Cannabinoid Receptors are Coupled to Stimulation of Insulin Secretion from Mouse MIN6 β -cells

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Key Words

MIN6 β -cells • Insulin secretion • Cannabinoids • Cyclic AMP • Calcium • Immunocytochemistry

Abstract

Endocannabinoid lipids are known to exert orexigenic effects via central cannabinoid CB1 and CB2 receptors, which have also been identified in islet endocrine cells. However, there is no consensus on whether the receptors are expressed by β -cells, nor what effect CB1 and CB2 receptor agonists have on insulin secretion. In the current study we have therefore used the mouse MIN6 β -cell line rather than primary islets, which are heterogeneous clusters of endocrine cells. Cannabinoid receptor and diacylglycerol lipase isoform mRNAs were detected in MIN6 cells by RT-PCR and immunocytochemistry was used to identify cannabinoid receptor expression by MIN6 cells. Changes in cyclic AMP and intracellular calcium were measured by immunoassay and microfluorimetry, respectively, and insulin secretion from perfused MIN6 pseudoislets was determined by radioimmunoassay. MIN6 β -cells express the cannabinoid synthesising enzyme diacylglycerol li-

pase and CB1 and CB2 receptors, which are coupled to inhibition of β -cell cyclic AMP generation and stimulation of intracellular calcium levels. Cannabinoid receptor activation with pharmacological agonists resulted in reversible elevations in insulin secretion at both 2mM and 20 mM glucose. Synthesis of endocannabinoids by β -cells may provide an additional mechanism for stimulation of insulin secretion through activation of β -cell CB1 and/or CB2 cannabinoid receptors.

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Introduction

The well-known appetite-inducing effects of cannabis are mediated by its principal psychoactive component, Δ^9 -tetrahydrocannabinol (THC), via activation of cannabinoid receptors [1]. The two major cannabinoid receptors, CB1 [2] and CB2 [3], were cloned in the 1990s and more recently GPR55 has been classified as a

cannabinoid receptor [4-7] even though it shows less than 15% homology with CB1 and CB2 receptors [4, 7]. The CB1 receptor is one of the most abundantly expressed GPCRs in the mammalian brain, and it is also expressed in a variety of peripheral tissues such as testes, heart and lung [8]. CB2 receptors show high expression in immune tissues and cells, with lower levels reported in peripheral tissues such as the pancreas [3, 9, 10].

THC-induced changes in feeding behaviour and body weight were first investigated in the late 1970s, when stimulatory effects of THC on appetite and food intake were demonstrated [11]. The locally produced endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), were also reported to promote hyperphagia [12], confirming the key involvement of the endocannabinoid system in the regulation of food intake. With the development of a CB1 receptor antagonist, rimonabant, which was marketed as an anti-obesity agent but withdrawn due to its psychological effects, it has become increasingly evident that this system, particularly the CB1 receptor, plays a regulatory role in metabolism and energy balance. Indeed, as well as being expressed in central regions regulating food intake, CB1 receptors are also found in metabolically important organs such as skeletal muscle [13], liver [14], adipose tissue [15] and the gastrointestinal tract [16].

CB1 and CB2 receptors have also been identified in endocrine cells of the pancreas [17], and this observation has been followed by a series of studies investigating the role of cannabinoids in regulating insulin secretion in vitro [18-23] and glucose homeostasis [24, 25]. However, there is currently no consensus on whether CB1 and CB2 receptors are expressed by islet β -cells or what effect their activation has on insulin secretion. Part of the problem may be that although β -cells are the majority cell type in islets, the presence of α - δ - and PP-cells, as well as non-endocrine cells, makes it difficult to provide convincing evidence of β -cell-specific roles for particular receptors when using whole islets. We have therefore used the mouse MIN6 β -cell line [26] to investigate the expression of CB1 and CB2 receptors by β -cells and the downstream signalling cascades to which they are coupled, and the profile of insulin secretory responses to cannabinoid receptor agonists.

