MATERIALS AND METHODS

2.1 The isolated tissue experiments

The isolated guinea-pig bronchial preparation is used as an *in vitro* model of functional pharmacology to investigate the effect of cannabinoids on the smooth muscle motility of the tissue in response to nerve or agonist stimulation. It is well known that cannabinoids can modulate neurotransmitter release from nerve endings. Neural responses (i.e. postjunctional responses) can be measured *in vitro* using EFS with parameters selectively activating nerves and not ASM. These responses should be completely blocked by the Na^+ channel blocker TTX (1 μM), confirming their neural origin. Furthermore, EFS of airways can result in a constrictor response that is not sensitive to adrenoceptor and muscarinic cholinoceptor antagonists and therefore termed an eNANC. The release of neuropeptides from C-fibres (eNANC response) can be modulated by cannabinoids which may influence neurogenic inflammation in the airways.

Additionally, cannabinoids can affect allergen-induced airway inflammation, the inflammation which contributes to the pathophysiological features of asthma. Asthma is a complex inflammatory disorder, characterized by elevated serum IgE, airway hyper-responsiveness, mucus hypersecretion, increased numbers and activation of inflammatory and immune cells within the airways, including eosinophils, Th2 lymphocytes, mast cells, neutrophils and macrophages. Mast cells as true immune effector cells were targeted in a guinea-pig, *in vitro* model of bronchial asthma. The acute antigen-induced airway constriction due to mast cell activation, represented the early bronchoconstriction phase of human asthma. The parallel control condition was provided by non-immunological mast cell activation.

2.1.1 Guinea-pig bronchial preparation

Heston guinea-pigs (700-1000 g) of either sex were sacrificied by cervical dislocation and exsanguination. The cervical part and the rib cage were cut open and the whole tracheobronchial tree together with the heart were gently removed and immersed in Krebs-Henseleit solution of composition in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5; pH=7.4, gassed with 95 % O_2 and 5 % CO_2. A dissection microscope was used to reduce the possibility of any damage to both main bronchi during the removal of unneeded tissues.

2.1.2 Apparatus setup and maintenance

Two main bronchial rings (4-5 mm) were mounted in two organ baths (10 ml) paying particular attention to the position of the strip of the bronchial smooth muscle so that it was not touched by the thread. The initial tension was 0.5 g and the Krebs-Henseleit solution in baths was thermostatically controlled and maintained at 37ºC and continuously gassed with 95 % O_2 and 5 % CO_2.

Changes in tension of the preparation were recorded isometrically in units of grams (g) using Dynamometer UF1 force transducers (Pioden Control., U.K.), connected to a PowerLab Chart Version 5.1 data-acquisition system (AD Instruments, U.K.) on a Dell computer.

In experiments where tissues underwent EFS, each tissue was placed between a pair of platinum (Pt) electrodes connected to a 6002 stimulator (Harvard Apparatus, U.K.) or S88 stimulator (Grass, U.S.A.) via a variable cycle timer (SRI, U.K.) (figure 2.1).

In our model of asthma, guinea-pigs were sensitized by intraperitoneal doses of ovalbumin (OVA, Sigma). Bronchial rings were prepared from OVA-sensitized as well as normal, non-sensitized guinea-pigs.

Since cannabinoids are lipophilic compounds they tend to stick to glassware. Due to this property, the organ baths and stimulation electrodes had to be washed with 2 M hydrochloric acid, and copious amounts of distilled water to achieve complete removal of any residual cannabinoid at the end of each experiment.

