Molecular Pharmacology

Supplementary Data

FTY720-P activates Sphingosine-1-phosphate receptor 2 and selectively couples to $G\alpha_{12/13}$/ Rho/ ROCK to induce myofibroblast contraction

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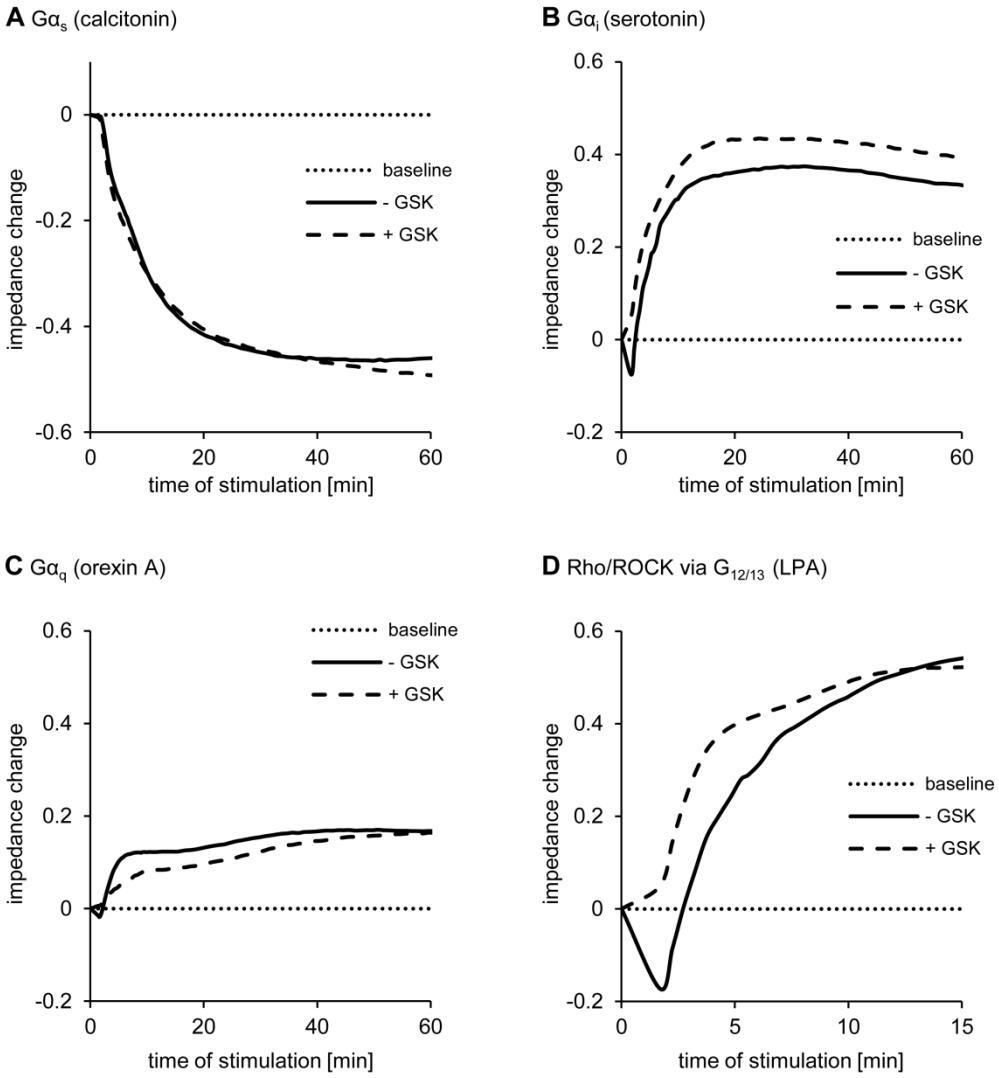
Supplementary Data S1:

Impedance assays capture $G\alpha_{12/13}$ Rho/ ROCK activation via GPCRs.

