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The influence of acetoacetate and butyrate on calcium influx and ATP concentrations in HT-29 cells

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The effects of acetoacetate and butyrate on Ca^{2+}-influx in HT-29 cells were unknown. Extracellular signals can be transferred to the intracellular environment of the cell via changes in the Ca^{2+}-concentration. Extracellular Ca^{2+} may enter the cell via Ca^{2+}-channels in the plasma membrane. Physiological processes occurring within the cell are dependent on Ca^{2+}-concentration, including enzyme activity. Intracellular Ca^{2+}-concentrations were measured using Fura-2/AM, a fluorescent intracellular Ca^{2+}-probe. Ca^{2+}-concentrations were measured immediately on application of the inducers to the cells, as well as after a 9 day incubation period. The effect of these inducers on the L-type voltage-operated Ca^{2+}-channels were determined using the whole-cell patch-clamp technique. To validate these results for the intestinal epithelial model, membrane current studies were performed on HT-29 cells grown on a polycarbonate membrane. ATP concentrations were measured, and the theoretical effect of the inducers on PDE 4 activity was determined. It was found that both acetoacetate and butyrate blocked Ca^{2+}-influx through the L-type voltage-operated Ca^{2+}-channels, resulting in the initial low Ca^{2+}-concentration ($p < 0.05$). The blockage effect is short-lived as after a 9 day incubation period in the presence of the inducers, Ca^{2+}-concentrations were higher than that of the HT-29 control sample ($p < 0.05$). ATP concentrations of the cells were decreased in the presence of the inducers ($p < 0.05$), whilst it was suggested that no interaction between the catalytic site of PDE 4 and the inducers existed.

1. Introduction

The second messenger calcium (Ca^{2+}) is important for the regulation of various physiological processes, including exocytosis, blood coagulation, and hormone and neurotransmitter secretion from secretory vesicles. It is derived from the endoplasmic reticulum (ER) in response to 1,4,5-triphosphates and moves from extra- to intra-cellular regions via Ca^{2+}-channels in the plasma membrane [1].

Increased intracellular Ca^{2+}-levels are capable of effecting a number of enzymes including protein kinase C and Ca^{2+}/Calmodulin-dependent protein kinases. Extracellular Ca^{2+} may enter the cell via Ca^{2+}-channels in the plasma membrane and/or is released from intracellular Ca^{2+}-stores (for eg. ER). Ca^{2+} can be released from the intracellular stores via either the inositol-1,4,5-triphosphate receptors or through ryanodine receptor (RyR) channels. Inositol-1,4,5-triphosphate binds to their receptors resulting in Ca^{2+}-release from intracellular stores in non-excitable cells, while RyRs are activated by Ca^{2+}-binding in excitable cells [2, 3].

Ca^{2+}-channels in the plasma membrane include receptor-activated Ca^{2+}-channels, voltage-operated Ca^{2+}-channels (VOCCs) or ligand-gated non-specific cation channels. These channels act to replace Ca^{2+}-stores of the ER. These channels are activated for very short periods (milliseconds) before being inactivated [4].

Crosstalk between cyclic AMP (cAMP) and Ca^{2+}-signal transduction pathways also exist in the form of a negative feedback loop. Influx of Ca^{2+} results from elevated cAMP levels that in turn decreases the rate at which cAMP is synthesised by inhibiting the activity of adenylyl cyclase. This would then affect the ATP concentrations in the cell. Furthermore, the cAMP-dependent protein kinase A (PKA) is capable of phosphorylating Ca^{2+}-ion channels, thereby effecting an altered ability of the channels to release Ca^{2+} into the cytosol. Both Ca^{2+}-concentration and activated PKA levels are dependent on ATP levels. In turn, ATP concentration is, in part, controlled by phosphodiesterase (PDE) activity [5, 6].

The HT-29 cell line is a neoplastic cell line derived from an adenocarcinoma of the colon. Under standard culture conditions, these cells grow as a monolayer of undifferentiated small intestinal cells. However, in the presence of certain compounds such as butyrate and acetoacetate, these cells undergo differentiation into enterocytes [7].