Materials and Methods

Materials

MIN6 cells were kindly provided by Dr Y Oka and Professor J-I Miyazaki (then at the University of Tokyo, Japan).

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine, trypsin-EDTA, Fura-2 AM, noradrenaline and ethidium bromide were purchased from Sigma-Aldrich (Dorset, UK). ACEA and JWH015 were from Tocris Biosciences (Bristol, UK). Fetal bovine serum (FBS) was from Invitrogen (Paisley, UK). RNeasy mini kits and gel extraction kits were obtained from Qiagen (West Sussex, UK) and PCR primers were from Operon Biotech (Cologne, Germany). Antibodies against CB1 and CB2 receptors were from Autogen Bioclear (Wilts, UK), the anti-insulin antibody was from Dako UK Ltd (Cambridgeshire, UK), and Texas Red- and FITC-conjugated secondary antibodies were from Thermo Scientific (Epsom, UK). The cyclic AMP Biotrak Enzymeimmunoassay kits were obtained from GE Healthcare (Buckinghamshire, UK). Standard PCR was carried out using a Px2 Thermal RT-PCR cycler (Thermo Scientific, Epsom, UK).

Cell culture

MIN6 β -cells (passage 25-43) were maintained in culture in DMEM supplemented with 2 mM glutamine, 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were seeded into 96-well plates for measurements of cyclic AMP generation, into 6-well plates for gene expression measurements, and configured as three dimensional 'pseudoislets' [27] in uncharged 10cm Petri dishes for insulin secretion experiments.

Detection of mRNAs by RT-PCR

RNA was isolated from MIN6 cells, reverse-transcribed into cDNAs as described previously [28] and cDNAs were amplified using primers (Table 1) for cannabinoid receptors 1 and 2, and for diacylglycerol (DAG) lipases α and β , which are responsible for generation of the endocannabinoid 2-AG from DAG. The PCR products were separated on 1.8% agarose gels, extracted using the Qiagen Gel Extraction kit and sequenced by the Molecular Biology Unit at King's College London.

Immunocytochemistry

Approximately 30,000 MIN6 cells were seeded onto ethanol-washed glass coverslips, fixed with 4% paraformaldehyde when adherent, permeabilised with 0.1% (v/v) Triton X-100 in PBS, blocked with 10% swine serum then incubated overnight at 4°C with antibodies directed against insulin (1/100 dilution) and CB1 or CB2 receptor antibodies (both at 1/200 dilution). Immunoreactive proteins were detected by incubation for two hours at room temperature with Texas Red- (insulin) or FITC- (cannabinoid receptors) conjugated secondary antibodies (1/50 dilutions) followed by visualisation under a Nikon TE2000 fluorescence microscope. Control protocols were performed by incubating cells in the presence of fluorophore-conjugated secondary antibodies in the absence of primary antibodies and no fluorescence was detected.

Cyclic AMP quantification

MIN6 cells were seeded at a density of 100,000 cells per well in 96 well plates, allowed to adhere overnight then pre-incubated for 2 hours in physiological buffer [29] containing 2 mM glucose. After incubation at 37°C for 30 minutes in 200 μ l of buffer supplemented with agents of interest the cells were

Gene Name	Primer Sequence	Annealing Temp.	No. of PCR Cycles	Product Size
Diacylglycerol lipase α (DAG-L α)	F:GGAGCAGGAGATGGTCCTGTC R:GATGGCAAAGTATGTGGGCTC	58°C	30 cycles	562bp
Diacylglycerol lipase β (DAG-L β)	F:AAGCGGCCAGATACATTACACC R:CTTCCAGGTGGATGATCCTGC	58°C	30 cycles	570bp
Cannabinoid receptor 1 (CB1R)	F: CCTGGGCTGGAACTGCAA R:CCGAAGACGTCATAC ACCATGA	58°C	30 cycles	351bp
Cannabinoid receptor 2 (CB2R)	F:GGATGCCGGGAGACAGAAGTGA R:CCCATGAGCGGCAGGTAAGAAAT	58°C	35 cycles	506bp

Table 1. Primer sequences. The table shows the primer sets, annealing temperature and number of cycles used in RT-PCR amplification of diacylglycerol lipase isoforms and cannabinoid receptors.

lysed and sonicated on ice. Cyclic AMP contents of cell lysates were determined using the cyclic AMP Biotrak Enzymeimmunoassay System, according to the manufacturer's protocols.

