Online data supplement:

G_i-coupled $GABA_B$ReceptorsCross-Regulate Phospholipase C and Calciumin Airway Smooth Muscle

Kentaro Mizuta[1,2], Fumiko Mizuta[1,2], Dingbang Xu[1], Eiji Masaki[2], Reynold A. Panettieri, Jr.[3] and Charles W. Emala[1]

Materials and Methods:

Measurement of $[Ca^{2+}]_i$

Confluent human airway smooth muscle cells in 96-well plates were incubated in modified Hanks' balanced salt solution (HBSS) (in mM: NaCl 138, KCl 5.3, $CaCl_2$ 2.5, $MgSO_4$ 0.4, $MgCl_2$ 0.49, Na_2HPO_4 0.34, $NaHCO_3$ 4.2, KH_2PO_4 0.44, dextrose 5.5, Hepes 20, pH 7.4) at 100 µl/well containing 5 µM Fluo-4 AM (DMSO vehicle final concentration of 0.5 %), 0.05 % Pluronic® F-127 (DMSO vehicle final concentration of 0. 25 %) and 2.5 mM probenecid for 30 min at 37°C. Once the cells were loaded, the cells were washed twice with modified HBSS containing 2.5 mM probenecid and left for an additional 30 min at room temperature to allow complete de-esterification of the intracellular AM esters. This buffer was exchanged (100 µl/well) just before starting the measurement of fluorescence. The fluorescence was then continuously recorded every 5 seconds at wavelengths of 485 nm excitation and 528 nm emission using a fluorescence microplate reader (Synergy™ HT; BioTek Instruments, Winooski, VT). Triplicate wells were simultaneously measured and values were averaged for each data point.After a stable baseline was established for the first 10 min, the cells were pretreated with inhibitors [CGP35348 (100 µM; 30 min), CGP55845 (10 nM; 30 min), U73122 (5 µM; 10 min), xestospongin C (20 µM; 30 min)] or vehicle (modified HBSS; 10 min). For the experiment using pertussis toxin (PTX), cells were pretreated with PTX for 3 hrs prior to load with fluorescent dye so that the pertussis toxin pretreatment duration would be 4 hrs.Then the cells were incubated with 100 µM baclofen for 5 min. Finally, the cells were incubated with 1 µM bradykinin and the fluorescence intensity was recorded for 10 min. In all studies the fluorescence intensity after treatments is presented as the change (ΔF) from baseline fluorescence (F_o). Experiments were repeated at least 6 times on human airway smooth muscle cells obtained from the same source.

In vitro effects of $GABA_B$ receptor agonist on guinea pig airway smooth muscle contraction

All studies were approved by the Columbia University's Institutional Animal Care and Use Committee. Force measurements were performed on closed guinea pig tracheal rings suspended in organ baths as previously described (1). Briefly, Hartley male guinea pigs (approx. 400g body weight) were anesthetized with 50 mg/kg i.p. pentobarbitol, the tracheas were removed promptly, dissected into closed rings comprised of 2 cartilagenous rings from which mucosa, connective tissue

and epithelium were removed. Silk threads were tied to the rings such that the threads were at each end of the posterior aspect of the ring (lacking in cartilage), approximiately 180^{o} from one another. One thread was attached to a fixed point at the bottom of 4 ml organ baths (Radnoti Glass Technology, Monrovia, CA) and the opposing thread was attached to a Grass FT03 force transducer (Grass-Telefactor, West Warwick, RI) coupled to a computer via Biopac hardware and Acknowledge 7.3.3 software (Biopac Systems, Inc., Goleta, CA) for continuous digital recording of muscle tension. The rings were suspended in 4ml of Krebs-Henseleit (KH) buffer solution (composition in mM: NaCl 118, KCl 5.6, $CaCl_2$ 0.5, $MgSO_4$ 0.2, $NaHCO_3$ 25, NaH_2PO_4 1.3, D-glucose 5.6) with 10μM indomethacin (DMSO vehicle final concentration of 0.01%) which was continuously bubbled with 95% O_2 and 5% CO_2 at pH 7.4, 37^{o}C. The rings were equilibrated at 1g of isotonic tension for 1 hr with new KH buffer added every 15 min. All tracheal rings were initially contracted with 10uM N-Vanillylnonanamide (capsaicin analogue) to deplete C-fibers of mediators. Following extensive buffer changes, resting tension was reset at 1.0g. All rings were pre-contracted with 2 cycles of cumulatively increasing concentration of acetylcholine (0.1 μM - 1 mM) with extensive buffer washes between and after these two cycles with resetting of the resting tension to 1.0 g. Tracheal rings were contracted with 80mM KCl to determine each rings maximal contractile force. To eliminate the contributions of neural depolarizations or histamine release from mast cells, all rings were pretreated with 1uM tetrodotoxin and 10uM pyrilamine. Tracheal rings were then contracted with substance P (1 μM) with or without the co-addition of 100uM baclofen with continuous digital measurements of muscle force. Muscle force induced by substance P in the absence or presence of baclofen was expressed as a percent of the maximum force generated in each ring by KCl.

Reference:

1. Jooste E, Zhang Y, Emala CW. Rapacuronium preferentially antagonizes the function of M2 versus M3 muscarinic receptors in guinea pig airway smooth muscle. *Anesthesiology* 2005;102:117-124.