The effect of butyrate and acetoacetate on many cell types has been well documented. Of particular interest is the effect of 10 mM butyrate on smooth muscle cells. 10 mM butyrate has been shown to cause an increase in intracellular acidification that may result from mitochondrial dysfunction, which in turn may alter Ca^{2+}-movement in smooth muscle cells. A similar effect has been demonstrated with acetoacetate, which decreases the SH-groups on mitochondria, resulting in their dysfunction [8].

In light of the importance of the Ca^{2+}-concentration to signalling pathways, the Ca^{2+}-concentrations of HT-29 cells in the presence of 5 mM butyrate and acetoacetate were measured. Since Ca^{2+}-release from intracellular stores into the plasma membrane appears to be ineffective in activating adenylyl cyclase, it was suggested that the Ca^{2+}-channels in the plasma membrane played a major role in maintaining the Ca^{2+}-concentration within the cell. In addition, the influence of the inducers of differentiation on the VOCCs was determined via the whole-cell patch-clamp technique using ventricular myocytes. Results from the patch-clamp technique were then validated for the intestinal model by performing membrane current studies on HT-29 cells that were grown on a polycarbonate membrane [9].

Due to the effect of both butyrate and acetoacetate on mitochondrial function, the ATP concentrations in HT-29 cells were measured. Theoretical effects of these compounds on PDE 4 activity were determined via computational chemistry.

2. Investigations and results

2.1. Intracellular Ca^{2+}-concentrations

On immediate application of the 5 mM solutions of the inducers (pH 7.4) to the HT-29 cells (Fig. 1), all the samples showed a decreased intracellular Ca^{2+}-concentration in comparison to the HT-29 control sample ($p < 0.05$ Mann-

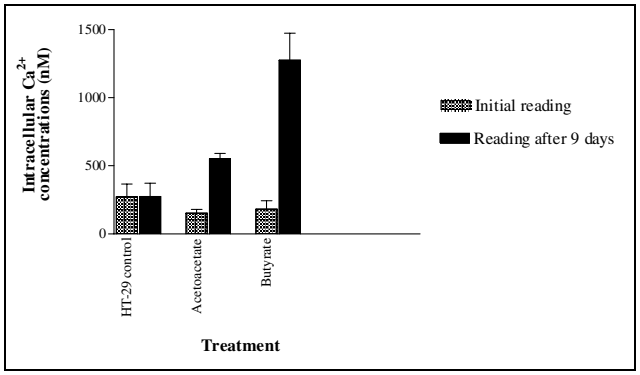

Fig. 1: $[Ca^{2+}]_i$ readings in HT-29 cells using Fura-2/AM. The cells were incubated in the presence of the inducers for 9 days at 37 °C for the 9 day reading

Whitney, n = 4). It was therefore suspected that the inducers had an inhibitory effect on the influx of Ca^{2+} from the extracellular milieu. This was possibly due to the blockage of one or more of the Ca^{2+}-channels in the plasma membrane. The butyrate sample showed the highest initial reading in comparison to the HT-29 control sample, followed by the acetoacetate sample. This indicated that butyrate exerted a greater effect on the channels than acetoacetate. After 9 days, all the samples showed significantly higher levels of Ca^{2+} ($p < 0.05$ Mann-Whitney, n = 4) than the HT-29 control.

After the 9 day incubation in the presence of the inducers, HT-29 butyrate had the highest intracellular Ca^{2+}-concentration ($p < 0.05$ Mann-Whitney, n = 4), followed by the acetoacetate sample. It was thus assumed that butyrate action on the channels was rapid yet short-lived in comparison to a decreased inhibition by acetoacetate that affected the channels for a longer period than butyrate did.

2.2. Effect of the inducers on Ca^{2+}-L-type channels in ventricular myocytes

Both acetoacetate and butyrate block the Ca^{2+}-channels. The maximum effect in both cases was noted after 3 min. 5 mM acetoacetate caused a 63% blockage of the peak inward current (Fig. 2A), while butyrate only caused a 50% blockage of the peak inward current (Fig. 2B). After a 5 min wash-out period, the current affected by acetoacetate returned to approximately that of the control current.

