**Activation of CFTR by EPAC, lubiprostone and EP₄ receptors in human bronchial epithelial cells**

**Introduction**

The chloride secreting channel cystic fibrosis (CF) trans-membrane conductance regulator (CFTR) is found in airway epithelial cells where the importance of its role is demonstrated in the disease cystic fibrosis. In this disorder, mutations in CFTR impair the secretion of chloride ions across cells, resulting in many symptoms of which respiratory disturbances can be particularly fatal (Sinn et al, 2011). Lubiprostone, a prostaglandin derived fatty acid, has been identified as an activator of CFTR in ovine (Cuthbert, 2010) and murine epithelia (Schiffhauer et al, 2013) with Cuthbert (2010) additionally reporting that it targets the prostanoid receptor EP₄. It is so far known that CFTR functions through the activation of the cAMP/PKA pathway (Namkung et al, 2010) but the molecular mechanisms following this remain undetermined. An alternative downstream target of cAMP is exchange proteins activated by cAMP (EPAC). The aim is to determine whether EPAC and lubiprostone work to activate CFTR, and if EP₄ receptors are involved in the latter using human bronchial epithelial cells.

**Method**

Three different protocols were carried out to assess activation of CFTR. In each, bronchial cells from the cell line 16HBE14o- were grown on semi-permeable inserts. The Ussing chamber technique was used to measure resistance and potential difference (PD or Vte) changes induced by 10µA current pulses. Five minutes using a blank insert were recorded each day as well as ensuring day matched controls were carried out. At the end of each experiment the response of the cells to the apical addition of CFTRinh172, a specific inhibitor of CFTR, was also measured. The readings were taken every minute until steady state with the various inhibitors and drugs was reached.

Protocol 1: The insert was incubated for 10 minutes before control measurements were recorded for 5 minutes and lubiprostone was added to the apical side. In test experiments a fresh insert was pre-incubated with the EPAC inhibitor ESI-09 on each side of the chamber and the procedure repeated.

Protocol 2: The control insert was stabilised for 30 minutes with basal readings taken during the last 5 minutes. Lubiprostone was added to the basolateral side. The test insert was incubated with forskolin and IBMX, two compounds that keep cAMP levels high by producing more and preventing its breakdown, prior to repetition of the procedure.

Protocol 3: For the control, the insert was left to stabilise for 10 minutes and then 5 basal readings were taken, followed by the apical addition of lubiprostone. The process was repeated with a new insert that was apically incubated with L161,982, an EP₄ antagonist.

It is known that chloride secretion increases short circuit (SCC or ISC) and this was calculated using Ohm’s law which states that current is equal to the potential difference divided by the resistance. Analysis of variance (ANOVA) and statistical t-tests will be used to determine the significance of the results.

**Results**

In the control, cells responded to lubiprostone by depolarising causing a positive increase in the transepithelial potential and short circuit current, an effect that was reversed by the addition of CFTR inhibitor (n=12). In accordance with this, the results from the analysis of variants show that there are significant differences in both Vte and ISC between the control and lubiprostone steady
state, and between the lubiprostone steady state and CFTR inhibitor. A similar response was observed in the cells pre-incubated with ESI-09 (n=13). This is shown in Graph 1. The unpaired t-tests reveal that there is no significant difference between the sets of data, as the values all lie above 0.05, signifying there is no impact on the changes in Vte or SCC when the inserts are pre-incubated with ESI-09.

![Changes in ISC for control and ESI-09 experiments](image)

Graph 1

The control for the second procedure also depolarised in response to the addition of lubiprostone, and this was reversed by CFTR inhibitor (n=6). The analysis of variants showed significant differences in Vte and SCC between the basal readings and lubiprostone steady state and lubiprostone steady state and CFTR inhibitor. In the presence of forskolin and IBMX the Vte and ISC recorded during the control were much higher showing a greater depolarisation, however there was no further effect when lubiprostone was added (n=7). This depolarisation was reversed by CFTR inhibitor, shown in Graph 2. Analysis of variants maintains there was no difference between the control and lubiprostone steady state but there was a change between the lubiprostone steady state and CFTR inhibitor. The two-tailed t-test values were above 0.05 showing that the Vte and shift were the same whether activated by lubiprostone or forskolin and IBMX.

