Redox regulation of the calcium activated and voltage gated potassium channel, BK: shaping the pancreatic beta cells action potential and calcium influx.

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Abstract

Large conductance calcium activated potassium channels (BK) are ubiquitous in the membranes of all excitable cells and are responsible for limiting action potential propagation and cell function by hyperpolarising action potentials, there is evidence that BK channels are important for the function of pancreatic β cells. Hydrogen peroxide (H_2O_2) is a reactive oxygen species commonly found in all cell types and known to have an effect on BK channels. In this study electrophysiology and fluorescence imaging were used determine the effect of hydrogen peroxide on BK channel activity and action potential shaping. In single-channel patches BK channels were activated by hydrogen peroxide with a bell-shaped relationship, 1 µM H_2O_2 was shown to be the most stimulatory concentration, identified by a 1000-fold increase in open channel probability ($p = 0.0280$). From whole-cell recordings, carried out to confirm activity across the whole cell and measure the voltage dependent effects, it was shown that this was a universal effect across the entire cell membrane with a 24% increase in potassium current amplitude ($p = 0.0224$), the effect of hydrogen peroxide acting on BK was confirmed by the blockers quinine and paxilline. Inside-out patch showed that hydrogen peroxide acted directly

on channels to stimulate channel opening ($p = 0.0141$). Current-clamp recordings showed that hydrogen peroxide had no significant effect on the shape of action potentials in terms of baseline membrane potential, peak amplitude; half-width and maximum rate of decay of spiking, stimulated by 2.5 and 10 mM glucose. Through calcium imaging it was observed that H_2O_2 had no significant effect on calcium influx. It was concluded that although hydrogen peroxide directly activates BK channels in β cells, this effect is insufficient to alter the shape of glucose-stimulated action potentials or calcium influx.

Introduction

Potassium channels

Ion channels are an important component for the function of cells, present on the membranes of all excitable cell types allowing them to generate electrical signals. Generally speaking they are proteins, forming pores across the cell membrane which allow for a number of physiological processes. One of the most important and widely studied functions of ion channels is control of the cells membrane potential, determined by the concentration of positive and negative ions within the cell (Levin, 2012). Ion channels are divided into different families depending on their role within the cell, hyperpolarising channels make the membrane potential more negative by allowing K^+ ions to flow out or Cl^- ions to flow into the cell. Depolarising channels make the membrane

potential more positive, included in this family are Na^+ and Ca^{2+} channels, or they may be nonselective allowing entry of both sodium and calcium ions.

Potassium channels are found on the cell membranes of almost all species (Kuo et al., 2005; Kuang, Purhonen and Hebert, 2015), they are roughly grouped into three classes: the voltage gated (K_v), inwardly rectifying (K_{ir}), and tandem pore domain (K2P) channels. A further class of ligand-gated channels (K_{ligand}) exist (Buckhingham et al., 2005).

The large-conductance voltage- and Ca($^{2+}$)-activated K(+) (BK, BK_{Ca}, MaxiK, Slo1, or $K_{Ca}1.1$), referred to as BK for the rest of this report, channels are composed of 4 homotetrameric α-subunits, composed of 6 regions (figure 1), resembling voltage gated K^+ channels; however they also contain a large cytoplasmic C-terminal domain and an extra transmembrane segment (Wallner, Meera and Toro, 1996). The C-terminus, containing around 1200 amino acids, has been identified to contain Ca^{2+} binding sites and regulatory domains, as well as key structural features such as ion gating, permeation and regions for protein or ionic modulation of the channel (Quirk and Reinhart, 2001; Contreras et al., 2013).

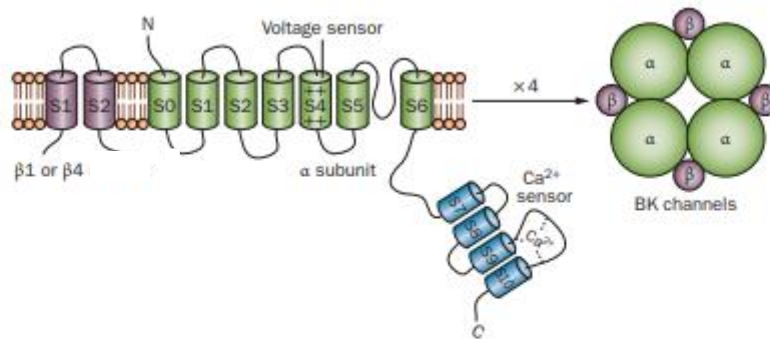

Figure 1. Illustration of the transmembrane architecture and subunit stoichiometry of BK expressed in smooth muscle cells. BK channels have 4 pore-forming α subunits consisting of 6 transmembrane segments and 4 regulatory β subunits made up of 2 segments. The voltage sensor is in the S4 domain (Petkov, 2011).

The α subunits of BK channels are encoded by a single *slo1 (KCNMA1)* gene, located at the chromosomal 10q22.3 region, which undergoes extensive alternative splicing creating high levels of functional diversity within the channel (Rohmann, Deitcher and Bass, 2009), allowing the channels to play many physiological roles including from the maintenance of smooth muscle tone to hearing and neurosecretion (Latorre et al., 2017). Analysis of BK channel alternative splicing has shown that constitutive exons are conserved among different species of the same phylum, while alternative exons are not and across phyla some sites appear to be more susceptible to alternative splicing than others (Fodor and Aldrich, 2009). The STREX (stress-axis hormone-regulated exon) complex is a key determinant of splicing in BK channels

and modifies the ability of the channel to respond to calcium, oxidation and phosphorylation shifts. Alternative splicing of the *slo1* gene is also important in regards to the localisation of the channel in the plasma membrane of intracellular organelles (Petrik and Brenner, 2007). Post translational modifications such as protein phosphorylation, lipidation, glycosylation (Meera et al., 1997), and ubiquitination (Liu et al., 2014); association with β subunits can also account for functional variability of the channel.

Genetic deletion of BK channel α subunits in mice; and gain of function mutations in humans are associated with neurological channelopathies such as ataxia and epilepsy, implicating alterations in BK channels in central nervous system dysfunction (Poschmann et al., 2015; Wang et al., 2017). Kyle *et al* (2013) determined cysteine string protein (CSPα) to be a key regulator of BK channel expression and current density. Mutations in this protein result in the hereditary neurodegenerative disorder adult-onset autosomal dominant neuronal ceroid lipofuscinosis (ANCL), which is characterised by dementia, seizures and loss of motor functions. Mutations in the CSPα N terminal J domain or central cysteine string region resulting in an increased level of BK expression. In this study it was also shown that BK channel expression was increased in the brain of CSPα null mice. From this it was determined that genetic loss or dysfunction of CSPα lead to a pathogenesis of

neurodegeneration as a result of altered neuronal membrane excitability due to abnormal BK channel expression on the cell surface.

The BK channels main physiological function is hyperpolarisation of the cell membrane resulting in closure of voltage gated ion channels and a decreased influx of Ca^{2+}, achieved as a result of membrane depolarisation and increased intracellular Ca^{2+}, they are responsible for this in a wide variety of cell types including hair, smooth muscle and neuronal cells. This leads to stabilisation of the membrane potential and decreased cell excitability (Yuan et al., 2010). The voltage dependence of BK changes in response to differing intracellular Ca^{2+} concentrations, although it is suggested that the voltage dependence of BK is independent from the Ca^{2+} dependent activation. Through cloning of BK it was found that the S4 transmembrane segment contains multiple regularly spaced arginine residues, a hallmark of the voltage sensitive domain (VSD) in voltage-gated potassium channels (Adelman et al., 1992). Mutation of the arginine residues in S4 to neutral amino acids reduced the steepness of the conductance-voltage relationship suggesting the S4 segment serves as a voltage sensor in BK. The voltage sensor in BK is moved to an active state in response to depolarisation, at positive voltages (30 – 80 mV) the probability of channels being open is much higher than at negative voltages (≤ −20 mV), where the voltage sensor is in the resting state (Cui, Yang and Lee, 2008). The voltage sensing domain is made up of transmembrane

segments S1-S4, whilst the S5-6 pore-gate domain opens and closes to control K^+ flow. The activation gate which changes conformation to allow for efflux of K^+ during channel activation is located on the cytosolic side of the S6 segment (Lee and Cui, 2010).

Four types of β subunit of the BK channel have been identified in mammals, each containing two transmembrane segments, short intracellular N and C terminal regions and an extracellular loop of cysteines. β subunits have all been shown to alter voltage dependence and gating; and Ca^+ sensitivity, with varying degrees of effect. The β1 subunit induces an increase in sensitivity to Ca^{2+}, a negative shift in voltage dependence, and a deceleration of the macroscopic kinetics of α subunits in BK channels (Cox and Aldrich, 2000). The β2 subunit also increases Ca^{2+} and voltage sensitivity and slows down the kinetics, but also imparts a rapid inactivation of the channel (Xia, Ding and Lingle, 2003). Conversely the β3 subunit has not been shown to alter calcium sensitivity or voltage dependence in either direction (Uebele et al., 2000). Hu *et al* (2003) suggest that differences between the four sequence variants (V1-4) in the four β3 isoforms (β3a, β3b, β3c and β3d) result in differing levels of potassium current voltage-dependence activation and inactivation. The β4 subunit shows a similar pattern to the first two subunits and alters Ca^{2+} sensitivity, however in a more complex fashion. At high Ca^{2+} concentrations (10 μM free calcium) the channel voltage sensitivity is increased, whereas at lower

concentrations it is decreased, observed as a reduction in open probability of the channel at low Ca^{2+} (Brenner et al., 2000). β4 has also been shown to slow down kinetics in relation to activation and repolarisation following plasma membrane depolarisation (Brenner et al., 2005) BK channels can be either heteromeric or homomeric and the β subunits construction is dependent on the tissue in which they are expressed, the β1 subunit primarily distributes in smooth muscle cells, while β2 and 3 are mainly, although not exclusively, neuronally expressed. β4 is expressed primarily in the brain (Behrens et al., 2000). The diversity of tissue expression of β subunits and their role in channel function is summarised in table 1.

β subunit	**Effect on channel function**		**Tissue expression**	**References**
	Ca^{2+} sensitivity	Voltage sensitivity		
β1	Increase	Decrease	Smooth muscle, aorta, trachea, kidney, urinary bladder, brain	(Contreras et al., 2013; Li et al., 2015; Orio and Latorre, 2005; Wallner et al., 1995)
β2	Increase	No significant decrease	Spleen, placenta, pancreas, heart, kidney, uterus, chromafin cells, brain, dorsal root ganglia	(Contreras et al., 2013; Li et al., 2015; Uebele et al., 2000; Zeng et al., 2007)

β3a	No effect	Increase	Spleen, placenta, pancreas, heart, kidney	(Contreras et al., 2013; Li et al., 2015)
β3b	No effect	Decrease	Spleen, pancreas, kidney, heart, brain, placenta, lung, liver, testes	(Contreras et al., 2013; Li et al., 2015; (Poulsen et al., 2009)
β3c	No effect	No effect	Spleen, prostate, placenta, liver, kidney, pancreas, ovary, brain, lung	(Contreras et al., 2013; Li et al., 2015; (Poulsen et al., 2009)
β3d	Unknown	Unknown	Spleen, testes, placenta, kidney, pancreas, brain, lung	(Contreras et al., 2013; Li et al., 2015; Poulsen et al., 2009)
β4	Inhibit channel at low Ca^{2+} ; activate channel at high Ca^{2+}	Decrease	Brain, neuronal tissue, kidney, and bladder smooth muscle.	(Behrens et al., 2000; Li et al., 2015; Shruti et al., 2012)

Table 1. Summary of BK channel β subunits and their characteristics in effecting channel function as well as the tissues in which each subtype has been found to be expressed.

β cell physiology

Insulin is a key hormone in glucose metabolism and in healthy subjects it is released precisely to meet the demands of the metabolism. It is secreted from β cells as a response to nutrients in the blood circulation, including fatty acids, amino acids and other monosaccharides; however the primary stimulus for insulin release is glucose. Glucose transporter 2 (GLUT2) mediates facilitated diffusion of glucose across the plasma membrane of β cells. Glucose is then phosphorylated by glucokinase, which is rate limiting due to its relatively low affinity for glucose. Subsequent glycolysis results in the production of pyruvate which is oxidised via the tricarboxylic acid cycle to produce ATP. Increase in the intracellular ATP/ADP ratio, leads to closure of K_{ATP} channels resulting in membrane depolarisation, opening of voltage dependent Ca^{2+} channels and influx of Ca^{2+}, leading to the eventual exocytosis of insulin containing granules (Suckale, 2008).