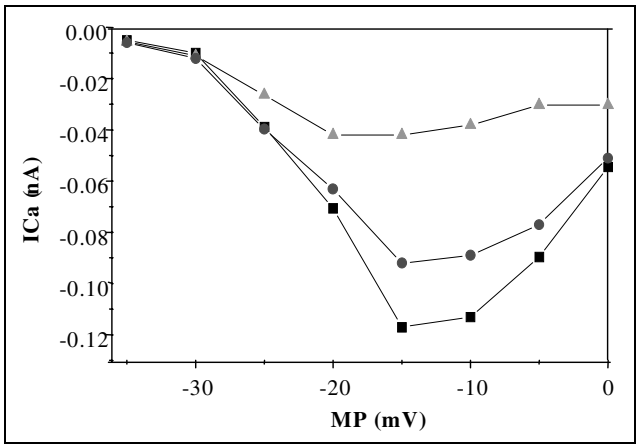

Fig. 2A: The current-voltage relationship of inward currents recorded with the addition of 5 mM acetoacetate. (■) = Control after 10 min (to ensure stable current). (●) = Wash-out period (▲) = 5 mM acetoacetate. ICa = inward current flow. MP = membrane potential

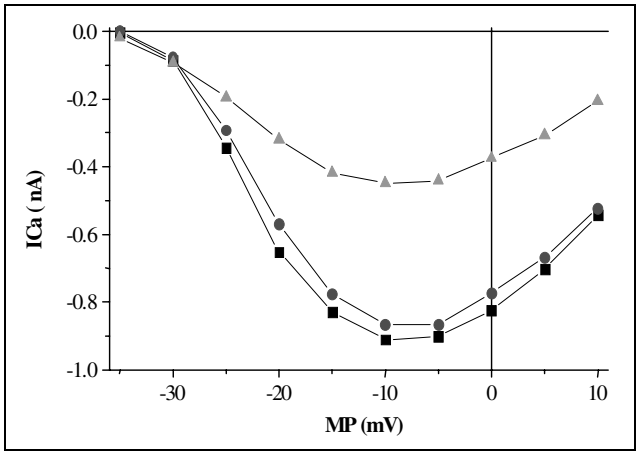

Fig. 2B: The current-voltage relationship of inward currents recorded with the addition of 5 mM butyrate. (■) = Control after 10 min (to ensure stable current). (●) = Wash-out period (▲) = 5 mM butyrate. ICa = inward current. MP = membrane potential

A 5 min wash-out period was sufficient to return the current to that of the control value after exposure to 5 mM butyrate.

2.3. Membrane current studies in HT-29 cells

As a positive control, 0.5 mM verapamil, a commonly used Ca^{2+}-channel blocker, was added to the cells and the current was monitored over a 10 min period.

On the addition of 0.5 mM verapamil to the membrane, the current decreased by 1.76 nA over the 10 min period (Fig. 3). This indicated a decrease in membrane current across the membrane. Similarly, when the inducers were added to the membrane, the current across the membrane recorded over a 10 min period followed the same trend as that of verapamil. The least effective inducer was butyrate (a decrease of 0.88 nA), whilst acetoacetate caused a decrease of 1.14 nA.

2.4. ATP concentrations

In comparison to the HT-29 control sample (Fig. 4) the next highest ATP concentration was found for the aceto-

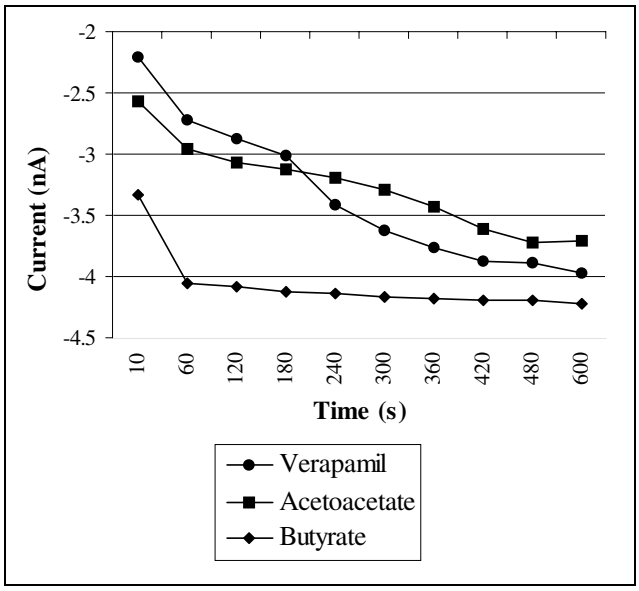

Fig. 3: Membrane current of HT-29 cells grown on a polycarbonate membrane. The cells were exposed to 5 mM concentrations of the inducers. Verapamil was added at a concentration of 0.5 mM. nA = nanoamperes

