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**OC01**

Interleukin-13 increases amiloride-insensitive water resorption in airway epithelia

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The airway epithelium provides the first defense against airborne pathogens and noxae. Its apical surface is covered by a thin fluid layer called airway surface liquid (ASL). Precise regulation of ASL volume is essential for respiratory function. Sodium resorption via amiloride sensitive epithelial sodium channels (ENaC) and chloride secretion via CFTR are key mechanisms of ASL homeostasis. Interleukin-13 (IL-13) is the major cytokine that drives chronic inflammation in allergic asthma (1) and has been demonstrated to increase chloride secretion in airway epithelia (2-4). Therefore, IL-13 is supposed to induce a pro-secretory epithelial phenotype. However, the interplay of ion channel alterations, water transport and ASL volume homeostasis during chronic IL-13 exposure is still not completely understood. In the present study, we investigated the effect of IL-13 on ion transport, ASL regulation and water transport in human tracheal epithelial cells (hTEpC).

Use of hTEpC was in accordance with national ethical standards. Cells were differentiated at air-liquid-interface and cultivated in presence of IL-13 or without as control epithelia for further 10 days. Ussing chamber experiments revealed decrease of amiloride sensitive short circuit current (Isc) and increase of CFTR and TMEM16A mediated Isc in IL-13 exposed epithelia. In line with Isc data, IL-13 treatment reduced mRNA transcript levels of b- and g-ENaC subunits and increased CFTR and TMEM16A expression. ASL volume and water resorption rates were determined using deuterium oxide dilution. ASL volumes slightly decreased upon IL-13 treatment to 0.65 (0.25-0.81) ul/0.33cm² compared to 0.98 (0.35-1.81) ul/0.33cm² (median (min-max)) in control epithelia (p=0.007, Mann Whitney test, n=12). Compensatory water resorption upon ASL expansion with 25 ul of isotonic NaCl solution markedly increased in IL-13 exposed cells compared to control epithelia with resorption rates of 0.86 (0.81-1.12) ul/(h*0.33cm²) and 0.54 (0.29-0.72) ul/(h*0.33cm²) (median (min-max)), respectively (p<0.0001, Mann Whitney test, n=18). IL-13 induced ASL volume and
Resorption rate alterations were abolished in the presence of the JAK inhibitor tofacitinib, which blocks JAK/STAT6 mediated IL-13 signaling. ENaC inhibition with amiloride prevented 45% of resorption in control epithelia, but only 11% of resorption of IL-13 treated epithelia. Inhibitors for chloride channels (NPPB), CFTR (CFTRinh-172), TMEM16A (T16Ainh-A01), the Cl-/HCO3--exchanger pendrin (YS-01), potassium channels (TEA, Ba2+) and calcium channels (diltiazem) did not affect water resorption in IL-13 treated epithelia. Elevated resorption rates in IL-13 exposed epithelia decreased to control levels in the presence of gadolinium (Gd3+), which among others blocks mechanosensitive ion channels. However, another inhibitor of mechanosensitive ion channels, GsMTx4 did not impact water resorption. Evidently, the Gd3+ sensitive pathway does not depend on mechanosensitive ion channels. Ussing chamber experiments confirmed a Gd3+ sensitive ISc fraction, which was higher in IL-13 treated epithelia with 1.93 (1.08-4.62) uA/cm2 compared to 1.05 (0.31-1.68) uA/cm2 (median (min-max)) in control epithelia (p=0.021, Mann Whitney test, n=9).

Our results demonstrate that IL-13 increases pro-secretory ion transport but reduces ASL volumes and increases water resorption capacity in human airway epithelia. In contrast to control cells, resorption is only marginally amiloride sensitive, but depends on a Gd3+ sensitive mechanism.


OC02

The bitter taste receptor agonist denatonium influences mouse tracheal epithelial ion transport

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Intro: The epithelial ion and fluid secretion as part of mucociliary clearance protects the pulmonary system from infections. In murine tracheal epithelium, we have previously shown that denatonium-induced activation of brush cells (BC) evokes phospholipase Cβ2 (PLCβ2)- and transient receptor potential melastatin 5 channel (Trpm5)-dependent release of acetylcholine.

Aims/Objective: We here elucidated a possible impact of BC on transepithelial ion transport.

Methods: Transepithelial short circuit currents (Isc) of freshly isolated tracheae of Trpm5+/+, Trpm5-/- or Trpm5-DTR mice were recorded using a modified Ussing chamber. BC-depletion in Trpm5-DTR mice was confirmed by whole-mount immunohistochemistry. Changes in [Ca2+]i were studied in HEK293 cells transfected with the taste receptor type 2 (Tas2R) 108 or Tas2R105. For each experiment a group size of n = 5 animals was designed, with each animal corresponding to one measurement. Data were subjected to the Kolmogorov-Smirnov test to examine normal distribution followed by the paired or unpaired Student’s t-test to delineate statistical significance. P values below 0.05 were considered statistically significant. Values are given as mean ± s.e.m. All animal care and experimental procedures were performed according to the German and European animal welfare law and they were approved by the German animal welfare committee.

Results: Application of the Tas2R agonist denatonium increased Isc dose-dependently (EC50 = 397 μM) in Trpm5+/+ and Trpm5-/- mice (EC50 = 40 μM). Denatonium (1 mM) significantly increased [Ca2+]i in Tas2R108- or Tas2R105-expressing cells (HEK293: 4.65 ± 0.20 AU; Tas2R105: 21.78 ± 1.35 AU; Tas2R108: 18.27 ± 0.99 AU; p<0.05). Inhibition of the bitter taste signaling cascade with gallein (Gβγ antagonist; from 8.49 ± 1.30 μA*cm⁻² to 5.89 ± 2.56 μA*cm⁻²), U73122 (PLCβ2 inhibitor; from 10.30 ± 1.48 μA*cm⁻² to 8.70 ± 1.71 μA*cm⁻²), 2-Aminoethoxydiphenyl borate (2-APB, inositol 1,4,5-trisphosphate receptor antagonist; from 12.90 ± 2.68 μA*cm⁻² to 1.02 ± 0.33 μA*cm⁻²) and Triphenylphosphine oxide (TPPO, Trpm5 inhibitor; from 11.54 ± 1.85 μA*cm⁻² to 2.49 ± 0.52 μA*cm⁻²) significantly decreased the denatonium-effect (p<0.05, n=5 for each set of experiments). In Trpm5-/- and Trpm5-DTR mice, in which BC were depleted by diphtheria toxin, the denatonium-effect was reduced compared to Trpm5+/+ mice (p<0.05, n=5; Trpm5+/+: 17.20 ± 3.21 μA*cm⁻²; Trpm5-/-: 9.96 ± 1.73 μA*cm⁻²; Trpm5-DTR 9.51 ± 1.22 μA*cm⁻²). Inhibition of cholinergic signaling with mecamylamine and atropine did not influence the denatonium-effect (p=0.12, n=5). Interestingly, application of denatonium led to ATP-release from BC as inhibition of the ATP-release channel pannexin1 (probenecid; from 16.38 ± 2.05 μA*cm⁻² to 5.55 ± 1.73 μA*cm⁻²), ATP-dependent K⁺ channels (glibenclamide; from 15.00 ± 4.30 μA*cm⁻² to 8.02 ± 1.94 μA*cm⁻²) and ATP-receptors (suramin; from 16.41 ± 3.42 μA*cm⁻² to 13.69 ± 2.71 μA*cm⁻²) significantly decreased the denatonium-effect (p<0.05, n=5 for each set of experiments). Additionally, the ENaC antagonist amiloride significantly reduced the denatonium-effect (from 12.56 ± 2.72 μA*cm⁻² to 5.66 ± 0.59 μA*cm⁻²; p<0.05, n=5).

Conclusions: Denatonium evokes ATP-release from BC via the canonical bitter taste signalling cascade. ATP acts on ENaC-mediated transepithelial ion transport. Thus, activation of BC results in a decreased absorption of the airway surface liquid.
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OC03

Nematode ASICs drive motor behaviour by maintaining intestinal lumen pH and interacting with calcium oscillations

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Acid-sensing ion channels (ASICs) are part of the conserved family of DEG/ENaCs which have shown to be one of the main proton-receptors in vertebrates. However, a challenge in vertebrates is in vivo linking physiological processes directly to behaviour, we therefore turn to the transparent nematode C. elegans which present an excellent model organism for a systems-level understanding of genetics, physiology and behaviour due to its complete connectome, vast behavioural repertoire and due to its transparency physiological processes can be easily observed in real time using optical indicators. In order to investigate how ASICs impact physiology and behaviour, we use the C. elegans intestine which act as an ultradian pattern generator though the generation of intracellular calcium oscillation (Thomas, 1990, Dal Santo et al., 1999), these in turn translate into motor output that are initiated by the secretion of protons from the intestine (Beg et al., 2008). Our expression analysis and electrophysiological data provided us with four ASIC candidate subunits, ACD-3, ACD-5, DEL-5 and FLR-1 expressed in the intestine. ACD-5 is open at neutral pH and becomes blocked at low pH when ectopically expressed in Xenopus oocytes (pH50 of 4.90, n = 5). Tagged ACD-5 localises to the intestinal apical membrane exposed to the acidic intestinal pH. In vitro evidence in HEK cells show that it can interact with the other subunit candidates. Double and triple mutants show a starved phenotype suggesting that these ASICs are likely to be involved in the pH-dependent nutrient uptake. Mutants show abnormal rhythmic motor behaviour that is initiated by intestinal calcium, meaning missed and weak muscle contractions and increased intervals between contractions. The median wild-type contractions occur every 48 seconds, while in acd-5 mutants they occur every 55 seconds, flr-1 single mutant the interval length is reduced to 33 seconds and for acd-3/del-5 double mutants or acd-5/acd-3/del-5 triple mutants to 39 and 35 seconds (n<15 per condition). A Kruskal-Wallis test was statistically significant p>0.0001, a post-hoc multiple comparison test showed that only the acd-5 mutant showed a statistically significant increase in interval length. By visualising pH fluctuations using fluorescent dye (KR35) (Bender et al., 2013) and calcium transients in live animals, we showed an overall reduction of acidity in the intestinal lumen of acd-5 mutants (9<n>24) as well as slower intervals of the intestinal calcium wave (n>10 animals, 5 consecutive intervals, Mann-Whitney U, p=0.439), this directly translates to slowing of rhythmic muscle contractions as described above. For our behavioural analysis, live calcium imaging of the intestinal cells and intestinal lumen pH we used 10-28 animals for each genotype. For calcium imaging and behavioural analysis, we
observed each individual animal for 5 consecutive cycles. The data did not follow a normal distribution and was therefore analysed using a non-parametric Mann-Whitney U test or Kruskal-Wallis test with Bonferroni correction. Our results suggest that the ACD-5 is indeed involved in pH maintenance in the intestine and that it interacts with calcium transients in the intestinal epithelium and that the interplay between pH and calcium oscillations translates into behaviour.


OC04

Effects of the skeletal Ryanodine Receptor Type 1 mutation on uterine artery vascular smooth muscle function and fetal growth in pregnant RyR1Y522S/+ mice

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Introduction

The type 1 ryanodine receptor (RYR1) is predominantly associated with excitation contraction coupling in skeletal muscle. Reports from malignant hyperthermia susceptible (MHS) patients with RYR1 mutations report mild abnormal bleeding phenotypes, typically characterised by menorrhagia, post-partum haemorrhage, and gum/post-operative bleeding[1]. This implies contribution of RyR1 in vascular smooth muscle and, the female reproductive system. Utilizing an established MHS mouse model we explored the hypothesis that RyR1 Y22S will impact pregnant uterine artery function and fetal/placental growth.

Methods

Female nulligravida C57/B6 heterozygous RyR1Y522S/+ knock-in mice (G2) and female wild-type mice (G3), aged 8 to 14 weeks were time-mated with wild-type males. Wild-type female mice were also mated with HTZ males (G1). Sighting of a copulatory plug was designated gestation day 0.5, at gestation day 18.5 females and fetuses were culled using Schedule 1 methods. Fetuses (G1 n=39, G2 n=45, G3 n=83) and placentas (G1 n=32, G2 n=38, G3 n=83) were separated, patted dry for weighing.
Uterine artery segments (2 mm) were dissected from excised pregnant uterus and mounted on DMT wire myographs. Vessels were pre-incubated with dantrolene (30µM) or dimethyl sulfoxide (DMSO control) and contracted with cumulative concentrations of phenylephrine (PE, 10nM-10µM) (DMSO G1 n=16, G2 n=12, G3 n=18; dantrolene G1 n=12, G2 n=10, G3 n=17) or pre-constricted with PE and relaxation induced by cumulative concentrations of carbachol (CCH, 10nM-10µM) (DMSO G1 n=15, G2 n=10, G3 n=14; dantrolene: G1 n=10, G2 n=9, G3 n=13).

Data was analysed using GraphPad PRISM, curves of best fit created using non-linear regression model and variables Maximum, Minimum, IC50 and Hillslope analysed. Data presented as mean ± SEM, analysed with one-way ANOVA for multiple groups and unpaired t-test for two groups, (n = number of animals).

Results

Fetuses from G2 mothers (0.7378g±0.0602, P=0.0007) and G1 mothers (0.6967g±0.037, P<0.0001) were smaller compared to fetuses from G3 mothers (0.9256g±0.0225). Placentas from G2 mothers were larger (0.1158g±0.0041) compared to placentas from G3 mothers (0.0950g±0.0015,P<0.0001) and G1 mothers (0.0966g±0.0026,P=0.0003). The number of fetal resorptions recorded in G2 mothers (median=2, n=3) were twice that of G3 and G1 mothers (median=1, n=5, n=7). G2 had smaller litter size (7.417±0.3361) compared to G3 mothers (8.4±0.2753,P=0.0333), G1 litter size (8.25±0.3708,P>0.2) was not significantly different from either G2 or G3. Neither PE inducted contraction or CCH relaxation of pregnant uterine artery was significantly different between three groups (P>0.05). Pre-treatment with dantrolene did not impact vessels contraction or relaxation. Data provided as a table.

Conclusions

Reduced fetal weight in litters from HTZ mothers indicates impaired fetal growth due to the presence of RYR1 Y522S in the litter. Increased placental weight may indicate a compensatory mechanism for reduced fetal weight. However, increased placental weight was only observed in HTZ mothers, suggesting the influence of maternal genotype and physiology on placental growth. Increased fetal resorptions and reduced litter size in HTZ mothers suggests that maternal RYR1 Y522S impacts fetal survival. The lack of changes in pregnant uterine artery function ex vivo suggests that RYR1 Y522S may impact fetal and placental growth via mechanisms excluding uterine artery function.