The release of insulin is directly linked to electrical activity in β cells, which is mediated by ion channels. The membrane potential at a resting state by K^+ conductance by K_{ATP} channels, following a reduction in K^+ conductance and membrane depolarisation to a membrane potential of approximately -50 mV in response to glucose. Voltage-dependent Ca^{2+} channels open leading to the instigation of action potentials mediated by Ca^{2+} in rodents and Na^+ currents in human cells when

glucose concentrations are above 10 mM (Pressel and Misler, 1990). Action potential repolarisation is regulated by voltage-dependent K^+ channels (K_v channels) and Ca^{2+}-regulated K^+ channels (K_{Ca} channels) of which BK falls into. Repolarisation is also mediated by the delayed rectifier channel (K_{DR}) which is activated following depolarisation due to the influx of Na^+, and allows for efflux of K^+; by acting in this manner K_{DR} is responsible for regulation of the duration of impulses and prevention of repetition of action potentials. β cells stimulated by glucose have a characteristic oscillatory electrical activity, with rhythmical burst periods, where Ca^{2+} action potentials are propagated as a result of extracellular calcium entry, interspersed with periods where the plasma membrane becomes too hyperpolarised for Ca^{2+} generated action potentials (interbursting), mediated by the open probability of K_{ATP} channels (Düfer et al., 2009). The length of the electrically active burst phase is also linked to the concentration of glucose, with a rising concentration leading to a longer active burst phase (Meissner and Schmelz, 1974).

Role of BK channels in β cell function

A number of studies carried out on BK channels have been on those expressed in rodent β cells and various insulinomas (Cook, Ikeuchi and Fujimoto, 1984; Li et al., 1999). Early models suggested that BK was responsible for glucose dependent electrical excitability in β cells (Atwater, Rosario and Rojas, 1983), however later studies determined

that BK played only a minor in the shaping of action potentials, carried out by potassium channels and that BK is not a major factor in glucose mediated electrical activity on pancreatic islets (Smith et al., 1990). Kukuljan, Goncalves and Atwater (1991) also found that the frequency of action potentials and the rate of their repolarisation in the presence of glucose, as well as the frequencies of slow oscillations in membrane potential were not affected when the selective BK channel blocker charybdotoxin (CTX) was used. Braun *et al* (2008) found that blocking BK channels in human islets via iberiotoxin increased the amplitude of action potentials but had no effect on the frequency, supporting previous work that concluded BK only had a minor role in shaping action potentials during bursting. It was also noted that Ca^{2+} activated BK channels played a key role in the repolarisation of β cells, with other potassium channels understood to be involved only playing a minor role. Braun *et al* (2008) also concluded that there a number of differences in human and mouse β cells and that BK plays in critical role in action potential shaping in human cells, whereas in mouse cells it is considered to have only a minor function. Sweet and Satin (2010) suggested that BK has an important role in modulation and shaping of action potentials and glucose stimulated insulin secretion in mouse β cells, and that it is similar to the role it plays in action potential regulation in human cells as suggested by Braun *et al*, similarly in this work, it was also found that blocking the channel, albeit with paxilline, another selective blocker of BK increased action potential amplitude

without affecting the frequency, which is contrary to the findings of Smith *et al* (1990) and Kukuljan, Goncalves and Atwater; Houamed (2010).

Based on the evidence that BK channels are a key component in shaping the action potential and Ca^{2+} influx, and the resulting role both play in insulin secretion, the channel has been identified as a therapeutic target for the treatment of type 2 diabetes, which affects over 3 million people in the UK (MacDonald et al., 2001). Other studies (Braun et al., 2008) have shown that blockade of BK channels in human islets increases insulin secretion, supporting the idea that BK is a potential pharmacological target for diabetes therapy.

Oxidative stress and β cells

Ion channels, contained on the organelle and plasma membranes of cells are targets for oxidative stress. Oxidative stress results in the fluctuation of ions across membranes and the subsequent increase in electrical excitability resulting in cell hyperexcitability, which results in insulin secretory failure in β cells, as a result of K_{ATP} inhibition (Remedi and Nichols, 2008). Oxidative stress also results in alteration of the resting potential, hormone secretion, muscle contraction and cell cycle regulation, due to hyperexcitability and immune attack.

Pancreatic β cells are particularly vulnerable to attack from reactive oxygen species (ROS). Elmorsy *et al* (2017) found that ROS impair mitochondrial respiration to inhibit bioenergetics resulting in apoptosis. Β cells are also susceptible to redox imbalance from oxidative stress (Tiedge et al., 1997). Reactive species are small molecules containing in their structure a nitrogen or oxygen atom. They may be free radicals with an unpaired electron or non-radical, they may also be anions or non-anions, each species has greatly varying reactivity, for example H_2O_2 is produced continuously *in vivo* and remains in a quasi-steady state (Chance, Sies and Boveris, 1979). At lower concentrations H_2O_2 is involved in signal transduction, cell development and proliferation and apoptosis, however it can be cytotoxic at higher concentrations, causing oxidative modifications, disrupting cellular homeostatis and elicit responses unrelated to those caused by lower concentrations, as observed by Antunes and Cadenas (2001) who found that increasing H_2O_2 concentrations from 0.7 μM to 1–3 μM induced apoptosis and further increases in concentration shifted the mode of cell death towards necrosis, in a Jurkat cell line. The cytotoxic capacity of H_2O_2 varies between cell types and is dependent on the ability of the cell to eliminate H_2O_2 (Gülden et al., 2010; Kaneto et al., 1995).

Other oxidative species which effect β cell function are reactive nitrogen species, which include nitric oxide (NO), produced by either constitutively active or inducible nitric oxide synthases, which has been

shown to initiate apoptosis via interleukin-1 (Kaneto et al., 1995). Superoxides (O^{2-}) have also been implicated in oxidative stress related β cell damage, generated by prolonged exposure to elevated glucose (Tang et al., 2007).

Diabetes mellitus pathogenesis can involve the loss of β cells via ROS generated intracellularly as a result of autoimmune response via inflammatory cytokines in type 1 diabetes or hyperglycaemic and hyperlipidaemic conditions in type 2 diabetes. Of the cytokines involved in β cell damage in both human and animal models interleukin 1β is the key component for cytotoxicity, with tumour necrosis factor α, another pro-inflammatory cytokine with cytotoxic properties involved in the process (Jorns et al., 2005). It has been theorised that interleukin 1β stimulates production of nitric oxides and that this is the main method of cytotoxicity induced by the cytokine (Eizirik and Mandrup-Poulsen, 2001). In the MIN6 pancreatic β cell line, Tsoubouchi *et al* (2004) determined that exposure to an elevated level of glucose for 2 hours induced increased ROS production, which was blocked by the NADH oxidase inhibitor diphenylene iodonium and protein kinase C (PKC) inhibitor calphostin C; however flavoprotein inhibitors showed no effect on ROS production. The sulfonylurea gilbenclamide was also found to stimulate ROS production, which was again blocked by diphenylene iodonium and calphostin C. The results from this study suggest that an increased level of glucose induces an increase in ROS production in

MIN6 and that the source of this is NADH oxidase dependent on PKC activation. It is suggested that PKC activation in pancreatic β cells probably arises through two different mechanisms; a rise in intracellular Ca2+ concentration and an increase in diacylglycerol (DAG) levels synthesized by the de novo pathway (Zawalich and Rasmussen, 1990). The stimulatory effect of the insulin secretagogue glibenclamide was blocked by the same inhibitors, further suggesting the involvement of PKC activated NADH oxidase and that this is another mechanism for β cell deterioration and the progression of diabetes.

In the heart oxidative stress is also a factor in reperfusion injury, which occurs as a result of restoration of blood supply to tissues following ischaemia. During ischaemia the deficiency of oxygen results in anaerobic glycolysis and disruption of ATP production affecting the ATP-dependent ion pumps sodium potassium (Na^+/K^+) ATPase and calcium (Ca^{2+}) ATPase. Overload of intracellular Na^+ and Ca^{2+} as a result of decreased pH also affects ion homeostasis, these factors combine to reduce cardiac contractility and induce cell death via necrosis and apoptosis, further ischaemic damage is prevented by restoration of the blood flow (Bulkley, 1987; Sanada, Komuro and Kitakaze, 2011). There is evidence ROS are produced during ischaemia due to the presence of residual O_2 and the impairment of the electron transport chain converting oxygen to oxygen free radicals (Zhu and Zuo, 2013). It remains possible that a similar mechanism may exit for

pancreatic β cells, as reperfusion injury following ischaemia has been observed in acute pancreatitis in rats (Hoffmann, Uhl and Messmer, 1996).

Reperfusion following ischaemia is vital in restoring tissue function however it can also include tissue damage as a result of ROS release. As aerobic respiration is restored, ROS, in particular superoxide, O^{2-} , are produced which results in further increased production of ROS from the mitochondria, generating a cycle of oxidative stress (Chen and Zweier, 2014). ROS also facilitate neutrophil infiltration, which causes ROS production by nitric oxides due to uncoupling of nitric oxide synthase which reduced nitric oxide production and increases nitric oxide synthase superoxide production (Silberman et al., 2008). In terms of β cell physiology hyperglycaemia is a common complication for patients undergoing ischaemia, with an increased mortality rate for diabetic patients. Su *et al* (2013) determined that hyperglycaemia exacerbated oxidative stress in cardiomyocytes as a result of increased thioredoxin-interacting protein (Txnip) expression, Thioredoxin is a ROS scavenger and a major contributing factor to maintain redox balance, acute hyperglycaemia was shown to reduce its levels through Akt and p38 activation modulation, increasing the levels of oxidative stress and amplifying cardiac injury following ischaemia and reperfusion.

To protect beta cells from damage caused by oxidative stress, a variety of different compounds can be used. Tang *et al* (2012) found that the superoxide dismutase mimetic tempol can be used to interrupt the positive feedback cycle between oxidative stress and endoplasmic reticulum stress during glucose induced cell damage. They also found that chemical chaperones such as 4-phenylbutyrate and tauroursodeoxycholic acid prevented glucose induced β cell damage by reducing mitochondrial superoxides in mice, however this study was only carried out over a 48 hour period of hyperglycaemia, which is very short term and not representative of glucose toxicity in human cells. Plant antioxidants such as phenols and flavonoids have also been shown to have potential to be used to protect against oxidative stress related conditions such as diabetes. More recently Bahar *et al* (2017) found that an extract of *Crassocephalum crepidioides* (Asteraceae) Benth. S. Moore, an edible herb found in many sub-tropical regions, reduced intracellular ROS production and subsequently reduced β cell apoptosis indicating a protective effect in a an INS-1 cell line.

Oxidative stress and BK channels

Oxidative stress is understood to play a role in the function of β cells, which have been shown to be susceptible to damage by ROS; however the effect of oxidative stress on BK channels is not fully understood.

There is discrepancy across the literature as to whether ROS are an activator or an inhibitor of BK. This is due to differences in experimental method and variations in the cellular environment. Kyle, Mishra and Braun (2017) found that increased NO signalling enhanced BK currents in rat cerebral myocytes. Dong *et al* (2008) also observed an increase in channel activation in human endothelial cells when treated with H_2O_2 (100 μM) or glucose oxidase. It was also concluded that H_2O_2 stimulates BK channels through the NO/sGC/cGMP pathway in cultured human endothelial cells. Earlier work by Barlow, El-Mowafy and White (2000) also found that H_2O_2 was a BK activator in cardiac smooth muscle cells. Application of 300 μM H_2O_2 caused a significant increase in channel open probability. They also suggest that H_2O_2 acts via the PLA_2-arachidonic acid signalling cascade to open channels.

Other studies have found, however, that H_2O_2 inhibits the activity of BK channels in vascular smooth muscle cells by inhibiting the ability of the channel to bind with Ca^{2+} by targeting a cysteine residue at the calcium binding site (Tang et al., 2004). However the H_2O_2 concentration (1.7 mM) in this study was much higher than those used in other studies. ; Zhang *et al* (2006) also found that oxidation of cysteine residues by 120 μM H_2O_2 reduced Ca^{2+} affinity and resulted in a decrease in the activity of the channel.

H_2O_2 is known to activate K_{ATP} channels caused hyperpolarisation of the plasma membrane; interestingly work looking at the effect of ROS on potassium channels in mouse pancreatic beta cell lines determined that the application of 1 mM H_2O_2 caused an increase in the K_{ATP} potassium current, which was inhibited by tolbutamide (a selective K_{ATP} blocker), however a small part of this component remained, resistant to tolbutamide. This component was determined to be sensitive to paxilline, the BK channel blocker. In subsequent experiments in BK knockout mice, the H_2O_2 induced current was completely abolished with tolbutamide. These findings support other work showing that ROS are an activator of BK channels; importantly this indicates that ROS are a BK activator in pancreatic β cells. In this work it was also determined that the loss of BK channels, impaired the secretion of insulin (Düfer et al., 2010), which presents an interesting opportunity to further examine the effects of ROS on BK, particularly in pancreatic cells, and how this could affect insulin secretion.

There is great discrepancy across the various studies carried out on the effect of ROS on BK, the aim of this work is to examine how ROS affect BK and the ways that ROS determine action potential shaping, using a variety of configurations of patch clamp electrophysiology allowing for recordings with a high single to noise ratio, supported by calcium and ROS imaging techniques to directly investigate the effects of ROS on the channel.