![Changes in ISC for control and FSK/IBMX experiments](image)

Graph 2

In the final procedure, the control shows a positive increase in transepithelial potential to lubiprostone that was then decreased upon addition of CFTR inhibitor (n=5). Cells pre-incubated with L161,982 also have a similar reaction to lubiprostone and CFTR inhibitor but it appears that only two thirds of the response achieved in the control is attained (n=7), as can be seen in Graph 3.
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Analysis of variants revealed significance between control and lubiprostone steady state and lubiprostone steady state and CFTR inhibitor for Vte and SCC measurements in both experiments. The t-tests however showed that the Vte was different for the responses to lubiprostone, but not for the responses to CFTR inhibitor between the two data sets. The t-tests also showed the SCC was significantly different both in response to lubiprostone and CFTR inhibitor when the two groups were compared, as the P values were less than 0.05. The results indicate that in the presence of L161,982 there is a response to lubiprostone and CFTR inhibitor but overall, this response is lessened.

Results that were more than two standard deviations from the mean were excluded from the graphs and statistical analysis. The measurements were all rounded to two decimal places to ensure the same precision and consistency. The error bars on the graphs represent the standard deviation about the mean.

Discussion

Based on the results from the statistical analyses, there was no difference between the control and the results from the pre-incubation with ESI-09, where a decrease in the response to lubiprostone would have been expected if Epac was indeed implicated in the signalling cascade. Therefore we can conclude that Epac is not involved in activating CFTR. Hoque (2010) similarly found that in intestinal cells the Epac dependent cAMP stimulation of chloride secretion was not carried out by CFTR. The role for PKA is acknowledged as important in the regulation of CFTR (Heqedus et al, 2009) however it is not alone in achieving full activation of CFTR. Studies have shown an involvement for protein kinase C required for PKA activation (Chappe et al, 2003), though at this point it is hard to speculate what else may be downstream cAMP in the activation of CFTR.

To assess the capacity of lubiprstone’s activation of CFTR basolaterally, the compounds forskolin and IBMX were used to alleviate cAMP levels to activate CFTR. If lubiprostone works via cAMP, upon its addition no further increase in PD and SCC was to be expected. Indeed, no further activation was recorded as the cAMP levels were already high, thereby deducing that lubiprostone works to activate CFTR via cAMP or alternatively, through a different pathway which cannot be activated if the cAMP signalling cascade is already initiated. Similar results are obtained when lubiprostone was administered apically.

The involvement of EP4 receptors in the lubiprostone activation of CFTR was also investigated following previously published data that suggests activation of this receptor triggers the cAMP
production required (Cuthbert, 2010). The statistical analysis revealed that in the presence of the specific inhibitor of EP₄ receptors, L161,982, the cells responded to lubiprostone and CFTR inhibitor as in the control, apart from the t-test results for changes in Vte to the response of CFTR inhibitor which showed no difference between the data series, an outcome that may be due to the small data set (n=5). To assess this, more data would need to be collected. However, the overall response was blunted compared to the control experiment indicating that lubiprostone works through EP₄. Other studies conducted in Xenopus oocytes have corroborated this (Norimatsu et al, 2012). Although using the maximum inhibitory concentration of L161,982, two thirds of a response was present, suggesting lubiprostone targets other receptors in its activation of CFTR.

The experience of working in a lab has been very valuable in helping me decide the type of career I would like to pursue after completing my degree, giving me a very clear idea of what a PhD would be like. I have learnt many technical skills and have been able to apply my knowledge learnt in lectures to my understanding of the practical’s and research I undertook. Although I have decided I do not want to work in a lab, the summer placement was very enjoyable and I feel that the research skills in particular have prepared me for final year, alongside the opportunity to develop my problem solving and general lab skills.

References