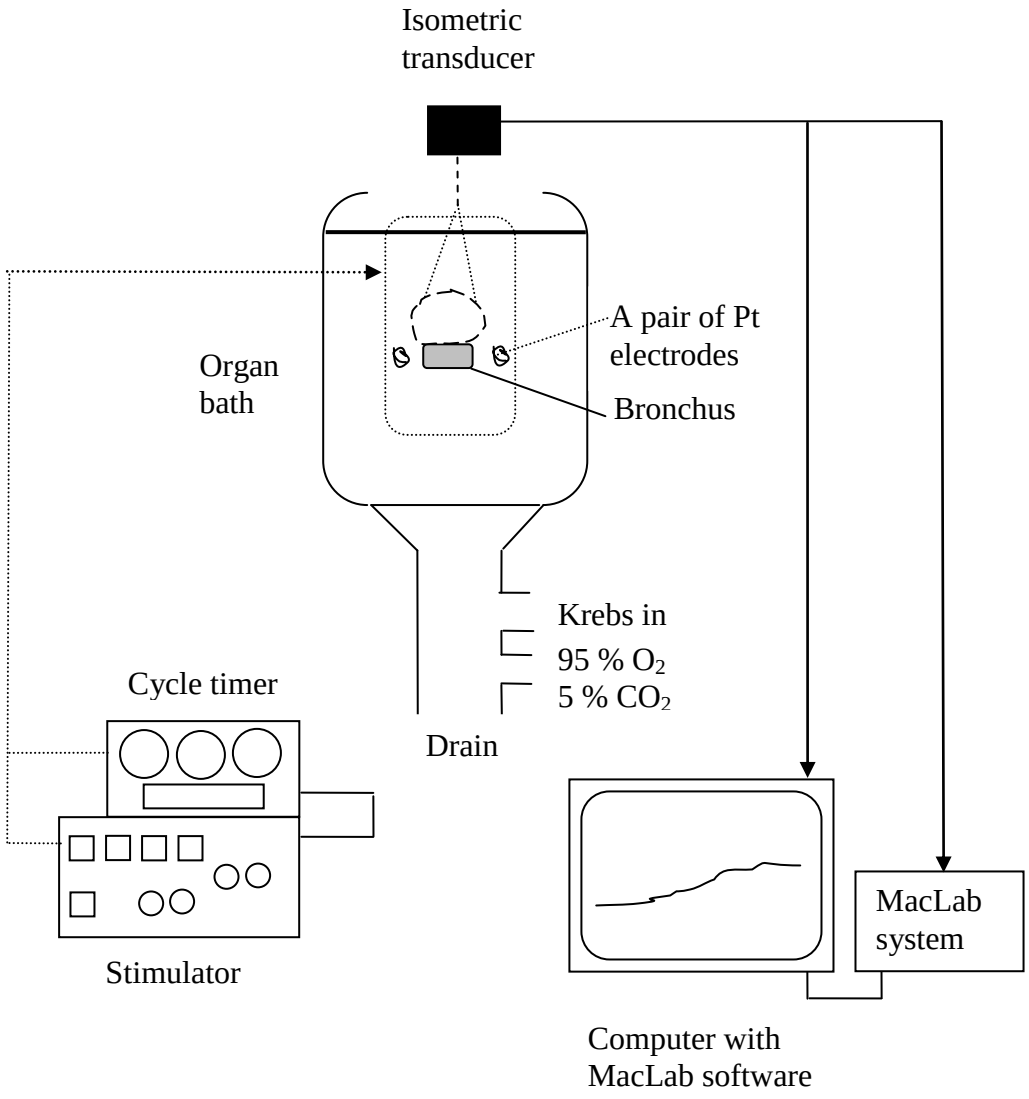

Figure 2.1 **Diagram of organ bath apparatus**

2.1.3 Experimental designs

In each experiment the tissue was equilibrated for 60 min. Before application of EFS or any drugs, the viability of isolated bronchi was checked using carbachol (CCh) at 300 nM or at 10 μM, applied at the end of an experiment.

Electrically evoked contractions

EFS parameters to evoke eNANC responses were selected on the basis of published work where cannabinoids have been shown to produce an effect (Yoshihara et al., 2004). Guinea-pig bronchi were stimulated at 10 Hz, 1 ms pulse duration for 30 sec every 30 min at 50 V intensity in the presence of atropine to block the excitatory cholinergic response, propranolol to block the inhibitory adrenergic response, and phosphoramidon to reduce the degradation of endogenous tachykinins (all at 1 μM). Some experiments were also carried out in the presence of indomethacin (10 μM) to avoid the effect of inhibitory prostaglandins. These NANC contractile responses additionally superfused with indomethacin appeared to be more stable with higher magnitudes of contraction. No drugs were added until two or three reproducible responses (control responses) were obtained to EFS. The time taken for the monophasic sustained contractions to stabilise was 4-6 hours. During this period at least one wash-out with Krebs-Henseleit solution containing atropine, propranolol, phosphoramidon, (indomethacin) was required. When EFS-evoked contractile responses did not become consistent within seven hours, no drug was tested. Cannabinoid agonists were applied 15 or 20 min before the next EFS stimulus. When competition studies were conducted, cannabinoid antagonists were added 20 min prior the addition of the agonist. None of the drugs employed in this study affected the baseline tone (data not shown).

Agonist-induced contractions

Cumulative concentration-response curves for cannabinoids were constructed only once on each tissue, and the contractile effect of each dose was allowed to plateau at its maximum before the next dose of the drug was applied. The time between each dose was 10-20 min. The vehicle controls were performed in parallel.

