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G_o but not G_{i2} or G_{i3} is required for muscarinic regulation of heart rate and heart rate variability in mice

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Abstract

Muscarinic receptor-mediated cardiac parasympathetic activity is essential for regulating heart rate and heart rate variability (HRV). It has not been clear which G_i/G_o protein is responsible for these effects. We addressed this question using knockout mice that lack G protein α_{i2}, α_{i3}, or α_o specifically. Unlike previously reported, our α_o-null mice had significantly more survivors with normal life span. Isolated hearts from α_o-null mice demonstrated much less sensitivity to the negative chronotropic effects of the muscarinic agonist carbachol to lower heart rate at baseline and a more profound effect under the stimulation of the β-adrenergic agonist isoproterenol. In the presence of parasympathetic activation indirectly produced by methoxamine, an α_1-adrenergic agonist, α_o-null mice showed markedly decreased HRV compared with wild-type control mice. These differences in heart rate and HRV were not observed in α_{i2}-null or α_{i3}-null mice. Our findings establish an essential role for α_o G protein in the anti-adrenergic effect of carbachol on heart rate regulation.

Keywords: G-proteins; Heart rate; Heart rate variability; Parasympathetic

Normal heart rate is controlled by cardiac sympathetic and parasympathetic nerves to achieve homeostasis [1,2]. Cardiac sympathetic system is mediated by β-adrenergic receptors that are coupled primarily to α_s G protein and responsible for cardiac activation including positive chronotropic, dromotropic, lusitropic, and inotropic effects. Cardiac parasympathetic system is mediated by muscarinic acetylcholine (M2) receptor that is coupled to α_i/α_o G proteins and responsible for negative chronotropic and dromotropic effects. In sinoatrial node pacemaker cells, α_s and α_o act simultaneously, with α_o being more potent, suggesting that parasympathetic inhibition of heart rate is much greater on a background of sympathetic stimulation [3]. Mutations in either α_{i2} or α_o to disrupt binding to regulator of G protein signaling results in enhanced muscarinic M2 receptor-mediated bradycardic responses in cardiocytes derived from embryonic stem cells [4]. However, how different α_i/α_o G proteins (α_{i2}, α_{i3}, and α_o) control whole heart rate has not been determined.

The opposing effects of sympathetic and parasympathetic systems cause beat-to-beat alterations in heart rate, a phenomenon called heart rate variability (HRV) [5]. Because HRV is a reflection of parasympathetic tone it predicts survival after heart attack [6]. It is not clear how different α_i/α_o G proteins control HRV.

To investigate the specificity of α_i/α_o G proteins in controlling heart rate and HRV, we created α_o-null mice and used an isolated heart perfusion method to study the alteration in muscarinic-induced chronotropic effects under

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basal conditions and in the presence of prior β-adrenergic stimulation, as well as telemetry implants to analyze their EKG. The results were compared with those from α_{i2}-null mice and α_{i3}-null mice previously reported [7,8].

Materials and methods

Animal model. Production of gene targeted mice: using previously published constructs for gene targeting [7,8], the α_o gene was inactivated in J1 cells cultured on mouse embryo fibroblasts. Targeted lines identified as previously described by Southern blot analysis [8], were injected into Balb/c blastocysts. Resulting chimeras passed the targeted mutation in the germline bred to Balb/c or 129SvEv. Heterozygotes were mated to obtain littermates that were either wild-type or homozygous for the gene inactivation in order to control for genetic background.

Western blot analysis. Plasma membranes from mouse cardiac ventricles were prepared, subjected to electrophoresis, and transferred to PVDF membranes as described previously [9]. Membranes were incubated with primary antibodies specifically recognizing α_o, α_{i2}, α_{i3}, β_{comm}, and α_s. The membranes were then incubated in secondary antiserums conjugated with horseradish peroxidase and detected as before [9].