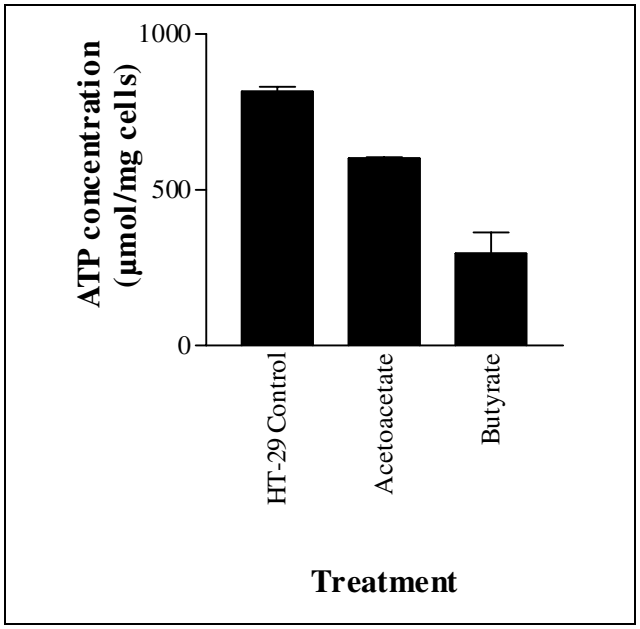

Fig. 4: ATP concentrations in HT-29 cells after a 24 h incubation period at 37 °C. The cells were treated with 5 mM inducers, pH 7.4

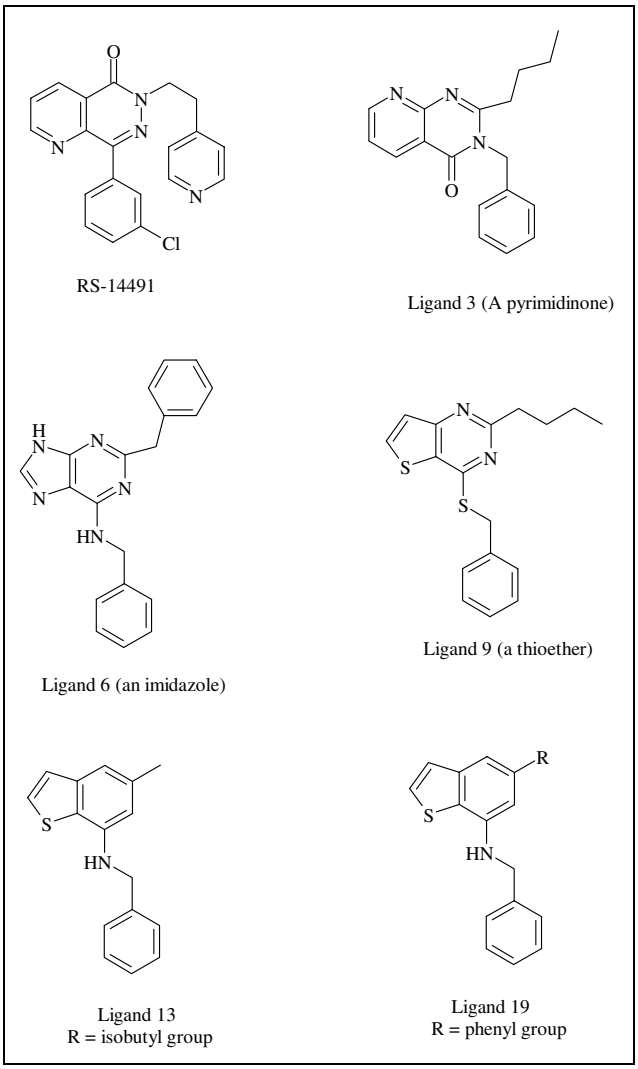

Fig. 5: PDE 4 inhibitors as used in the standard curve for the validation of the PDE 4 model [17]

acetate sample, followed by the butyrate sample ($p < 0.05$, Mann-Whitney, $n = 6$). It appears that the energy demand of these cells in the presence of the inducers is reduced, since the cells are ketogenic in nature [10].

