$G\alpha_{12}$ Facilitates Methacholine-Induced Shortening in Human Airway Smooth Muscle By Modulating Phosphoinositide 3-Kinase-Mediated Activation In A RhoA-Dependent Manner

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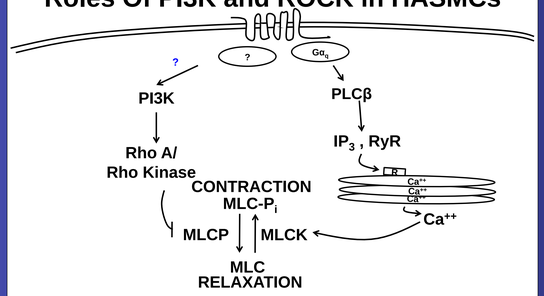
Abstract

RATIONALE: Phosphoinositide 3-kinase (PI3K)-dependent activation of rho kinase (ROCK) is necessary for agonist-induced human airway smooth muscle cell (HASMC) contraction, and inhibition of PI3K promotes bronchodilation of human small airways[1]. The upstream mechanisms driving agonist-mediated PI3K/ROCK axis activation, however, remain unclear. Given the capacity of G12 family proteins to activate ROCK pathways in other cell types[2], the role of G12 proteins in modulating M3R-stimulated PI3K/ROCK activation and HASMC contraction was examined. **METHODS:** siRNA and pharmacological inhibitors, as well as overexpression of a regulator of G-protein signaling (RGS) protein that limits $G\alpha_{12}$ activation, were used in HASMCs. Phosphorylation of AKT, myosin phosphatase targeting subunit-1 (MYPT1), and myosin light chain-20 (MLC) was measured. $G\alpha_{12}$ coupling was evaluated using co-immunoprecipitation and serum response element (SRE)-luciferase reporter assay. HASMC contraction was evaluated using magnetic twisting cytometry (MTC). Human precision-cut lung slices (hPCLS) were utilized to evaluate bronchoconstriction. **RESULTS:** Knockdown of $G\alpha_{12}$ attenuated carbachol-induced activation of AKT, MYPT1, and MLC phosphorylation. $G\alpha_{12}$ coimmunoprecipitated with the M3R, and p115RhoGEF-RGS overexpression inhibited carbachol-mediated induction of SRE-luciferase reporter. p115RhoGEF-RGS overexpression inhibited carbachol-induced activation of AKT and HASMC contraction. Moreover, pharmacological inhibition of RhoA blunted carbachol-mediated activation of PI3K. Lastly, RhoA inhibitors induced dilation of hPCLS. **CONCLUSIONS:** $G\alpha_{12}$ plays a crucial role in HASMC contraction via RhoA-dependent activation of the PI3K/ROCK axis. Inhibition of RhoA activation induces bronchodilation in hPCLS, and targeting $G\alpha_{12}$ signaling may elucidate novel therapeutic targets in asthma. Taken together, these findings provide alternative approaches to the clinical management of airway obstruction in asthma.

Roles Of PI3K and ROCK in HASMCs



Hypothesis

Since G12 family proteins regulate smooth muscle tone by activating Rho Kinase, we hypothesize that $G\alpha_{12}$ may regulate HASMC contraction by activation of the PI3K/ROCK axis.

M3 muscarinic acetylcholine receptor couples to $G\alpha_{12}$ in HASMCs

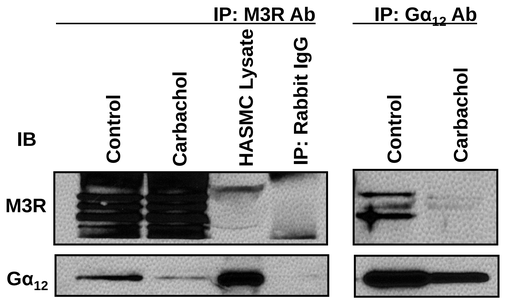


Figure 1 –HASMCs were stimulated with carbachol (10 μM, 1 min) and lysates were immunoprecipitated with anti-M3R or anti-$G\alpha_{12}$ antibody and then probed as indicated. Immunoblot is representative of five independent experiments.

$G\alpha_{12}$ siRNA attenuates M3R-mediated activation of the PI3K/ROCK/MLC axis in HASMCs