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Characterisation of T1019PfsX38 hERG mutation reveals changes in channel gating dynamics

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Loss-of-function mutations in the hERG K+ channel cause long QT syndrome type II (LQT2) (Curran et al. 1995). This increases the incidence of cardiac arrhythmias and sudden death in affected individuals. More than 1000 mutations were reported to cause LQT2, but only a fraction of them has been functionally characterised. T1019PfsX38 (c.3054delC) is a mutation which was identified in an extended LQT2 family from Oman (Al Senaidi et al. 2014). It affects the C-tail of hERG and causes a severe phenotype in homozygote individuals and a mild phenotype in heterozygote individuals. We sought to determine if T1019PfsX38 affects hERG channel function in a heterologous expression system. We utilised the whole-cell configuration of the patch clamp technique to study wild-type-(WT-) and T1019PfsX38-hERG channels that were transiently expressed in human embryonic kidney 293 (HEK293) cells. All electrophysiology experiments were conducted at room temperature and holding potential of -80 mV. The intracellular solution contained in mM: 130 KCl, 5 MgCl2, 5 EGTA, 5 Na2ATP and 10 HEPES (pH 7.2). The extracellular solution contained in mM: 140 NaCl, 4 KCl, 2.5 CaCl2, 1 MgCl2, 10 D-glucose and 5 HEPES (pH 7.45). The voltage dependences of current activation and inactivation were determined by fitting the current-to-voltage relationships using the Boltzmann equation $I = (I_{\text{max}} / 1 + \exp((V_{\text{mid}} - V) / k)) + C$. The results are mean ± SD of n observations; n is the number of cells. Statistical analyses were performed using Student’s t test. Both WT- and T1019PfsX38-hERG produced considerable currents when expressed in HEK293 cells. Depolarising test potentials from -40 to 60 mV elicited inwardly rectifying currents at positive voltages in both conditions (n > 7). In addition, both variants produced strong tail currents upon repolarisation (n > 7). The densities of these tail currents after repolarisation from 20 mV were similar for both variants (WT-hERG, $I(density) = 70.43 ± 28.98$ pA/pF, n = 5; T1019PfsX38-hERG, $I(density) = 78.90 ± 36.14$ pA/pF, n = 6; P > 0.05). There were no differences in the voltage dependences of WT- and T1019PfsX38-hERG channel activation (WT-hERG, $V_{\text{mid}} = 4.56 ± 7.07$ mV, k = 8.09 ± 1.32, n = 5; T1019PfsX38-hERG, $V_{\text{mid}} = 1.96 ± 5.34$ mV, k = 9.73 ± 2.04, n = 6) (P > 0.05) or inactivation (WT-hERG, $V_{\text{mid}} = -44.68 ± 5.98$ mV, n = 5; T1019PfsX38-hERG, $V_{\text{mid}} = -47.29 ± 6.43$, n = 6) (P > 0.05). The time constants of current recovery from inactivation were equal for both variants at all tested potentials (n ≥ 6) (P > 0.05). However, at hyperpolarising potentials (-100 to -140 mV), the time constants of current deactivation were > 8-fold higher for WT- (n = 6) than T1019PfsX38-hERG (n = 7) (P < 0.05). Therefore, the modified C-tail by T1019PfsX38 changes the dynamics of hERG channel gating by accelerating deactivation at hyperpolarising potentials. These results have potential implications for the design of LQT2 mutation specific therapies.


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OC06

Cholinergic control of ATP-mediated interkleukin-1β release in monocytes involves endothelial NO synthase

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Objective: The pro-inflammatory cytokine interleukin-1β (IL-1β) is important for host defense against infections. High systemic IL-1β levels, however, contribute to the pathogenesis of numerous life-threatening inflammatory diseases. Therefore, mechanisms controlling IL-1β release are of substantial clinical interest. Recently, we identified a cholinergic mechanism that inhibits the ATP-mediated release of IL-1β by human monocytes via nicotinic acetylcholine receptor (nAChR) subunits α7, α9 and/or α10. We also discovered novel nAChR agonists that trigger this inhibitory function in monocytic cells without eliciting ionotropic functions at conventional nAChRs. Here, we investigate the metabotropic signaling pathway that links nAChR activation to the inhibition of the ATP-sensitive P2X7 receptor (P2X7R).

Methods: Lipopolysaccharide-primed human monocytic U937 cells, primary human monocytes (isolated from whole blood by negative selection) and peripheral blood mononuclear cells (PBMC) isolated from wild-type and endothelial nitric oxide synthase (eNOS) gene-deficient mice were used. All experiments on human blood samples (performed in accordance with the Helsinki Declaration) and on animals were approved by the local ethics committee. Animals received human care according to NIH “Guide for the Care and Use of Laboratory Animals”. Cells were stimulated with the P2X7R agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine-5’-triphosphate (BzATP) and IL-1β was measured in cell culture supernatants by ELISA. A panel of nAChR agonists (e.g. nicotine, acetylcholine), eNOS inhibitors (L-NIO, L-NAME), NO-donors (SNAP, SIN-1) and the siRNA technique were used. Patch-clamp and intracellular Ca²⁺ imaging experiments were performed on P2X7R-overexpressing HEK cells.

Results: In human monocytic U937 cells the inhibitory effect of nAChR agonists was prevented by eNOS inhibitors (n = 4, P ≤ 0.05, Kruskal Wallis test followed by Mann Whitney rank sum test). Furthermore, the BzATP-induced IL-1β release was blunted in U937 cells treated with siRNA to silence eNOS expression. In the same line, in PBMC from eNOS gene-deficient mice the inhibitory effect of nAChR agonists was absent (n = 5, P ≤ 0.05, Friedman test followed by Wilcoxon signed-rank test), suggesting that nAChRs signal via eNOS to inhibit BzATP-induced IL-1β release. In patch-clamp...
and Ca2+ imaging experiments, BzATP induced ionotropic P2X7R responses. In line with these data, the BzATP-induced P2X7R activity was abolished in presence of the NO donor SIN-1, suggesting that nitrosylation of P2X7R inhibits its ionotropic function. The inhibitory effect of SIN-1 on BzATP-mediated IL-1β release was confirmed in freshly isolated human monocytes (n = 6, P ≤ 0.05, Friedman test followed by Wilcoxon signed-rank test).

**Conclusion:** We provide evidence that activation of monocytic nAChRs induces metabotropic signaling, which involves eNOS activation, inhibition of the P2X7R most likely via receptor nitrosylation and, thus, inhibition of ATP-mediated IL-1β-release. This signaling pathway might be protective against damage-mediated sterile inflammation.

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**PC01**

Activation of tracheal brush cells promotes *Trpm5*-dependent innate immune responses in *Pseudomonas aeruginosa* infection

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Previously, we have shown that transient receptor potential channel M5 (*Trpm5*) expressing tracheal brush cells (BCs) are cholinergic chemosensors that elicit respiratory reflexes triggered by *Pseudomonas aeruginosa* quorum-sensing-associated metabolites (QSM).

Here, we investigate the impact of *Trpm5* function on the disease outcome in *P. aeruginosa* (PA) pneumonia.

Changes in [Ca2+]i levels in BCs in response to the PA product PQS (3,4-dihydroxy-2-heptylquinoline) were studied on tracheal explants of *Trpm5*-GCaMP3-mice. Plasma extravasation and neutrophil recruitment was studied 30 minutes after Evans Blue (EB) application followed by inhalation of either PQS or supernatants of various PA strains. An experimental infection model was established using the mucoid NH57388A strain isolated from a cystic fibrosis patient to inoculate *Trpm5*+/+ and *Trpm5*−/−.
mice. Values are presented as mean ± SEM. Data were subjected to the Kolmogorov-Smirnov-test to examine normal distribution followed by either paired or unpaired Student’s t-test, Mann-Whitney-, Kruskal-Wallis-test or ANOVA to delineate statistical significance. P-values below 0.05 were considered statistically significant. All animal care and experimental procedures were performed according to the German and European animal welfare law and they were approved by the German animal welfare committee.

In BCs PQS increased the [Ca^{2+}] level from 0.97 ± 0.01 AU to 1.73 ± 0.16 AU (n=40 cells from 3 mice, Student’s paired t-test p<0.001). Inhalation of either PQS or bacterial supernatants induced a significant increase of EB extravasation (PQS: from 3.98 ± 0.4 AU (vehicle) to 16.08 ± 0.86 AU (1 µM), 14.31 ± 0.87 AU (10 µM) and 14.98 ± 1.06 AU (50 µM); PA103: from 6.17 ± 0.65 AU to 18.99 ± 2.01 AU; NH57388A: from 3.98 ± 0.4 AU to 14.15 ± 1.51 AU; n=10-15 rings/3 mice, ANOVA p<0.05) and enhanced recruitment of neutrophils to the tracheal extraepithelial space (PQS: from 5.33 ± 1.15 to 17.64 ± 3.85 (1 µM), to 27.56 ± 5.39 (10 µM) and to 15.7 ± 5.31 neutrophils/tracheal ring (50 µM); PA103: from 1.97 ± 0.39 to 6.0 ± 0.78 neutrophils/ring; NH57388A: from 1.97 ± 0.39 to 4.8 ± 0.94 neutrophils/ring; n=10-15 rings/3 mice, ANOVA (PQS) and Mann-Whitney-test (supernatants) p<0.05). Interestingly, also supernatants of a QSM-deficient strain (D8A6) led to EB extravasation (from 3.98 ± 0.4 AU to 12.58 ± 0.52 AU; n=15 rings/3 mice, ANOVA p<0.01) but this effect was reduced (ANOVA p<0.01) compared to the QSM-competent strain PA103. Additionally, an increased number of neutrophils was observed in lungs after stimulation with PQS or NH57388A supernatant (PQS: from 26.31 ± 1.63 to 44.08 ± 2.29 neutrophils/square (50 µM); NH57388A: from 11.94 ± 0.71 to 18.8 ± 1.02 neutrophils/square; n=20 squares/3 mice, ANOVA p<0.01). Infection with NH57388A led to higher weight loss (87.22 ± 1.44 % (Trpm5^{+/+}) to 81.67 ± 1.65 % (Trpm5^{-/-}) remaining bodyweight) and mortality in Trpm5^{-/-} mice (n=13 mice, Student’s unpaired t-test p<0.05). Lungs of Trpm5^{-/-} mice exhibited more damaged areas compared to healthy lungs (n=3 mice).

Our results provide evidence for a BC-dependent activation of neurogenic inflammation in order to rapidly eliminate inhaled pathogens via neutrophil recruitment ameliorating infection outcome.

Acknowledgements :- This work was supported by the German Research Foundation (DFG SFB TRR 152 projects P11 and Z02 to UB, project P15 to VC and TG and project P22 to GKC).

PC02

The cardiotonic steroids digoxin and marinobufagenin modulate tight and gap Junctions through the Na^+, K^+ - ATPase.

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Cardiac glycosides are a group of compounds widely known for their action known for their inhibitory activity of the Na’, K’ ATPase, which made them useful as therapy against cardiac disease in cardiac tissue. Some of them have been found to be endogenously produced (ECG).

Besides inhibiting the ATPase, cardiotonic steroids can bind to the ATPase triggering signaling cascades that influence several physiological processes of cells. Thus, the Na’, K’ ATPase has acquired importance as a receptor that modulates the physiology of the cell. We have previously shown that ouabain modulates characteristic structures of epithelia such as tight junctions, gap junction, cilia, and adherens junctions.

In this work, we study the influence of digoxin and marinobufagenin, two others endogenously expressed cardiac glycosides, on Gap junctional Intercellular Communication (GJIC) as well as the degree of transepithelial tightness due to tight junction integrity (TJ) of epithelial cells, using MDCK-II confluent monolayers. We evaluated GJIC by dye transfer assays and tight junction integrity by transepithelial electrical resistance (TER) measurements, as well as immunohistochemistry and western blot assays of expression of claudins 2 and 4. All studies were performed by triplicate. For TER assays, western blot and Immunocytochemistry we performed n=9, while for dye transfer assays we kept n>30 (usually between 50 and 120), being n the individual cells injected on each assay. Statistical significance was estimated via ONE-WAY analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison or Student’s t-test.

We found that both digoxin and marinobufagenin improve GJIC and significantly enhance the tightness of the tight junctions, as evaluated from TER measurements. Immunofluorescence assays show that both compounds promote enhanced basolateral localization of claudin-4 but not claudin 2, while densitometric analysis of western blot assays indicate a significantly increased expression of claudin 4. These changes, induced by digoxin and marinobufagenin on GJIC and TER, were not observed on MDCK-R, a modified MDCK cell line that has a genetically induced insensitive α1 subunit, thus suggesting that Na’, K’ ATPase acts as a receptor mediating the actions of both ECG. Plus, the fact that the effect of both cardiac glycosides was suppressed by incubation with PP2, an inhibitor of c-Src kinase, PD98059, an inhibitor of mitogen extracellular kinase-1 and Y-27632, a selective inhibitor of ROCK, and a Rho-associated protein kinase associated to cell to cell junctions regulation, indicate altogether that the signaling pathways involved include c-Src and ERK1/2, as well as Rho-ROCK.

These results widen and strengthen our general hypothesis that a very important physiological role of ECGs and the Na’, K’ ATPase is the control of the epithelial phenotype and the regulation of cell-cell contacts.

This work has been published as Ogazon Del Toro et al., 2019. Influence of The Cardiac Glycosides Digoxin and Marinobufagenin on Ephitelial Physiology. Cardiol Res Pract. Dec 30;2019:8646787.
Differential Expression of Store Operated Calcium Entry Complex Proteins in Human Skin and Sweat Gland Cell Lines

Acknowledgements: CONACyT - Comisión Nacional de Ciencia y Tecnología, México

Reference 1: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7024086/
Reference 3: https://linkinghub.elsevier.com/retrieve/pii/S0270-9295(05)00053-7
Background: Several studies have identified the membrane receptors and proteins involved in the control of calcium signalling and ion transport in skin and eccrine sweat gland cells. Thus, Store-Operated Ca\textsuperscript{2+} Entry (SOCE) has been identified as the major mechanism for Ca\textsuperscript{2+} import from the extracellular to intracellular cell space in both keratinocytes and eccrine sweat gland secretory coil cells\textsuperscript{1,2}. SOCE involves the detection of Ca\textsuperscript{2+} store depletion in the endoplasmatic reticulum (ER) by stromal interaction molecule (STIM) proteins, which then translocate to the plasma membrane to activate the Ca\textsuperscript{2+}-selective channel Orai. In contrast, the signalling pathways utilised by apocrine glands are unresolved. This study aims to determine and compare the expression and activity of STIM and Orai SOCE proteins in human-derived skin and sweat gland cell lines.

