et al. 2010).

In chromaffin cells, Goz inhibits granule fusion to the plasma membrane by controlling the priming of granules, which therefore regulates catecholamine secretion (Gasman et al. 1997). The most prominent result from the present study was the 51% significant decrease in granule numbers and changes in the electron density of the granules in cGoz−/− mice as compared to control mice. A finding similar to this was not reported in the previous studies on β cells or chromaffin cells, which suggests that either Goz plays various roles in the vectorial transport of secretory products within secretory cells or that it plays different roles in different cell types.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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