2.5. Computational modelling

A computational model of PDE 4 was developed to screen for PDE 4 activity. This model was validated by docking a set of known PDE 4 inhibitors (Fig. 5) into the proposed active site. Statistical analysis of the relationship between the energy calculated for the interaction between the inhibitor and the model, and the IC_{50} concentrations of the inhibitors were determined (Fig. 6). An r^2 of 0.8598 was obtained. Since $r^2 \neq 1$, we are unable to extrapolate this interaction to physiological conditions. The same procedure was followed for the inducers, which were individually docked into the PDE 4 catalytic site. The average energies of the interaction between PDE 4 and the inducers were obtained (Table).

The obtained energies does not fall within the range of the standard curve, but it can be assumed that the activity of the inducers on PDE 4 is minimal. This implies that no decrease in PDE 4 activity is initiated by the presence of the inhibitors.

3. Discussion

One of the main pathways by which extracellular signals are transferred to intracellular sites is via changes in the cytosolic free Ca^{2+}-concentrations. Normal intracellular Ca^{2+}-concentration is approximately 10^{-7} M, 20000 times less than the extracellular concentration [11].

The effect of the inducers on intracellular Ca^{2+}-concentrations in HT-29 cells was unknown. The fluorescent Ca^{2+}-probe, Fura-2/AM, was used to measure the intracellular Ca^{2+}-concentrations. Initial readings were taken immediately after the addition of 5 mM inducers to the cell samples. On the whole, the emission at 340 and 380 nm reached baseline levels after 3 min, although readings were taken over a 10 min period. A reading after 9 days in the presence of the inducers was also taken. Butyrate appeared to inhibit the influx of Ca^{2+} to a greater extent than acetoacetate on initial application of the inducers to the cells (Fig. 1). After 9 days in the presence of the inducers, the Ca^{2+}-concentration in the presence of butyrate showed higher levels than that of acetoacetate (Fig. 1).

Due to the decreased intracellular Ca^{2+}-concentrations on initial application of the inducers to the samples, the effect of the inducers on the L-type channels were examined. Ca^{2+}-release from intracellular stores was not considered, as release from these stores appears ineffective in generating a signal resulting in increased intracellular Ca^{2+}-concentration [9].

The whole-cell patch-clamp technique was used to record ionic currents under voltage-clamp conditions from single cells isolated by enzymatic dispersion from ventricles of rat heart. During recording of the currents, voltage and

Table: Energies and derived IC_{50} values obtained for the respective inducers

Inducer	Average complex energy (kcal/mol)	Derived IC_{50} values (µM)
Acetoacetate	−305.37 ± 16.52	26.304
Butyrate	−198.87 ± 1.72	47.819

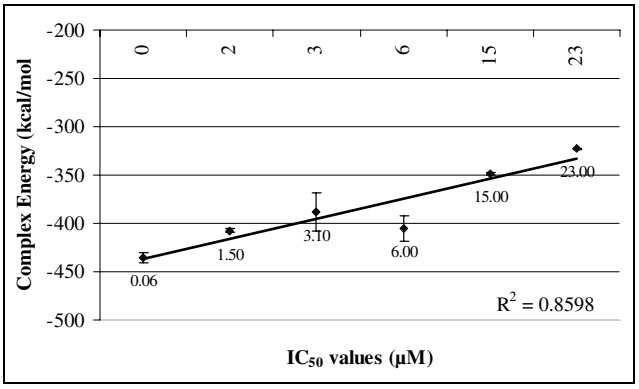

Fig. 6: Standard curve for the validation of the PDE 4 model based on docking of a set of known PDE 4 inhibitors. The following ligands correspond with the IC_{50} values: 0.06 μM = RS-14491; 1.50 μM = ligand 13; 3.10 μM = ligand 19; 6.00 μM = ligand 9; 15.00 μM = ligand 3 and 23.00 μM = ligand 6

time domains were chosen for optimal current flows through the L-type Ca^{2+}-channels with exclusion of influences from other currents. The current-voltage relationship of inward currents was recorded at different test potentials (−35 mV to +10 mV in 5 mV steps) (Figs. 2A and 2B).