Materials and Methods: Expression of SOCE-associated genes and proteins were determined by qPCR, western blot analysis and immunofluorescence (N=3). SOCE was induced by Ca\textsuperscript{2+} addition after Ca\textsuperscript{2+} depletion from the ER with thapsigargin (Tg), an inhibitor of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA). Employing keratinocytes (HaCaT), eccrine (NCL-SG3) and apocrine (ASG5)\textsuperscript{3} cell lines, intracellular Ca\textsuperscript{2+} responses were studied using Ca\textsuperscript{2+} imaging techniques (n\textsubscript{HaCaT} = 14-28 cells; n\textsubscript{NCL-SG3} = 20-37 cells; n\textsubscript{ASG5} = 10-20 cells; N=3). The magnitude of the Ca\textsuperscript{2+} responses was determined by calculating the area under the Tg-evoked curve.

Results: qPCR demonstrated significantly different levels of SOCE protein gene expression in the skin and sweat gland cell lines used. Low levels of STIM1 (P<0.001) and Orai1 (not significant) were detected in ASG5 cells compared to HaCaT and NCL-SG3 cells, whereas STIM2 was significantly higher in ASG5 versus HaCaT cells (P<0.001), and Orai3 was higher in ASG5 versus NCL-SG3 cells (P<0.05). Western blot analysis revealed STIM2 and Orai3 to be the predominant SOCE proteins expressed in the apocrine ASG5 cell line relative to the STIM1 levels of HaCaT cells (P<0.05) and Orai1 levels of both HaCaT (P<0.005) and NCL-SG3 cells (P<0.05). In HaCaT cells, SOCE proteins were mainly located in the plasma membrane, whilst in the NCL-SG3 cell line the majority of proteins were confined to cytoplasmic areas, with STIM1 and 2 predominantly located in the ER region. However, in ASG5 cells, STIM2 and Orai3 proteins were detected in the plasma membrane region, whereas STIM1 and Orai1 proteins were localized in cytoplasmic areas. Furthermore, intracellular Ca\textsuperscript{2+} concentration measurements demonstrated that activation of SOCE with 3\textmu M Tg induced a smaller Ca\textsuperscript{2+} response in apocrine ASG5 cells compared to both HaCaT cells (P<0.0001) and eccrine NCL-SG3 cells (P<0.05).

Conclusions: This study confirms previous observations that keratinocytes and eccrine gland cell types express high levels of both STIM1 and Orai1. It also shows comparatively, that cells derived from the apocrine gland express relatively higher amounts of STIM2 and Orai3, suggesting a novel role for these proteins in intracellular signalling in this tissue.

Reference 1 :- Tomita N-T, Putney JW, J Cell Sci 126, 606-612; 2012
Modulation of impaired uterine contractility by Transient Receptor Potential cation TRPV4 and TRPC4 channels

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Impairment of uterine contractility underlies the most common problems in modern obstetrics that still cannot be properly treated, such as preterm uterine contractions [1], weak uncoordinated labor contractions (dystocia) [2] and postpartum hemorrhage [3]. Thus, it is important to identify new molecular targets that can modulate myometrium contractility and be used for the development of novel therapeutical approaches. TRP channels demonstrate a wide range of activation stimuli, ion selectivity and variability in the regulation of their functioning [4]. TRPV4 and TRPC4 are being gated in a different manner (they are primarily mechanosensitive and receptor-operated channels, respectively). However, they both contribute to the increase of the intracellular Ca²⁺ concentration and therefore may serve as novel targets for regulating uterine contractility.

We used in vitro recording of the contractile force of myometrium strips isolated from either pregnant rats (18-22 days) or from women at term (38-40 weeks, tissue obtained during elective cesarean section) with full compliance with the World Medical Association’s Declaration of Helsinki and local bioethical policies (protocol #4 of 10.10.2018). Statistical comparison of the data was made by using the Mann-Whitney test or Student’s t-test in accordance with the type of data distribution.

Application of the TRPV4 agonist GSK1016790A (0.3 μM) to the human myometrium increased muscle tension with no phasic contractions in one group of samples (n=5). Other examined strips (n=4) responded with phasic activity with some peaks more than 3.5 times higher. Average contraction force amplitude was increased up to 1.16±0.04 compared to 1.00 reference value (p<0.05). When treated with oxytocin (10 nM) in the presence of the TRPV4 blocker HC-067047 (1 μM) one group (n=4) demonstrated only 33% increased force of contractions (1.33±0.22; p<0.05) compared to the response to oxytocin alone, where the amplitude was 5.3±1.48. In the second group of muscle strips (n=4), phasic contractions were attenuated, while muscle tension was decreased by 51% (p<0.05).

In rats, activation of TRPC4 by its potent direct agonist (-)-englerine A (1 nM) leads to a 150% increase of the amplitude of spontaneous myometrial contractions (2.52±0.64; n=9, p<0.05). This was similar to the effect evoked by either oxytocin or carbachol. No significant changes of contractile activity were observed in response to (-)-englerine A (1-100 nM) either in human myometrium from patients with normal laboring (N=3, n=14), or in preparations from patients with dystocia that were
responsive to oxytocin (n=6). In contrast, (−)-englerine A (30 nM) evoked phasic contractions (Me=1.27, Q1=0.91, Q3=1.62) in preparations from patients with dystocia that showed weak responses to oxytocin (n=6).

Thus, activation of TPRV4 and TRPC4 modulates the contractile response of the myometrium and the studies focused on the use of TRPV4 inhibitors for the blockade of mechanosensitive response are promising. Further investigation of TRPC4 agonists as an additional contribution to signalling via oxytocin receptors is also warranted.


Acknowledgements :- We would like to thank to Dr. David Beech for his kind supply of (−)-englerine A for this investigation. This work was supported by grants of the Department of Targeted Training of National Academy of Science of Ukraine (#7B-2018) and Ministry of Education and Science of Ukraine (#19BF036-01)
**Methods:** The experiments were conducted on human kidney cells (HK-2) and differentiated primary human skeletal muscle cells (myotubes). The abundance of total and phosphorylated proteins was estimated by immunoblotting. The metabolic status was assessed by measuring the lactate production, the cellular ADP and ATP content, and oxygen consumption rate. One-way ANOVA with Dunnett’s or Bonferroni test were used for statistical analysis. **Ethics statement:** Procedures on primary human myotubes were approved by National medical ethics committee and accorded with the ethical standards and principles of the World Medical Association’s Declaration of Helsinki.

**Results:** AMPK activators AICAR and A-769662 did not affect the basal phosphorylation of Tyr10 in HK-2 cells (n=16 replicates), while they suppressed it by more than 50% in human myotubes (n=8 donors, \( P<0.05 \)). Epidermal growth factor (EGF) increased Tyr10 phosphorylation by 250% in HK-2 cells (n=18 replicates, \( P<0.0001 \)) and by 86% in human myotubes (n=8 donors, \( P<0.0001 \)), which was opposed by inhibitors of tyrosine kinases (genistein), Src kinases (PP2), and EGF receptor (EGFR, gefitinib) (Fig. 1). In HK-2 cells, AMPK activators decreased the EGF-stimulated phosphorylation of Tyr10 as well as EGFR (Tyr1173), which suggested that AMPK blocked NKA phosphorylation by suppressing the activation of EGFR. Ouabain (100 nM), a pharmacological NKA inhibitor (Fig. 1), did not affect the basal phosphorylation of Tyr10, but it increased the EGF-stimulated Tyr10 phosphorylation by 98% (n=12 replicates, \( P<0.0001 \)) without altering the phosphorylation of EGFR (Tyr1173). Ouabain acted in a dose-dependent manner (30-1000 nM) to decrease the lactate production by up to 24% (n=6 replicates, \( P<0.05 \)) as well as the basal and maximal (uncoupled) oxygen consumption rate, respectively, by up to 16% and 38% (n=6 replicates, \( P<0.0001 \)). The ADP:ATP ratio was concomitantly increased by up to 26% (n=9 replicates, \( P<0.01 \)), which indicated inhibition of NKA by ouabain did not improve cellular energy status.

**Conclusions:** In summary, AMPK activation suppressed the phosphorylation of Tyr10, while NKA inhibitor ouabain promoted it. Collectively, our results suggest a new mechanism by which AMPK regulates NKA, while highlighting a link between regulation of energy metabolism and NKA-mediated ion transport in kidney and skeletal muscle.
Figure 1: The phosphorylation of Tyr10 (Y10) of the NKA α-subunit is suppressed by AMPK and stimulated by EGF via EGFR and Src tyrosine kinase pathway. Legend: AICAR, A-709662 – pharmacological activators of AMPK; gefitinib – EGFR inhibitor; geranstein – tyrosine kinase inhibitor; PP2 (4-amino-5-[4-chlorophenyl]-7-[t-butyl] pyrazolo[3,4-d]-pyrimidine) – Src kinase inhibitor. Abbreviations: AMPK – AMP-activated protein kinase; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; NKA – Na,K-ATPase; P – protein phosphorylation; Y10 – Tyr10.

The inhibitory action of single-walled carbon nanotubes on TRPC4-mediated muscarinic cation current in mouse ileal smooth muscle cells

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Single-walled carbon nanotubes (SWCNTs) possess many unique physical and chemical properties, which make them promising nanostructured materials for biomedical use [1]. SWCNTs can interact with a variety of biological macromolecules, such as receptors and ion channels. It has been previously shown that SWCNTs can block different types of K⁺ channels heterologously expressed in mammalian cells [2]. Here we aimed to study the effects of SWCNTs on the muscarinic cation current, termed mICAT, using both molecular modelling and electrophysiological studies. This current is recognized as the principal component of cholinergic excitation-contraction coupling in gastrointestinal smooth muscles, which is mainly carried by TRPC4 channels [3,4].

SWCNTs of 0.5-2 nm in diameter and 1-5 μm in length were synthesized by means of the arc-discharge technique between two graphite electrodes in He atmosphere (700 mbar). SWCNTs were carboxylated by HNO₃ (3 M) treatment during 2 h at T=373 K to improve their hydrophilicity. Molecular interactions between SWCNTs and TRPC4 channel protein were simulated using CHARMM-GUI software, while calculations were done using gromacs 2020. Ileal myocytes were freshly isolated from three month-old male BALB/c mice by enzymatic tissue digestion using collagenase type 1A. Patch-clamp recordings of whole-cell currents were carried out at room temperature using symmetrical Cs⁺ containing (125 mM) solutions with 10 mM BAPTA/4.6 mM CaCl₂.
mixture in the pipette solution to 'clamp' \([\text{Ca}^{2+}]\), at 100 nM for \(\text{mICAT}\) isolation. Steady-state \(I-V\) relationships of \(\text{mICAT}\) were measured by slow (6 s duration) voltage ramps from 80 to -120 mV, which were applied every 30 s. Values are presented as means ± S.E.M.

Using molecular docking and molecular dynamics simulations we found that SWCNTs could form stable bonding with TRPC4 channels. Depending on the size of SWCNTs, three optimal binding models were considered, all of which predicted channel pore hindrance for cation entry. For direct tests using patch-clamp recordings, \(\text{mICAT}\) was activated by GTPγS (200 µM) infusion via patch-pipette, thus bypassing M2 and M3 receptors. Under these conditions, the current slowly reached a peak amplitude in about 5-7 min, at which point SWCNTs (10 µg/ml) were applied to the external solution. This resulted in slow current inhibition with mean time constant of 180±31 s (n=5). Mean inward \(\text{mICAT}\) amplitude was -650.4±146.1 pA in control decreasing to -269.2±84.0 pA in the presence of SWCNTs (paired \(t\)-test, two-tail P value of 0.03; n=5). Notably, \(\text{mICAT}\) was inhibited to similar extent in the whole range of membrane potentials and the inhibition was associated with some acceleration of current deactivation during voltage steps from -40 to -120 mV (mean time constant was 35.2±3.3 ms in control reducing to 18.1±3.1 ms in the presence of SWCNTs; paired \(t\)-test, two-tail P value of 0.018; n=5) suggesting a shortening of open dwell time of the channel in the presence of SWCNTs.

We conclude that SWCNTs inhibit TRPC4 channel in a voltage-independent manner by shortening its open state, consistently with their interaction occurring within the plane of TRPC4 channel pore as predicted by \textit{in silico} molecular dynamics and docking simulations.


Acknowledgements :-

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PC07

Prostaglandin E2 increases intercellular communication through gap junction channels

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Gap Junctions are channels that connect two cells, allowing the interchange of small molecules such as ions and second messengers. They are critical to maintain cell homeostasis and participate in several physiological processes. They are regulated by several endogenous compounds, among them prostaglandins.

Prostaglandins are a group of lipids that produce diverse physiological and pathological effects. Prostaglandin E2 (PGE2) stands out for the wide variety of functions in which it participates. To date, there is little information about the influence of PGE2 on gap junctional intercellular communication (GJIC) in any type of tissue, including epithelia. In this work, we set out to determine whether PGE2 influences GJIC in epithelial cells (MDCK cells). To this end, we performed dye (Lucifer yellow) transfer assays to compare GJIC of MDCK cells treated with PGE2 and untreated cells. All assays were performed as triplicate and n maintained above 30 (being n the number of individual cells injected). Statistical analysis, consisted of Analyses of Variance (ANOVA) test, followed by different tests depending on the normality of data (see published work) Our results indicated that (1) PGE2 induces a statistically significant increase in GJIC from 100 nM and from 15 min after its addition to the medium, (2) Dye transfer assays in the presence of cycloheximide or actynomicine such effect does not require the synthesis of new mRNA or proteins subunits but rather trafficking of subunits already synthesized (demonstrated by the use of nocodazole and brefeldin A), and (3) Assays using commercial inhibitors demonstrate that such effect is mediated by the EP2 receptor, which, in turn, triggers a signaling pathway that includes activation of adenylyl cyclase and protein kinase A (PKA). These results widen the knowledge regarding modulation of gap junctional intercellular communication by prostaglandins.
Possible Involvement of Ionotropic Receptor Gene Modulations in Mesio-Temporal Lobe Epilepsy

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AIM: The Mesio-Temporal Lobe Epilepsy (MTLE) syndrome is the most common form of intractable epilepsies, that is characterized by the recurrence of focal seizures occurring in mesio-temporal limbic structures and is frequently related with hippocampal sclerosis and drug resistance. The aim of this study was to examine possible ion channel modulations in MTLE by using bioinformatics tools, by examining the expression levels of genes, obtained from intrahippocampal kainate-injected MTLE mouse model.

METHODS: GSE88992 dataset obtained from GEO (Gene Expression Omnibus) database was re-examined for this research. In the dataset, total RNA samples derived from normal (n=9) and kainate-induced (n=8) hippocampus tissues of C57BL/6J male mice are recruited. After the gene expression levels in the dataset were re-analysed in the R program, gene set enrichment analyses were performed in Gene Ontology (GO) and ENRICHR tools.

APPROACH FOR STATISTICAL ANALYSIS: Expression levels of genes, commonly implicated to play a role in several biological processes, in GSE88992 dataset are re-analysed in R biostatistics program. Based on Benjamini-Hochberg correction analysis, adjusted p-values <0.05 were accepted as significant.