Isolated heart preparation. Hearts were excised, the aortic root cannulated to a 20-gauge needle and perfused retrograde with a modified Krebs–Henseleit buffer (in mM): 120 NaCl, 4.5 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 0.5 EDTA, 25 $NaCO_3$, and 10 dextrose (pH 7.4 when gassed with 95%O_2/5%CO_2 at 37 °C). The entrance of the pulmonary veins into the left atrium was then cannulated and the perfusion was subsequently switched to the working mode [10]. The pulmonary artery was cut to allow measurement of coronary flow. Left ventricular pressures were monitored continually through indwelling cannulae coupled to pressure transducers and linked to a MacLab A/D system and a Macintosh computer with the sampling rate set at 2 kHz. LVP and heart rate were monitored in the working heart mode for 5–10 min to ensure stability of the preparation before initiation of the experiment. After a 10-min stabilization period, the dose–response to carbachol was determined in the absence or presence of 1 μM isoproterenol; stable beating rate was then quantified after each drug dose addition.

In vivo EKG. Telemetry monitor implants (Data Science, model TA10ETA-F20) were placed in mice. After recovery for at least 24 h, heart rate was monitored by telemetry using a MacLab digitization and a Power PC computer. Heart rate variability (HRV) was analyzed using MacLab software. Data were collected at baseline for at least 15 min and continuously following administration of methoxamine. Data were analyzed for HRV and heart rate when stabilized 5–15 min after addition of methoxamine.

Statistics. Mean ± standard error (SE) values were analyzed using Prism (GraphPad Software Inc.). Statistical comparisons between groups were performed by Student's *t* test. Dose–response curves were compared by Two-way ANOVA and Bonferroni post-tests. Groups were considered significantly different if *P* values were $\leqslant 0.05$.

Results

Generating α_o-null mice

Inactivation of the α_o gene was confirmed by Western blot (Fig. 1A). There was no apparent change of other G-protein subunits, including α_{i2}, α_{i3}, β_{comm}, and α_s, in response to α_o inactivation (Fig. 1A). Mice homozygous for the α_o targeted gene exhibited perinatal mortality, but in contrast to previous reports [11,12], a significant percentage in the present study had normal life spans (Fig. 1B). We were therefore able to study healthy, fertile, adult mice for alterations in heart rate control. Improved animal husbandry in decreasing stress is likely the reason for better survival rate, although difference in genetic background can not be ruled out.

Response to carbachol in heart rate is significantly reduced in mouse heart lacking α_o

At baseline, carbachol dose–response curve in α_o-null mouse hearts demonstrated a shift to the right for a half log unit when compared with WT (Fig. 2A, lower two curves), showing decreased response to carbachol. When isoproterenol was present, the extent of slowing was much reduced and the effect of lacking α_o was not overcome even by very high doses of carbachol (Fig. 2A, upper two curves). These results indicate that α_o-mediated pathways are critical to negative chronotropic effects of carbachol, particularly in the presence of isoproterenol. To establish whether this is specific to α_o, we tested α_{i2}-null and α_{i3}-null mice using the same protocol. Neither α_{i2}-null nor α_{i3}-null mouse heart displayed significantly different responses to carbachol than WT at baseline or with isoproterenol stimulation (Fig. 2B and C). Mutants lacking both α_{i2} and α_{i3} were not born, implying embryonic lethality.

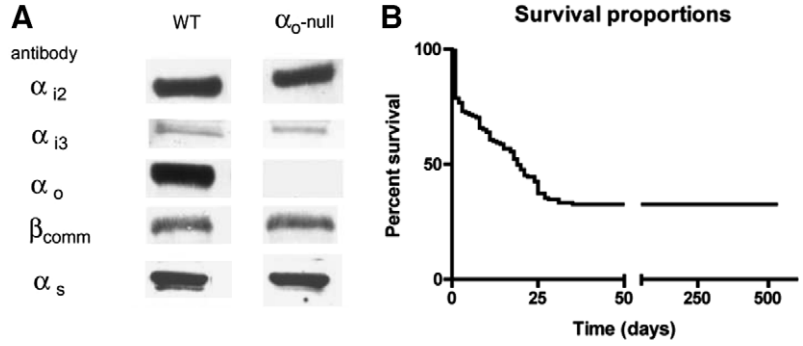

Fig. 1. Inactivation of α_o in mice. (A) Western blots of hearts from α_o-null mice and control wild-type mice. Antibodies against α_o, as well as α_{i2}, α_{i3}, β_{comm}, and α_s were used to verify the deletion of α_o and no compensatory changes in other G proteins. (B) Survival of α_o-null mice. Mice that survived the critical period lived nearly 2 years. The numbers of animals were $n = 25$ at 100 days, 18 at 150 days, and 12 at 200 days. The decrease in number of animals was due to sacrifice for experiments.