A repetitive experimental design was chosen where two cumulative concentration-response curves were constructed to drugs [NKA (0.1 nM-10 μM), histamine (1 nM-100 μM), and CCh (10 nM-10 μM)] per one tissue. The first concentration-response curve to NKA or histamine or CCh was followed by repeated wash. After 40 min the tissues were exposed either to CBD, its vehicle, absolute ethanol (0.01 % or 0.1 %) or various drugs. Following 20 min incubation, NKA or histamine or CCh was again cumulatively added to the bath. Tissues were then repeatedly washed over a 40-60 min period and at the end single concentration of CCh (10 μM) was added to determine the maximum contractile capability of each tissue.

In the non-repetitive experimental design, only one cumulative concentration-response curve was performed after application of the drug of interest. CCh (10 μM) was added to determine the maximum contractile capability of each tissue at the end of experiment.

Antigen-induced contractions- immunological response

The model of allergic asthma was kindly provided by Dr. Cliff Whelan (University of Hertfordshire, U.K.). Sensitization to OVA was performed under a licence granted under the Animals (Scientific procedures) Act 1986 (Licence no. PIL 70/5952 and 70/9775). Heston guinea-pigs (700-1000 g) of either sex received an intraperitoneal injection of OVA (10 μg) and aluminium hydroxide (100 mg, BDH chemicals) suspended in normal saline (1 ml). After 14 days, animals were challenged with OVA

(1 mg/ ml) intraperitoneally. To protect animals against fatal anaphylaxis, mepyramine (1 mg/ kg) was given 15 min before the challenge. 21 days later both main bronchi were dissected and mounted into organ baths with Krebs solutions containing indomethacin (10 μM). In order to test the immune response, only one bronchial ring was exposed to cumulative doses of OVA (1-100 μg/ ml) and the second bronchial ring was tested in the presence of a drug of interest and OVA. The contractile effect of each OVA dose was allowed to plateau at its maximum before the next dose of the drug was applied. The time between each dose was 3-10 min. At the end, the responsiveness to CCh (10 μM) was monitored.

Compound 48/80-induced contractions- non-immunological response

Similarly to immunological stimulus, only one bronchial ring was exposed to cumulative doses of compound 48/80 (1-300 μg/ ml), the mast cell degranulator which releases histamine. The second bronchial ring was tested in the presence of a drug of interest with compound 48/80. The contractile effect of each compound 48/80 dose was allowed to plateau at its maximum before the next dose of the drug was applied. The time between each dose was 5-10 min. At the end, the responsiveness to CCh (10 μM) was monitored.

2.1.4 Analysis of data from isolated tissue experiments

Values are expressed as g contractions ±standard error of the mean (sem), or as a percentage of the maximum contraction induced by CCh (10 μM) or histamine (100 μM) ±sem. Changes in the amplitude of eNANC responses to cannabinoids were expressed as a percentage ratio of the mean amplitude of the contraction after addition of the drug, to the mean amplitude of stable contractions before the addition of the drug. Mean values of the two sets of data were compared using Student's unpaired two-tailed *t* test. Data obtained with OVA, compound 48/80 and repetitive design were evaluated using Student's paired two-tailed *t* test. Curves were fitted using non-linear regression analysis using GraphPAD Prism statistical software (GraphPAD Software, C.A., U.S.A.).

2.1.5 Drugs used in the study

Name	*Main cellular function*	*Supplier*	*Solvent*
Anandamide (AEA) N—(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide	Endogenous cannabinoid, CB_1/CB_2 and $TRPV_1$ receptor agonist	Tocris-Cookson (U.K.)	Ethanol
Atropine Endo-(±)-α-(hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester	mACh receptor antagonist	Sigma (U.K.)	Distilled water
(-)-Cannabidiol (CBD) 2-[(1R,6R)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol	Weak CB_1 receptor antagonist and $TRPV_1$ receptor agonist	Tocris-Cookson (U.K.)	Ethanol
Capsaicin (CPS) 8-Methyl-N-vanillyl-trans-6-nonenamide	$TRPV_1$ receptor agonist	Sigma (U.K.)	Ethanol
Capsazepine (CPZ) N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide	$TRPV_1$ receptor antagonist	Tocris-Cookson (U.K.)	Ethanol
Carbachol (CCh) Carbamylcholine chloride	ACh receptor agonist (nACh/mACh)	Sigma (U.K.)	Distilled water
Compound 48/80	Potent histamine releasing agent	Sigma (U.K.)	Distilled water
Histamine 2-(4-Imidazolyl)ethylamine	H_1 and H_2 receptor agonist	Sigma (U.K.)	Distilled water
Indomethacin N-p-chlorbenzoyl-5-methoxy-2-methylindole-3-acetic acid	Inhibitor of cycloxygenase products	Sigma (U.K.)	Ethanol
Mepyramine N-(4-Methoxyphenyl)methyl-N′,N′-dimethyl-N-(2-pyridinyl)-1,2-ethanediamine maleate salt	H_1 receptor antagonist	Sigma (U.K.)	Distilled water
MK886 3-[3-*tert*-Butylthio-1-(4-chlorobenzyl)-5-isopropyl-1H-indol-2-yl]-2,2-dimethylpropionic acid, sodium salt hydrate	Inhibitor of 5-lipoxygenase products	Sigma (U.K.)	DMSO