RESULTS: Gene expression levels on the dataset indicated that some gap junction proteins (GJ- A1, C3, D2), most of potassium voltage-gated channel proteins (KCN- A2,4,6; B1; AB1-3; C1-3; D1-2; E3; G1,2,4; H3,5,8; Q2,3,5; S1-3; V1; N1; T1-2; U1; MA1; J3,9,11,12), cyclic nucleotide-gated channels (HCN2, CNGA4), transient receptor potential cation channels (TRP-C1,2,4,5; M2,7), mucolipin 1 (MCOLN1), sodium channels (SCN- 1A, 2A1, 2B, 3A, 4B, 8A, M1; NALCN), some cholinergic receptors (CHR-N A3,4,5,7, B2), gamma-aminobutyric acid (GABA) A receptors (GABR-A1,3,5; B1-3; D; G2-3), glutamate receptors (GRIA 1-4; GRIK 1-5; GRIN- 1, A, 3A, 2B, 2C), some voltage-sensitive chloride channels (CLCN-2,6), some anoctamin receptors (ANO- 3,4,8), ryanodine receptor genes (RYR1-3) were down-regulated (p<0.05); and GJ- A3,4, B1,2, C1; KCN- A4, D3, F1, Q1, J8,14; TRP-V4, M6; CHR-N A1, B1, E, G; GABRA2; CLCN-3,5; ANO-1,6 genes were up-regulated (p<0.05) in the MTLE mice group, compared with normal group (Table 1).

CONCLUSION: Results from this in silico analysis indicate imbalances in the expression levels (down- and up-regulation) of genes encoding ionotrophic receptors, implicating involvement of impaired ion channel modulations in the pathogenesis of MTLE.
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<td>transient receptor potential cation channel, subfamily C, member 4</td>
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<td>PKD2</td>
<td>polycystic kidney disease 2</td>
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<td>SCN2A1</td>
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<td>SCN8A</td>
<td>sodium channel, voltage-gated, type VIIIi, alpha</td>
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<td>CACNA1B</td>
<td>calcium channel, voltage-dependent, N type, alpha 1B subunit</td>
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<td>NALCN</td>
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<td>CHRNA7</td>
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Table 1. Differential expressed genes about ionotropic receptors results (FC: fold-change; Red and italic labels represent down-regulated genes and blue labels represent up-regulated genes.)


Acknowledgements: - I would like to express my gratitude to my supervisor, Prof. Dr. Ahmet AYAR, who has never withheld his scientific and spiritual support.

PC09

Potassium-Selective Stretch-Activated Ion Channels are Present in Human Atrial Fibroblasts: Exploring a Possible Role in Mechano-Induced Fibrosis

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1Institute for Experimental Cardiovascular Medicine, University Heart Center Freiburg-Bad Krozingen, Freiburg im Breisgau, Germany 2Medical Center and Faculty of Medicine, University of Freiburg, Freiburg im Breisgau, Germany 3Spemann Graduate School of Biology and Medicine (SGBM) and Faculty of Biology, University of Freiburg, Freiburg im Breisgau, Germany 4Centre for Integrative Biological Signalling Studies (CIBSS), University of Freiburg, Freiburg im Breisgau, Germany

Prolonged and abnormal mechanical stimuli, as described in atrial fibrillation (AF), may be sensed and transduced by stretch-activated ion channels (SAC). K+-selective SAC (SACk) were described in cardiac myocytes and non-myocytes. In murine ventricular fibroblasts, SACk play a central role in
pathological tissue remodelling. However, the exact function(s) and relevance of SAC\textsubscript{K}, in particular in human, are not understood, and they are not currently seen as treatment option for targeting tissue remodelling.

We hypothesize that SAC\textsubscript{K} are present in human atrial fibroblasts, that their activity and/or expression is altered in AF, and that they are causally involved in the control of extracellular matrix production, as well as in proliferation, migration and phenoconversion of atrial fibroblasts.

In a newly established cell line of immortalized human atrial fibroblasts, mRNA expression of SAC\textsubscript{K}, namely two-pore-domain K\textsuperscript{+} and big conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, was detected (n=6; N=3). To study functional expression of SAC\textsubscript{K} in this cell line, a combination of patch clamp recordings and pharmacological interventions was used. We identified currents sensitive to arachidonic acid (n=10; N=3; \(p=0.006\)) and to cytoskeletal integrity (n=42; N=3; \(p<0.001\)), as shown by an increase in open probability upon patch excision, suggesting a presence of SAC\textsubscript{K}. However, inter-cell variability of SAC\textsubscript{K} activity was large, possibly due to a heterogeneous population of fibroblasts and myofibroblasts in the cell culture. We therefore enriched the cell culture with either phenotype and found a larger stretch-activated current in fibroblasts (SD-208 treated) compared to myofibroblasts (TGF-\(\beta\)1 treated; n=29 and 21, respectively; N=3; \(p=0.039\) at \(-40\) mmHg and \(0.012\) at \(-50\) mmHg).

In conclusion, we obtained evidence for SAC\textsubscript{K} activity in human atrial fibroblasts, and for their implication in fibroblast phenoconversion. Future work will assess whether SAC\textsubscript{K} are cause for, or consequence of, phenoconversion, and whether they affect mechano-induced fibrosis. If so, this could have an impact on the pathophysiology of AF, and make SAC\textsubscript{K} a target for pharmacological intervention to slow, reduce or reverse tissue remodelling in AF.

Acknowledgements :- ED acknowledges support by Amgen Inc. ED, UR, PK, and RP are members of SFB1425, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation DFG #422681845).

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**PC10**

**Decreased Intrinsic Excitability Potentiation of CA1 Pyramidal Neurons Induced by Neonatal Blockade of NMDA Receptors**

Ernesto Griego\(^1\), Emilio J. Galván\(^1\)

\(^1\)Department of Pharmacobiology. Center for Research and Advanced Studies (CINVESTAV), Mexico City, Mexico

Transient hypofunction of N-methyl-D-aspartate receptors (NMDARs) during postnatal development may underlie cognitive symptoms of schizophrenia (Uehara et al., 2009; Hernández-Frausto et al., 2019), which may also involve dysregulation of voltage-dependent channels that affect intrinsic excitability potentiation (I–E potentiation), a non-synaptic form of neural plasticity involved in memory formation. Here, we aimed to identify electrophysiologic alterations in CA1 pyramidal cells (CA1 PCs) from animals neonatally treated with the NMDA receptor antagonist, MK-801 (0.2 mg/kg)
or with saline solution (n = 46 cells / 22 animals for each condition). The animal procedures followed the guidelines of the Mexican Official Norm for use and care of laboratory animals (NOM-062-ZOO-1999) and the Bioethics Committee of CINVESTAV (Protocol 0090-14). Patch-clamp whole-cell recordings from MK-801-treated animals revealed altered electrophysiologic properties (R_N, τ_membr, and I_Rheo). We also found an upregulation of the Ba^{2+}-sensitive K^+ inward-rectifier conductance. These alterations were accompanied by an increase in the fast-inactivating K^+ current (I_{K-fast} increment = 132.73 % compared to control; P = 0.031) and slow-inactivating K^+ currents (I_{K-slow} increment = 141.32 % compared to control; P = 0.043; Mann-Whitney U tests; n = 8 cells for each condition). These changes in potassium currents were accompanied by a decrease in the firing frequency (F_{1, 10} = 38.58; two-way ANOVA; P < 0.001).

Because our results show that MK-801 reduces R_N, upregulates K^+ currents, and reduces the excitability of CA1 PCs, we hypothesized that MK-801-treated cells would have impaired I–E potentiation. Trains of theta stimulation paired with a postsynaptic action potential triggered I–E potentiation, and increased the AP discharge (baseline: 1.4 ± 0.2; at 45 min after paired stimulation: 5.4 ± 0.5; Student’s t-test; t_{(5)} = 3.651; P = 0.021), the somatic R_N (baseline: 128 ± 11 MΩ; at 45 min after stimulation: 208. ± 14.7 MΩ; Student’s t-test; t_{(5)} = 9.269; P > 0.001), and decreased the AP threshold (baseline: -39.4 ± 1.4 mV; at 45 min after PS: -48.8 ± 0.9 mV; Student’s t-test; t_{(5)} = 10.130; P < 0.001) in control cells. By contrast, MK-801-treated cells expressed blunted I–E potentiation. No changes were detected in AP discharge (baseline: 1.2 ± 0.2; at 45 min after stimulation: 2.2 ± 0.5; Student’s t-test; t_{(5)} = 2.762; P = 0.064) and minor changes were detected in the somatic R_N (baseline: 103.6 ± 10.3 MΩ; at 45 min after stimulation: 146.2 ± 6 MΩ; Student’s t-test; t_{(5)} = 4.508; P = 0.012) and the AP threshold (baseline: -38.8 ± 1 mV; at 45 min after stimulation: -44.6 ± 1.4 mV; Student’s t-test; t_{(5)} = 3.965; P = 0.017). In control cells, I–E potentiation was accompanied by strengthening of the glutamatergic transmission (EPSC at 45 min after stimulation: 246.3 ± 11% of baseline; Student’s t-test; t_{(5)} = 13.522; P < 0.001), but not in MK-801-treated cells (EPSC at 45 min after PS: 125.7 ± 10% of baseline; Student’s t-test; t_{(4)} = 2.553; P < 0.063).

Our results offer mechanistic evidence that may contribute to the understanding of the role of ion channels in schizophrenia pathophysiology.


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PC11

Novel CACNA1I/Cav3.3 calcium channel variants associated with Hemiplegic Migraine
Familial hemiplegic migraine (FHM) is a severe neurogenetic disorder for which three main causal genes, CACNA1A, SCN1A, and ATP1A2, encoding for Cav2.1, Nav1.1 and the K/Na ATPase, respectively, have been implicated (Russel et al., 2011). However, studies by our group and others show that more than 80% of referred diagnostic cases of hemiplegic migraine (HM) are negative for exonic mutations in FHM genes, invoking the involvement of other genes in the disorder (Maksemous et al., 2019).

Using whole exome sequencing data from 187 HM cases confirmed to be negative for mutations in known FHM genes, rare CACNA1I gene variants encoding the T-type calcium channel Cav3.3 were identified. Some novel CACNA1I variants appeared in multiple probands and at higher rates than expected based on minor allele frequency data from large control cohorts derived from gnomAD and UK Biobank databases. Burden testing of CACNA1I variants showed a statistically significant increase in allelic burden in the HM case group compared to gnomAD (OR = 2.30, P = 0.00005) and the UK Biobank (OR = 2.32, P = 0.0004).

The biophysical properties of whole-cell currents mediated by five recombinant Cav3.3 variants bearing changes in functionally defined extracellular regions (Fig. 1A) were recorded from HEK293 cells using patch clamp electrophysiology (Fig. 1). In comparison to Cav3.3-WT (208±14.9pA/pF, n=17), the current density of variants D302G (121±18pA/pF, n=11; p=0.0008), R307H (104±10pA/pF, n=11; p< 0.0001) and Q1158H (133±10pA/pF, n=21; p<0.0001) were significantly lower; whereas variants R111G (153±35pA/pF, n=5; p=0.1101) and M128L (196±31pA/pF, n=11; p=0.6902) were expressed at current levels comparable to WT. Cav3.3-R307H (n=6, p=0.0002) and -Q1158H (n=11; p=0.0009) channels displayed ~2-fold slower current kinetics than those mediated by WT (Fig. 1B). The half-activation voltages of activation (act V0.5) of Cav3.3-M128L (-23±1 mV, n=12; p=0.0216), R307H (-20±1mV, n=6; p=0.04914) and Cav3.3-Q1158H (-18±0.3mV, n=9; p=0.0025) displayed statistically significant shifts from Cav3.3-WT activation (-21±1mV, n=15). Furthermore, Ca,3.3-Q1158H channels displayed a depolarizing shift in voltage dependence of half maximal inactivation, SSI V0.5 (-43±1mV, n=9; p= 0.0072) and shallower slope (d(x) 8±0.3; p=0.026) compared to Cav3.3-WT (V0.5 -47±1mV, d(x) 7±0.1, n=15). Finally, while Cav3.3-wt currents were strongly inhibited at acidic extracellular pH (I_{pH6.5}/I_{pH7.4} = 0.5±0.02; n=9; paired t test p<0.0001) and enhanced by alkalinisation (I_{pH8.0}/I_{pH7.4} = 1.3±0.03; n=6, paired t test p=0.0003), both conditions had weaker effects on currents mediated by the R307H (I_{pH6.5}/I_{pH7.4} 0.7±0.04, n=7; p=0.04914) and Q1158H (I_{pH6.5}/I_{pH7.4} = 0.8±0.04, n=7; p=0.04914) variants.

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T-type calcium channels, including Cav3.3, are abundantly expressed in the thalamocortical system as well as the trigeminal and dorsal root ganglia. The multiplicity of biophysical changes observed and direct connection between the trigeminal nerves and cortical areas related to FHM symptoms, such as those controlling learning/speech, motor, visual and nociceptive functions (Noseda et al., 2011), suggest a potential for disruptions in neuronal excitatory/inhibitory balance to trigger symptoms of Hemiplegic Migraine in CACNA1I variants carriers.


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The SLC26 (solute carrier 26) anion transporter family is involved in diverse processes in the body. Specifically, mammalian SLC26A5 (prestin) mediates ultrafast cellular length changes of cochlear outer hair cells, a process known as electromotility, whereas non-mammalian SLC26A5 orthologs are transport-active. The uncoupled chloride transporter SLC26A9 is involved in gastric acid regulation and chloride transport in airway epithelia. While the exact molecular mechanism underlying transport and electromotility has remained unknown, we hypothesized before [1] that both transport and electromotility of the different SLC26A5 isoforms arise from a similar molecular process.

Recently, experimental structures of mammalian [2] and bacterial homologs [3] revealed dimeric organization of SLC26 family proteins, with each monomer organized into two major helix bundles: the core domain containing the substrate binding site and the scaffold domain positioned at the dimer interface. Here, we report on a combined structural and functional approach to elucidate the structural dynamics that generate both electromotility and anion transport.

Using extensive molecular dynamics simulations based on the recent cryo-EM structure of inward-facing murine SLC26A9 combined with homology modeling of prestin, we developed and refined an ensemble of structures that represent various putative functional states of prestin and murine SLC26A9, revealing an elevator-like movement of the core domain, while the scaffold domain remains static.