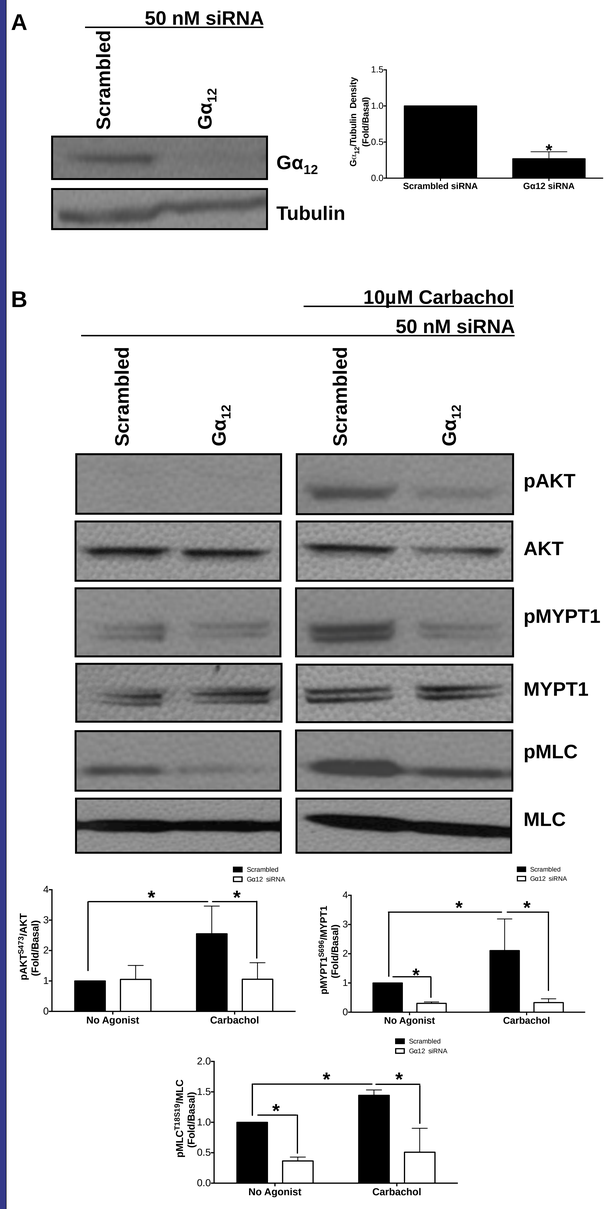
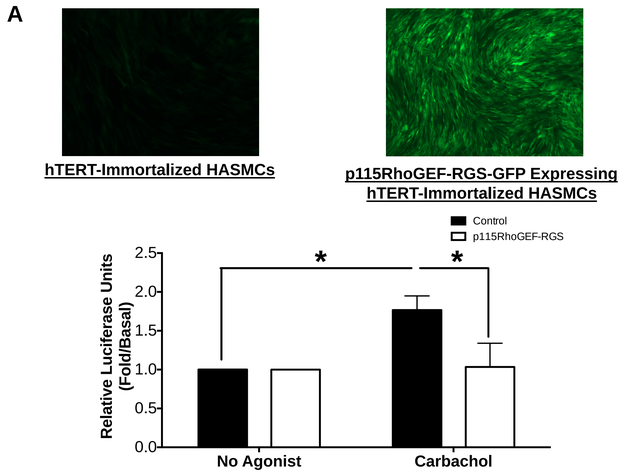


Figure 2 – (A) Effect of $G\alpha_{12}$ or scrambled siRNA on protein expression. Data normalized to tubulin expression in the same samples. (B) Effect of carbachol on AKT, MYPT1, and MLC phosphorylation at S473 (pAKT), T696 (pMYPT1), and S19 (pMLC) after transfection with $G\alpha_{12}$ or scrambled siRNA. pAKT, pMYPT1, and pMLC data were normalized to total AKT (AKT), total MYPT1 (MYPT1), and total MLC (MLC).

p115RhoGEF-RGS expression attenuates M3R-mediated SRE-luciferase reporter induction, PI3K activation, and HASMC contraction



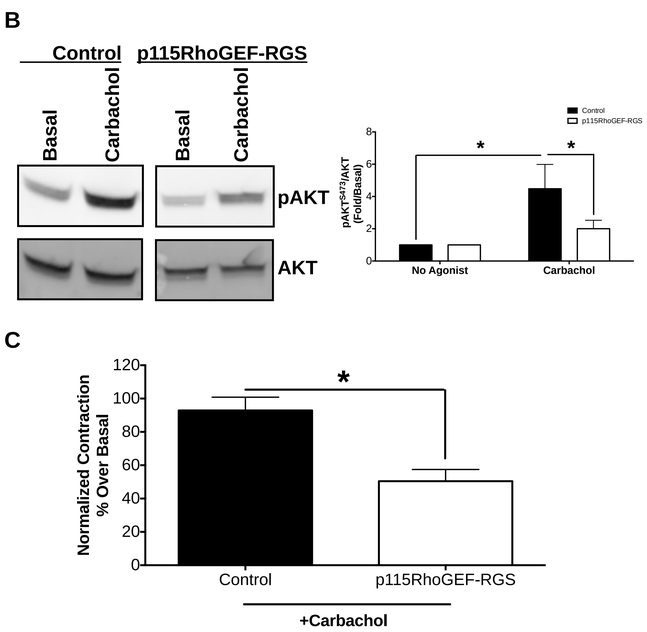


Figure 3 – (A) hTERT-immortalized HASMCs expressing p115RhoGEF-RGS and control hTERT-immortalized HASMCs were infected with SRE-luciferase reporter. After carbachol stimulation (10 μM, 6 h), cells were lysed and SRE-luciferase reporter activity was measured. (B-C) HASMCs expressing p115RhoGEF-RGS were stimulated with carbachol and assayed for AKT phosphorylation using immunoblot, and contraction using magnetic twisting cytometry. Data are representative of five independent experiments (n = 6, mean ± SD); statistical comparisons analyzed by unpaired t-test are denoted by lines between tested conditions *P < 0.05.