Role of BK in action potential shaping

The role of BK in action potential shaping is unclear; however it is clear that H_2O_2 is present as a modulator of the process. The discord across the various literature in this area may be a consequence of the variety of experimental conditions present and the variation of oxidative stress cells are subjected to with an experimental regimen, the variation of the ability of different cell types to protect against oxidative stress is also a key factor in the general differences across previous work. Given this I intend to examine the effect of H_2O_2 across a wide range of concentrations from physiological to supraphysiological in both MIN6 cell lines and primary cells isolated from mouse pancreatic tissue. Single channel patch-clamp will allow for investigation into the effects of H_2O_2 on the channel, inside out and whole cell configurations of patch clamp will allow for further study into whether hydrogen peroxide acts directly on the channel and will provide some insight into the mechanism of how ROS affect BK channels and the resulting effect on action potentials. Calcium imaging will allow for investigation into the movement of calcium in glucose stimulated cells and the involvement of H_2O_2 in the calcium currents during action potential stimulation. Imaging will also be used to determine the effect of H_2O_2 on the mitochondrial membrane potential using rhodamine 123, following glucose-induced action potential stimulation and subsequent oxidative stress. These will

allow for exploration into modulation of the channel and how the action potential can be shaped as a result.

Methods

All materials sourced from Sigma Aldrich, Gillingham, UK unless stated otherwise.

Cell culture

Ethical approval

Animal care and experimental procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986). All animals were killed by cervical dislocation

Primary cell culture

Primary cell culture was carried out using pancreatic tissue isolated from C57BL/6 mice grown for 9-10 weeks. On death, mice were wetted down with ethanol prior to tissue extraction to minimise spread of fur and provide an extra degree of sterility. Isolated pancreatic tissue was collected and transferred to a media of Hanks buffer (5.6 mM KCl, 137 mM NaCl, 1.2 mM $NaH2PO_4$, 10 mM HEPES, 2.6 mM $CaCl_2$, 1.6 mM

$MgCl_2$, 4.2 mM $NaHCO_3$, pH 7.4) supplemented with 500mg/L glucose and 5g/L bovine serum albumin. This media was used to prepare a digestion solution of 1mg/ml type V collagenase. The tissue was then macerated with sprung scissors in a scintillation vial and incubated at 37°C for 15 minutes, and shaken for 15 seconds every five minutes. The digestion was diluted with 20ml sterile filtered (0.22 µm pore size) isolation media and allowed to settle. The suspension was then washed three times using vacuum pump to remove media. The final digestion was then transferred to petri dish and examined under a dissection microscope to locate pancreatic islets, which were collected using 10 µL finnpipette into a separate dish.

Islets were then transferred into a sterile Eppendorf tube (1.5 ml) and spun down by micro-centrifuge at 1000 rpm for seven seconds. The supernatant was removed and the pellet was washed with sterile PBS for two minutes then spun down for a second time. The PBS was removed and the pellet incubated with 200 µL 10% trypsin-EDTA solution at 37°C for 180 seconds. Then cells were then titrated 7 times using the same pipette and the action of trypsin halted with the addition of 800 µL RPMI 1640 (Gibco, Loughborough, UK) media supplemented with 10% fetal calf serum and 2% penicillin streptomycin. The suspension was titrated again and 100 µL droplets placed on Nunc 35 mm petri dishes (Fisher, Loughborough, UK) and incubated for 3 hours, after which 2.5 mL of the same media was then added slowly from the

side of the dish and cells left to incubate for 24 hours at 37°C and 5% CO_2 prior to experimental work.

Secondary cell culture

Cell line studies were carried out using mouse insulinoma 6 (MIN6) cells cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, and grown to a confluency of ~90% in Nunc 75 cm^2 flasks (Fisher, Loughborough, UK) incubated at 37°C and 5% CO_2. Trypsin-EDTA solution (10%) was used to remove cells from the surface of flasks. Cells were seeded into Nunc petri dishes and incubated for 24 hours at 37°C and 5% CO_2 prior to carrying out experimental work. The advantage of using a cell line for β cell research is that it allows for generation of large numbers of phenotypically homogenous cells (Daunt, Dale and Smith, 2006).

Electrophysiology

Pipettes were pulled from Harvard GC150TF-15 thin walled borosilicate glass capillaries (Harvard Apparatus, Cambridge, UK) using a PC-10 Dual-Stage Glass Micropipette Puller (Narishige International, London, UK) and the tips coated in sticky wax (Kerr Dental, Peterborough, UK). Prior to experiments pipettes were fire polished using a MF-900 microforge (Narishige) to a resistance between 5-7 MΩ.

For cell-attached recordings pipettes were filled with a solution of 146 mM KCl, 2.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM HEPES and 200 μM tolbutamide to block K_{ATP} activity. Cell attached patch clamp experiments cell were bathed in a calcium free bath solution which contained 143 mM KCl, 1.2 mM $NaH2PO_4$, 10 mM HEPES, 3.8 mM $MgCl_2$, 4.2 mM $NaHCO_3$, pH 7.4 supplemented with 10 mM glucose. This solution electrochemically clamps the resting membrane potential to 0 mV. Currents were recorded with a pipette potential of +70 mV, which clamps the membrane potential to -70 mV, and filtered at 2 kHz with an 8 pole Bessel. H_2O_2 was prepared at concentrations of 0.001-100 μM in the bath from solution.

Inside-out patch experiments were initially carried out the same bath and pipette solutions as cell-attached patch-clamp, Once a cell-attached seal was formed, a bath solution of 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.32 mM $CaCl_2$ which produced 100 nM free calcium, 1mM ATP disodium salt and 10 mM HEPES was perfused into the bath and the membrane was excised by retraction of the pipette. Treatments were prepared in the same ATP solution and a holding potential of 70 mM was again applied following formation of a seal. For inside-out recordings currents were filtered at 5 kHz.

Whole cell recordings were taken using the ATP solution in the pipette and Hanks solution in the bath. Voltage-gated currents were elicited by

a voltage step protocol, with the membrane potential increased by 10 mV increments, from a holding potential of -70 mV at a frequency of 10000 Hz and step duration of 240 ms. Currents were filtered at 5 kHz and leak subtracted. To confirm changes in peak amplitude were due to H_2O_2 acting on BK the channel was blocked by the well characterised specific blockers of K_{DR} and BK; quinine and paxilline, based on the data of Smith *et al* (1990) 10 μM quinine blocks only 9% of BK (K_d 100 μM) compared to 86% of K_{DR} (K_d = 4 μM) with a selective block of K_{DR}. Their effects were observed both in and absent of the presence of H_2O_2. Paxilline was diluted from a 10 mM stock prepared in DMSO, so a DMSO control was also used for whole-cell investigations.

Perforated patch pipettes were tip filled with a solution of 76 mM K_2SO_4, 10 mM KCl, 10 mM NaCl, 55 mM Sucrose, 10 mM HEPES, 1 mM $MgCl_2$, pH 7.4 and backfilled with the same solution but which also contained amphotericin B prepared in DMSO to 200 μg/mL, this produced sufficient perforation of the membrane as defined by an access resistance of < 30 MΩ, to allow currents to be recorded within 5-10 minutes. In perforated current clamp recording of the K^+ current, series resistance and capacitance was recorded before compensation of voltage offset. To investigate the role of BK in action potential shaping, current clamp was used with perforated patch to study the transmembrane voltage changes resulting from glucose induced action potentials, and how they can be modulated by oxidative stress. 1 μM

H_2O_2 was identified as the most effective concentration for BK activation from the prior single channel studies. Paxilline (100 nM) was again used as a blocker of BK, along with 100 nM charybdotoxin (Tocris), another selective blocker of BK channels and 400 μM tetraethylammonium (TEA) another well characterised inhibitor of BK.

Results were determined by determining the means of the treatment values and the means of control values taken prior to treatment perforated patch currents were filtered at 5 kHz. Recordings were made using an Axopatch-1D (Axon Instruments, Scottsdale, AZ, USA) patch clamp amplifier and a Digidata 1440A low-noise digitizer controlled by Pclamp 10 software (Molecular Devices, Sunnyvale, CA, USA). All electrophysiology experiments were performed at room temperature (20-25°C). Solutions prepared with 15 MΩ-cm dH_2O.

Imaging

Changes in cytosolic calcium concentration $[Ca^{2+}]_i$ and mitochondrial membrane potential ($\Delta\Psi_{mit}$) were carried out as using the protocol described by Daunt, Dale and Smith (2006). Imaging was carried out by seeding MIN6 cells onto Ø22 mm size 0 glass cover slides sterilised using ethanol, transferred to 6 well plates and allowing 48 hours for cells to settle following addition of 3 mL cell suspension to each well. Determination of calcium concentrations was carried out on cells loaded with 1 μM Fluo-4 AM (Thermo Fisher, Loughborough, UK) fluorescent

dye, diluted in Hanks, for 30 minutes. $\Delta\Psi_{mit}$ analysis was carried out using using a 1mg/mL Rhodamine 123 dye, loaded onto cells for ten minutes at room temperature as loading at a higher temperature reduced the localisation of the dye, causing loading into other cellular organelles other than the mitochondria.

Images were captured using images were captured at 1 Hz with a Coolsnap HQ2 camera (Photometrics, Tucson, AZ, USA) with a Zeiss XBO 75 xenon short-arc lamp (Zeiss, Oberkochen, Germany). Recordings were captured Imaging Workbench ver 6.0 software (INDEC Biosystems, Los Altos, CA, USA) Regions of interest were drawn around cells corrected for background fluorescence by subtraction, and the average fluorescence intensity calculated. ROIs were from both single cells and cell clusters. For each experimental group, samples were pooled from multiple visual fields from at least 4 different preparations. Initially the cells were washed by perifusing hanks at for 4 minutes before carrying out treatments, cell were continually perifused at 32°C.

For $[Ca^{2+}]_i$ only cells that responded to 10 mM glucose with an increase in steady-state $[Ca^{2+}]_i$ greater than 10% compared to basal were chosen for further analysis.

To calibrate $[Ca^{2+}]_i$, fluorescence changes were calibrated by a one-point method that involved permeabilization of the cells with saponin 1mg/mL with the maximum fluorescence value observed taken as Fmax. To calculate $[Ca^{2+}]_i$ the following equation (equation 1) was used:

$$[Ca^{2+}]_i = Kd \times \frac{F}{Fmax - F}$$

Where F is the background corrected fluorescence, and Kd is the dissociation constant of Fluo-4: 345 nM (6). To quantify changes in calcium metabolism, the mean (μ) and standard deviation (σ) of $[Ca^{2+}]_I$ were calculated for the last 60 seconds of any given treatment, a period over which $[Ca^{2+}]_I$ attained a steady state for all treatments For data comparison, $\mu[Ca^{2+}]_I$ and $\sigma[Ca^{2+}]_I$ were normalised as percentage of that measured for glucose in the same ROI, using equation 2:

$$\left(\frac{Treatment - Glucose}{Glucose - Basal}\right) \times 100$$

For $\Delta\Psi_{mit}$, Only cells that responded to 1 μM FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), a mitochondrial protonophore which collapses $\Delta\Psi_{mit}$ and results in the redistribution of the dye from the mitochondria into the cytosol and is associated with a fluorescence

increase (Elmorsy *et al.*, 2017), were chosen for further analysis. ROI were normalised as a as percentage of that measured for glucose in the same ROI, using the previous equation. Imaging analysis was carried out using Origin 6.1 software with custom scripts written in Labtalk (Originlab, Northampton, MA, USA).

Statistics

All statistical analysis was carried out using PRISM 7 software (GraphPad Software Inc., San Diego, CA), normality of data sets was determined by D'Agostino & Pearson test. Data is expressed as means ± SEM., with *n* respresenting the number of values represented. Test used are detailed in the text where a $p < 0.05$ was deemed statistically significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, **** $p \leq 0.0001$.

Results

Single channel studies

The effect of hydrogen peroxide on MIN6 BK channels was determined by cell attached patch-clamp. Traces were recorded for three minutes in the three periods totalling nine minutes, the final three minutes is represented. Prior to analysis recordings were Gaussian filtered at 1080 Hz to remove noise. Initially a control record was taken prior to a 2

minute perifusion of H_2O_2. Values are expressed as a ratio of the probability of open channels (NPo) to the NPo of the control ± SEM. Fig 2A shows that in the presence of glucose little to no single-channel activity was observed, on subsequent perifusion of H_2O_2 single channel activity consisting of an inward current of 14 pA ± SEM (n = 8). Lifetime characteristics indicative of BK channel activity were observed (Smith et al., 1990)

A significant increase (Spearman's rank, r = 0.302, 95%, p = 0.037) in NPo when H_2O_2 was increased from 0.001 μM until 1 μM (fig 2B) - following which further increases in concentration showed a decreased activity. 10 μM showed the lowest probability of BK being open out of the concentrations studied. 100 μM H_2O_2 also resulting in a lower action than all concentrations below 1 μM, the relationship between NPo and H_2O_2 concentration was overall observed to be bell-shaped (Figure 2).