To probe this model, we first performed a cysteine accessibility scan along the core-scaffold interface of both electromotile mammalian prestin and the transport-active zebrafish SLC26A5, as well as human SLC26A9. Accessibility of individual amino acid positions suggested a major reorientation between core and scaffold domains that exposes positions close to the central binding site towards the extracellular solution. Secondly, we directly measured voltage dependent movement of the core domain by voltage clamp fluorometry both from mammalian prestin and from the zebrafish SLC26A5. Fluorescence changes of an environmentally sensitive dye attached to the core domain (figure 1) displayed the same voltage dependence as electromotility [4] in both orthologues, indicating that conformational rearrangement of the core domain is directly associated with prestin’s mechanical activity. Moreover, extracellular application of the divalent sulfate anion shifted the voltage...
dependence of the fluorescence signal change reversibly to less depolarized voltages (from 101.1±19.0 mV to 62.1±23.0 mV, n=16, p<0.001; mean±s.d., student’s t-test), concomitantly with a shift of reversal potential of transport currents from -41.4±10.2 mV to -87.9±8.6 mV (n=10, p<0.001), indicating involvement of the core domain movement in anion transport. Third, cross-bridging of pairs of cysteines engineered into the core/scaffold domain interface of mammalian prestin supports the hypothesis of a translational movement of the core domain relative to the scaffold domain.

Together these findings show for the first time that both electromotility of prestin and transport by other members of the SLC26 family may result from a conserved elevator-like movement.

Reference 1: Schaechinger TJ and Oliver D (2007) PNAS, 7693-7698
Reference 2: Walter JD et al. (2019), eLife
Reference 3: Geertsma ER et al. (2015), Nat Struct Mol Biol., 803-808
Reference 4: Albert JT et al. (2007), J Physiol. 451-461

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PC13

Targeting G protein-gated inwardly rectifying potassium (GIRK) channels in the Central Nervous System

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G protein-gated inwardly rectifying potassium (Kir3/GirK) channels are essential for maintaining resting membrane potential and inhibitory neurotransmission, controlling neural excitability by hyperpolarization in response to different G protein-coupled receptors (GPCR) activation. In fact, GirK channels are downstream targets for various neuromodulators, hormones and neurotransmitters acting at respective GPCR: GABA<sub>B</sub>, adenosine A<sub>1</sub>, 5HT<sub>1A</sub>, endocannabinoid CB<sub>1</sub>, D<sub>1</sub> and D<sub>2</sub>-like and other receptors, contributing to the general homeostasis and particular synaptic plasticity processes, learning, memory and pain signaling. Given the growing evidence of relationships between GirK channels and neurologic and psychiatric conditions, we hypothesized that to integrate the last findings related to physiology, pathophysiology and pharmacology of these channels will make a valued reference work on the evolving science of pharmacology for both researchers and clinicians, and would define the relevance of GirK channels as a potential target in central nervous system (CNS) disorders that currently do not have an adequate treatment. So, our main aim was to update the knowledge on the functions of this channel, as well as understand the current pharmacology used in the therapeutic treatments targeted on GirK channels. A search for original and review articles on PubMed and Google Scholar was performed, using relevant search terms. Articles were included (448 in total) if they explored at least one of the following categories regarding GirK channels: a) channel structure and function, (b) brain distribution, (c) G protein regulation, (d) pharmacology, and (e) implication in human diseases. We excluded the articles somewhat irrelevant to the brain disorders, such as those that looked at GirK in spinal cord, heart and endocrine cells. GirK channels, tetramers composed by four essential subunits, are broadly distributed in the brain with different expression patterns in different neurons and subcellular compartments. Although many natural compounds and well-known drugs are found to modulate GirK channels, only few selective agents have been discovered. Behavioral and genetic studies suggest a critical role for the appropriate functioning of the CNS and excitatory-inhibitory balance maintenance, as well as their involvement in many neurologic and psychiatric conditions, such as Parkinson’s and Alzheimer’s diseases, mood disorders, attention deficit hyperactivity disorder, schizophrenia, epilepsy, alcoholism and drug addiction. Finally, we conclude that GirK channels are a very promising tool to treat these conditions that already are or will become a global public health problem.
Epilepsy-causing \textit{KCNT1} variants increase $\text{K}_{\text{Na1.1}}$ channel activity by disrupting the activation gate, which is partially restored by wild-type subunit coexpression.

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Gain-of-function pathogenic variants of \textit{KCNT1}, the gene encoding the sodium-activated potassium channel subunit $\text{K}_{\text{Na1.1}}$, are associated with several refractory early-onset epilepsies. Upwards of 50 distinct missense pathogenic variants have been identified across the transmembrane and intracellular domains of the channel, and all result in increased $\text{K}_{\text{Na1.1}}$ activity, with one exception.

The majority of patients are heterozygous, though there is a paucity of information about how pathogenic variants increase channel activity and also the behaviour of heterotetrameric channels comprised of both wildtype (WT) and variant subunits. To better understand these, we selected seven disease-causing variants from across the spectrum of disorders
and involving different protein domains and studied their functional properties. Whole-cell electrophysiology was used to characterise CHO cells expressing homomeric and heteromeric $K_{Na1.1}$ channel assemblies carrying (AD)SHE- and EIMFS-causing variants in the presence and absence of 10 mM intracellular sodium.

Whilst WT $K_{Na1.1}$ channels have an activation midpoint of 21.77 ± 4.35 mV (n=6), homomeric variant channels displayed a 20-40 mV hyperpolarising shift in this value (n= 5-9, p<0.05 for all mutants). Unlike WT $K_{Na1.1}$, which displays virtually no current in the absence of sodium (2.93 ±1.08 pA/pF at +10 mV, n=6), all homomeric variant $K_{Na1.1}$ channels exhibited voltage-dependent activation in the absence of sodium, with current amplitudes ranging from 26.21-241.64 pA/pF at +10 mV (n=5-9). Activation midpoints were depolarised compared to those in the presence of sodium however (p<0.05 for all mutants, except one). Activation energy, or the zero-voltage free energy difference between the inactive and open state of the channel ($\Delta G_0$) could be calculated from the activation curves of WT and variant $K_{Na1.1}$ channels. All homomeric pathogenic variants had a negative $\Delta G_0$ value (-0.53- -0.02 kcal/mol), unlike the WT $K_{Na1.1}$ channel (0.49 ± 0.10 kcal/mol), indicating that the open state is more favourable, and can thus be described as destabilising the inactive conformation. In the absence of sodium the $\Delta G_0$ values for homomeric variants were positive (-0.16+0.68 kcal/mol), suggesting that removal of sodium effectively stabilises the inactivated conformation. Heteromeric variant $K_{Na1.1}$ channels had distinct activation kinetics to homomeric WT and variant $K_{Na1.1}$ channels, with some residual activity in the absence of sodium (5.86-72.48 pA/pF at +10 mV, n=5-9). In general, WT and variant $K_{Na1.1}$ activation followed a single exponential, with time constants that were unaffected by voltage or sodium. One variant had a significantly faster time constant at +10 mV compared to WT $K_{Na1.1}$ (p<0.05, n=6 for both). Mutation of a threonine in the selectivity filter to cysteine disrupted voltage-dependent activation and potassium selectivity but retained sodium-dependence.

Our findings are consistent with a model of K$_{Na1.1}$ channel gating that involves a sodium-dependent activation gate and a voltage-dependent selectivity filter gate. As a common mechanism, all disease-causing K$_{Na1.1}$ variants lowered the energetic barrier associated with the sodium-dependent transitions between the activated and inactivated states. Destabilisation of the inactivated conformation of the channel structure can explain how disease-causing amino acid substitutions in diverse regions of the channel structure all cause gain-of-function.

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PC15

Functionally enhanced but not codon optimized CFTR cDNAs increased Cl⁻ transport and airway-surface-liquid height when expressed in Cystic Fibrosis airway epithelial cells
In Cystic Fibrosis, loss of Cl⁻ secretion leads to dehydration of the airway surface liquid (ASL) and development of respiratory disease. Class I (no protein) and Class VII (no mRNA) CFTR mutations are responsible for severe lung disease in ~10% of Cystic Fibrosis patients. These types of CFTR mutations are untreatable with current pharmaceuticals. Gene therapy and gene editing practices offer a potential benefit to these patients. However, the techniques are low efficiency and have not yet translated into improved lung function in vivo. We therefore investigated the potential for increased functional restoration of Cl⁻ transport using CFTR cDNAs that were codon optimized to increase protein expression (hCAI) or activity (K978C) or both (h^K978C).

Human CF bronchial epithelial cells (CFBE) were obtained from endobronchial brushings or post-transplantation CF lungs as previously described (1) in accordance with ethical approvals obtained by The University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #03-1396). Cells were cultured on permeable supports and maintained at air-liquid interface (ALI) in a modified bronchial epithelial growth. Transepithelial short circuit current (Isc) was measured across ALI cultures 28-35 days after seeding cells. HEK293T were obtained from ATCC and cultured in RPM1 + 10% bovine serum. CFTR cDNAs were prepared using site directed mutagenesis of wild type CFTR (WT) and high codon adaptive index CFTR (hCAI), cloned down-stream of a SFFV promoter and biscistronic GFP, verified by sanger sequencing. Airway surface liquid (ASL) and protein localisation were analysed using fluorescent microscopy (2); protein abundance by western blotting. Anion transport of CFTR in HEK293T cells was measured by fluorescence quenching assay using halide sensitive YFP-H148Q/I152L (YFP).

Transient transfection of HEK293T with hCAI and h^K978C produced more CFTR protein than WT or K978C (14+/−3 and 10.5+/−2 fold change respectively, p<0.05, n=3). The use of the halide sensitive YFP quenching assay showed hCAI and h^K978C also displayed more halide transport (0.23+/−0.04 and 0.30+/−0.02mM/s) than WT (0.06+/−0.01 mM/s, p<0.05, n=3). Treatment with the small molecule potentiator VX770 increased Cl⁻ transport via hCAI to levels observed with h^K978C, indicating a similar mechanism of potentiation. However, in CFBE cultured at ALI, K978C produced >2 fold more Isc attributable to CFTR and increased ASL height compared to WT (slopes I∞ vs % transduced and ASL height vs % transduced, -1.56 vs 0.75, 0.86 vs 0.58 respectively, p<0.05), while hCAI and h^K978C showed little change across all functional readouts (all n=6-8 from 3 donors). Further investigation showed that protein from both codon optimized cDNAs was mislocalised. hCAI was found at the apical and basolateral membrane; h^K978C was mislocalised and rapidly degraded.

These findings highlight the importance of the use of physiologically relevant cells to determine efficacy of CFTR cDNA in gene therapy. For the first time, this data shows that codon optimised CFTR cDNA is unsuitable for standard gene therapy practices employing high activity promoters. However,
the use of K978C cDNA in gene therapy and gene editing practices may allow for more potent recovery of function in CF lung disease than WT cDNA.


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PC16

Vitamin D receptor activation exerts anti-secretory actions in colonic epithelial cells

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Background: The vitamin D receptor (VDR) is a nuclear receptor that is expressed in many tissues throughout the body, but is particularly abundant within the colon. VDR is activated by the active form of vitamin D, calcitriol, but is also known as a receptor for the secondary bile acid, lithocholic acid. VDR has previously been shown to have anti-inflammatory and barrier promoting actions in the large intestine. However, there is little known of the role VDR plays in regulating colonic epithelial transport function. Interestingly, previous studies from our laboratory have shown the nuclear bile acid receptor, farnesoid X receptor (FXR), to exert anti-secretory actions in colonic epithelial cells. The current study aims to investigate a role for VDR in regulating colonic Cl- secretion and whether it may be a target for the treatment of diarrhoeal diseases.

Aim: To investigate the effects of VDR activation by calcitriol on colonic epithelial Cl- secretion

Methods: Polarised monolayers of T84 cells were used as a model of the colonic epithelium. Protein expression of VDR was measured by western blotting. Calcitriol (1 - 20 nM) was used to activate VDR and the VDR target protein, CYP24A1, was used to verify activation of the receptor by qRT-PCR. Expression of the FXR target protein, FGF19 was used to verify that calcitriol is specific to VDR. The effects of VDR activation by calcitriol (1 - 100 nM) on chloride secretion was determined using the Ussing chamber technique. Changes in short-circuit current (Isc) induced by the cAMP-dependent agonist, forskolin (FSK; 10 µM) or the Ca2+-dependent agonist, carbachol (CCh; 100 µM) were assessed.
Results: VDR was found to be expressed in T84 cell monolayers as determined by western blotting. Treatment of T84 cells with calcitriol (1 - 20 nM) significantly \((n = 3, \ *p<0.05)\) increased expression of CYP24A1, indicating activation of VDR by the agonist. Calcitriol did not alter the activity of FXR, indicating specificity of the agonist for VDR. In Ussing chambers, the FXR agonist, GW4064, inhibited Cl- secretory responses to both FSK and CCh, as previously reported \((n = 7, \ ***p<0.001)\). Calcitriol \((10 - 100 \text{ nM})\) was also found to significantly inhibit FSK-stimulated Cl- secretory responses by 37.9 ± 4.7 % \((p < 0.01)\) and 32.6 ± 7.2 % \((p < 0.05)\), respectively \((n = 7)\), while 100 nM calcitriol significantly reduced CCh-induced responses by 31.4 ± 6.5 % \((n = 7, \ *p \leq 0.05)\). Analysis of the time course over which calcitriol exerts its effects revealed its antisecretory actions to be apparent after 24 - 48 hrs \((n = 5, \ p \leq 0.05)\).

Conclusion: VDR is expressed in colonic epithelial cells where its activation by its natural ligand, calcitriol, inhibits agonist-induced Cl- secretory responses. Future studies will aim to determine a possible role for VDR in mediating anti-secretory actions of luminal bile acids, such as LCA. Since Cl-secretion is the primary driving force for fluid secretion in the intestine, our data suggest that VDR may serve as a target for the development of new anti-diarrhoeal agents.

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Objective: We studied AQP3 dependent mechanisms of transepithelial water transport upon apical exposure of human airway epithelia to hyperosmotic solutions. We aimed on the role of AQP3 in transepithelial water transport and its modulation by changes in ASL osmolality.

Methods: NCI-H441 and primary nasal epithelial cells (NEC) were cultivated at air liquid interface and apically exposed to iso- and hyperosmotic solutions. ASL volumes were quantified via deuterium oxide dilution method. mRNA and protein expression were investigated using semi-quantitative qPCR and immunocytochemistry, respectively. Use of human cells was conducted in compliance with local ethical standards.

Results: ASL volume measurement revealed a secretory phase upon exposure to hyperosmotic solutions, followed by resorption of the excess volume starting 4 and 8 hours after exposure to 6% NaCl and 20% mannitol solutions, respectively. mRNA levels for AQP3 transiently increased 4 – 8 hours after exposure in both NCI-H441 and NEC. Immunocytochemistry experiments revealed AQP3 accumulation at the lateral membrane of H441 cells. The fraction of AQP3-positive cells increased from 13% (4%-19%) to 36% (24%-42%; p=0.0003) and 33% (26%-36%; p=0.0017) after 24 hours in epithelia exposed to 6% NaCl and 20% mannitol solutions, respectively (median (min-max); n=8; Kruskal-Wallis test with Dunn’s multiple comparison test). Genetic knockdown of AQP3 did not affect fluid secretion, but significantly reduced resorptive transport rates between 4 and 48 hours from 0.13 (0.10-0.16) µl/(0.33cm²*h) to 0.09 (0.06-0.12) µl/(0.33cm²*h) for 6% NaCl (p=0.03; F-test from linear regression; n=11). For 20% mannitol transport rates were slightly reduced from 0.19 (0.16-0.22) µl/(0.33cm²*h) to 0.16 (0.12-0.19) µl/(0.33cm²*h) between 8 and 48 hours after exposure, but did not differ significantly (p=0.173; F-test from linear regression; n=11) (all shown as best fit (95% CI)).