RhoA inhibition blunts M3R-mediated PI3K activation

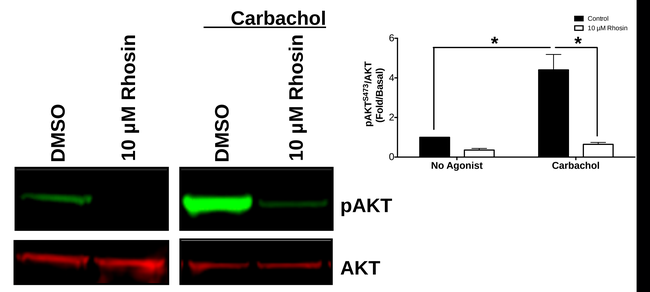


Figure 4 – Effect of rhosin (10 μM, 30 min) on carbachol-induced (10 μM, 10 min) AKT phosphorylation at S473 (pAKT).

RhoA Inhibitors Induce Bronchodilation of Human Small Airways

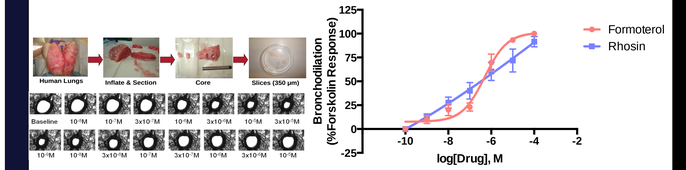
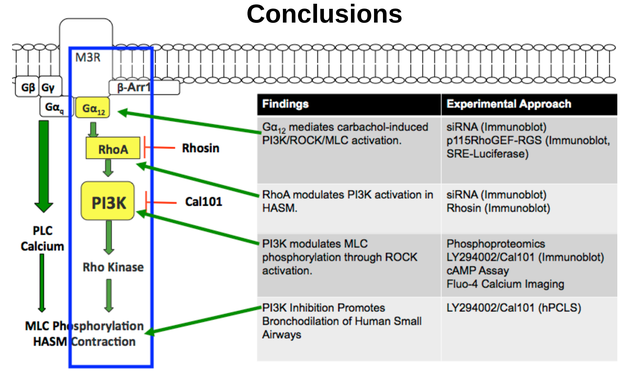


Figure 5 – Airways were preconstricted to carbachol (10^{-8}–10^{-4} M) prior to dilation to rhosin or formoterol (10^{-10}–10^{-4} M). Data were normalized to maximum forskolin response (10 μM) that was given after max dose of formoterol or rhosin. Each data point is expressed as mean ± SEM. Each group contains 2 airways from each of the three donors (6 total airways).

Conclusions



Findings	Experimental Approach
$G\alpha_{12}$ mediates carbachol-induced PI3K/ROCK/MLC activation.	siRNA (Immunoblot) p115RhoGEF-RGS (Immunoblot, SRE-Luciferase)
RhoA modulates PI3K activation in HASM.	siRNA (Immunoblot) Rhosin (Immunoblot)
PI3K modulates MLC phosphorylation through ROCK activation.	Phosphoproteomics LY294002/Cal101 (Immunoblot) cAMP Assay Fluo-4 Calcium Imaging
PI3K Inhibition Promotes Bronchodilation of Human Small Airways	LY294002/Cal101 (hPCLS)

Significance

$G\alpha_{12}$ plays a crucial role in HASMC contraction via RhoA-dependent activation of the PI3K/ROCK axis. Inhibition of RhoA activation induces bronchodilation in hPCLS, and targeting $G\alpha_{12}$ signaling may elucidate novel therapeutic targets in asthma.

References

1. Koziol-White, C. J., Yoo, E. J., Cao, G., Zhang, J., Papanikolaou, E., Pushkarsky, I., Andrews, A., Himes, B. E., Damoiseaux, R. D., Liggett, S. B., Di Carlo, D., Kurten, R. C., and Panettieri, R. A. Jr. (2016) Inhibition of PI3K promotes dilation of human small airways in a rho kinase-dependent manner. British Journal of Pharmacology, 173: 2726–2738. doi: 10.1111/bph.13542.
2. Riobo, N.A., and Manning, D.R. (2005). Receptors coupled to heterotrimeric G proteins of the G12 family. Trends Pharmacol. Sci. *26*: 146–154.