Single-cell calcium microfluorimetry

Approximately 30,000 MIN6 cells were seeded onto 2.2 cm circular acid/ethanol washed sterile glass cover-slips. After maintenance overnight under standard tissue culture conditions cells were loaded for 40 minutes at 37°C with 5 μ M Fura-2 AM and perfused with physiological buffer [29] in the absence and presence of the CB1- and CB2 receptor-selective agonists ACEA and JWH015. Changes in intracellular Ca²⁺ were quantified as previously described [28].

Insulin secretion and radioimmunoassay

MIN6 cells were seeded into 96-well plates at a density of 10,000 cells per well, maintained in culture overnight to allow cell adherence, then pre-incubated in a physiological buffer [29] supplemented with 2 mM glucose for 2 hours. Cells were incubated with 200 μ l of 10 μ M ACEA or JWH015 at 37°C for 30 minutes after which 100 μ l of the incubation buffer was retrieved, and the insulin content was measured by radioimmunoassay [30]. For dynamic measurements of insulin secretion MIN6 cell 'pseudoislet' clusters [27] were perfused at a flow rate of 0.5 ml/min at 37°C in a temperature-controlled environment. Perfusate samples were collected every 2 min for the duration of the experiments and insulin secretion was determined by radioimmunoassay.

Statistical Analyses

All numerical data are expressed as means $\pm$ SEM of multiple experiments. Student's t tests were used to compare two groups and ANOVA was used for multiple comparisons followed by Bonferroni's test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

Results

Expression of CB1 and CB2 cannabinoid receptors by MIN6 β -cells

It can be seen from Fig. 1A that products of the appropriate sizes were amplified from MIN6 cell cDNA using primers for mouse CB1 (351bp) and CB2 (506bp) receptors and for the α - and β -isoforms of diacylglycerol lipase (DAG lipase), the enzyme responsible for synthesis of the endocannabinoid 2-AG. Amplicon sequencing confirmed the identities of the products, with >98% homology between predicted sequences and those of the amplified products. As expected, no amplicons were obtained when non reverse-transcribed mRNA was used instead of the cDNA template. Immunocytochemical analysis indicated that cannabinoid receptor mRNAs are translated into proteins in MIN6 β -cells, where CB1 and CB2 receptor immunoreactive proteins were identified in insulin-positive cells (Fig. 1B). Proteins of the correct molecular weights (64kDa for CB1 and 35kDa for CB2) were also detected by western blotting (data not shown).

Effect of cannabinoid receptor agonists on intracellular cyclic AMP levels in MIN6 β -cells

Cannabinoid receptors are coupled to the inhibition of cyclic AMP generation in a variety of tissues [8], and we have recently reported that agonists selective for CB1 and CB2 receptors (ACEA and JWH015, respectively) decrease cyclic AMP levels in mouse islets [23]. In the current study 10 μ M ACEA or 10 μ M JWH015 caused significant reductions in MIN6 cell cyclic AMP levels in the presence of 20 mM glucose, as did 10 μ M of the

Fig. 1. Cannabinoid receptor and diacylglycerol lipase expression by MIN6 cells. (A): Products of RT-PCR reactions are shown under UV light after electrophoretic separation on 1.8% agarose gels. Amplicons of the correct sizes coding for DAG-lipase α - and β - isoforms and CB1 and CB2 cannabinoid receptor subtypes were amplified from MIN6 cell cDNA. Messenger RNA was used as a negative control. Results are representative of data obtained from 6 separate MIN6 cell preparations. (B): Fixed MIN6 cells were immunoprobed with rabbit antibodies directed against CB1 (upper panel) or CB2 (lower panel) receptors and immunoreactivity was revealed using a FITC-conjugated donkey anti-rabbit secondary antibody (green). The sections were also probed with an anti-insulin primary antibody and Texas Red-conjugated secondary antibody (red). Fluorescent images were then merged to illustrate receptor co-localisation to insulin-expressing β -cells (yellow). Results are representative of two separate MIN6 cell preparations.