Conclusion: Our study shows that AQP3 is upregulated upon exposure of airway epithelia to hyperosmotic solutions and accumulates at the lateral membrane. Genetic modification of AQP3 does not impact secretion, but fluid reabsorption. Probably, AQP3 could be a potential target to modulate ASL adjustment in the context of hyperosmotic solutions.

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PC18

Species dependent differences in cystic fibrosis transmembrane conductance regulator (CFTR) channel gating and response to the potentiator ivacaftor

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Cross-species comparative studies are a powerful approach to understand the function of cystic fibrosis transmembrane conductance regulator (CFTR), the epithelial anion channel defective in cystic fibrosis (CF) (1, 2). Here, we investigate the single-channel properties and effects of the CFTR potentiator ivacaftor on human, pig, sheep, ferret and mouse CFTR heterologously expressed in mammalian cells. Using the patch-clamp technique, we studied CFTR Cl⁻ channels in excised inside-out membrane patches from C127 and CHO cells stably expressing human and mouse CFTR, respectively, and CHO cells transiently expressing ferret, pig and sheep CFTR. To amplify the size of channel openings, we used a large Cl⁻ concentration gradient ([Cl⁻]i, 147 mM; [Cl⁻]o, 10 mM) and clamped voltage at –50 mV; temperature was 37 °C. In all cases, CFTR generated Cl⁻-selective currents regulated by protein kinase A-dependent phosphorylation and intracellular ATP. However, the single-channel properties and the response of CFTR orthologues to ivacaftor differed notably. Although ferret, pig and sheep CFTR exhibited a bursting pattern of channel gating similar to human CFTR, there were differences in current amplitude (i) and open probability (P_o). At 1 mM ATP, pig CFTR (–0.84 ± 0.02 pA, n = 7; means ± SEM) and sheep CFTR (–0.85 ± 0.02 pA, n = 6) had a larger i than that of human CFTR (–0.67 ± 0.01 pA, n = 7), whereas that of ferret CFTR (–0.24 ± 0.02 pA, n = 6) and mouse CFTR (–0.23 ± 0.02 pA, n = 4) were markedly reduced. The P_o of pig CFTR (0.63 ± 0.05, n = 7), sheep CFTR (0.45 ± 0.02, n = 6) and ferret CFTR (0.44 ± 0.07, n = 6) were higher than that of human CFTR (0.33 ± 0.01, n = 7). By contrast, mouse CFTR predominantly resided in a sub-conductance state with a small amplitude and rarely opened to the full open state (P_o, 0.05 ± 0.01, n = 4). To examine the ivacaftor-sensitivity of these CFTR orthologues, ivacaftor (0.01 – 10 µM) was acutely added to the intracellular solution. For ferret CFTR (n = 4 – 6), the shape of the concentration-response relationship resembled that of human CFTR (n = 3), whereas pig CFTR (n = 2) and sheep CFTR (n = 3) were potentiated at higher concentrations. Interestingly, ivacaftor did not potentiate mouse CFTR (n = 5 – 6) at all concentrations tested. To determine whether these species differences could be attributed to variations in residues within the ivacaftor-binding site, we performed an in silico alanine scan (3) of the ivacaftor-bound human CFTR cryo-EM structure (4), and aligned the CFTR orthologue sequences. This structural analysis predicted that replacement of phenylalanine at position 931 with leucine in mouse, pig and sheep CFTR reduces ivacaftor binding. Additionally, mouse CFTR contains a phenylalanine at position 304, resulting in a loss of the hydrogen bond made by tyrosine in human CFTR (5). We conclude that both Y304 and F931 contribute to ivacaftor binding, with Y304 playing an essential role in the binding pocket.

Reference 5 :- Yeh HI et al. (2019). J Gen Physiol 151, 912-928.

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Introduction: Created as a cystic fibrosis (CF)-like model, overexpression of the β-epithelial sodium channel (ENaC) subunit elicits Na⁺ hyperabsorption and dehydration of the airway surface liquid (ASL) (Mall et al., 2008). βENaC-overexpression in transgenic (tg) mice generates mucus plug pathology, inflammation and emphysema similar to chronic obstructive pulmonary disease (COPD). As ASL dehydration worsens COPD/emphysema in humans, we hypothesized whether ASL dehydration-driven emphysema in βENaC-tg mice resembles the progressive phenotype of human COPD using longitudinal micro-computed tomography (µCT).

Methods: βENaC-tg and wild type littermates were anesthetized with 2 % isoflurane and scanned using µCT at 7, 10, 14, 21, 28, 48, 56, 72, 84, 98, 112, 126, 140, 168, 196, 224 and 415 days old. Quantitative lung biomarkers such as aerated lung volume and density were derived from longitudinal µCT data. Lung function analysis, bronchoalveolar lavage (BAL) and haematoxylin and eosin (H&E) staining of lung tissue sections were performed at 58 (±1), 226 (±1), and 416 (±1) days for cross-validation. Data are presented as means ± SEM and analysed for statistical significance using 2-way ANOVA with Sidak’s multiple comparison test as appropriate, n = number of animals.

Results: µCT-derived aerated lung volumes were significantly larger for βENaC-tg mice (0.42 ± 0.02 ml, n = 7) by comparison to controls (0.24 ± 0.01 ml, n = 2, p < 0.0001) after just 21 days old, and the aerated lung density of βENaC-tg mice was significantly lower than controls at every timepoint (p = 0.0009, 2-way ANOVA), indicative of emphysema. µCT analysis suggested that emphysema develops rapidly in βENaC-tg mice younger than 56 days before stabilising after 72 days equating to a transient age-related phenotype. Lung function data supported a stabilising emphysema phenotype with no significant difference between lung elastance (Est) and tissue elasticity (H) of 58 (±1) day old (Est: 9.47 ± 0.36 cmH₂O/ml, n = 7, H: 14.66 ± 0.34 ml/cmH₂O, n = 7) and 416 (±1) day old βENaC-tg mice (Est: 8.64 ± 0.52 cmH₂O/ml, n = 6, p = 0.9933, H: 12.59 ± 0.56 ml/cmH₂O, n = 6, p = 0.21).
Furthermore, H&E staining evidenced destruction of alveoli, confirming emphysema in βENaC-tg mice, and that the severity was consistent between 58 (±1), 226 (±1), and 416 (±1) days old. Furthermore, neutrophil levels in the BAL were elevated in 58 (±1) day old β-ENaC-tg mice (8.1x10⁴ ± 2.1x10⁴ cell/ml, n = 7, p = 0.0012) but declined significantly by 224 (±1) days old (2.1x10⁴ ± 0.5x10⁴ cell/ml, n = 6, p = 0.0012) suggesting a downregulation of the inflammatory response at later stages.

Conclusion: Although designed as a CF-like pathology model, there are many similarities between the βENaC-tg and COPD phenotype. Longitudinal µCT recorded the progression of emphysema, with the presence of emphysema and an early neutrophilic inflammatory response confirmed by lung function, histology and BAL analysis. Together, the βENaC-tg phenotype provides an appropriate model for the development of severe COPD which then stabilises between 58 and 224 days old, defining a window for therapeutic intervention in this model.


PC20

DEVELOPMENT OF IN VITRO TRANSCRIBED mRNA THERAPEUTICS FOR CYSTIC FIBROSIS

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Cystic fibrosis (CF) is a recessive disease that affects approximately 10,000 people in the UK. The disease is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Absent/non-functional CFTR leads to an imbalance of sodium, chloride and bicarbonate ion transport, and production of thick, sticky mucus in the lung which results in chronic bacterial infection and inflammation [1]. Gene replacement therapy with viral/non-viral vectors has been explored in the last 25 years but all failed to show significant clinical efficacy [2]. In vitro transcribed (IVT) mRNA has emerged in the last few years as a new approach for protein replacement. IVT mRNA offers potential advantages of greater efficiency of expression as mRNA delivery is only required to the cytoplasm rather than the nucleus, and safety as there is no risk of genomic integration [3].

We are developing CFTR IVT mRNA therapy for CF to replace wild type CFTR protein using receptor-targeted nanocomplex (RTN) formulations that consist of liposomes epithelial receptor targeting peptides and the nucleic acid. Our approach was to optimise RTN nanoparticles for transfection of primary cystic fibrosis bronchial epithelial (CFBE) cells, at submerged culture and air-liquid interface
(ALI) cultures, as well as in mouse lung, using reporter IVT mRNAs. Finally we assessed delivery efficiency of CFTR mRNA in CFBE cells at submerged culture and ALI culture by immunoblotting.

We first optimised the RTN formulations, comparing combinations of three different cationic liposomes and five peptides. As a result, we identified a novel formulation for mRNA delivery that achieved almost 100% cellular uptake efficiency and 90% transfection efficiency, compared to a maximum of approximately 20% with a plasmid reporter. The same formulation was able to deliver the mRNAs in ALI cultured cells and mouse lungs. CFTR mRNA was successfully delivered to primary normal human bronchial epithelial cells (NHBE) and CF bronchial epithelial (CFBE) cells. In addition, we found that co-delivery of the commercial drug, corrector of CFTR, Lumacaftor (VX-809), improved the expression or stability of CFTR protein in CFBE cells in submerged culture by approximately 2 to 2.5-fold. Moreover, CFTR protein expression was shown to be upregulated in ALI culture of CFTR cells transfected with RTN CFTR mRNA by measurement of net ion channel by Ussing Chamber. The ΔIs was 3.5 ± 1.2 (SD) (n=3) in the cells transfected with CFTR mRNA before/after Forskolin added while it was 0.23 ± 0.49 (SD) (n=2).

In conclusion, IVT mRNA of CFTR delivered by RTNs is a promising novel therapeutic for cystic fibrosis. In addition, the flexibility of lipoplex allows co-delivery of CFTR mRNA with Lumacaftor, which significantly improved CFTR expression.

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PC21
Aquaporin-3 silencing impairs barrier function of airway epithelia

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The airway epithelium separates the respiratory tract from the organism. Its apical surface is shielded by a liquid layer that is called the apical or airway surface liquid (ASL). Its volume must be tightly controlled to ensure lung function. Therefore, the airway epithelium must act as a barrier to prevent leakage from the interstitial compartment to airway surface. At the same time, it has to regulate the transepithelial transport of water and solutes to adjust ASL volume. Besides ion transport, we previously demonstrated that ASL adjustment also depends on aquaporin-3 (AQP3). AQP3 silencing has been shown to influence colonic barrier function (1), but this has not been investigated for the respiratory epithelium yet. This study aims on the effect of AQP3 on barrier function of the respiratory epithelium.

NCI-H441 cells cultivated at air/liquid-interface (ALI) conditions were used as a model for the distal airway epithelium. AQP3 knockdown (KD) was generated by stable expression of shRNA. Wild type cells (WT) and cells that stable express non-targeting shRNA (NC) were used as controls. To examine
the permeability of the tight junctions the transepithelial electrical resistance (TEER) was measured on day 0, 3, 5, 7 and 9 after ALI establishment. In WT and NC cells, TEER rises from day 3 and reaches a steady state from day 5 onwards. A rise in TEER was also observed in KD cells. However, when compared to WT and NC cells, the increase in TEER was delayed and occurred from day 5 onwards and did not reach steady state until day 9. TEER on day 7 was 406 (186-585) W*cm² in WT, 570 (75-667) W*cm² in NC and 233 (52-929) W*cm² in KD cell, (all as median (min - max)). TEER differences between KD vs WT and NC cells were significant (p = 0.007 and p < 0.0001, respectively; two-way ANOVA with Bonferroni correction; n=20). Determination of apparent permeability coefficient (P_{app}) revealed elevated paracellular permeability for fluorescein and FITC labeled 4 kDa-dextran in KD cells with P_{app} of 2.0*10^{-6}(1.3*10^{-6}-5.4*10^{-6}) cm/s compared to 4.2*10^{-7}(2.1*10^{-7}-9.1*10^{-7}) cm/s in WT and 2.9*10^{-7}(6.5*10^{-8}-4.8*10^{-7}) cm/s in NC cells (p<0.0001 for KD vs WT and KD vs NC, for both fluorescein and 4 kDa-dextran, two-way ANOVA with Bonferroni correction; n=16). P_{app} of 20 kDa-dextran did not differ between WT, KD and NC. On day 7, mRNA transcript levels determined via RT-PCR showed no alterations of tight junction gene expression between WT, KD, and NC. Immunofluorescence staining of ZO-1 and claudin-4 revealed continuous tight junction structures in all investigated cells.

Our results demonstrate that AQP3 silencing impairs barrier function in airway epithelia without apparent effects on tight junction gene expression and integrity. However, TEER time course experiments indicate different dynamics of tight junction formation in KD cells compared to WT and NC cells. Altered organization of claudins at the tight junctions may account for the observed functional differences.


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**PC22**

**Regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) by the nuclear bile acid receptor, farnesoid X receptor.**

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**Introduction and Aims:** CFTR, a transmembrane Cl⁻ channel important in regulating intestinal fluid and electrolyte secretion, is implicated in the pathogenesis of a number of intestinal disorders. At physiological concentrations, bile acids, acting via the nuclear receptor, farnesoid X receptor (FXR),
inhibit colonic epithelial CFTR expression. Dietary phytochemicals have been reported to have the capacity to modulate FXR signalling. Here, we set out to investigate mechanisms underlying FXR regulation of epithelial CFTR expression, and the potential for therapeutically targeting the receptor with dietary phytochemicals.

**Methods:** Polarised monolayers of T\textsubscript{84} colonic epithelial cells were treated with the FXR agonist, GW4064 (5µM), in the absence or presence of a plant phytochemical, designated here as KFS1 (5µM). CFTR and FXR expression were measured by qRT-PCR and immunoblotting. Expression of NF-kB, FOXA1, HNF1A, and CDX2, transcription factors that regulate CFTR expression, were measured by qRT-PCR. Nuclear translocation of NF-kB was measured by immunoblotting. Electrophysiological studies of T\textsubscript{84} cells were conducted in Ussing chambers. Studies on human colonic enteroids were carried out with ethical approval from Johns Hopkins University School of Medicine Institutional Review Board, while studies of murine colonic epithelial enteroids were conducted with approval from the Institutional Review Board of the University of California San Diego.