Blockage of the peak inward current was measured from the current-voltage relationship as the percentage change from the control at the membrane potential where the current reached a maximum (eg. at −10 mV for butyrate, Fig. 2B).

The patch-clamp technique allows for noise from other ion channels to be avoided, thus allowing the effects of inducers on the group of channels to be investigated.

At 5 mM concentrations, butyrate had the least effect on the channels (50%) (Fig. 2B), while acetoacetate blocked the influx by 63% (Fig. 2A). This was in contrast to the Ca^{2+}-concentrations that showed decreased levels in comparison to acetoacetate on immediate application of the compounds to the cell samples. This may be explained by the difference in cell types used. It is also suggested that the affinity that acetoacetate and butyrate has for the channels is short-lived, since only 5 min was needed for the wash-out period before the current returned to that of the control value in both samples.

The patch-clamp experiments were done on excitable ventricular cells, which, in many ways, is not representative of the non-excitable intestinal epithelia. To validate this data for our intestinal cell model, the membrane current of HT-29 cells grown on a polycarbonate membrane was recorded in the presence of the respective inducers (Fig. 3).

These results confirm findings by the patch-clamp technique, i.e. that the inducers are capable of blocking Ca^{2+}-influx from the external environment via the VOCCs. This provides support for the initial low levels of Ca^{2+} noted in the cells (Fig. 1). The increase in intracellular Ca^{2+}-concentration observed after the 9 day exposure period to the inducers (Fig. 1) may result from of one of three factors. Firstly, as the inducers are metabolised in the cells, more inducer is diffused into the cells, resulting in decreased blockage of the Ca^{2+}-channels, since the inducers do not have a high affinity for the channels. Secondly, the negative feedback loop between cAMP and Ca^{2+} may come into play. A third possibility is the release of Ca^{2+} from an intracellular store such as the ER, to replenish Ca^{2+} in the cell. However, the third possibility may not be feasible since only Ca^{2+} that enters the cell from the extracellular milieu is capable of regulating adenylyl cyclase activity and not Ca^{2+} released from intracellular stores [12].

The first two possibilities may thus negate the blockage effect of the inducers on the channels, resulting in an influx of Ca^{2+} into the cells.

When small changes were applied to the membrane holding voltage, substantial changes in the Ca^{2+}-concentration was observed, and this influx into HT-29 cells is dependent on membrane voltage. As the pathway by which Ca^{2+} entered HT-29 cells was unknown, this study has provided further insight into the mechanism of the influx of Ca^{2+} [13].

HT-29 cells are ketogenic in nature. In HT-29 cells, the SCFAs are used in preference to glucose as an energy and carbon source. Results by Graz and Cowley (1997) show that HT-29 cells convert SCFAs to acetoacetate, which is then stored in the cells. This leads to an increase in the total energy state of the cells, resulting in differentiation. This acetoacetate production may eliminate the need for high cellular ATP concentrations. Thus, the known effects of SCFAs on energy metabolism in HT-29 cells are decreased ATP concentrations, which result in the expression of biochemical markers such as AP. Results for the HT-29 cells shown in Figure 4 are in agreement with this theory [10].

As can be noted from the results obtained for the theoretical interaction between PDE 4 and the inducers, it is suggested that the inducers do not interact with the catalytic site of PDE 4 to effect a reduction in its activity. This suggestion is, however, lacking in experimental evidence.

Since the effect of the inducers on Ca^{2+}-concentration and PDE 4 activities in HT-29 cells were not known, this study has provided further insight into, not only the effect on Ca^{2+}-concentration, but also into the mechanism by which Ca^{2+} is fluxed into the cell. Furthermore, theoretical evidence suggests that the inducers do not interfere with PDE 4 activity.

4. Experimental

4.1. Routine cell culture

The HT-29 cells (obtained from Highveld Biologicals, Kelvin, South Africa) were routinely maintained in 25 cm^2 flasks (Corningware, Cambridge, U.S.A.) in Dulbecco's modification of Eagle's minimal essential medium (DMEM) (Highveld Biological, Kelvin, South Africa), supplemented with 10% heat inactivated foetal calf serum (FCS) (Highveld Biological, Johannesburg, South Africa) at 37 °C. The medium was replaced every 24 h. The cells were sub-cultured at 75% confluence with 0.25% trypsin (Highveld Biological, Johannesburg, South Africa) and 1 mM EDTA (BDH Chemicals, Poole, England) in 1 mM phosphate-buffered saline (PBS) at pH 7.4.