adrenergic agonist noradrenaline (NA; Fig. 2). It can also be seen from Fig. 2 that incubation of MIN6 cells in the presence of 10 μ M forskolin, an activator of adenylate cyclase, led to significant elevations in cyclic AMP and this was also significantly inhibited by ACEA, JWH015 and NA (58.5 $\pm$ 9.3%, 64.9 $\pm$ 12.6% and 73.7 $\pm$ 10.4% inhibition respectively, n = 16-18, P <0.001 versus 20 mM glucose + 10 μ M forskolin).

Effect of cannabinoid receptor agonists on intracellular Ca^{2+} levels in MIN6 β -cells

Fig. 3 shows that activation of both CB1 and CB2 receptors resulted in reversible increases in $[Ca^{2+}]_i$ levels in Fura-2-loaded MIN6 β -cells. Thus, it can be seen from Fig. 3 (panel A) that 1 μ M ACEA stimulated a rapid, reversible increase in intracellular Ca^{2+} in MIN6 β -cells, and subsequent exposure to 10 μ M ACEA resulted in a further reversible increase. The cells responded to activation of CB2 receptors by 1 μ M JWH015 with a small increase in $[Ca^{2+}]_i$ and subsequent exposure to 10 μ M JWH015 led to a larger Ca^{2+} response (Fig. 3B). The cells did not show any loss of function after exposure to 10 μ M ACEA or 10 μ M JWH015 as they were able to mount an appropriate elevation in $[Ca^{2+}]_i$ in response to the purinergic agonist, ATP (Fig. 3A and 3B).

To determine whether the cannabinoid receptor agonists stimulated increases in Ca^{2+} in β -cells via ion

Fig. 2. MIN6 β -cell cannabinoid receptors are coupled to inhibition of adenylate cyclase. Groups of 100,000 MIN6 cells were pre-incubated for 1 hour in a physiological buffer supplemented with 2 mM glucose followed by a 30-minute incubation at 37°C with reagents as shown. The cyclic AMP content of the cell extracts was measured by enzyme immunoassay and data are presented as mean $\pm$ SEM, n =16-23. NA, ACEA and JWH015 significantly ($***P$ <0.001) inhibited cyclic AMP accumulation at 20mM glucose and forskolin (FSK) potentiated cyclic AMP generation ($**P$ <0.01 versus 20 mM glucose). Results shown here are representative of 3 separate experiments.

Fig. 3. MIN6 β -cell cannabinoid receptors are coupled to calcium entry via voltage-gated calcium channels. Fura-2-loaded MIN6 cells were exposed to the test agents shown by the grey bars, in a physiological buffer supplemented with 2 mM glucose. ACEA (A) and JWH015 (B) induced reversible increases in $[Ca^{2+}]_i$ (solid lines). The voltage-gated calcium channel inhibitor nifedipine (10 μ M) markedly reduced elevations in Ca^{2+} in response to the cannabinoid receptor agonists, but not to the purinergic agonist ATP (dotted lines). Changes in $[Ca^{2+}]_i$ in individual cells are expressed as 340 nm/380 nm ratiometric data (means $\pm$ SEM $n=21-36$), and each panel is representative of at least 2 separate experiments.