**Results:** Treatment of T\textsubscript{84} monolayers with GW4064 significantly downregulated CFTR mRNA to 0.51 ± 0.06 fold after 12 hrs (n=12; p<0.001) and protein levels to 0.28 ± 0.06 fold after 48 hrs, compared to controls (n=8; p<0.001). Electrophysiological studies in Ussing chambers showed that GW4064 treatment for 48 hrs inhibited Cl\textsuperscript{-} secretory responses to the Ca\textsuperscript{2+}-dependent agonist carbachol (CCh;100mM) and the cAMP-dependent agonist, forskolin (FSK;10mM) by 79.9 ± 7.5 % and 74.2 ± 8.9 %, respectively. Transcriptomic analysis of human colonic enteroids revealed FXR to be robustly expressed in secretory (crypt-like) cells and that its activation also induced CFTR downregulation. FXR activation did not alter expression or phosphorylation of the p65 subunit of NF-kB, or inhibit its translocation to the nucleus. GW4064 downregulated FOXA1 mRNA expression by 33.2 ± 5.2% after 3 hrs (n=4; p<0.05), but had no effect on HNF1A or CDX2 expression. Treatment with the phytochemical, KFS1 (5 mM;24hrs), upregulated FXR mRNA and protein expression in T\textsubscript{84} cells and enhanced GW4064-induced downregulation of CFTR mRNA by 0.28 ± 0.05 fold (n=8; p<0.01) and protein by 0.25 ± 0.11 fold (n=4) after 24 hours. Similarly, KFS1 significantly upregulated FXR mRNA expression 2.3 ± 0.2 fold (n=4; p<0.01) compared to controls in murine colonic epithelial enteroids and enhanced GW4064-induced downregulation of CFTR mRNA 0.5 ± 0.1 fold (n=4; p<0.05) compared to GW4064 alone. Finally, KFS1 enhanced FXR inhibition of agonist-induced Cl\textsuperscript{-} secretory responses across T\textsubscript{84} cells mounted in Ussing chambers.

**Conclusion:** FXR regulates colonic epithelial CFTR expression and function by a mechanism which appears independent of NF-kB, but which may involve FOXA1. By virtue of their ability to upregulate FXR expression, and thereby enhance its antisecretory actions, plant extracts containing KFS1 have excellent potential to be developed as targeted nutraceuticals for the treatment and prevention of intestinal disease.

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Influence of lauric acid on the relaxation of corpus cavernosum in streptozotocin induced diabetic male wistar rats

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Introduction: Penile erection involves the release of acetylcholine by parasympathetic nerves, which stimulates nitric oxide (NO) production by the endothelium. NO then causes relaxation of the corpus cavernosum and penile arteries (1). Diabetes mellitus is a risk factor for erectile dysfunction (2) and commonly prescribed medications for erectile dysfunction has shown unsatisfactory results in diabetics (3). Plant products are being considered as viable alternative therapy however, the mechanism of action of their active constituents need to be investigated. Lauric acid (LA) is the most abundant component of coconut oil, which is renowned for its therapeutic action (4). This study thus sought to investigate the relaxant effect of LA on the corpus cavernosum in diabetic male rats.

Method: 28 male rats (12 weeks old) were divided into seven groups of four each (n=4). Group 1: Normal control, Group 2: Diabetic untreated, Group 3: Diabetic treated with LA (90 mg/Kg), Group 4: Diabetic treated with LA (180 mg/Kg), Group 5: Diabetic treated with LA (360 mg/Kg),  Group 6: Diabetic treated with coconut oil (1.42 mL/Kg), and Group 7: Diabetic treated with sildenafil (20 mg/Kg). Diabetes was induced by an intraperitoneal injection of streptozotocin (65 mg/Kg). After 4 weeks of treatment, the rats were sacrificed by cervical dislocation, and then strips of corpus cavernosum were extracted. Following pre-contraction with phenylephrine (10^{-7}) or KCl (60 mM), percentage relaxation of the cavernosal strips to cumulative doses of acetylcholine and sodium nitroprusside (SNP) were then evaluated. Results were presented as mean ± S.E.M. Data was analyzed using ANOVA and Turkey post-hoc tests. Values with p < 0.05 were considered significant.

Result: Compared to Group 1, relaxation response of phenylephrine pre-contracted corpus cavernosum to acetylcholine was significantly reduced (p < 0.05) in all other groups except in Group 7. Compared to the Group 1, relaxation response of KCl pre-contracted tissues to acetylcholine was significantly reduced (p < 0.05) in all other groups except in Group 3. There was a significant reduction (p < 0.05) in relaxation response of phenylephrine pre-contracted tissues to SNP in Group 2, Group 5 and Group 6, compared to Group 1. Relaxation response of the tissues to SNP following pre-contraction with KCl was significantly reduced (p < 0.05) in Group 2 compared to Group 1.

Discussion: Reduced relaxation response to acetylcholine in cavernosal tissues of diabetic rats indicates endothelial dysfunction. The relaxation response was however improved in KCl pre-contracted tissues of diabetic rats by lauric acid (90 mg/Kg). KCl causes contraction by promoting the
influx of Ca$^{2+}$ through voltage gated channels (5) Inhibition of this process by lauric acid can account for the improved relaxation.

Improved relaxation response to SNP, an NO donor, in tissues of diabetic rats by lauric acid suggests it boosts the mediatory molecules involved in the relaxant effect of NO. The improved relaxation response in KCl pre-contracted tissues by all the doses of lauric acid including coconut oil, which is rich in lauric acid, further proves lauric acid also enhanced relaxation by inhibiting the action of KCl.

Figure 1: Effect of Lauric acid on the dose-relaxation response of Phenylephrine pre-contracted cavernosal tissues to Acetylcholine in Diabetic Male Wistar rats.

NC - Normal control; DM-UT - Diabetic untreated; DM + LA 90 - Diabetic treated with Lauric acid (90 mg/kg); DM + LA 180 - Diabetic treated with Lauric acid (180 mg/kg); DM + LA 360 - Diabetic treated with Lauric acid (360 mg/kg); DM + CO - Diabetic treated with coconut oil; DM + Sild - Diabetic treated with Sildenafil. a - significant compared to NC (p < 0.05); b - significant compared to DM-UT (p < 0.05); c - significant compared to DM + LA 90; d - significant compared to DM + LA 180; f - significant compared to DM + CO (p < 0.05); g - significant compared to DM + Sild
Figure 2: Effect of Lauric acid on the percentage relaxation of Acetylcholine in KCl precontracted cavernosal tissues in diabetic male wistar rats

NC - Normal control. DM-UT - Diabetic untreated. DM + LA 90 - Diabetic treated with Lauric acid (90 mg/kg). DM + LA 180 - Diabetic treated with Lauric acid (180 mg/kg). DM + LA 360 - Diabetic treated with Lauric acid (360 mg/kg). DM + CO - Diabetic treated with coconut oil. DM + Sild - Diabetic treated with Sildenafil. a - significant compared to NC (p < 0.05); c - significant compared to DM + LA 90 (p < 0.05). g - significant compared to DM + Sild (p < 0.05).
Figure 3: Effect of Lauric acid on the percentage relaxation of Sodium nitroprusside in Phenylephrine pre-contracted cavernosal tissues in diabetic male wistar rats

NC - Normal control; DM-UT - Diabetic untreated; DM + LA 90 - Diabetic treated with Lauric acid (90 mg/kg); DM + LA 180 - Diabetic treated with Lauric acid (180 mg/kg); DM + LA 360 - Diabetic treated with Lauric acid (360 mg/kg); DM + CO - Diabetic treated with coconut oil; DM + Sild - Diabetic treated with Silidenafli. a - significant compared to NC (p < 0.05); b - significant compared to DM-UT (p < 0.05); c - significant compared to DM + LA 90 (p < 0.05); e - significant compared to DM + LA 360; f - significant compared to DM + CO (p < 0.05); g - significant compared to DM + Sild (p < 0.05)
Figure 4: Effect of Lauric acid on the percentage relaxation of Sodium nitroprusside (SNP) in KCl precontracted cavernosal tissues in diabetic male wistar rats

NC - Normal control; DM-UT - Diabetic untreated; DM + LA 90 - Diabetic treated with Lauric acid (90 mg/kg); DM + LA 180 - Diabetic treated with Lauric acid (180 mg/kg); DM + LA 360 - Diabetic treated with Lauric acid (360 mg/kg); DM + CO - Diabetic treated with coconut oil; DM + Sild - Diabetic treated with Sildenafil. a - significant compared to NC (p < 0.05); b - significant compared to DM-UT (p < 0.05); g - significant compared to DM + Sild (p < 0.05)

The acidic breast cancer microenvironment increases Na⁺ entry into cancer cells through voltage-gated sodium channels.

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The voltage-gated sodium channel Nav1.5 is aberrantly expressed in breast cancer where it increases tumour growth and invasion (1). Here, we determined Nav1.5 protein expression in a breast cancer tissue microarray by immunohistochemistry. High Nav1.5 expression correlated with reduced survival (hazard ratio 1.37, P=0.013, n=1482 patients, log-rank test) and greatly increased metastasis (hazard ratio 1.78, P=0.0001, n=1482 patients, log-rank test). Given that breast cancer fatality is usually due to metastasis, this finding suggests Nav1.5 may have prognostic value.

Tumours have an acidic extracellular pH (2). We asked whether this could impact on Nav1.5 channels. Using whole cell patch clamp recording, we measured Na⁺ currents in the MDA-MB-231 breast cancer cell line while altering the extracellular pH. The persistent Na⁺ current was more than doubled (0.31 ± 0.04 pA/pF to 0.71 ± 0.11 pA/pF, mean ± SEM, P=0.002, n=10 cells, paired t test) in low extracellular pH (pH 6.2 compared to pH 7.2). This increased Na⁺ current into cells in acidic conditions could help to explain the elevation in Na⁺ observed in solid tumours in vivo (3, 4).

We wanted to find out whether it was the extracellular or the intracellular pH which was most important for regulating this change in persistent Na⁺ current. Using the fluorescent H⁺ indicator BCECF-AM, we showed that intracellular pH followed extracellular pH changes in our experimental conditions (a 1.2 pH unit reduction in extracellular pH caused a 0.9 ± 0.25 pH unit reduction in intracellular pH, mean ± SEM, P=0.006, n=5-6 experimental repeats, unpaired t test). We next altered the pH of the intracellular recording solution in whole cell patch clamp experiments. This did not affect the gating of the channels, so it is likely extracellular acidity which increases the persistent Na⁺ current carried by Nav1.5.

We wished to measure the extracellular pH in breast tumour tissue. We used ion-selective microelectrodes in ex-vivo tissue slices from the MDA-MB-231 xenograft mouse model. The mean tumour pH was 6.9 ± 0.1 (mean ± SEM; n=9 tumours) which is significantly more acidic than the reported extracellular pH in healthy tissue (pH 7.4) (5). When looking at the slices in greater detail, we showed that extracellular pH is lower where cells are dividing. Regions with lower extracellular pH (pH 6.8 ± 0.1 vs pH 7.0 ± 0.1, mean ± SEM, P=0.008, n=9 tumours, paired t test) tended to be more proliferative, judged by immunohistochemical Ki67 staining (20.1 ± 7.0 vs 6.9 ± 2.4 % positive cells, mean ± SEM, P=0.025, n=9 tumours, paired t test) and less apoptotic, judged by immunohistochemical activated caspase 3 staining (2.1 ± 0.7 vs 10.9 ± 3.5 % positive cells, mean ± SEM, P=0.034, n=9 tumours, paired t test).
We have shown that tumour Na\textsubscript{v}1.5 expression is strongly linked to metastasis and poor prognosis, and these channels will admit more Na\textsuperscript{+} into cancer cells in acidic conditions. We also showed that breast tumours are acidic, particularly where cells are proliferating rapidly. This may explain the elevation in Na\textsuperscript{+} seen in many types of cancer.


Acknowledgements :- This work was funded by grants from Breast Cancer Now, Cancer Research UK and the Physiological Society. The tissue microarray and accompanying data were provided by the Breast Cancer Now Tissue Bank.

All human tissue and animal work was undertaken in accordance with the ethical standards according to the Declaration of Helsinki, and according to national and international guidelines, following approvals from the relevant local research ethics committees.

PC25

Upregulated antimicrobial peptide expression protects \textit{slc26a3}\textsuperscript{-/-} mice from gut inflammation despite dysbiosis.

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\textbf{Introduction}: 

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Anion transporter SLC26A3 (DRA- Down Regulated in Adenoma) is localized on the apical membrane of the colonic mucosa and is functionally involved in the absorption of luminal chloride (Cl\(^-\)) in exchange for bicarbonate ions (HCO\(_3^-\)). Deletion or mutations in this gene, results in an acidic, chloride rich diarrhea disturbed mucus barrier and a predisposition for intestinal inflammation in humans and mice.

**Objective:**

Our study was conducted to investigate the composition of the microbiota in the cecum and colon of wt and slc26a3\(^{-/-}\) littermates and subsequently, the inflammatory potential and host responses to these shifts.

**Methods:**

The luminal and the mucosa-adherent colonic microbiome of cohoused slc26a3\(^{-/-}\) mice and wt littermates was analysed by 16S rRNA gene sequencing at multiple time points (Weeks 5, 8, 12, 16 and 20 for luminal and Early – 4- 9 weeks, Mid – 10- 15 weeks, Late - 16- 20 weeks for mucosa-adherent microbiome). After sacrifice at the designated time points, the upregulation of defensive host strategies was assessed by qPCR. Oral fecal microbiome transfer (FMT) from cohoused slc26a3\(^{-/-}\) and wt littermates to germfree mice was performed to assess the effect of slc26a3\(^{-/-}\) microbiome on the development of inflammation in the germ-free mice. Post FMT, the recipient microbiome was analysed, and the colonic mucosa was investigated by immunohistochemistry (IHC), qPCR, and histology for signs of inflammation.

**Results:**

The colonic luminal (n = 5 pairs of wt and slc26a3\(^{-/-}\) /timepoint) and adherent microbiome (Early - n= 5-7, Mid – n=3-4 and Late – n=4-6 pairs) of slc26a3\(^{-/-}\) mice was highly abnormal from early age with a dramatic loss in the alpha diversity. Notably, there was an overall loss of short chain fatty acid producers such as Lachnospiraceae (luminal, n=25 pairs, *** p= 0.0001; mucosal, n=14 pairs, * p<0.05) and a strong increase of in bacterial families such as Bacteroidaceae (Luminal, n=25 pairs, **** p<0.0001; Mucosal, n=14 pairs, * p<0.05). This was accompanied by a strong increase in IL22 (n=20-25/group, * p<0.05), thereby possibly driving the increase in the expression of several antimicrobial peptides, such as Reg3\(\beta\) (n=21-26/group, * p<0.05) and Relm\(\beta\) (n=22-27/group, **** p<0.0001). Transfer of the highly abnormal microbiome into germ-free mice did not result in intestinal inflammation. Instead, the diversity in the recipient microbiome normalized over time.