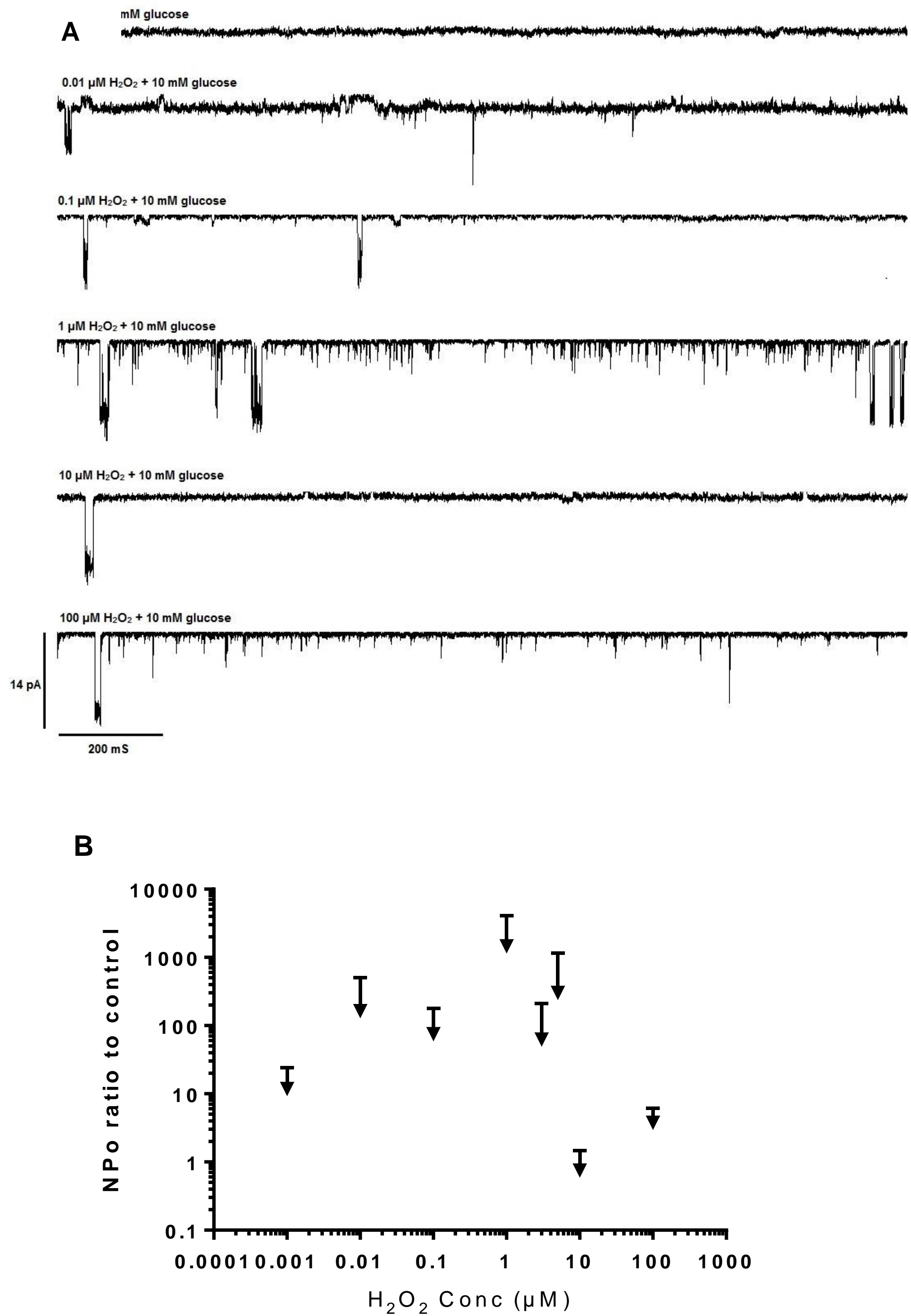

Figure 2. Hydrogen peroxide activates BK channels in MIN6 in a concentration dependent manner when examined using cell-attached

patch-clamp. **A** Representative activity of BK in different single MIN6 cells in response to the perifusion of the varying concentrations of H_2O_2 indicated, vertical bars represent peak amplitude and horizontal bars represent time. Inward flickers are characteristic of BK channel openings **B** The relationship is shown between H_2O_2 concentration and mean NPo after 9 minutes following perifusion of H_2O_2. Error bars represent ± SEM. *N* = 3-8

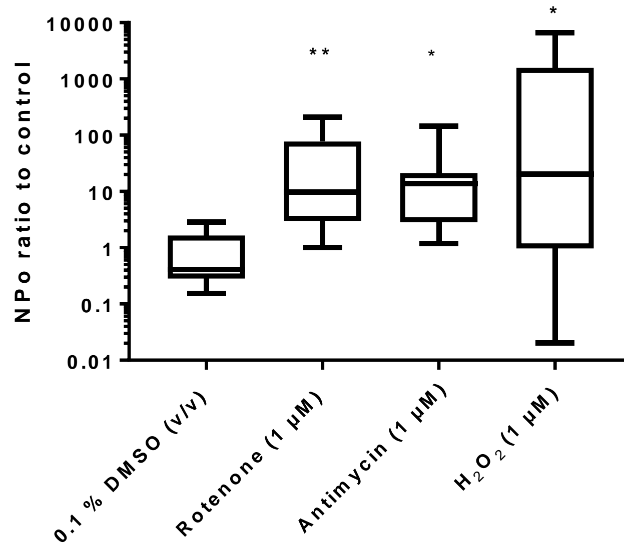

Figure 3. Comparison of BK activators to H_2O_2. MIN6 cells were exposed to rotenone, antimycin and H_2O_2 by a 2 minute perifusion then the final 3 minutes of a nine minute period were taken and the NPo determined. Rotenone and H_2O_2 were significantly different to the control, *p ≤ 0.05 **p ≤ 0.01, error bars express ± SEM, *n* = 8.

Rotenone and antimycin have both been observed to produce free radicals, rotenone by inhibiting the mitochondrial complex 1, leading the incomplete electron transfer to oxygen leading to the formation of reactive oxygen species (Heinz et al., 2017); and antimycin which also inhibits mitochondrial electron transfer leading to ROS production (Park et al., 2007) . were both used as positive controls (both 1 µM) in dimethyl sulfoxide (DMSO) in single-channel patch recordings. Figure 3 shows that rotenone, antimycin and 1 µM H_2O_2 (Kruskal-Wallis; $p = 0.0089$, $p = 0.0162$, $p = 0.0280$ respectively) increased the probability of BK channels being open relative to the control of DMSO.

Inside-out patch clamp studies

To determine if there was a direct effect of 1 µM H_2O_2 on the BK channels inside out patch experiments were carried out on MIN6 cells as this exposes the cytosolic surface of the membrane directly to treatments. ATP bath solution was used as a vehicle control and 10 mM $CaCl_2$ was used as a positive control. Both H_2O_2 or $CaCl_2$ (Kruskal-Wallis $p = 0.0141$ and $p = 0.0247$ respectively) significantly increased activity relative to the control solution (fig 4a), this indicates that 1 µM hydrogen peroxide acts in a direct manner to open BK channels present in MIN6 cells.

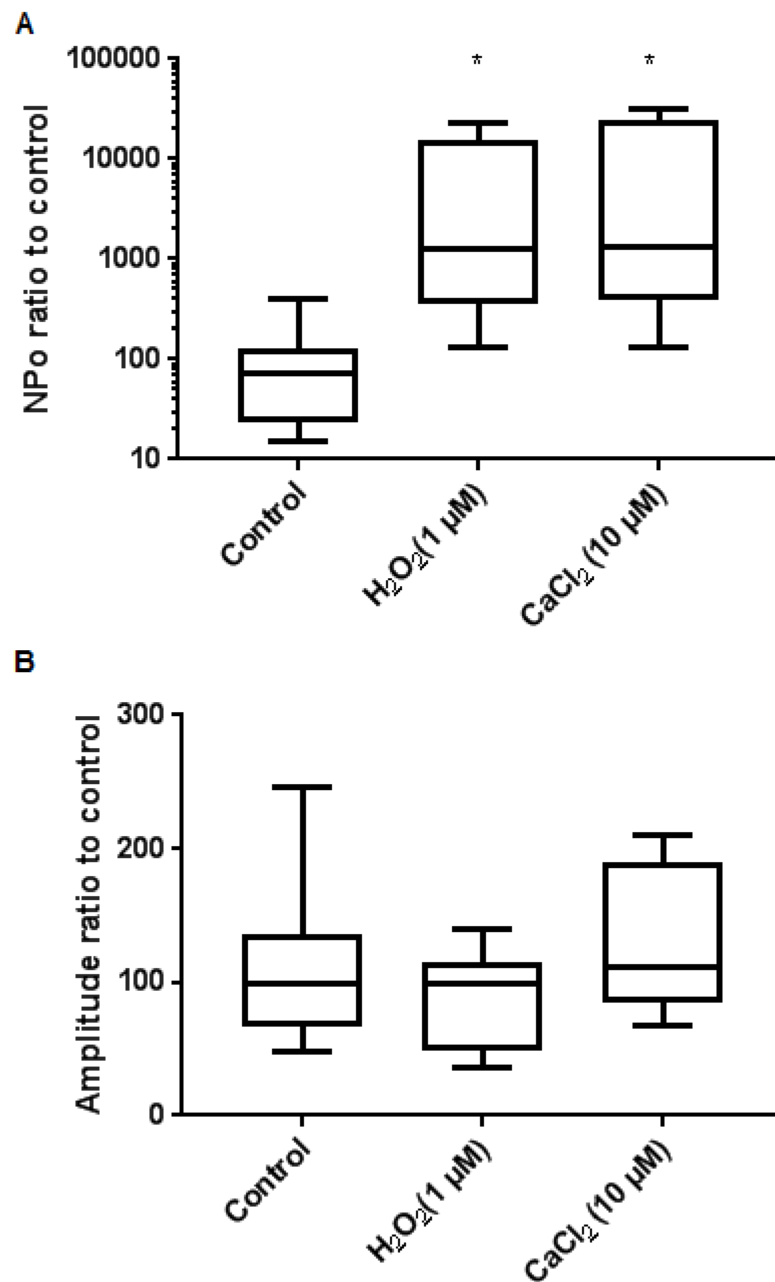

Figure 4. Investigation into the direct of effect of H_2O_2 on BK channels in MIN6 cells. **A** Following excision of the patch treatments were perifused for 2 minutes then K^+ currents were recorded for nine minutes, the activity for the final three minutes is represented. **B** Amplitude of K^+ currents determined by inside out patch, following

treatment of cells with ATP solution (control), 10 μM $CaCl_2$ and 1 μM H_2O_2, normalised to control runs prior to treatment *$p \leq 0.05$, n = 4-7. Data shown as mean ± SEM.

H_2O_2 showed no significant effect on the amplitude of K^+ currents as observed by inside-out patch (fig 4b), in relation to the control solution amplitudes, the mean amplitudes as percentages of matched pairs to the control value. for H_2O_2 was 88.64%±15.55 compared to 111.8%±21.84 for the ATP solution vehicle control showing very little change between the two solutions. (Kruskal-Wallis, p H_2O_2 = >0.9999 and $CaCl_2$ = 0.4655). This is an overall indication that BK has little influence on the amplitude of K^+ currents in MIN6.

Whole-cell patch-clamp studies

Whole-cell voltage-clamp experiments investigated the effect of 1 μM H_2O_2 on the amplitude of all active potassium currents in MIN6 cells.

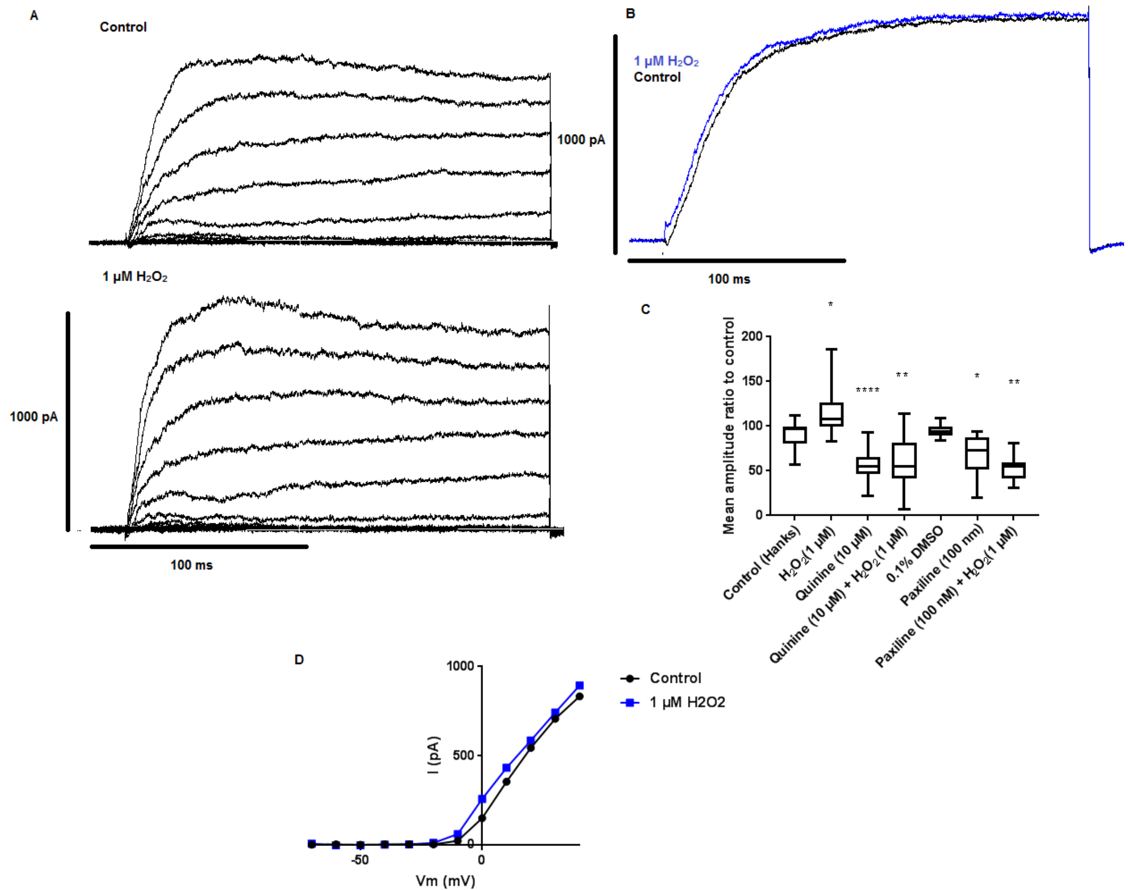

Figure 5. Effect of H_2O_2, quinine and paxilline on whole-cell potassium currents. **A.** Representative traces of control potassium currents and currents in response to H_2O_2, following a voltage step protocol from -70 to 40 mV. Data taken from a single MIN6 cell. Bars represent amplitude and time span. **B.** Comparison traces between potassium currents before and after exposure to 1 µM H_2O_2. Currents taken from applied membrane potential of 0 mV, from a single cell. Bars represent amplitude and time span. **C** Mean amplitude was measured at a steady state over 10 ms; values are representative of the amplitude at a membrane potential of 0 mV in response to addition of 1 µM H_2O_2 (n =

14); 10 μM quinine and 100 nM paxilline with (*n* = 20 and 15) and without (*n* = 10 and 9) the presence of H_2O_2. Quinine and H_2O_2 results were compared to a control of bath solution, paxilline results compared to bath solution with the addition of 0.1% DMSO. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001. Data shown as mean ± SEM. **D** Peak I/V relationships representing the currents observed in prior to and following addition of 1 μM H_2O_2.