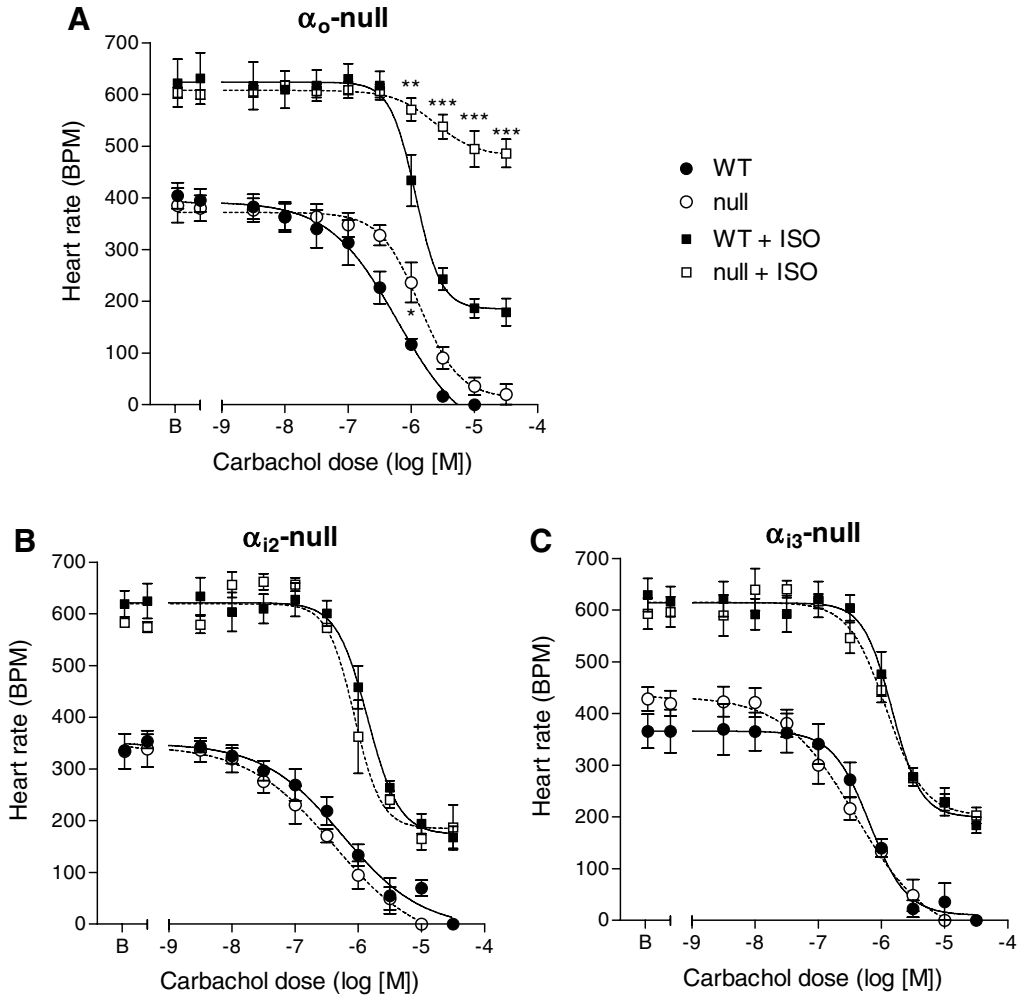

Fig. 2. Hearts from α_o-null mice show decreased sensitivity to muscarinic agonist. (A) Negative chronotropic responses to carbachol in isolated hearts from α_o-null and littermate control (WT) mice. Hearts were perfused *ex vivo* in working or ejecting mode as described in methods. Carbachol dose–response curves in the absence and presence of isoproterenol (1 μM, squares) were determined by increasing addition of carbachol. $n = 5$ for each group. By Two-way ANOVA, $P = 0.0071$ WT vs. null; $P < 0.0001$ WT + ISO vs. null + ISO. Bonferroni post-tests, $^{*}P < 0.05$ vs. WT; $^{**}P < 0.01$ vs. WT + ISO; $^{***}P < 0.001$ vs. WT + ISO. (B,C) Negative chronotropic responses to carbachol in isolated hearts from α_{i2}-null and α_{i3}-null mice respectively. No statistical significance was detected.