**Conclusion:**


The dysbiotic microbiome, low mucosal pH and absence of firm mucus layer in slc26a3−/− colon is accompanied by a very strong upregulation of several antimicrobial proteins. This could possibly explain the surprisingly low inflammatory burden in the gut of these mice.

**Ethical standards and permissions:**

All experiments involving animals were approved by the Hannover Medical School committee on investigations involving animals and an independent committee assembled by the local authorities (Authorization number: 33.14-42502-04-14/1549). The authors confirm that all ethical standards were conformed with and the mice were all humanely treated.

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**PC26**

**Differential Roles of Beta-cell IP3R and RyR ER Ca2+ Channels in Tunicamycin-Induced Disruption of Beta-cell Ca2+ Homeostasis**

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Pancreatic beta cells maintain glucose homeostasis by secreting insulin following a rise in plasma glucose. Insulin secretion is a Ca2+- dependent process. The endoplasmic reticulum (ER) helps to regulate the cytosolic Ca2+ level, thereby playing a role in Ca2+-induced insulin release. ER stress, triggered by the accumulation of unfolded proteins in the ER, can lead to ER Ca2+ depletion that can contribute to beta-cell deterioration and an increased risk of type-2 diabetes. We sought to determine the effects of tunicamycin (TM)-induced ER stress on ER Ca2+ channels, inositol 1,4,5-triphosphate receptors (IP3Rs) and ryanodine receptors (RyRs), and consequent alterations in beta-cell Ca2+ homeostasis. We found that TM-treatment caused RyR1 upregulation (>50%) and IP3R1 downregulation (~50%) in insulin-secreting INS-1(832/13) cells through qPCR. To determine the functional roles of these changes in TM-induced beta-cell dysfunction, we treated mouse islets with either the RyR1 inhibitor dantrolene (Dan) or the IP3R inhibitor xestospongin C (XeC) along with TM. Beta cells treated with TM exhibited reduced ER Ca2+ (~17%) and increased cytosolic Ca2+ (70% increase in % oscillation) in subthreshold glucose compared to controls through FRET and fluorescence Ca2+ imaging respectively. These alterations plus beta-cell apoptosis triggered by TM were inhibited by Dan, whereas XeC had little or no effect. Silencing RyR1 using a siRNA also blocked the TM-induced cytosolic Ca2+ oscillations in subthreshold glucose. Each experiment was repeated at least three times. Taken together, these results suggest that RyR1 plays a critical role in mediating
the disturbed cellular Ca\textsuperscript{2+} homeostasis seen in response to the induction of ER stress. Targeting RyR1 may thus represent a novel pharmacological approach for ameliorating diabetes.

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**PC27**

Screening Novel Inhibitors of the Neutral Amino Acid Transporter B\textsuperscript{0}AT1 (SLC6A19), a Potential Target to Treat Metabolic Diseases

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B\textsuperscript{0}AT1 (Slc6a19) is a sodium dependent neutral amino acid transporter responsible for the secondary active transport of neutral amino acids across the brush border membrane of the kidney and intestine. The surface expression of B\textsuperscript{0}AT1 requires either collectrin or angiotensin converting enzyme 2 in the kidney and intestine respectively [1, 2]. A Slc6a19 KO mouse showed neutral aminoaciduria in urine as observed in Hartnup disorder, a benign medical condition that is caused by mutation in the Slc6a19 gene. Further, characterisation of these mice revealed that lack of B\textsuperscript{0}AT1 improves glucose tolerance and enhances fat metabolism [3]. This would suggest the pharmacological inhibition of B\textsuperscript{0}AT1 using chemical compounds could lead to new drugs to treat type 2 diabetes mellitus (T2DM). More recently, it has also been suggested as a target to treat phenylketonuria [4].

High throughput screening of a compound library was performed in order to identify inhibitors of B\textsuperscript{0}AT1, which yielded three novel inhibitors E4, E18 and CB3, with IC\textsubscript{50} values of <10µM [5]. We improved previously identified inhibitors of B\textsuperscript{0}AT1 by medicinal chemistry and identified a third generation of inhibitors. Fifty analogues were screened using fluorescence based membrane potential assay (FLIPR). Modified compounds inhibited B\textsuperscript{0}AT1 with higher affinity (IC\textsubscript{50} values ranging from 0.7µM-2 µM)
FIGURE 1: Inhibitors E4, CB3, and E18 were identified by high-throughput screening. Inhibition of BcAT1 activity was tested in CHO-BC cells using membrane potential assay (TPLR) which inhibited transport of Hoechst (1.5μM) with an IC₅₀ values of 6.7±0.76μM, 1.93±0.30μM, 13.4±0.89μM, respectively. (n=3, x=3)

FIGURE 2: Properties of third generation inhibitors of BcAT1 identified by medicinal chemistry (Analogues of E4). Inhibition of BcAT1 activity was tested in CHO-BC cells using membrane potential assay (TPLR) which inhibited transport of Hoechst (3.5μM) with an IC₅₀ values of 0.7μM and 2μM, respectively. (n=3, x=3)


PC28

Store-operated Ca^{2+} entry in uterine endometrial cancer
Globally, uterine endometrial carcinoma (EC) is the sixth most common malignancy in women. EC is usually diagnosed at an early stage due to patient-reported postmenopausal bleeding, resulting in excellent 5-year survival rates. However, prognosis for women with advanced or recurring EC is relatively poor [1].

Ca²⁺ signals are generated by the interplay of ion channels, receptors, buffers, pumps and exchangers, characteristic of each cell type, termed the “Ca²⁺-toolkit” [2]. We investigated changes in transcription of 196 genes encoding Ca²⁺-toolkit proteins between EC tumours (n = 546) and normal adjacent tissues (n= 35), using The Cancer Genome Atlas accessed via the UALCAN resource [3]. Of these genes, all encoding proteins involved in store-operated Ca²⁺ entry (SOCE) displayed significant differences in expression level between normal and tumour tissues. For example, both Orai1 (normal: median 22.8 transcripts per million (TPM) (range: 13.0-34.0) versus tumour: median 31.3 (2.5-71.7) TPM, p <1 x 10⁻¹² by Student’s t-test) and Orai2 (normal: median 6.9 (2.3-9.5) TPM versus tumour: median 31.3 (2.5-71.7)TPM, p <1.7 x 10⁻⁶) were significantly upregulated, whereas Orai3 was significantly downregulated (normal: median 28.6 (11.4-46.7) TPM versus tumour: median 23.0 (3.8-49.9) TPM, p = 0.02) in tumour tissue. By Kaplan-Meier analyses, differential expression of Orai1 or Orai3 had no significant effect on survival, but patients with tumour expression of Orai2 in the upper quartile displayed significantly poorer survival than those with expression in the lower quartile (p = 0.005).

To test the effects of an Orai antagonist, GSK-7975A [4], on SOCE in the human EC cell-line RL95-2, cells were imaged by fluorescent videomicroscopy using the Ca²⁺ fluorophore, fura-2. The endoplasmic reticulum was depleted of Ca²⁺ in nominally Ca²⁺-free HEPES-buffered saline using thapsigargin, then SOCE was monitored by re-addition of extracellular Ca²⁺. At a concentration of 10 μM, pre-addition of GSK-7975A significantly decreased the initial rate of rise in fura-2 ratio relative to vehicle-treated cells (0.50 ± 0.25/min (n = 6) versus 1.69 ± 0.41/min (n =6), mean ± SEM, p = 0.03).

To assess the effect of GSK-7975A on the ability of RL95-2 EC to form colonies, an important characteristic of cancer, cells were seeded at low-density (3x10³ cells/well) on 12 well plates and were incubated with a range of concentrations of the Orai antagonist for 2 weeks. Cells were then stained with crystal violet, photographed and colonies were quantified using ImageJ. GSK-7975A inhibited colony formation with a log IC₅₀ of -5.305 (± 0.137) M (4.95 μM, n = 3) [4] and at 100 mM caused maximal inhibition (0.08 ± 0.05% of integrated colony density of vehicle treated cells).

These findings suggest that SOCE operates in RL95-2 cells and plays a role in EC clonogenesis. We will verify this using other Orai antagonists and RNA knockdown approaches. In addition, we will examine participation of SOCE in other aspects of RL95-2 cell biology, including proliferation, adherence and cell-death. Potentially, these investigations will determine if SOCE is a suitable target for the development of new therapeutic approaches for the treatment of EC.

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Effects of obesity and diabesity on calcium signaling in ventricular myocytes from the Zucker rat
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Introduction: Obesity is a major risk factor for type 2 diabetes mellitus. Cardiovascular disease is the major cause of morbidity and mortality in diabetic patients. The electro-mechanical function of the heart is frequently compromised in obesity and diabesity.

Aims & Objectives: The effects of obesity and diabesity on calcium signaling in left ventricular myocytes from Zucker fatty (ZF), Zucker diabetic fatty (ZDF) compared to Zucker lean (ZL) control rats have been investigated.

Methods: Experiments were performed in left ventricular myocytes from ZL, ZF and ZDF rats (Charles River, UK). Myocytes were isolated by enzymatic and mechanical dispersal. Myocyte shortening, intracellular Ca²⁺ and L-type Ca²⁺ current were investigated with video imaging, fluorescence photometry and whole cell patch clamp techniques, respectively. Ethical approval was obtained from the UAE University Animal Research Ethics Committee and experiments were performed in accordance with institutional guidelines. Results were expressed as the mean ± SEM of ‘n’ observations. Statistical comparisons were performed using one-way ANOVA followed by post hoc Bonferroni test. P values of less than 0.05 were considered significant.

Results: In 18 ZL, 21 ZF and 21 ZDF rats body weights were 434±12, 718±17 and 619±42 g, respectively and non-fasting blood glucose were 118±5, 135±6 and 333±28 mg/dl, respectively. Time to peak (TPK) shortening was significantly (p<0.05) prolonged in myocytes from ZDF (158.6±3.1 ms, n=87 cells) compared to ZF (130.3±2.6 ms, n=92 cells) and ZL controls (126.5±3.1 ms, n=79 cells). Time to half (THALF) decay of the Ca²⁺ transient was significantly (p<0.05) prolonged in myocytes from ZDF (1.044±0.038 sec, n=86) compared to ZF (0.977±0.043 sec, n=92) and ZL controls (0.962±0.040 sec, n=78). TPK of caffeine-evoked Ca²⁺ transient was prolonged in myocytes from ZDF (575.46±30.96 ms, n=82), compared to ZF (468.81±29.73 ms, n=70) and ZL controls (377.82±29.41 ms, n=72). THALF decay of the caffeine-evoked Ca²⁺ transients was also significantly prolonged in myocytes from ZDF (2.051±0.076 sec, n=80) compared to ZF (1.638±0.075 sec, n=70) and ZL controls.
(1.459±0.06 sec, n=72). Amplitude of shortening and amplitude of Ca\(^{2+}\) transients were not significantly altered in myocytes from ZDF and ZF compared to ZL controls. Amplitude of L-type Ca\(^{2+}\) current was not significantly altered in myocytes from ZDF and ZF compared to ZL controls. Amplitude of shortening and the Ca\(^{2+}\) transients were well preserved in ZDF and ZF myocytes compared to ZL controls.

**Conclusions**: Defects in the uptake and release of sarcoplasmic reticulum (SR) Ca\(^{2+}\) might partly underlie the altered time course of shortening and Ca\(^{2+}\) transient in ventricular myocytes from ZDF rat. Further functional and structural studies are ongoing to investigate Ca\(^{2+}\) uptake and release mechanisms of the SR.

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**PC30**

**Ablation of Na\(^{+}/H\(^{+}\) exchanger-3 prevents tissue iron loading in the Hfe mouse model of hereditary hemochromatosis**

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Several common hereditary disorders are associated with toxic iron overload which leads to liver cirrhosis, cardiomyopathy, and endocrine disorders. Hereditary hemochromatosis arises from mutations in the genes coding for any of several proteins involved in iron sensing (e.g. Hfe), hepcidin production (e.g. hemjuvelin), or hepcidin action, resulting in elevated iron absorption relative to the iron need. Divalent metal-ion transporter-1 (DMT1) is a H\(^{+}\)-coupled Fe\(^{2+}\) transporter and the principal or only mechanism by which nonheme iron is taken up at the intestinal brush border (Shawki *et al.* 2015). DMT1 is a validated therapeutic target in treating iron overload. We have shown that Na\(^{+}/H\(^{+}\) exchanger-3 (NHE3) is required for adequate iron absorption, via its physiological role in generating at the intestinal brush border an acidic microclimate that energizes DMT1-mediated H\(^{+}\)-coupled Fe\(^{2+}\) transport (Shawki *et al.* 2016), so we have explored here the contribution of NHE3 to pathological iron loading. We tested the hypothesis that ablation of NHE3 activity would prevent pathological iron loading. We examined the effect of ablating the SLC9A3 gene coding for NHE3 in the Hfe mouse model of hereditary hemochromatosis. We collected blood and tissues from mice under isoflurane anesthesia (5% by vaporizer, to effect) and then euthanized the mice, following a protocol approved by the University of Cincinnati IACUC. We measured tissue iron levels, hematological and blood-iron variables, and the hepatic expression of *Hamp1* (coding for hepcidin) by using qPCR in male and female FVB/N mice, age ≈ 120 d, fed a normal diet (NIH-07). We examined four genotypes in both sexes: wildtype mice, NHE3 knockout (NHE3\(^{-/-}\)), Hfe knockout (hemochromatosis disease model, Hfe\(^{-/-}\)), and double knockout (NHE3\(^{-/-}\) | Hfe\(^{-/-}\)) (n = 12–26 mice per group). We chose α = 0.05, and analyzed our data (mean, SD) by using multifactorial ANOVA. We found that ablation of NHE3
prevented the liver iron loading characteristic of the Hfe$^{-/-}$ mouse model, an effect that was independent of sex (Fig 1). We observed a similar effect on iron loading in the spleen. Ablation of NHE3 produced no sign of overt anemia. Hepatic expression of hepcidin was depressed in NHE3$^{-/-}$ and in Hfe$^{-/-}$ mice relative to wildtype mice, and remained low in the NHE3$^{-/-}$ | Hfe$^{-/-}$ mice, providing a plausible explanation for the observation that, despite correction of the tissue iron levels in NHE3$^{-/-}$ | Hfe$^{-/-}$ mice, iron levels remained high in the readily accessible serum iron pool (typically 0.1% of total body iron). We conclude that ablation of NHE3 prevents pathological tissue iron loading in the Hfe mouse model of hemochromatosis. Pharmacological blockade of NHE3 may offer a means of inhibiting iron absorption in hereditary hemochromatosis.


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