In order to validate impedance assays for studying potential FTY720-P-mediated $G\alpha_{12/13}$/ Rho/ ROCK activation, we first established the specificity of the ROCK inhibitor GSK269962 in impedance assays using agonist-receptor pairs with well-established subtype coupling in well-defined cell systems and followed the impedance responses in the absence (solid line) or presence of GSK269962 (dashed line) (Suppl. Fig. 1). Stimulation of CHO-K1 empty vector cells with calcitonin (0.5 nM), induced a strong decrease of impedance over 1 h, which was not changed in the presence of GSK269962 (Suppl. Fig. 1A). When CHO-K1 empty vector cells were stimulated for 1 h with serotonin (100 nM), we observed a rapid and stable increase of impedance. This response was not altered by preincubation with GSK269962 (Suppl. Fig. 1B). Orexin A (10 nM), which we used to study $G\alpha_q$ coupling (Smart et al., 1999), induced an increase of impedance over 1 h in CHO-cells recombinantly expressing the orexin 2 receptor (CHO-Ox2 cells). This increase was not blocked in the presence of GSK269962 (Suppl. Fig. 1C). In order to study $G\alpha_{12/13}$ signaling, we stimulated CHO-K1 empty vector cells with LPA (1 μM), a well-described activator of Rho/ ROCK signaling via $G\alpha_{12/13}$ protein coupling (Kozasa et al., 2011; Xiang et al., 2013). LPA induced two response phases within 15 min. First, a decrease of impedance for ~3 min was observed. This decrease was then followed by an increase of impedance over baseline (Suppl. Fig. 1D). The LPA-induced first response phase was suppressed by the ROCK inhibitor GSK269962 demonstrating that the early impedance decrease, but not the later impedance increase, reflects Rho-kinase activation, probably by $G\alpha_{12/13}$ signaling. The LPA-induced second phase of the impedance response was blocked by pre-treatment with pertussis toxin (data not shown) and is therefore caused by the activation of the $G\alpha_i$ pathway. Taken together, these data show that GSK269962 does not inhibit $G\alpha_s$, $G\alpha_i$ or $G\alpha_q$, but can specifically be used for the characterization of $G\alpha_{12/13}$ activation by GPCR agonists using impedance technology.



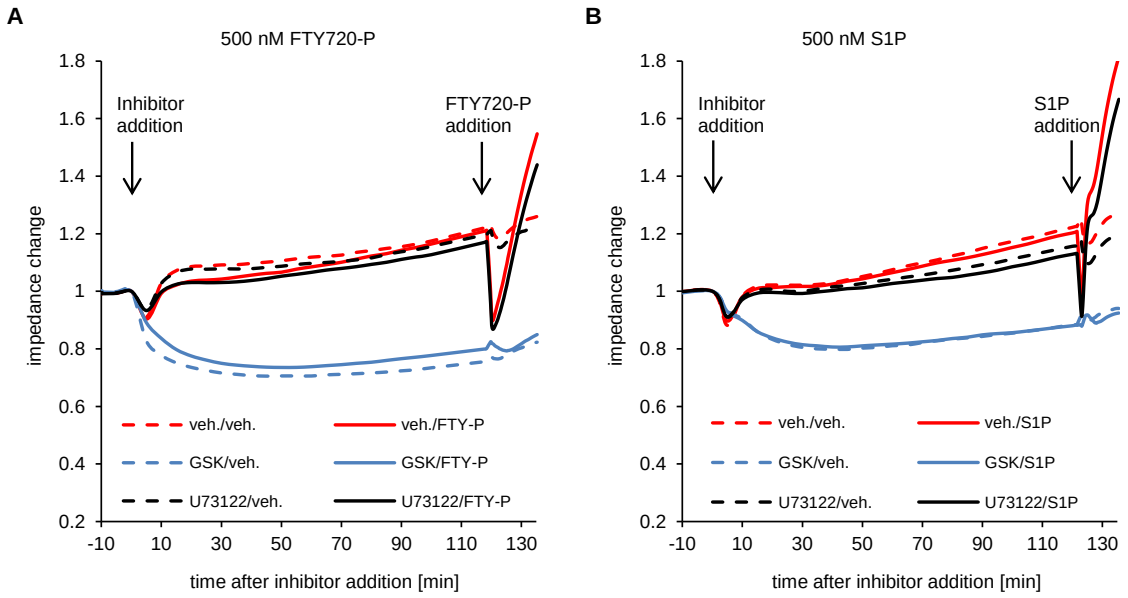
Supplementary Figure S1: Establishing analysis of $G\alpha_{12/13}$ Rho/ ROCK activation in impedance assays using the ROCK inhibitor GSK26996.

CHO-K1 empty vector cells (A, B, D) or CHO-Ox2 cells (C) were preincubated with vehicle or GSK269962 (40 nM) for 2 h before stimulation with 0.5 nM calcitonin (A), 100 nM serotonin (B), 10 nM orexin A (C) or 1 µM LPA (D). Impedance responses were then followed. Data show representative experiments (n=2-3).

Supplementary Data S2:

Impedance responses to GSK269962 and U73122

Pharmacological inhibitors can have major impact on impedance responses by themselves, e.g. by being cytotoxic. Such major effects can distort subsequent agonist-induced impedance responses making their interpretation difficult. To exclude such pre-treatment effects, we analyzed the impedance response to the inhibitors. Supplementary Figures S2A, B show the effect of GSK269962 and U73122 pre-treatment on basal impedance followed by the response to either FTY720-P or S1P or vehicle. The Rho/ ROCK inhibitor GSK269962 caused a moderate decrease in basal impedance while the PLC-β blocker U73122 had no effect on basal impedance.