Figure 5 shows that H_2O_2 significantly increased the amplitude of whole-cell K^+ currents (Sidak's test, p = 0.0224) by 24% (figure 5). Quinine significantly decreased whole-cell K^+ current amplitude (p = <0.0001) relative to that of the control, by 35%. Paxilline in the absence of H_2O_2 significantly (p = 0.0422) decreased whole-cell K^+ current amplitude relative to the DMSO control, indicating that quinine is a more effective blocker of background BK channel activity, however there was also no significant difference between quinine and paxilline (p = 0.3324). In the presence of H_2O_2 both paxilline (p = 0.0018) and quinine (p = 0.0070) at the concentrations used blocked whole-cell K^+ currents significantly, relative to their respective controls. Quinine reduced amplitude by 29%, treatment with paxilline reduced currents by 41% relative to DMSO, in the presence of 1 μM H_2O_2. Drugs were added in a solution containing 1 μM H_2O_2 following the acquisition of control data.

Perforated patch-clamp studies

In perforated patch voltage-clamp experiments, using the same voltage step protocol as that used in whole-cell, the cell remains metabolically intact (Smith et al., 1990) enabling the cell to retain intracellular secondary messengers and intracellular calcium. Comparisons of 1 μM H_2O_2 with a control of hanks and 100 nM paxilline in the presence of H_2O_2 compared to 0.1% DMSO in Hanks. 1 μM H_2O_2 proved to significantly increase whole-cell K^+ currents (figure 6) in comparison to control values taken prior to treatment; (Paired t test, $p = 0.0283$). Paxilline in the presence of Paxilline 1 μM H_2O_2 did not produce a significant decrease in whole-cell K^+ currents relative to the controls. (Paired t test, $p = 0.6528$). No significant difference was also observed between the hanks control solution and the DMSO control (Unpaired t test, 0.4488).

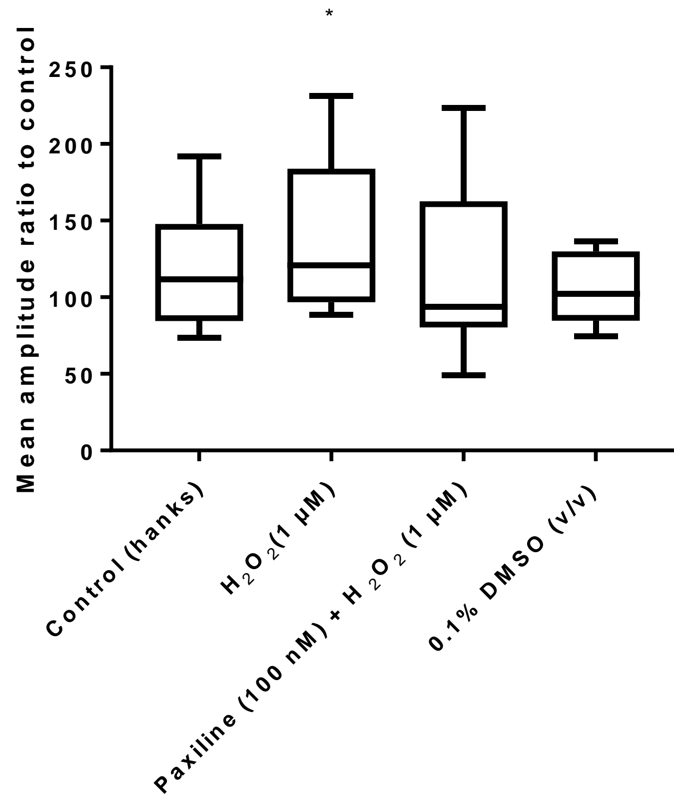

Figure 6. Effect of H_2O_2 and paxilline on potassium currents determined by perforated patch clamp. The mean amplitude was determined at a steady state over 10 ms, values are representative of the amplitude at 0 mV membrane potential following addition of H_2O_2 and a mixture of H_2O_2 and paxilline (n = 10). Mean of H_2O_2 was compared to the mean of hanks solution, paxilline in the presence of H_2O_2 was compared to 0.1% DMSO in hanks, *$p \leq 0.05$.

The changes in baseline membrane potential, peak height (antipeak), half width of spikes and the maximum rate of decay of action potentials stimulated by 10 mM glucose were examined (figure 7). Figure 7 shows that out of the treatments tested only TEA had any significant effect to shape action potentials, in the parameters evaluated. TEA significantly increased the peak voltage against the control (unpaired t test, $p = 0.0259$) and the rate of decay of spikes ($p = 0.0435$).

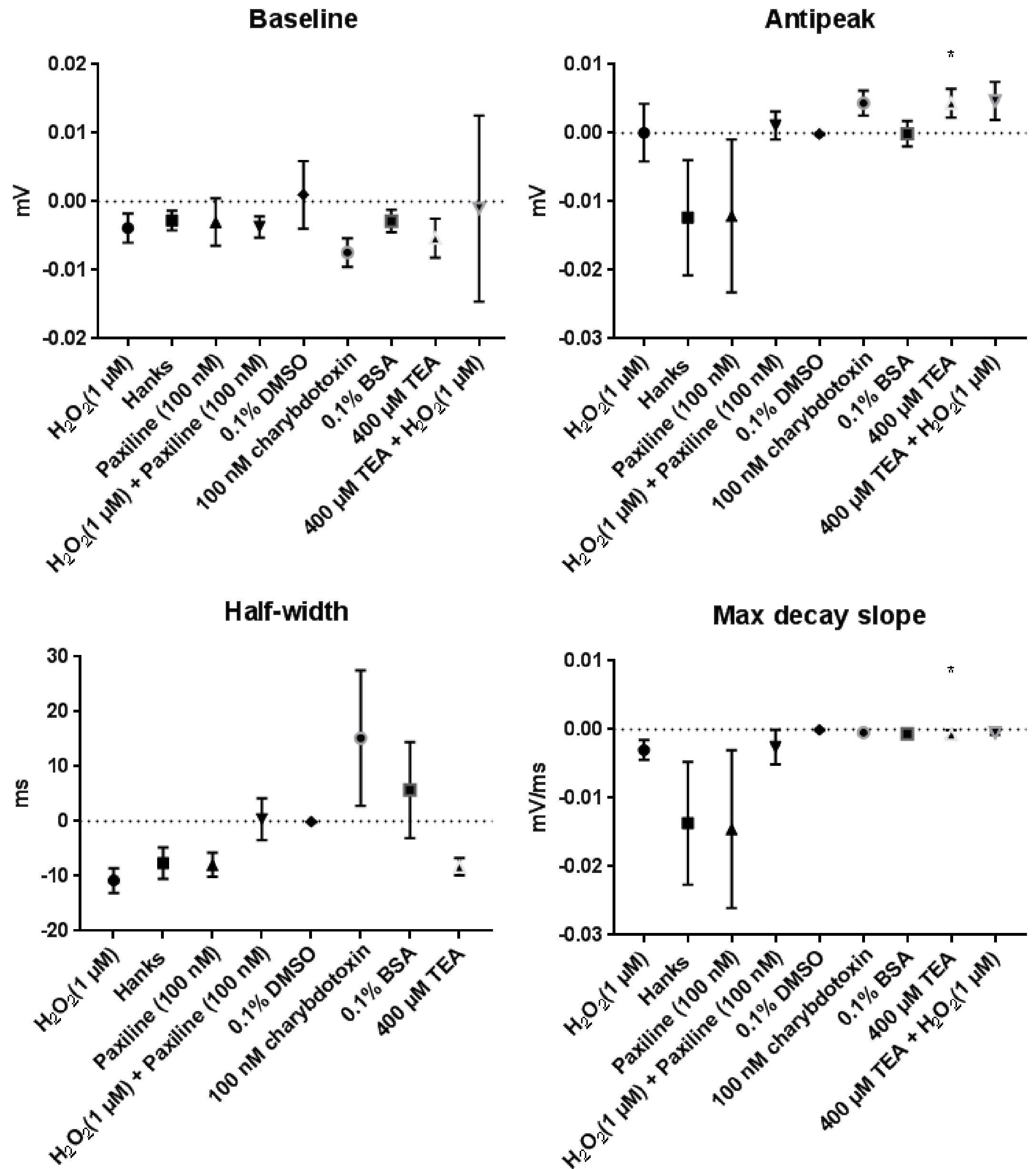

Figure 7. Effect of various treatments on parameters of 10 mM glucose stimulated action potential spiking in MIN6 cells. The mean (± SEM) baseline voltage, antipeak voltage, half-width and maximum rate of decay of action potentials following perifusion of treatment are displayed. Treatments (n = 3-9) were compared to their relative controls

by unpaired t test to determine statistical significance, *$p \leq 0.05$. Data shown as mean ± SEM.

Although not statistically significant differences between control values and H_2O_2 were observed (figure 7). The frequency of spiking was observed to be shorter, also H_2O_2 was observed to decrease the half-width of action potentials, as displayed from the histogram in figure 8, where there is a clear shift from the control values to hydrogen peroxide, with the peaks shifting from 21 to 16 bins. An increase in peak voltage was also observed although not statistically significant above the control.

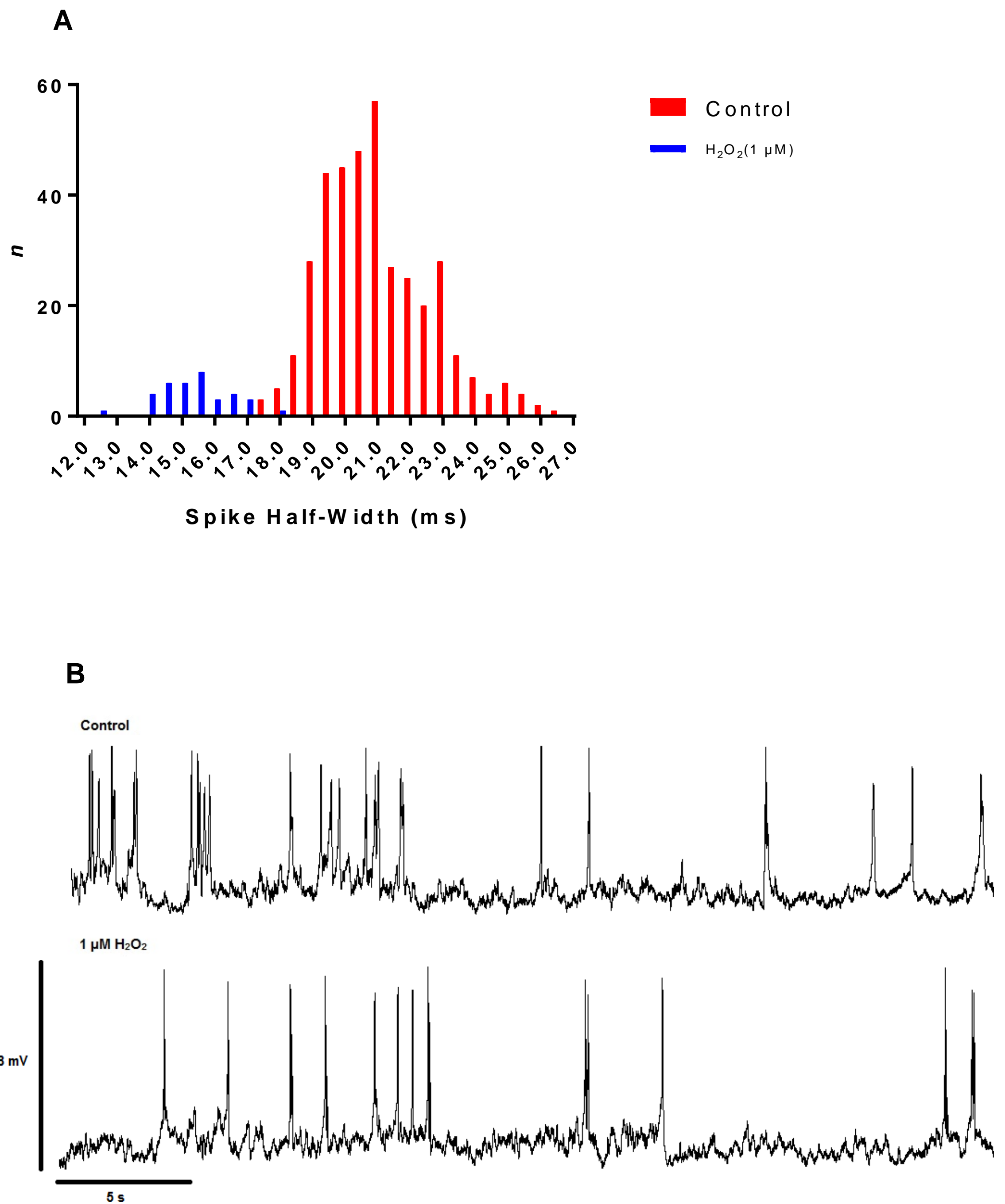

Figure 8. Effect of H_2O_2 on glucose induced action potential spiking. **A** Histogram representative of half-width of spikes detailing the distribution of values taken from a single cell. **B** Traces representative of action

potentials from a single MIN6 cell stimulated by 10 mM glucose under control conditions prior to treatment and in the presence of 1 μM H_2O_2.