4.2. Preparation of media

The differentiation-induction compounds, butyrate and acetoacetate, were prepared as 5 mM solutions (pH 7.4) in DMEM. This represented the *in vivo* concentrations of these compounds in the intestine. The solutions were filter sterilised using a 0.2 μm syringe filter. The solutions were added to the cells 2 days after seeding.

4.3. Ca^{2+}-concentrations using cell suspension

Intracellular Ca^{2+}-concentrations were measured as previously described [14].

4.4. Effect of butyrate and acetoacetate on Ca^{2+}-channels in ventricular myocytes of rats

The whole-cell patch-clamp technique was performed on excitable, ventricular cells as previously described. The cells were then exposed to 5 mM solutions of the respective inducers (pH 7.4), and the test potentials were increased in 5 mV steps from −35 mV to +10 mV. The inducers were washed out of the system for 5 min in the suspension solution [15].

4.5. Membrane current studies

The patch-clamp technique was performed on excitable ventricular cells, as opposed to the non-excitable HT-29 cells. In order to validate data obtained from the patch-clamp technique, membrane current studies on the HT-29 cells were performed. HT-29 cells were grown on a Costar Transwell polycarbonate membrane for 24 days with the medium being replaced every 2 days. The membrane was then inserted into a vertical diffusion chamber (Corning Costar, Cambridge), and DMEM was added. −80 mV was applied across the membrane to inactivate Na^+-channels. 0.5 mM verapamil was added and the current (nA) across the membrane was recorded over a period of 10 min. 5 mM solutions of the respective inducers were then added to the chamber. The chamber was washed with PBS between runs. The current across the membrane was recorded over the 10 min interval.

4.6. ATP concentration assay

The HT-29 cells were incubated in the presence of the inducers for 24 h, as this time was sufficient to illustrate the immediate response of the cells to the inducers. After the incubation period, the cells were trypsinised and pelleted via microcentrifugation. The pellets were resuspended in 200 µl deionised H_2O. 60 µl of the suspension was transferred to a clean Eppendorf, and 60 µl 12% trichloroacetic acid was added. The mixture was vortexed for 7 s and left on ice for 5 min. The mixture was centrifuged at 3000 g for 7 min. 50 µl of the supernatant was then assayed using the adenosine-5′-triphosphate kit as per manufacturers specifications [16].

4.7. Computational modelling for the interaction of butyrate and acetoacetate with the catalytic region of PDE 4

The amino acid sequence of a segment of the catalytic region of PDE 4A was obtained from the Brookhaven data bank. Standard docking protocols were used on HyperChem® software. Molecular dynamics (MD) was performed *in vacuo* as a source of initial conformation for energy minimisation studies. The initial structure of the protein was correlated using amber force fields. MD was run for 1 ps (1000 steps at 0.5 fs intervals), in conjunction with a temperature increase to 325 K. MD simulation lasted 10 ps. The energy of the molecule was minimised with regard to all the Cartesian coordinates of the molecule via the steepest descent followed by a Newton-Raphson method. The corresponding kinetic energy of the system (K) was recorded in kcal/mol. Potential energy surface (z) obtained from minimisation of the energy during simulation was recorded. The average potential energy of simulation period (y) is thus recorded, where $y = K + z$.

To validate the PDE 4 model, a set of inhibitors acting at the NH_2-terminal region was used to dock into the catalytic site. The statistical analysis between the energy calculated for interaction and the biological response (IC_{50}) was determined [17].

The inducer structures were generated and corrected for MM+ (Molecular Mechanics) force fields. The energies of the molecules were minimised. The inducers were then merged into the proposed catalytic site of the PDE 4 molecule. The energy of the inducers within the active site was minimised, after which the energy of the enzyme around the inducers was minimised. When the energy of the complex was steady, the complex was subjected to MD. All parameters, such as temperature and pressure are taken into account. MD was run for 50 ps and the average energy for the interaction was obtained.

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