Methysergide [8b(S)]-9,10-Didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide maleate	5-HT_1 and $5HT_2$ receptor antagonist	Tocris-Cookson (U.K.)	Distilled water
Neurokinin A (NKA)	NK_2 receptor agonist	Tocris-Cookson (U.K.)	Distilled water
Ovalbumin (OVA) Chicken egg albumin	Allergen	Sigma (U.K.)	Distilled water
Palmitoylethanolamide (PEA) N-(2-hydroxyethyl) hexadecanamide	Putative endogenous cannabinoid (independent of CB_1/CB_2 action	Tocris-Cookson (U.K.)	Ethanol
Phenylmethanesulfonyl fluoride (PMSF) Benzylsulfonyl fluoride	Inhibitor of serine proteases (FAAH)	Sigma (U.K.)	Ethanol
Phosphoramidon N-(α-rhamno-pyranosyl-phos-phono)-L-leucyl-L-tryptophan disodium salt	Inhibitor of the neutral endopeptidase	Sigma (U.K.)	DMSO
Propranolol DL-propranolol-(1-[isopropylamino]-3-[1-naphtyloxy]-2-propanol hydrochloride	β-adrenoceptor antagonist	Sigma (U.K.)	Distilled water
SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride	CB_1 receptor antagonist/inverse agonist	Sanofi-Recherche (France)	Ethanol
SR144528 {N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide	CB_2 receptor antagonist/inverse agonist	Sanofi-Recherche (France)	Ethanol
SR140333B (S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl]}-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride	NK_1 receptor antagonist	Sanofi-Synthelabo (France)	Ethanol
SR48968C N-[(2S0-4(4-acetylamino)-4-phenyl-1-piperidinyl]-2-(3,4-dichlorophenyl)butyl]-N-	NK_2 receptor antagonist	Sanofi-Synthelabo (France)	Ethanol

ethylbenzamide succinate			
Substance P (SP)	NK_1 receptor agonist	Tocris-Cookson (U.K.)	Distilled water
Δ^9-Tetrahydrocannabinol (Δ^9-THC)	Weak CB_1/CB_2 agonist	Sigma (U.K.)	Ethanol
Tetrodotoxin (TTX)	Voltage dependent Na^+ channel blocker	Tocris-Cookson (U.K.)	Citrate buffer
URB597 [3'-Carbamoyl-biphenyl-3-yl-cyclohexylcarbamate]	Potent and selective FAAH inhibitor	Alexis Biochemicals	Ethanol
Virodhamine (VIR) O-(2-aminoethyl)-5Z,8Z,11Z,14Z-eicosatetraenoate	Endogenous cannabinoid, partial agonist/antagonist at the CB_1 receptor (EC_{50}=1.9 μM), full agonist at the CB_2 receptor (EC_{50}=1.4 μM)	Tocris-Cookson (U.K.)	Ethanol
WIN55212-2 mesylate (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone mesylate	CB_1/CB_2 receptor agonist	Tocris-Cookson (U.K.)	Ethanol
WIN55212-3 mesylate [(3S)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone monomethanesulfonate	Optical isomer of (R)-(+)WIN55212-2	Tocris-Cookson (U.K.)	Ethanol

2.2 Cell cultures

2.2.1 Human bronchial epithelial 16HBE cells

The 16HBE cell line was obtained from Dr. Ad Nelemans, School of Pharmacy, Groningen University, The Netherlands. Cells were grown in modified Eagle's media (GIBCO/Invitrogen, U.K.), to which 200 mM L-glutamine, 10 % fetal bovine serum (FBS), penicillin/streptomycin (200 U/ ml and 200 μg/ ml respectively) and gentamycin (50 μg/ ml) were added. T-75 or T-25 culture flasks and 6-well plates (with 4-5 pieces of fragmented cover slips/ well) were coated with growth promoting media (see below) containing DMEM/F12 media, fibronectin, collagen and bovine serum albumin. These conditions promote the phenotype of the native epithelium. Cultures were maintained at 37°C in a 5 % CO_2 humidified tissue culture incubator and the feeding medium was refreshed every three days.

