Investigation into the mechanisms by which CFTR is activated using an Ussing chamber to measure short circuit current.

Introduction

Cystic fibrosis is caused by mutations in the gene coding for cystic fibrosis transmembrane conductance regulator (CFTR). The channel located on the apical membrane of airway epithelial cells transports chloride into the lumen followed by water increasing the airway surface liquid volume, depletion of which occurs in cystic fibrosis patients leading to bacterial accumulation in mucous plugs. The main cause of death is the insufficient amount of functioning lung tissue. (Boucher, 2004) To advance treatment of cystic fibrosis it is important to understand the regulatory mechanisms of the functioning channel in order to activate inactive channels present in the cell membranes of sufferers with certain mutations. (Derichs, 2013) We tested different drugs that act on CFTR in order to investigate the mechanisms that regulate the channel. Lubiprostone used in the treatment of some constipation is thought to target CLC2 channels on the apical membrane of transport epithelia. Cuthbert measured short circuit current (SCC) on airway epithelia of sheep. It was suggested Lubiprostone also acts through EP₄ receptors which activates Gₛ in turn stimulating adenylate cyclase to produce cAMP needed for CFTR phosphorylation and activation by protein kinase A (PKA). L-161982 is an EP₄ receptor antagonist. (Cuthbert, 2010) ESI-09 is an exchange proteins directly activated by cAMP (EPAC) inhibitor. EPAC may be involved in the activation of CFTR due to its activation by cAMP (Cheng, 2008) Forskolin (FSK) and IBMX are used to activate CFTR. 16HBE14o-cells were mounted in an Ussing chamber in order to measure the transepithelial potential (Vte) and short circuit current.

Methods

In all experiments an Ussing chamber was used to measure Vte and SCC. Transepithelial potential was measured with and without current injected every 60 seconds. 10µA of current was injected for 10 seconds each time. A daily blank was recorded at steady state. Day-matched controls were recorded. In each experiment the tissue was allowed to reach steady state before the experiment began. After each drug was added Vte was allowed to reach steady state or left until current had been injected 10 times.

In the first control the tissue was left for 10 minutes then current was injected over 5 minutes. 1µM of Lubiprostone was added to the apical side. At steady state 10µM of CFTR inhibitor was added to the apical side. In the experiment 10µM of ESI-09 was added to both sides and left for 10 minutes. 5 minutes of control readings were then taken. Lubiprostone and CFTR inhibitor were added as above.

In the second control the tissue was left for 30 minutes, in the last 5 minutes current was injected. 1µM of Lubiprostone was added basolaterally. At steady state 10µM of CFTR inhibitor was added apically. In the experiment 10µM of Forskolin (FSK) and 100µM of IBMX were added to both sides and left for 30 minutes. In the last 5 minutes current was injected. Lubiprostone and CFTR inhibitor were added as above.

In the third control the tissue was left for 10 minutes then current was injected for 5 minutes. 1µM Lubiprostone was added apically. At steady state 10µM CFTR inhibitor was added apically. In the
experiment 2.5µM of L-161982 was added apically and left for 10 minutes then current was injected for 5 minutes. Lubiprostone and CFTR inhibitor were added as above.

**Results**

For all experiments the change in Vte induced by current injection was measured. Resistance (R) of the insert could then be calculated and from the Vte and R, SCC could be calculated. Differences in Vte and SCC between steady state compared with control and steady state compared with CFTR inhibitor were also calculated. SCC is compared between inserts without ESI-09 and those pre-exposed to ESI-09 in Graph 1. In both, depolarisation occurs on addition of Lubiprostone and repolarisation occurs with addition of CFTR inhibitor.

**Graph 1**- comparison of SCC between activation by Lubiprostone with activation by Lubiprostone post exposure to ESI-09

SCC is compared between inserts without FSK and IBMX and those pre-exposed to FSK and IBMX in Graph 2. The control shows depolarisation and repolarisation with addition of Lubiprostone and CFTR inhibitor respectively. There is no increase in SCC when Lubiprostone is added in those inserts pre-exposed to FSK/IBMX but CFTR inhibitor causes repolarisation.

**Graph 2**- comparison of SCC between activation by Lubiprostone with activation by Lubiprostone post exposure to FSK and IBMX
SCC is compared between inserts without L-161982 and those pre-exposed to L-161982 in Graph 3. In both, depolarisation occurs on addition of Lubiprostone and repolarisation occurs with addition of CFTR inhibitor.

**Graph 3** comparison of SCC between activation by Lubiprostone with activation by Lubiprostone post exposure to L-161982

**Discussion**

An ANOVA test was performed on each set of data between the control and Lubiprostone steady state and then between Lubiprostone steady state and CFTR inhibitor for Vte and SCC. In experiment one, if Lubiprostone acts via EPAC to activate CFTR, the response to Lubiprostone will be reduced by ESI-09. The ANOVA tests showed there was a significant difference between the control and Lubiprostone steady state and again for Lubiprostone steady state and CFTR inhibitor for Vte and SCC. With ESI-09 added the ANOVA test gives the same result so depolarisation and repolarisation of the membrane still occurred with addition of Lubiprostone and CFTR inhibitor respectively. A t-Test was carried out to compare control Lubiprostone steady state Vte and ESI-09 Lubiprostone steady state Vte, no significant difference was found. No inhibition by ESI-09 occurred suggesting EPAC isn’t involved.

In the second experiment, Lubiprostone if thought to increase cAMP levels, activating CFTR and depolarising the basolateral membrane. Therefore there should be no further increase in Vte or SCC when pre-incubated with FSK and IBMX as levels of cAMP will already be at maximum so no further depolarisation can occur. The ANOVA test showed there was a significant difference between the control and Lubiprostone steady state and again for Lubiprostone steady state and CFTR inhibitor for Vte and SCC. When pre-exposed to FSK and IBMX there is no significant difference between control and Lubiprostone steady state for both Vte and SCC as the membrane was already depolarised by FSK and IBMX so could depolarise no further. There was a significant difference between Lubiprostone steady state and CFTR inhibitor for Vte and SCC as FSK and IBMX increase cAMP which activates CFTR, therefore inhibition repolarises the membrane. A t-test was carried out to compare control Lubiprostone steady state Vte and FSK/IBMX Lubiprostone steady state Vte, no significant difference was found. Therefore there was no further depolarisation when pre-incubated with FSK/IBMX suggesting Lubiprostone increases cAMP levels.
In the third experiment, if Lubiprostone acts via EP₄ receptors to increase cAMP then the response will be reduced by L161,982. The ANOVA tests showed there was a significant difference between the control and Lubiprostone steady state and again for Lubiprostone steady state and CFTR inhibitor for Vte and SCC. The same result occurred with L161,982 added so there is still a response to Lubiprostone and CFTR inhibitor. A t-Test was carried out between control Lubiprostone steady state Vte and L161,982 Lubiprostone steady state Vte and found there was a significant difference. This was the same for SCC. There is a partial inhibition when pre-exposed to maximum inhibitory concentration of L161,982 suggesting there may be other EP₄ receptors present on the apical membrane. With an n=5 for control and n=7 for L161,982 more data will be needed to confirm the result.

References


Reflection

I have really enjoyed the opportunity to complete a research project in Dr Robson’s laboratory at the university. I found the research we were doing really interesting and felt we had support. The experience has given me more confidence to apply for masters and PhDs and having the research experience will give me an advantage when applying. The time spent in the lab has reinforced that I want to go into research and I’d recommend anyone thinking of going into research to consider a placement.