Fig. 4. Effect of cannabinoid receptor agonists on basal insulin secretion from MIN6 β -cells. MIN6 cells configured as pseudoislets were perfused with a physiological buffer containing 2 mM glucose and supplemented as shown. 10 μ M ACEA (A) and JWH015 (B) induced significant increases ($P<0.01$) in insulin secretion at 2 mM glucose. Data are presented as a percentage of insulin secretion at 2 mM glucose (means $\pm$ SEM, $n=4$), and each panel is representative of at least 2 separate experiments.

influx through voltage-gated calcium channels (VGCC), further experiments were carried out in the presence of an L-type VGCC blocker, nifedipine (10 μ M). Fig. 3A (dotted line) demonstrates that the presence of 10 μ M nifedipine abolished the increase in $[Ca^{2+}]_i$ caused by 1 μ M ACEA and reduced the response to 10 μ M ACEA, as well as delaying the onset of the Ca^{2+} elevation.

Increases in $[Ca^{2+}]_i$ in response to CB2 receptor activation by 10 μ M JWH015 were fully inhibited by nifedipine but there was a small, short-lived response to 1 μ M JWH015 (Fig. 3B, dotted). Similar results were obtained when experiments were carried out in Ca^{2+} -free buffers that had been supplemented with 10 μ M EGTA, with >95% reduction in $[Ca^{2+}]_i$ in response to 10 μ M ACEA and

Fig. 5. CB1 and CB2 receptor agonists potentiate glucose-stimulated insulin secretion from MIN6 β -cells. MIN6 pseudoislets were perfused with buffers supplemented with 2mM glucose for the first 10 minutes and with 20 mM glucose thereafter. Twenty-minute exposure to 10 μ M ACEA (A) and JWH015 (B) induced a significant ($P<0.01$), transient potentiation of glucose-induced insulin secretion. Data are presented as a percentage of insulin secretion at 2 mM glucose (means $\pm$ SEM, $n=4$), and each panel is representative of at least 2 separate experiments.

JWH015 ($P<0.01$). It can be seen from Fig. 3A and 3B that blockade of VGCC by nifedipine did not cause a significant reduction in the amplitude of the $[Ca^{2+}]_i$ increase in response to the purinergic agonist ATP, with an average response of $82\pm7\%$ of the ATP-stimulated $[Ca^{2+}]_i$ increase observed in control cells, but removal of extracellular Ca^{2+} caused a $75\pm4\%$ reduction in the ATP-induced $[Ca^{2+}]_i$ response.

Effect of cannabinoid receptor agonists on insulin secretion

ACEA and JWH015 significantly stimulated acute (30 minute) insulin secretion from MIN6 β -cells at 2 mM glucose in batch incubation experiments. Thus, 10 μ M ACEA and 10 μ M JWH015 caused basal insulin secretion at 2 mM glucose to increase to $241\pm20.5\%$ and $203\pm17.1\%$ basal, respectively ($n=19-23$, $P<0.001$ versus secretion at 2mM glucose alone). As these static incubation protocols do not give any information about the time-course and reversibility of insulin secretion, cannabinoid-induced secretory profiles were investigated in perfusions. In these experiments MIN6 cells were configured as three-dimensional clusters because they show functional characteristics more closely resembling primary islets when formed as 'pseudoislets' [27]. The stimulatory effects of ACEA and JWH015 seen in static incubation experiments were confirmed in perfusion studies, which also revealed differences in the secretory

profiles in response to these two agonists at 2 mM glucose. Fig. 4A indicates that ACEA caused a rapid, but transient increase in insulin secretion, and the MIN6 cells showed a significant ($P<0.01$) secretory response to 20 mM glucose after exposure to ACEA. Insulin secretion in response to JWH015 at 2 mM glucose was also rapid in onset, but it had a biphasic profile and elevated insulin output was observed for the duration of exposure to JWH015 (Fig. 4B). Insulin secretion was readily reversible upon removal of JWH015 from the perfusion medium, and the MIN6 pseudoislets then responded to an increase in glucose concentration from 2 mM to 20 mM, as was also seen in the perfusions with ACEA (Fig. 4A). ACEA and JWH015 also increased insulin secretion from MIN6 β -cells at 20mM glucose, and the secretory profile to both agonists was very similar. Thus it can be seen from Fig. 5 that ACEA (panel A) and JWH015 (panel B) stimulated rapid increases in insulin secretion equivalent to approximately 50% of the magnitude of that obtained with 20 mM glucose alone, and secretion returned to pre-stimulatory levels within 10 minutes, despite the continued presence of the agonists.