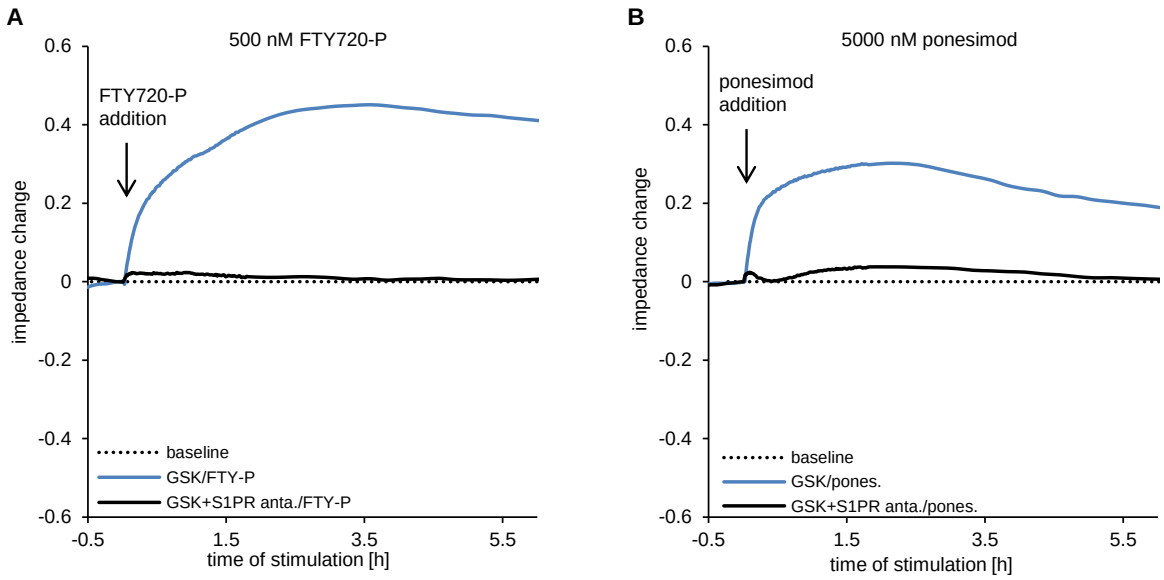
Supplementary Figure S2: Impedance responses to GSK269962 and U73122.

CHO-S1P$_2$ cells were pre-incubated with vehicle (red traces), GSK269962 (40 nM, blue traces) or U73122 (5 µM, black traces) for 2 h before stimulation with 500 nM FTY720-P (A) or 500 nM S1P (B) or vehicle (A, B) and impedance responses were followed. Data represent impedance raw traces normalized before inhibitor addition.

Supplementary Data S3:

Analysis of the residual impedance response to S1P receptor agonists in myofibroblasts pre-treated with GSK269962.

To test whether the S1P receptor agonist-induced "residual" impedance increase in myofibroblasts pre-treated with GSK269962 was due to the activation of S1P receptor subtypes by the agonists, GSK269962-pre-treated cells were co-treated with a mixture of $S1P_1$ antagonist W146 (Avanti Polar Lipids, Inc., Alabaster, AL), $S1P_2$ antagonist JTE-013 and $S1P_3$ antagonist TY-52156 [Murakami et al., 2010; synthesized by Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland)] before stimulating the cells with 500 nM FTY720-P (Supplementary Figure S3A) or 5000 nM ponesimod (Supplementary Figure S3B). This pre-treatment fully abolished the impedance increase caused by FTY720-P or ponesimod in presence of GSK269962, demonstrating that the "residual" response uncovered after GSK269962 treatment was indeed due to S1P receptor subtype activation.



Supplementary Figure S3:

Analysis of the residual impedance response to S1P receptor agonists in myofibroblasts pre-treated with GSK269962.

NHLF-derived myofibroblasts were pre-incubated for 2 h with GSK269962 (40 nM) or a combination of GSK269962 (40 nM) $S1P_1$ antagonist W146 (3 µM), $S1P_2$ antagonist JTE-013 (0.2 µM) and $S1P_3$ antagonist TY-52159 (1.25 µM) (called "GSK + S1PR anta.") followed by stimulation with FTY720-P (500 nM, A) or ponesimod (5000 nM, B). Impedance responses were followed for 6 h.

References

Kozasa T, Hajicek N, Chow CR and Suzuki N (2011) Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. *J Biochem* **150**(4): 357-369.

Murakami A, Takasugi H, Ohnuma S, Koide Y, Sakurai A, Takeda S, Hasegawa T, Sasamori J, Konno T, Hayashi K, Watanabe Y, Mori K, Sato Y, Takahashi A, Mochizuki N and Takakura N (2010) Sphingosine 1-phosphate (S1P) regulates vascular contraction via S1P3 receptor: investigation based on a new S1P3 receptor antagonist. Mol Pharmacol 77(4): 704-713.

Smart D, Jerman JC, Brough SJ, Rushton SL, Murdock PR, Jewitt F, Elshourbagy NA, Ellis CE, Middlemiss DN and Brown F (1999) Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br J Pharmacol* **128**(1): 1-3.

Xiang SY, Dusaban SS and Brown JH (2013) Lysophospholipid receptor activation of RhoA and lipid signaling pathways. *Biochim Biophys Acta* **1831**(1): 213-222.