Confluent monolayers were subdivided and plated twice a week in T-75 (T-25) flasks. Cells used for the experimentation (patch clamping) were plated on 6-well plates. The optimal seeding density was $2x10^5$-$3x10^5$ cells/ well. 16HBE cells were counted using the haemocytometer. The cell viability was regularly checked by trypan blue exclusion test (only cells with clear cytoplasm were considered as healthy whereas cells with blue cytoplasm were excluded). Prior to each subculture, the cell monolayer was washed twice with warm sterile phosphate buffered saline (PBS) and incubated for approximately 10 min in the presence of 0.25 % trypsin/ethylene diamine tetracetic acid (EDTA). After cell detachment, the trypsinization was stopped by addition of 9 ml (4 ml) of the complete medium. Cells were harvested, diluted and

plated to the appropriate density following incubation as described. Cells were patched after 2-3 days in culture.

Some experiments were carried out in the absence of FBS to test the possible influence of this growth factor on the cell's electrophysiological function. 16HBE cells were plated as described above, omitting FBS from the incubation medium. These conditions provided cell survival not more then 24 hours in 6-well plates. Cells were patched after 12 hours in culture.

Growth promoting media:

DMEM/F12	100 ml
Human fibronectin (1 mg/ ml)	1 ml
Collagen I, bovine (3 mg/ ml)	1 ml
Bovine serum albumin (BSA, 1 mg/ ml)	10 ml

2.2.2 Chinese hamster ovary cells

The Chinese hamster ovary (CHO) cell line transfected with the human CB_1 or CB_2 receptor (CHO-hCB_1/CHO-hCB_2) was kindly provided by GlaxoSmithKline, Stevenage. These cells served as positive controls for analysis using polymerase chain reaction analysis and western blotting in order to examine the presence or absence of both CB receptors at transcript level (CB_1mRNA/CB_2mRNA) and protein level. The incubation medium for CHO-hCB_1 and CHO-hCB_2 cells was DMEM/F12 medium (GIBCO/Invitrogen, U.K.), supplied by 2 mM L-glutamine, 10 % FBS and antibiotics, G418 (Geneticin) 1 mg/ ml and Hygromycin B 0.6 mg/ ml. G418 and Hygromycin B were essential for the selection and maintenance of cells transfected with CB receptors. Cells were grown in T-150 flask and instead of 0.25 % trypsin/EDTA solution, neat Versene (GIBCO/Invitrogen, U.K.) was used. 5 min of the incubation period was sufficient to lift cells off of the flask. Versene solution appeared to be less harsh than trypsin and it did not damage the transfected cells (Begg, personal communication).

2.3 Patch clamp technique

Two German electrophysiologists, Erwin Neher and Bert Sackmann, awarded the Nobel Prize in 1991, are considered to be the pioneers of patch clamp technique.

The technique involves voltage clamp and current clamp where the activity of ion channels is monitored. The ion channels can be studied in the intact cell, or the patch can be pulled away from the cell allowing an access to the cytosolic side of the membrane. On that basis there are four possible configurations each with their own characteristics: cell-attached patch, whole-cell, inside-out and outside-out excised patch.

Successful patch recording requires a tight seal (Gigaseal= 10^9 Ω) between the glass pipette (with the tip diameter of about 1 μm) and the cell membrane (figure 2.3).

Voltage clamp measures changes in current flow through the membrane. It employs an electronic feedback system that keeps the cell at a fixed membrane potential by injecting current as needed to hold the voltage constant (the holding potential set by the experimenter). The opposite current then represents the accurate ion flow over the membrane under investigation.

Current clamp applies current to measure the change in membrane potential which reflects the membrane response to ions leaving or entering the cell. This system does not require the feedback circuit.

Due to the patch clamp technique, ion channels can be studied at different levels: either whole cell (activity of ion channels all together using whole-cell configuration) or individual ion channels (single channel recording using cell-attached patch, inside-out or outside-out excised configuration). In addition the experimenter can manipulate

the extra- and intracellular fluid of the patched cell during a recording permitting the investigation of biochemical processes within a cell (Molleman, 2003).