Decreased HRV in mice lacking α_o

HRV was determined from at least 1000 R–R intervals between successive beats before and after administration of methoxamine to stimulate the parasympathetic system. This system was chosen in order to compare results with the study on I_{KACh} inactivation [13]. As an α_1-adrenergic agonist methoxamine causes vasoconstriction and increases blood pressure leading to activation of the carotid sinus baroreceptor and hence reflex activation of the parasympathetic system. The basal heart rate was not significantly different between α_o-null animals and their WT littermates and methoxamine lowered the heart rate in both genotypes (Fig. 3A), consistent with low parasympathetic tone in mice [14]. There was a markedly diminished response to methoxamine in HRV in the α_o-null animals, but not in α_{i2}-null or α_{i3}-null mice (Fig. 3B). Spectral analysis of HRV data indicated that the difference between α_o-null and WT was significant after administration of methoxamine, in all three components, high frequency (HF), low frequency (LF), and very low frequency (VLF) (Fig. 3C).

Discussion

Gene inactivation studies have established the requirement for the I_{KACh} channel in parasympathetic slowing heart rate and increases in HRV. It was estimated that about 50% of the negative chronotropic effect required an intact I_{KACh} channel [13]. We now show that α_o-mediated pathways, which are not required for I_{KACh} activation [7,12] but do regulate I_f, are also required for normal negative chronotropy in heart rate and parasympathetic stimulated increases in HRV. These results indicate that activation of I_{KAch} through the remaining pertussis toxin-sensitive G-proteins is not capable of maintaining normal negative chronotropic responses on its own. Therefore, at