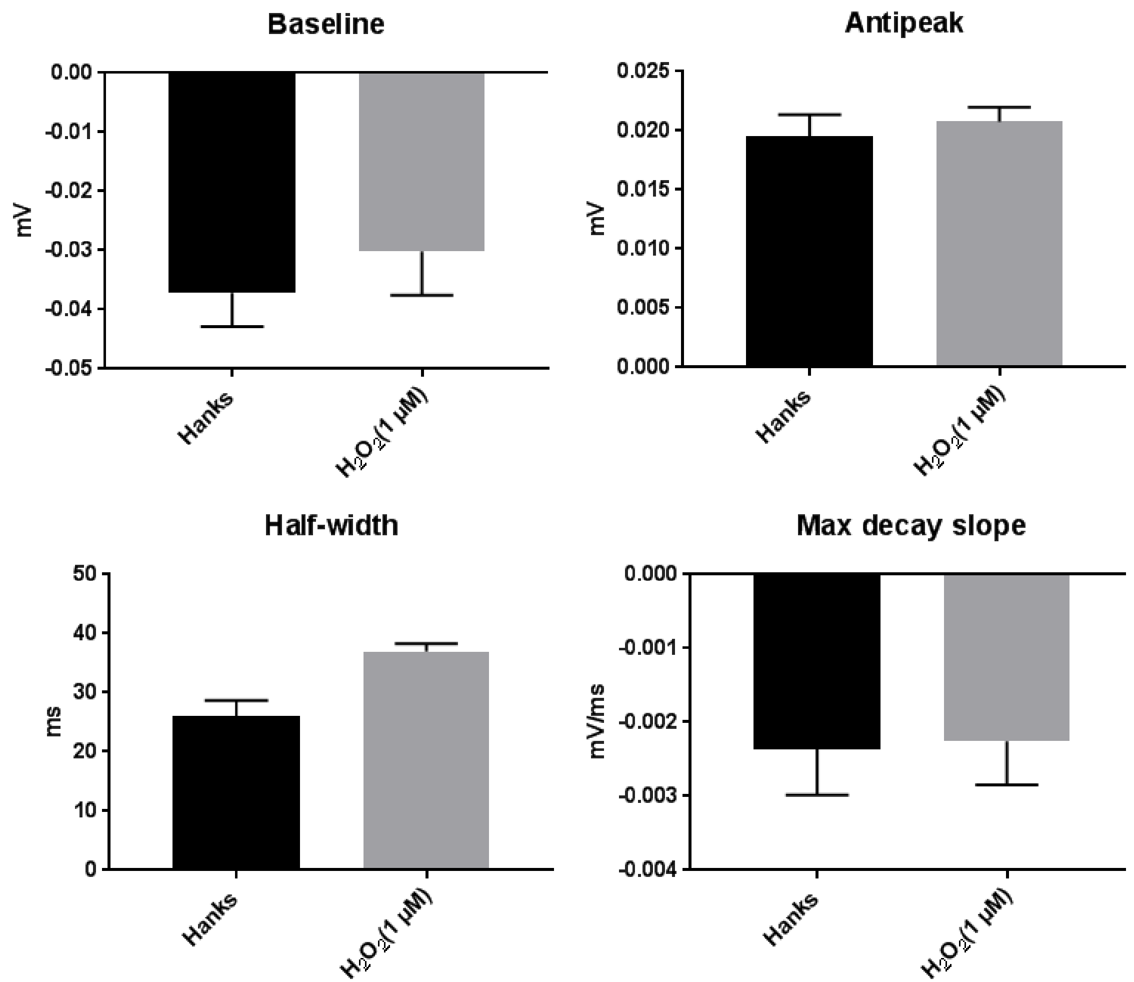

Figure 9. Effect of 1 μM H_2O_2 on parameters of 2.5 mM glucose stimulated action potential spiking in MIN6 cells. The mean (± SEM) baseline voltage, antipeak voltage, half-width and maximum rate of decay of action potentials following perifusion of treatment are

displayed. H_2O_2 (n = 6) was compared to a Hanks vehicle control by unpaired t test to determine statistical significance. Data shown as mean ± SEM.

Changes in baseline membrane potential, peak height (antipeak), half width of spikes and the maximum rate of decay were also examined when action potentials were stimulated with a lower 2.5 mM glucose concentration(figure 9). From this it was found that treatment of cells with 1 µM H_2O_2 produced no significant effect on the baseline membrane potential, relative to the Hanks vehicle control (Unpaired t test, p = 0.7862), antipeak (p = 0.6315), half-width (p = 0.1474) or maximum rate of decay (p = 0.8469).

Calcium imaging

The effect of H_2O_2 on the influx of calcium during glucose induced action potentials was examined using fluo-4 imaging. Cell were continually perifused, initially with hanks until a steady state was maintained then action potentials were induced by 10 mM glucose, only cells that elicited a glucose response ≥ 10% of the basal value were selected for analysis.

Initially it was evident that glucose produced an increase in fluorescence that was significantly different to that of the basal level ($p = 0.0002$), as did H_2O_2 ($p = 0.0009$), both determined by Kruskal-Wallis test. The control however did not produce a significant change from the basal level (figure 10), it was also noted that there was no significant difference between glucose and H_2O_2. There was no significant difference between the vehicle control and 1 μM H_2O_2 (Mann-Whitney, $p = 0.5342$), suggesting that hydrogen peroxide at the concentration used has no significant effect on Ca^{2+} during glucose induced action potential in MIN6.

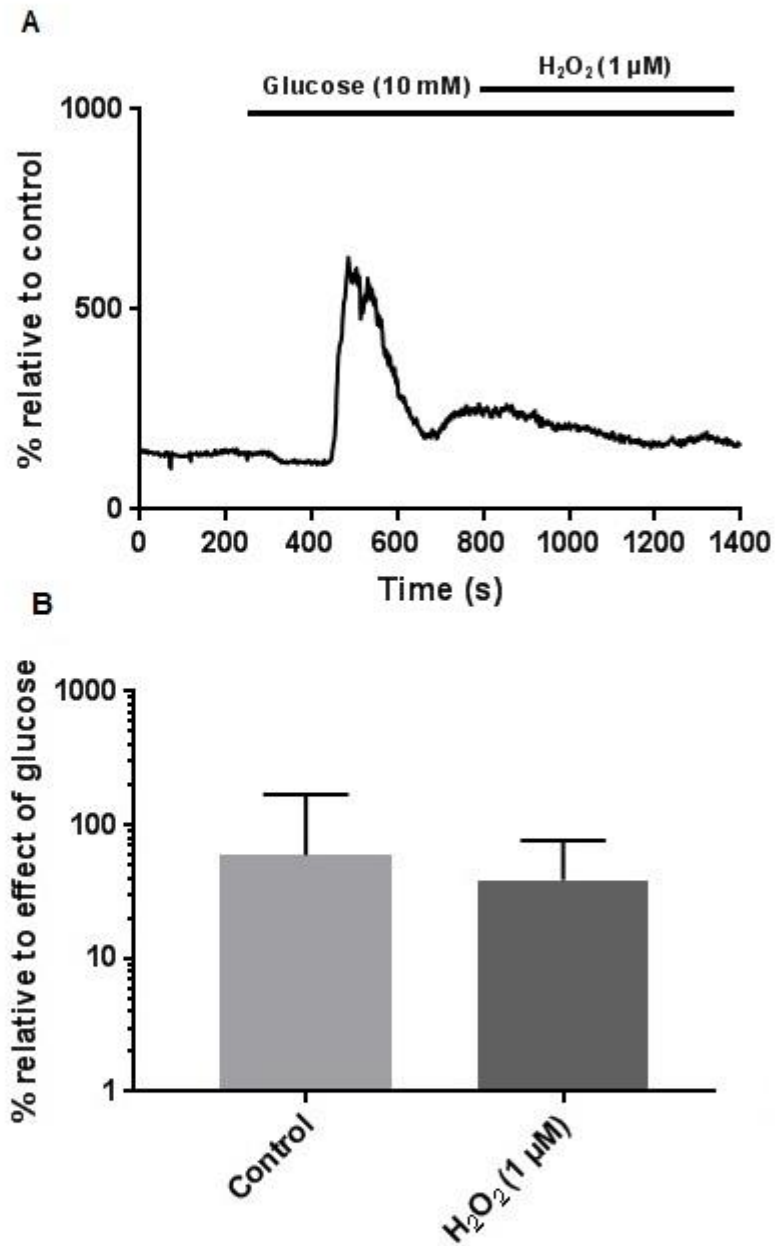

Figure 10. Effect of H_2O_2 on glucose induced calcium influx. **A** Representative effects of 10 mM glucose and 1 μM H_2O_2 on the signal emittance taken from a single MIN6 cell, relative to a control of 1mg/mL saponin with treatments applied for the duration of the bars. The influx of calcium in response to glucose is represented by the peak at 500

seconds. **B** Comparison between H_2O_2 and vehicle control with values represented as a % relative to the stimulatory effect of glucose, taken at an established steady state in the presence of glucose ± SEM (*n* = 14-79).

Determining changes in mitochondrial membrane potential

Rhodamine 123 allowed for further examination into the functional effects of H_2O_2 by studying how it affects $\Delta\Psi_{mit}$, following stimulation of action potential by 10 mM glucose. Cells were continually perifused with glucose cells which elicited a glucose response of ≥ 10% were selected for further analysis.

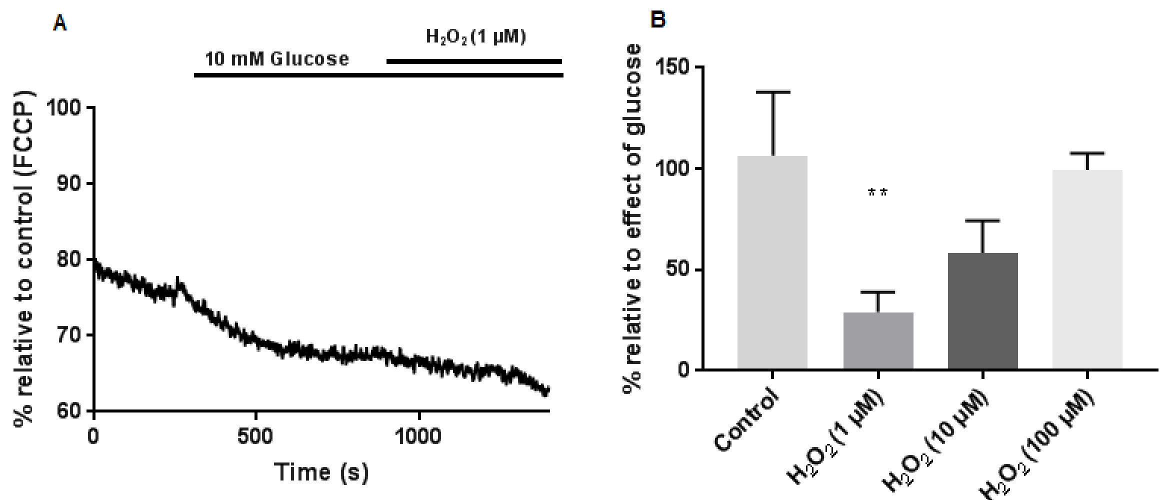

Figure 11. Effect of H_2O_2 on mitochondrial membrane potential during glucose induced action potentials. **A** Representative effects of 1 mM glucose and 1 μM H_2O_2 on the signal emittance taken from a single MIN6 cell, treatments applied for the duration of the bars. Glucose induced a decrease in membrane potential, which was further decreased with the addition of 1 μM H_2O_2 **B** Effect of vehicle control (10 mM glucose solution), 1, 10 and 100 μM H_2O_2 on mitochondrial membrane potential following activation of action potential by glucose. Values are expressed as a % relative to the effect of glucose, which induces quenching of fluorescent signals in MIN6 cells. Error bars represent ± SEM, (n = 7-36), **p ≤ 0.01.