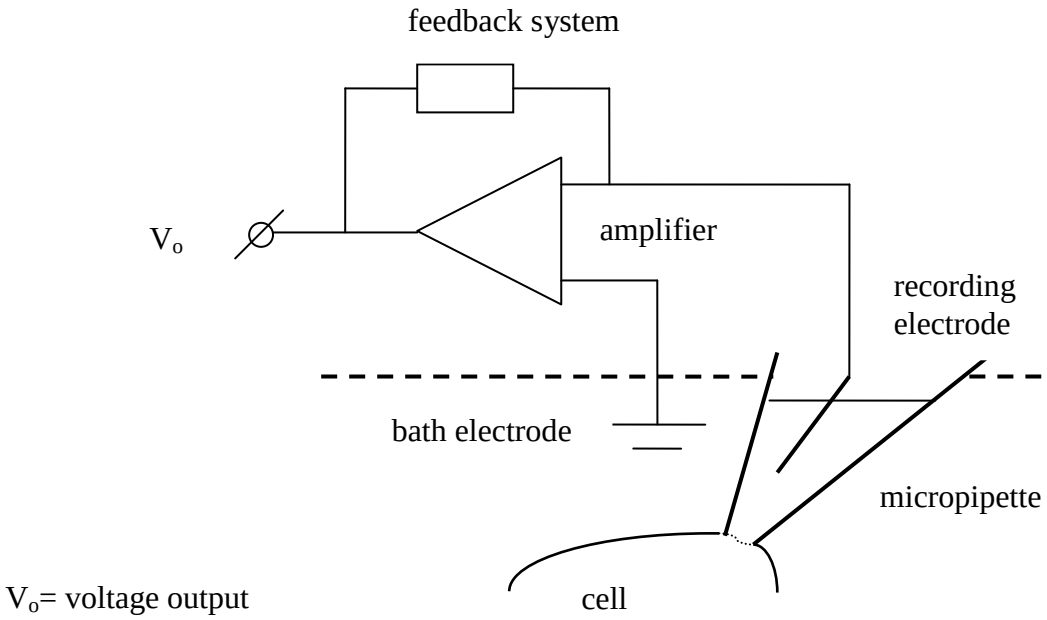

Figure 2.3 **Single electrode measurement (Molleman, 2003; Sherman-Gold, 1993).**

2.3.1 Procedure

Every experiment started with the preparation of micropipettes. They were made from borosilicate glass capillaries with an internal filament (Harvard Apparatus; GC150TF-10) that underwent pulling (P80/PC; Sutter Instrument Co., U.S.A.), fire-polishing (MF-830; Narishige, Japan) and then filling with the intracellular solution (ICS; see below).

The complete micropipette (pipette resistance= 2-4 MΩ) was mounted into the pipette holder, which was connected to the pre-amplifier (probe). The movement of the pipette with the recording electrode was provided by a course manipulator (MC35A; Narishige, Japan) and a fine micromanipulator (MHW-3; Narishige, Japan).

To visualise the cells in the bath filled with extracellular solution (ECS; see below), an inverted microscope was used (TE 200, Nikon, U.K.). The superfusion system was performed by gravity flow. Currents and voltages were recorded using an Axopatch 1D patch clamp amplifier and Digidata 1200 digital interface (Axon Instruments, U.S.A.).

The experiments were carried out at room temperature, with ECS of the following composition in mM: NaCl, 125; KCl, 6; $MgCl_2$, 2.5; NaH_2PO_4, 1.2; Hepes, 20; D-Glucose, 11; Sucrose, 67; $CaCl_2$, 1.2; pH adjusted to 7.35 with NaOH, 2.5 M and ICS containing (mM): NaCl, 5; KCl, 142; $MgCl_2$, 1.2; Hepes, 20; D-Glucose, 11; K-ATP, 5; Na-GTP, 0.1; pH adjusted to 7.2 with KOH, 1 M. All drugs were administered in the ECS, superfused at a rate of 4 ml/ min. If cannabinoids were used, the apparatus was washed with HCl (0.1 M), followed by absolute ethanol (Fischer, U.K.) and distilled water for the complete elimination of the cannabinoid after each experimentation.