Discussion

The role played by cannabinoid receptors in regulating energy balance is well-established [1, 31, 32] and, given

the important role played by insulin in fuel homeostasis, this has led to investigation of cannabinoid receptor expression and function in islets. However, to date there is no consensus on which cannabinoid receptors are expressed by different cells within islets, nor what effect cannabinoid receptor activation has on insulin secretion. Part of the difficulty is that the published studies have investigated receptor expression by mouse [18, 19, 23, 25, 33], rat [25, 33] and human [25, 33] islets, all of which are composed of a variety of endocrine cells of which β -cells account for approximately 80% (mice and rats) or 50-60% (humans) of the total cell populations. There have also been reports of cannabinoid signalling in RINm5F insulinoma cells [18, 20], but this rat cell line secretes glucagon and somatostatin as well as insulin [34] and insulin is present in only approximately 50% of RINm5F cells [35]. To overcome these potential problems the current study examined cannabinoid receptor expression by the MIN6 β -cell line, which was generated by insulin promoter-driven SV40 T antigen expression in mouse β -cells [26], and which does not secrete glucagon or somatostatin.

Detection of mRNAs coding for α - and β - isoforms of DAG lipase in MIN6 cells indicate that these β -cells are equipped with the enzymes necessary for synthesis of the endocannabinoid 2-AG from DAG, which can be generated in islets by phospholipase C activation or synthesised de novo from glucose [36]. The existence of a local β -cell endocannabinoid system was confirmed by identification of CB1 and CB2 receptor mRNAs in MIN6 cells, and their appropriate translation was established by immunocytochemistry. The immunocytochemical analysis also revealed that all MIN6 cells expressed insulin and we could not detect glucagon- or somatostatin-expressing cells (data not shown), confirming that the MIN6 cells are a pure β -cell population.

Previous studies have indicated that CB1 receptors are expressed by mouse islet α -cells [17, 25], β -cells [23, 37] or δ -cells [33], while CB2 receptors are reported to be localised to α - and β -cells [17, 25, 37, 38], and there is only limited agreement between studies from different groups. Our data demonstrating that MIN6 β -cells express CB1 and CB2 receptors are consistent with identification of CB1 receptor mRNA in the β TC6 mouse cell line [33], and with our recent report that CB1 and CB2 receptor immunoreactive proteins are expressed by β -cells of primary mouse islets [23].

Exposure of MIN6 cells to CB1 and CB2 cannabinoid receptor agonists, ACEA and JWH015, significantly inhibited cyclic AMP generation in response

to glucose and the adenylate cyclase activator forskolin, providing evidence that MIN6 β -cell cannabinoid receptors are coupled to G_i as they are in other cell types, including mouse islets [23, 39]. In addition to inhibiting cyclic AMP synthesis, cannabinoids have been reported to elevate intracellular Ca^{2+} levels in some cell types including neurons [40, 41] and RINm5F insulinoma cells [20]. However, this is also an area lacking consensus in terms of responses by mouse islets. Thus, one study has indicated that CB2 receptor activation reduced the amplitude of mouse islet Ca^{2+} oscillations [17], another demonstrated that CB1 receptor agonists inhibited Ca^{2+} oscillations in dissociated mouse islet cells [19] and we reported stimulatory effects of both CB1 and CB2 receptor agonists in mouse islet cells [23]. However, it is not clear in these earlier studies if islet non- β -cells contributed to the observed responses. The simplest way of assessing β -cell-specific effects is to use a β -cell line, so in the current study we used Fura-2-loaded MIN6 β -cells to determine the effects of cannabinoid receptor activation on intracellular Ca^{2+} levels. ACEA and JWH015 stimulated reversible increases in Ca^{2+} in MIN6 cells, indicating that β -cell CB1 and CB2 cannabinoid receptors are coupled to elevations in Ca^{2+} as well as inhibition of cyclic AMP generation. The stimulation of $[Ca^{2+}]_i$ by ACEA and JWH015 was dependent on activation of VGCC, as has been reported in neuroblastoma cells [41], since stimulatory effects were markedly reduced or abolished in the presence of nifedipine. These observations are in contrast to the effects of the phospholipase C (PLC)-coupled purinergic agonist ATP, which showed maintained Ca^{2+} elevations in the presence of nifedipine, suggesting that cannabinoids do not activate PLC in β -cells. The reduction in ATP-induced Ca^{2+} elevation in the presence of EGTA but not nifedipine most likely reflects that a full response to ATP requires adequate refilling of intracellular stores with extracellular Ca^{2+} via a non-VGCC-dependent mechanism, such as capacitive calcium entry.