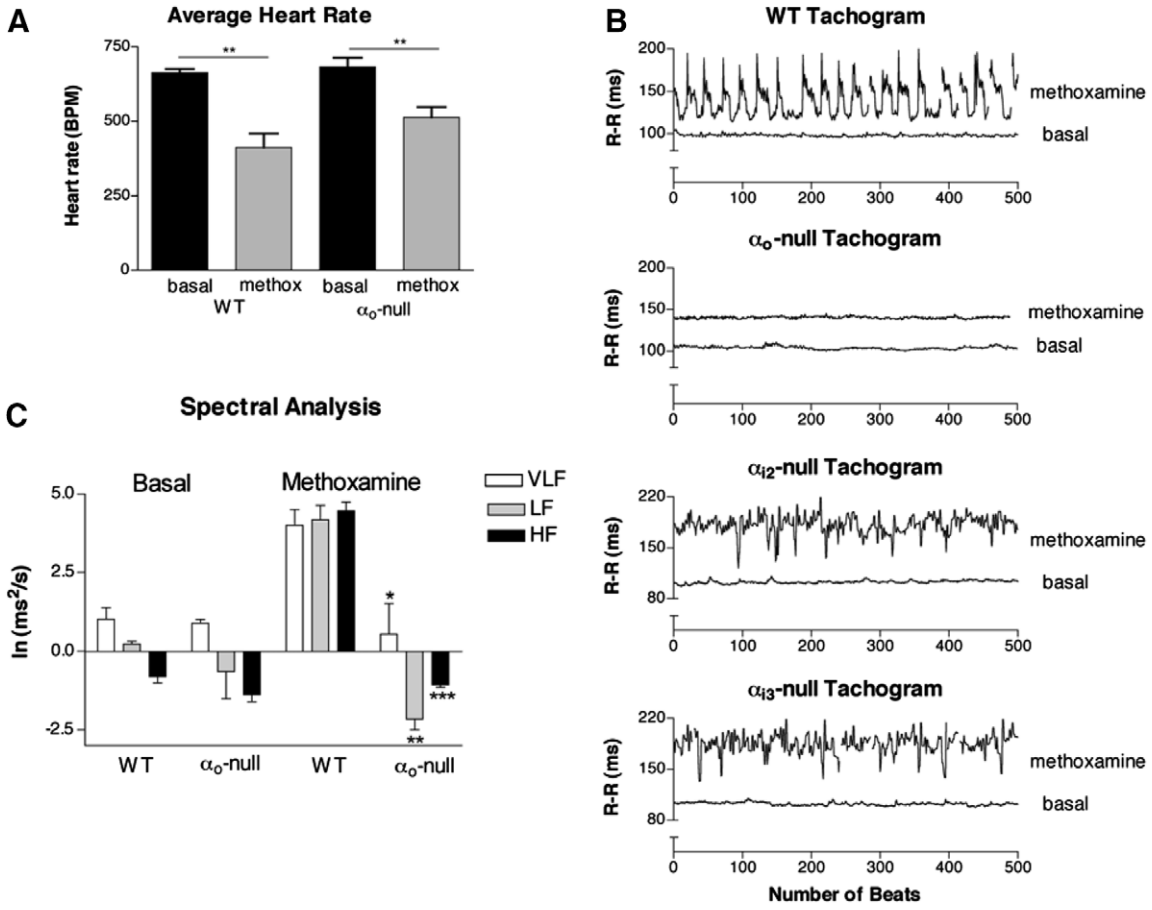

Fig. 3. Diminished HRV in α_o-null mice responding to methoxamine. (A) *In vivo* heart rates before and after methoxamine. Heart rates were measured using implantable EKG telemetry devices at baseline and after the injection of 6 mg/kg methoxamine. $n = 4$ for each group. $^{**}P < 0.01$. (B) Decreased HRV in the α_o-null mice compared to their WT littermates after methoxamine stimulation. Mice were chronically maintained EKG telemetry implants as described in methods. Data of the R–R intervals between successive beats before and after addition of methoxamine were collected continuously. (C) Quantification of HRV data in α_o-null and WT mice. Measurement of the power spectra was broken into three components: high frequency (HF) 1.5–4 Hz; low frequency (LF) 0.4–1.5 Hz; and very low frequency (VLF) <0.4 Hz. $^{*}P < 0.02$ vs. WT VLF; $^{**}P < 0.002$ vs. WT LF; $^{***}P < 0.0001$ vs. WT HF.

least two pathways are required, one leading to activation of I_{KACh} mediated by α_{i2} and α_{i3} released $\beta\gamma$ [7] and the other likely leading to inhibition of hyperpolarization-activated current I_f, which could require α_o but not α_{i2} or α_{i3}.

The exact contributions of these two pathways may differ depending on the system studied. In the intact heart, both pathways appear to be important in regulating heart rate and HRV. Both I_{KACh} and α_o inactivation (and hence likely effects of I_f), markedly affected the muscarinic negative chronotropic effects. Here, we show the importance of α_o under ex vivo conditions where complicating effects on blood pressure and sympathetic or parasympathetic nerve activity have been eliminated. A detailed interpretation of the meaning of the whole animal experiments is difficult in that, like the previous study [13], alterations in blood pressure or effects of knockouts on central nervous system function were not evaluated. However, there is a clear decrease in the HRV in α_o-null animals. Inactivation of I_{KACh} nearly eliminated HRV at baseline whereas α_o inactivation had no effect. When stimulated with methoxamine, both I_{KACh} and α_o inactivation caused marked decreases in HRV with I_{KACh} effects being slightly more pronounced. The effect on HRV is consistent with the decreased sensitivity to muscarinic stimulation by carbachol seen in the isolated perfused hearts. The contribution of the α_o dependent pathway may increase with high sympathetic activity or with other β-adrenergic stimulation compared to resting conditions.

These results establish a critical role for α_o-containing heterotrimers in the parasympathetic regulation of heart rate and HRV.

Conflict-of-interest

None.

Acknowledgments

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