2.3.2 Experimental protocol

After at least three days of the cell growing on cover slips, current recordings were made from 16HBE cells using the whole-cell configuration of patch clamping. In this configuration, the patch of membrane under the pipette tip is ruptured which enables the pipette solution and the electrode to make direct electrical contact with the cytosol. The advantages are quick display of ion channel population and possible control of cytosolic enviroment. These macro-currents can be evoked by potential changes via a step pulse protocol, or by a receptor agonist added to the ECS.

Bronchial epithelial cells were kept at the holding potential of -30 mV which is close to the resting membrane potential for these cells (Koslowsky et al., 1994; Kunzelmann et al., 1994). The voltage step protocol was applied from -30 mV to +120 mV, in 10 mV increments to evoke the opening of voltage-gated ion channels. In addition to the initially used voltage clamp, the membrane voltage of 16HBE cells was continuously recorded in current clamp mode. The technique was chosen to determine whether cannabinoids can affect membrane current or membrane potential in the 16HBE cell line. ATP was used as a positive control. The following cannabinoid ligands were monitored: AEA, VIR (endocannabinoids), and CP55940, WIN55212-2 (non-selective CB_1/CB_2 agonists).

2.3.3 Analysis of data from electrophysiological experiments

If applicable, values are expressed as means± sem.

2.3.4 Drugs used in the study

Name	*Main cellular function*	*Supplier*	*Solvent*
Anandamide (AEA) N—(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide	Endogenous cannabinoid, CB_1/CB_2 and $TRPV_1$ agonist	Tocris-Cookson (U.K.)	Ethanol
ATP Na_2 (ATP) Adenosine 5'-triphosphate disodium salt	Purinergic receptor agonist/activator of K^+_{Ca}	Sigma (U.K.)	Distilled water
Caesium chloride (Cs^+)	Non-specific K^+ channel inhibitor	BDH (U.K.)	ICS
CP55940 (-)-cis-3-[2-OH-4-(1,1-dimethylheptyl0phenyl]-trans-4-3(3-hydroxypropyl)cyklohexanol	CB_1/CB_2 receptor agonist	Pfizer (U.K.)	Ethanol
EGTA Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'	Ca^{2+} chelator	Sigma (U.K.)	ICS
Tetraethylammonium bromide (TEA)	K^+ channel inhibitor	Sigma (U.K.)	ICS
Virodhamine (VIR) O-(2-aminoethyl)-5Z,8Z,11Z,14Z-eicosatetraenoate	Endogenous cannabinoid, partial agonist/antagonist at the CB_1 receptor (EC_{50}=1.9 μM), full agonist at the CB_2 receptor (EC_{50}=1.4 μM)	Tocris-Cookson (U.K.)	Ethanol
WIN55212-2 mesylate (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone mesylate	Optical isomer of (R)-(+)WIN55212-2	Tocris-Cookson (U.K.)	Ethanol

2.4 Polymerase chain reaction

The conventional polymerase chain reaction (PCR) method has been performed in the laboratory of Prof. Anwar Baydoun (University of Hertfordshire, U.K.). This technique was applied to determine the possible mRNA expression of CB_1 and CB_2 receptors in the 16HBE cell line. In parallel, CHO-hCB_1/hCB_2 cells have been used as positive control cells.

The basic principle is in the amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The technique employs deoxynucleotide triphosphates (dNTPs, to provide both energy and nucleosides for the synthesis of DNA), thermostable DNA polymerase (to carry out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single-stranded template), two oligonucleotide primers (each complementary to opposite strands of the DNA region), template (single-stranded DNA, cDNA), and buffer containing Mg^{2+} (necessary for the action of DNA polymerase) (figure 2.4). The first step towards construction of cDNA is the preparation of high quality, intact RNA.