Insulin secretion from islet β -cells is under paracrine influences from α - and δ -cells, which provide stimulatory and inhibitory inputs respectively. Thus, the reports of inhibition [17, 19, 21] or stimulation [21, 23] of insulin secretion from islets in response to cannabinoid receptor activation might reflect complex paracrine interactions, whose outcome depends on the islet cell type expressing cannabinoid receptors and relative changes in Ca^{2+} and cyclic AMP levels in those cells. The contribution of non- β -cells to the net effect of cannabinoids on insulin secretion from whole islets is exacerbated when secretion

experiments are performed in static incubations [17, 19, 21] where secretory products from α - and δ -cells can accumulate over the duration of the experiment. In addition, if agonists have only a short-lived effect on secretory output this may not be seen when secretion is measured at a fixed time-point at the end of the incubation period. Thus, to overcome the potential problems associated with islet paracrine signalling and potentially transient effects of cannabinoids on insulin secretion, we used MIN6 cells configured as three dimensional pseudoislets in dynamic perfusion experiments to identify the time-course and reversibility of ACEA and JWH015 effects on insulin secretion.

These experiments indicated that both agonists caused reversible increases in insulin secretion at substimulatory (2 mM) and maximal stimulatory (20 mM) glucose concentrations. The stimulation by ACEA at 2 mM and 20 mM glucose was transient, with a return to pre-stimulatory insulin secretion levels before the ACEA was withdrawn. JWH015 caused a biphasic, prolonged stimulation of insulin secretion at 2 mM glucose, but its stimulatory effects at 20mM glucose were comparable in profile to those of ACEA. The sustained insulin secretory response to JWH015 at 2 mM glucose is unusual, but we have previously observed a similar profile of insulin secretion from human islets perfused in the presence of an insulintropic plant extract at 2 mM glucose [42], indicating that some stimuli are capable of inducing prolonged initiation of insulin output in the absence of a glucose stimulus. The stimulation of insulin secretion by ACEA at 2 mM glucose is in contrast to our experiments with mouse islets where it was without effect, but it might reflect expression of CB1 receptors by islet

δ -cells [33], with a stimulation of somatostatin output blocking the stimulatory effect in islets but not in MIN6 β -cells.

In summary, the experiments described in this paper have demonstrated that MIN6 β -cells express CB1 and CB2 receptors, and DAG lipase α - and β - isoforms, which are responsible for synthesis of the endocannabinoid 2-AG from DAG. These components of the endocannabinoid signalling system have also been identified in islets [21, 38, 43] and our data suggest that they may act as local regulators of insulin secretion through cannabinoid receptor-mediated elevation in β -cell Ca^{2+} levels. In this context, it is interesting to note that it has been reported that the lipase inhibitor orlistat inhibited both islet DAG lipase activity and glucose-stimulated insulin secretion [43], which is consistent with DAG lipase-mediated 2-AG synthesis by β -cells exerting a stimulatory effect on insulin secretion. Furthermore, in addition to its physiological stimulus-secretion coupling role, the cannabinoid transduction cascade may also have a pathophysiological function to stimulate insulin-dependent lipid deposition through enhanced insulin output in response to increased levels of 2-AG, as seen in obesity [18].

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