The decrease in membrane potential in response to H_2O_2 was observed to be concentration dependent. It was determined that 1 μM H_2O_2 significantly decreased (figure 11) the membrane potential compared to that of the 10 mM glucose vehicle control (Ordinary one-way ANOVA, p = 0.0095). At this concentration H_2O_2 elicited a further decrease in membrane potential relative to that of the control solution of 70% (mean). Conversely treatment with 10 μM and 100 μ M H_2O_2 did not elicit a significant change in membrane potential relative to the control (p = 0.4365 and 0.9966 respectively).

Observations from mammalian tissue studies

To confirm whether the same effects noted in MIN6 cells could be observed in mouse β cells, a small number of select experiment were carried out. Three single-channel patch-clamp experiments were carried out and NPos taken before and prior to perifusion of 1 μM H_2O_2, from these experiments downward flickers indicative of Bk channels and displaying similar characteristics to those taken from earlier MIN6 single-channel studies were observed (figure 12).

An I/V experiment was also conducted to determine the relationship between decrease the membrane potential and the resulting change in amplitude of potassium currents. The Pearson correlation coefficients of the control and 1 μM H_2O_2 were 0.941 and 0.9801 respectively and the relationship between voltage and current was found to be statistically significant for each ($P = <0.0001$ for both factors), indicating that changes in the membrane potential result in changes in amplitude. From the I/V curve it can be observed that the values from H_2O_2 treatment represent higher amplitudes at a positive membrane potential and show a greater inward current at negative membrane potentials, the current is inverted for both control and H_2O_2 at -20 mV. The control line of best fit intercepted the line at 0.6129 and had a slope of 0.07695, whereas H_2O_2 had an intercept of 1.673 and a slope of 0.1089.

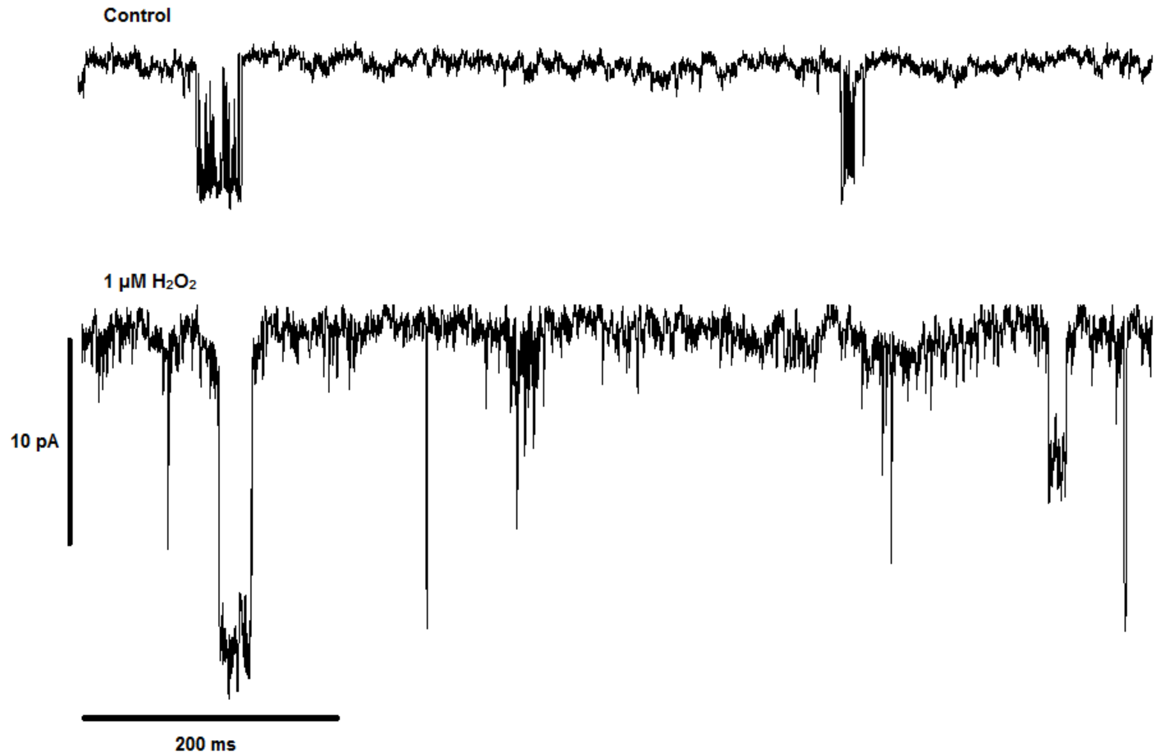

Figure 12. Effect of H_2O_2 on single channel currents in β cells isolated from C57BL/6 mice. Representative current traces taken from a single mouse β cell, prior to and following addition of H_2O_2, bars are indicative of the amplitude and time-span of channel events. Inward flickers are characteristic of BK channel opening.

Current clamp experiments were also carried out on mouse β cells examining the effects of 1 µM H_2O_2 on action potential shaping, from this it was noted that there was no observable difference between H_2O_2 and its control in terms of baseline shift, antipeak, half-width and maximum decay slope; similarly to that observed in MIN6.

Discussion

Previous work has observed that supraphysiological concentrations (>100 nM)(Sies, 2017), of H_2O_2 opens BK channels in arterial smooth muscle cells via the phospholipase$_2$-arachidonic acid signalling cascade, this was observed by Barlow, El-Mowafy and White (2000) who found that 300 μM was capable of activating Bk channels. H_2O_2 has also been shown to modulate BK channel activity in smooth muscle cells via interleukin-1β and that H_2O_2 (40-120 μM) stimulated BK channel activity when examined using whole cell patch-clamp electrophysiology (Gao et al., 2010). More recently Dan and Fei (2014) investigated the effects of incrementally increasing H_2O_2 concentration across a 1-4 μM range in the outer hair cells of guinea pig cochlea and determined that the potassium current amplitude and peak density increased in a concentration dependent fashion when studied using whole cell electrophysiology. In human embryonic kidney cells examined in the cell-attached configuration of patch clamp 100 μM H_2O_2 was shown to modulate the activity of BK channels and activate the cells via the phospholipase A2-AA signalling cascade, data that again supports the idea that ROS are an activator of BK channels (Liu et al., 2009).

Other work has observed removal of BK activation in the presence of H_2O_2 in vascular smooth muscle cells as result of cysteine modification

the α-subunit of the channel, by H_2O_2 at supraphysiological concentrations (Tang et al., 2004). Lu *et al* determined, using whole-cell electrophysiology that in diabetic mice ROS inhibited Akt signalling, and facilitated the forkhead box O subfamily transcription factor-3a (FOXO-3a)/ F- box–only protein (FBXO) pathway, causing degradation of the β_1 subunit of vascular BK channels and a decrease in channel current density; inhibition of this protein pathway prevented degradation, supporting the claims by previous work that ROS are an inhibitor of BK channels in both chronic and acute conditions. Excessive production of ROS is thought to lead to development of diseases such as diabetes and arteriosclerosis. It has been determined that generation of ROS in human embryonic kidney cells impair the function of BK channels. Lu *et al* (2006) examined that H_2O_2 caused oxidation of a cysteine residue in the channel, situated at the calcium binding site, leading to inhibition of BK channels. Wang, Crager and Pugazhenthi (2012) observed that under exposure to oxidative stress MIN6 and human islets cells lost a significant amount of β cell function. H_2O_2 at 200 μM led to autophagy and induction of apoptosis, resulting in cell death. In the study it was also found that overexpression of the enzyme manganese superoxide dismutase (MnSOD), a free radical scavenger, protected MIN6 cells from apoptosis induced by cytokines.

From the work carried out it is clear that hydrogen peroxide has a stimulatory effect on BK channels present in a MIN6 cell line, and whilst

there is a suggestion of its capability to activate the channel in mouse β cells the work done here is inconclusive and requires further investigation. Single-channel patch-clamp studies showed a significant increase in the open probability of channels when exposed to 1 μM H_2O_2, in relation to vehicle controls. The relationship between H_2O_2 and BK activation in MIN6 was observed as a concentration dependent 'bell-shaped' curve. 1 μM H_2O_2 produced the greatest degree of BK activation, whereas higher concentrations elicited a much lower response. This is somewhat contradictory to what Liu *et al* (2009) discovered as their work does agree with the conclusion that H_2O_2 is an activator of BK, resulting in an increase in K^+ currents in cell-attached patches, however in their work they found that 100 μM was a strong activator, and the level of activation also increased up to 400 μM, however human embryonic kidneys cells were used in their work as opposed to MIN6. In another study Liu *et al* (2010) also found that 100 μM was a significant activatory concentration of BK in single-channel studies in the same cell line. Zhang *et al* (2012) also determined that treatment with 50 μM H_2O_2 also resulted in a significant increase in the levels of Bk activation, in cell-attached patch configuration. In β cells Düfer *et al* (2011) also determined that H_2O_2 had a stimulatory effect on BK mediated K^+ currents. 200 μM tolbutamide in the pipette was deemed sufficient enough to block K_{ATP} mediated K^+ currents.

There is an overriding theory on the mechanism through which BK is activated by H_2O_2; Dong *et al* (2008) suggest that NO production is stimulated as a result of increased intracellular Ca^{2+}, which in turn stimulates PKC via the secondary messenger cGMP. Feng *et al* (2011) also observed that BK is activated by 100 μM H_2O_2 in human dermal fibroblasts and the increase in K^+ currents were mimicked by a PKC activator. It was also determined that the intracellular levels of PKC were significantly increased by H_2O_2 in a concentration dependent fashion, further supporting this theory of the method of BK activation by H_2O_2.

The effect of rotenone and antimycin on BK channels has been examined through a multitude of different methods. Rotenone is an inhibitor of the mitochondrial respiratory chain, working by uncoupling the transfer of electrons to prevent formation of NADH (Bednarczyk et al., 2013), in the present study it was determined that 1 μM rotenone had a significant activatory effect on BK channels by increasing the chances of channels being in an open state, which was also found to be the case for H_2O_2 in the same experiment, further supporting the idea that BK is activated by H_2O_2 Antimycin is also an inhibitor of mitochondrial respiration by inhibiting ubiquinol cytochrome c oxidoreductase (complex III). It was shown that also significantly caused an increase in the open probability of channels in relation to the DMSO vehicle control. Other studies have shown that antimycin is an

activator of BK channels (Boveris and Chance, 1973). The mechanism through which antimycin acts has been described by Chen *et al* (2003) and it is speculated to modify the mitochondrial complex III which results in an increase in ROS production from the Q_o site, oriented towards the intermembrane space. The use of both positive controls is further indication that Bk channels are stimulated by ROS.

After confirming that H_2O_2 had a stimulatory effect on BK channels in MIN6, inside-out patch- clamp was used to determine whether H_2O_2 had a direct effect on BK, due to the cytosolic region of the channel being exposed to the environmental conditions created by H_2O_2. From the inside-out patch experiments it was determined that H_2O_2 had a significant stimulatory effect, as did the $CaCl_2$ control which was expected due the increased calcium level present in the solution. This further supports the previous findings from the single-channel patch-clamp experiments that H_2O_2 activates BK. This also creates a suggestion that H_2O_2 acts directly on the channel, as opposed to through any secondary messenger which is contradictory to previous work (Dong et al*.,* 2008; Feng et al, 2011). It is possible that H_2O_2 creates a shift in the voltage dependency of BK channels, making them become more sensitised to voltage changes and increasing the probability of an open channel state, although this would need to be investigated further by examining the open probability at different voltages, at 30, 50 and 70 mV.

Conversely to the work carried out in the present study, other work has found that the increase in open channel probability caused by H_2O_2 is attenuated when measured by inside-out patch, in comparison to those measured by cell attached patches, indicating that H_2O_2 acts through a secondary messenger when stimulating BK channels. Zhang *et al* (2011) found that BK channels were activated in single-channel configuration with the intracellular components present; whilst once patches were excised in inside-out configuration the BK current was absent, indicating the importance of intracellular components in BK activation, when treated with 50 μM H_2O_2. Liu *et al* (2011) again found that BK channels were not activated in inside-out patches, but were in cell-attached patches when treated with 300 μM H_2O_2. Earlier work by Barlow and White (1998) also found that the same concentration activated BK in single-channel patches and activation was absent in inside-out patches. Liu *et al* (2010) suggest that a lack of activation in inside-out patches may be due to oxidative modification of cysteine residues in BK channels by ROS, or other membrane proteins that regulate BK channel function. In other studies (Papreck et al., 2012), consistent with the finding of the present study, H_2O_2 was found to activate BK in the inside out configuration but at a 32-326 mM concentration. It is noted that the concentration in this study used to activate BK in the inside out configuration (1 μM) was far lower than those used in other comparative work, however it's activation at this

concentration is consistent with the findings from the single-channel experiments carried out in the present study.

In work presented it was suggested that BK acts directly on the channel due to the level of increase of open channel probability. It can also be seen that there was no obvious change in the degree of H_2O_2 mediated activation of BK between inside-out and single-channel patch-clamp configurations, when observing the channel activity in MIN6. This further supports the idea that H_2O_2 acts directly on the channel and could indicate that it acts on a site located on the extracellular side of the channel, as the membrane components are preserved in single-channel patches.

Alongside the effect of H_2O_2 on the NPo, the effect on the amplitude of the K^+ currents was also observed using inside out patch, from this it was determined that H_2O_2 had no significant effect on the amplitude of BK mediated K^+ currents in MIN6 cells , when observed by inside-out patch, compared to the vehicle control. The positive control of 10 mM $CaCl_2$ also showed no significant effect in relation to the control, indicating that a high level of extracellular calcium does not result in a significant increase in amplitude of BK K^+ currents and that this lack of effect is also seen when cells are exposed to H_2O_2, in MIN6, when held at a constant membrane potential, in inside out patches.

Whole-cell patch clamp experiments further supported the previous conclusions from single-channel and inside-out studies that 1μM H_2O_2 is an activator of BK channels in MIN6. From the whole-cell experiments carried out it was determined that BK K^+ currents were significantly increased by H_2O_2 across the hole of the cell, in relation to the vehicle control. It was also determined that the currents were highly sensitive to treatment with 10 μM quinine, which is known to block BK currents in β cells (Bokvist, Rorsman and Smith, 1990), and that this effect was maintained when quinine was applied in tandem with H_2O_2. K^+ currents also proved to be sensitive to another proven BK blocker, paxilline (Shruti et al., 2012), which produced a significant inhibitory effect relative to the vehicle control when used alone to block background BK, and also produced a significant block when used in combination with H_2O_2, like quinine, and was capable of reversing the stimulatory effect of H_2O_2. The decline of current amplitude when both quinine and paxilline were applied to the cell as a result of channel block confirmed the role of BK when H_2O_2 stimulated an increase in current amplitudes.

The voltage step protocol used to analyse whole cell currents showed an increase in amplitude of K^+ at a steady state following 10 mV incremental increases in membrane potential. This is consistent with the finding of Feng *et al* (2012) who determined that H_2O_2 significantly increased BK K^+ current amplitudes when examined via the whole-cell

configuration of patch clamp in human dermal fibroblasts. Lai and Huang (2014) also confirmed that 1 μM H_2O_2 stimulates BK channels in the whole-cell configuration in guinea pig cochlea.

The failure of H_2O_2 to reverse the blockage of both quinine and paxilline suggests that they both act irreversibly and have a mechanism which results in a closed channel block. This is supported by Zhou and Lingle (2014) who suggest that paxilline allosterically alters the intrinsic closed-open equilibrium constant favouring occupancy of closed states. They also claim however that paxilline has a stronger affinity for channels that are in the closed state which could imply that it's time to take an effect on the channel is less than that of H_2O_2 as it produced a strong block when used in the presence of H_2O_2, which opened BK channels without the presence of a blocker. It could also be suggested that the lesser degree of blocking caused by paxilline alone was a result of channels at an open state due to the membrane potential of the cell being disrupted by the whole-cell method, a phenomenon which has been observed previously (Perkins, 2006). Other studies have determined that the binding of quinine to cause block in Slo3 channels (a close relative of BK) is not dependent on the open state (Wrighton, Muench and Lippiat, 2015), which is supported by the findings from the present study as quinine alone was found to be the strongest blocker out of those used, relative to the control; and in the presence of H_2O_2 the block was less than that of quinine alone, possibly indicating some

minor activation of channels by H_2O_2 and that quinine may not act as rapidly as paxilline. It must be noted however that quinine is a non-selective blocker of BK and has been shown to selectively block K_{DR} (Smith et al.,1990), in future investigations this experiment should be repeated with the use of another selective blocker of BK such as TEA or iberiotoxin (Haghdoost-Yazdi, Janahmadi and Behzadi, 2008). From the I/V plot of whole-cell K^+ currents it can also be observed that the currents were increased by 1 μM H_2O_2, in further work the membrane potential should be increased further past 40 mV to gain a fuller appreciation of the relationship between voltage and current in relation to β cell K^+ currents and determine whether the currents plateau at higher voltages or continue to increase in a linear fashion

To examine whole-cell currents without fully excising the plasma membrane whole-cell patch-clamp was another configuration used to determine the effect of 1 μM H_2O_2 on MIN6 BK channels. Similarly to the other configuration 1 μM H_2O_2 caused a significant increase in the whole-cell amplitude of currents. This is supported by the findings of Dong *et al* (2008) who found that there was no difference between whole-cell and perforated-patch and that H_2O_2 stimulated BK channels in both configurations. It was however observed that paxilline had no significant blocking effect in the presence of H_2O_2, in contrast to its effect in standard whole-cell patches. It is possible that attenuated effects of block in perforated patch are a result of excessive perforation

by amphotericin resulting in the cell entering conventional whole-cell configuration, this could also be a result of oxidative-stress related damage around the seal, occurring in perforated-patches.

After confirming the stimulatory effect of H_2O_2 on BK channels in MIN6, perforated patches were also used to examine the effect of H_2O_2 on action potential shaping, allowing pulses to be investigated over a prolonged period as the integrity of the cell membrane was retained. Action potentials were stimulated by addition of 10 mM glucose to the media. From the investigations carried out it was determined that 1 μM H_2O_2 had no significant effect on the shaping of action potentials based on the parameters of baseline, peak, half-width and maximum slope of decay. However it was also observed that addition of H_2O_2 caused a complete block of action potentials after a short period of exposure (1-2 mins), indicating that BK was activated in the patches and cells because hyperpolarised, this effect was also determined to be irreversible. Also the effect was not deemed to be significant histograms showed a shift in distribution and a shortening of the half length of action potential following exposure to H_2O_2.

It was also observed that only TEA of the BK blockers applied to cells had any significant effect on the shape of action potentials, significantly increasing the peak and maximum decay slope. Paxilline showed no significant effect which is consistent with the perforated whole-cell

patch-clamp experiments carried out in the present study. The selective BK channel blocker charybdotoxin, which has been observed to block BK channels in other cell types at the concentration used (Gu et al., 2014; Sun et al., 2015) produced no significant effect on action potential shape, however Kukuljan, Goncalves and Atwater (1991) found that in pancreatic β cells charybdotoxin had no significant on the shape on glucose induced action potentials which is consistent with the findings here.

TEA produced a significant effect on increasing the peak and decay slope of action potential stimulate via glucose in MIN6. TEA is a non-selective BK channel blocker which is noted for its effect on other K^+ channels (Li et al., 2011; Yellen, 1984), which presents a possible explanation for the lack of effect observed when cells were treated with H_2O_2. The findings from the present study suggest that BK plays a minor role in the shaping of action potentials in β cells when they are exposed to a fully stimulated by a high concentration of glucose. Other studies have shown that in β cells the repolarisation of spikes was mainly a result of activity of the 8-pS delayed rectifying K^+ channel (Smith et al., 1990). The effect of H_2O_2 on action potential shaping in β cells has not been fully clarified, however Ostrowski *et al* (2014) found that in neuronal cells application of H_2O_2 resulted in an initial reduction of action potential firing due to the depolarising current, which was reversed with washout of H_2O_2 and subsequent additions of H_2O_2 then

resulted in sustained hyper-excitability, albeit in neuronal cells. Similarly in the present study the initial reduction of action potential firing was observed, however following depolarisation of the cell spiking could not be recovered. In their study Ostrowski *et al* used a substantially higher concentration (500 μM) of H_2O_2 then that used in the present work, which may have resulted in the differing effects that were observed.

A possibility for the absence of observed effect was that at the glucose concentration used action potentials were being fully stimulated, which meant 1 μM H_2O_2 was insufficient to cause a significant effect, this was further investigated by stimulating action potentials with a lower concentration of 2.5 mM glucose. Following statistical analysis it was found that 1 μM H_2O_2 produced no significant change in the shape of action potentials stimulated at this concentration, which is consistent with the previous findings. To further investigate the possibility of H_2O_2 being capable of altering action potential shape a higher range of concentrations should be examined to see if any significant effect exists, however this does also present problems of cytotoxicity with the use of supraphysiological concentrations of H_2O_2.

H_2O_2 had no significant effect on the influx of calcium during glucose stimulated action potentials determined by fluorescent imaging, relative to the effect of glucose on cells. Addition of glucose to the media resulted in an initial spike in fluorescence, followed by an increased

steady state over the basal level, indicating that action potentials were stimulated which was supported statistically. Subsequent addition of 1 µM H_2O_2 applied constantly to the media did not produce any additional significant effect on the influx of Ca^{2+}. This is consistent with the findings from the current-clamp experiments which also found that H_2O_2 had no significant effect on action potential shaping. Although in neuronal as opposed to β cells, Benhassine and Berger (2008) found that activation of BK channels resulted in a decrease in calcium influx via glutamate receptors and reduced cellular excitability; although not significant a decrease in the mean cellular calcium was observed in cells treated with H_2O_2 relative to the mean of control cells, potentially indicating that activation of BK in β cells also reduces influx of calcium. This is also an indication that 1 µM H_2O_2 activated BK in cells at this state.

It was determined that 1 µM H_2O_2 significantly reduced $\Delta\Psi_{mit}$, relative to the vehicle control following stimulation of action potentials by glucose. This effect was determined as a ratio of the initial effect of glucose, which caused membrane potential depolarisation as a result of action potential stimulation, observed with the use of Rhodamine 123, where the signal is quenched by an increase in the level of dye. Increasing the concentration of H_2O_2 to 10 and 100 µM resulted in an insignificant decrease in membrane potential relative to that of the control. There was a greater decrease in membrane potential relative to the effect of

glucose observed as the concentration of H_2O_2 concentration was decreased, indicating a concentration dependent relationship between the two variables; this could be further investigated to determine the minimum and maximum H_2O_2 concentrations to potentiate a decrease in $\Delta\Psi_{mit}$. The findings from the present study are supported by Tada-Oikawa *et al* (2003), who found that increased H_2O_2, derived from rotenone caused a decrease in $\Delta\Psi_{mit}$ in a human leukaemia cell line. Earlier work by Konno and Kako (1991) also found that H_2O_2 was capable of reducing $\Delta\Psi_{mit}$, however only a high concentrations (5 mM) and gradually over time, their work was carried in cardiomyocytes however, which have been shown to have well-developed defence mechanisms against oxidative stress (Fisher-Wellman, Bell and Bloomer, 2009) compared to the lesser-developed mechanism of β cells. The results from the present study also support the single channel findings that 1 μM is the most stimulatory concentration of H_2O_2 for BK channels in MIN6. The decrease in mitochondrial membrane potential potentiated by H_2O_2 could indicate a presence of intracellular calcium prior to addition of H_2O_2 resulting in a further depolarisation of the cell (Chalmers and McCarron, 2008).

Some small scale studies were finally carried out on pancreatic β cells extracted from mouse islets. From this, replication of the stimulatory activity of 1 μM H_2O_2 was observed in the mammalian tissue using single-channel patch-clamp. The channel openings observed displayed

the characteristics of BK channels that had been previously observed in single-channel studies and identified in other work (Smith et al., 1990). This presents an opportunity to explore the relationship between H_2O_2 concentration and channel open probability in murine cells, similarly to the work carried out in single-channel patches using MIN6, which would allow for investigation into any variations between the two models.

The I/V plot of H_2O_2 against control also demonstrated a shift towards higher amplitudes at positive holding potentials and lower amplitudes at negative potentials, indicating increased excitability as a result of H_2O_2 treatment. Düfer *et al* (2010) also observed a stimulatory effect of H_2O_2 in murine β cells, consistent with the observations from the present study.

The effect of H_2O_2 on glucose stimulated action potential shaping in murine β cells was also observed using perforated patch and as with the observations from MIN6 no immediate effect was found; however it should be noted that these were only initial observations due to the small number of experiments carried out due to availability of animals to isolate cells from, and it cannot be fully concluded whether the effects seen across the experiments carried out on BK channels in MIN6 are replicated in murine cells until further work has been undertaken which would allow for a full statistical analysis of the two groups of data between the cell types. Further work would also involve determining

whether the direct stimulatory effect of H_2O_2, observed in MIN6, is replicated in murine cells.

Conclusion

To summarise, the investigations show that H_2O_2 activates BK channels in a 'bell-shaped' concentration dependent manner in single-channel patches, most effectively at 1 μM. Inside-out patches showed that it does this by acting on the protein directly as shown by the activation of BK in the absence of other cytosolic components. Whole-cell recordings showed that 1 μM H_2O_2 increased K^+ amplitudes, whilst BK was blocked by both quinine and paxilline to confirm its activity. Perforated patch recordings showed no significant increase or block in whole-cell amplitude, in contrast to the previous findings. 1 μM H_2O_2 also had no significant effect on action potential shape when stimulated by 2.5 and 10 mM glucose, as well as calcium influx. However 1 μM H_2O_2 did display a significant effect in decreasing the mitochondrial membrane potential during spiking stimulated by glucose, this effect was reduced with an increased concentration of H_2O_2.

These results may prove insightful in the development of potential therapeutic antioxidants used in the treatment of diabetes mellitus; future considerations for this work would involve further investigations into the effect of H_2O_2 on mouse β cells and how it compares to the

results found from MIN6; and determining the mechanism of H_2O_2 in stimulating BK channels.

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