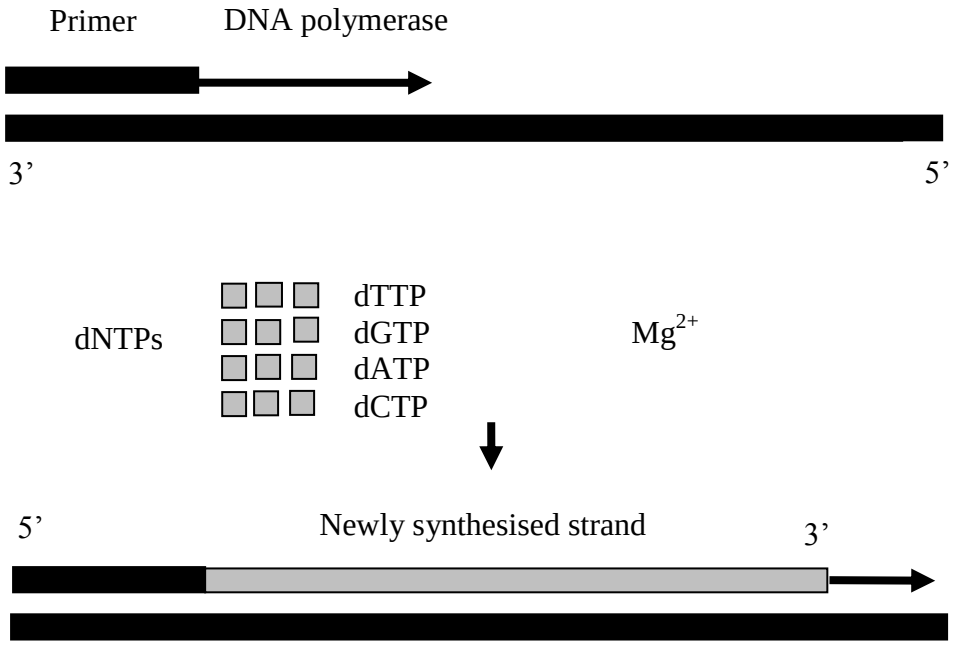

Figure 2.4 **Primer extension.**

2.4.1 Isolation of total RNA and determination of the RNA concentration

For PCR reactions, the total RNA of 16HBE cells and CHO-hCB_1/hCB_2 was extracted by the RNA-STAT-60^{TM} Reagent (Tel-Test). The reagent is an improved version of the popular single-step method reported by Chomczynski and Sacchi for total RNA isolation (Chomczynski and Sacchi, 1987). It is a mono-phase solution containing phenol and guanidinium thiocyanate which effectively dissolves DNA, RNA, and protein from a single biological sample. After the addition of chloroform and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the inter-phase containing DNA and an organic phase containing proteins. Each component can then be isolated after separating the phases. Total RNA/mRNA isolation by the RNA STAT-60 includes the following steps: homogenisation, RNA extraction, RNA precipitation and RNA wash.

Homogenisation

The monolayer of both 16HBE and CHO-hCB_1/hCB_2 cells were washed with sterile PBS and cells were lysed directly on the culture T-75 flask by adding 2 ml of RNA STAT-60 reagent. After scraping, the cell suspension underwent trituration and separation into aliquots. To ensure the complete dissociation of nucleoprotein complexes, cell aliquots were left at room temperature for 5 min.

RNA extraction

The essential step involves adding 200 µl of chloroform per every 1 ml of RNA STAT-60 reagent. The sample was shaken vigorously for 15 sec and incubated for max 15 min at room temperature. Later the homogenate was centrifuged at 13,000 RPM for 15 min at 4ºC. Centrifugation separates the mixture into 3 phases: low red

phenol chloroform or organic phase (containing protein), an inter-phase (containing DNA) and colourless upper aqueous phase containing the RNA.

RNA precipitation

The upper phase was carefully transferred (avoiding sucking of the inter-phase) into a fresh eppendorf tube (1.5 ml). Then isopropanol (500 µl per every 1 ml RNA-STAT 60) was added, the sample was incubated for 5-10 min at room temperature and again centrifuged at 13,000 RPM for 10 min at 4ºC. The RNA precipitate always formed a white pellet at the bottom of the tube.

RNA wash

After centrifugation the supernatant was removed and the RNA pellet was washed with 1 ml of 75 % ethanol per every 1 ml of the RNA STAT-60 used for the initial homogenisation. The sample was vortexed, centrifuged at 7,500 RPM for 5 min at 4ºC and briefly dried for 5-10 min in air at room temperature. At the end the product was eluted in 75 µl of diethylpyrocarbonate (DEPC) treated water. The purified RNA was stored at -70ºC and under this condition no degradation of RNA is detectable up to 1 year.

Once the total RNA was isolated, aliquoted samples underwent quantitation, purity and integrity tests.

Quantitation of RNA

The concentration of RNA was determined by measuring the absorbance in a spectrophotometer (Biophotometer, Eppendorf, Germany). RNA samples were diluted 1:100 by mixing 2 µl of sample with 198 µl of DEPC treated water. The diluted samples were poured into quartz cuvetts and the absorbance measured at wavelengths of 260 (A_{260}) and 280 (A_{280}) nm (DEPC treated water was used as a blank). The required sample concentration was automatically calculated.

Purity of RNA

To determine the quality of the extracted RNA, the A_{260}/A_{280} ratio was checked. Pure samples with a low protein or phenol contamination should have a ratio greater than 1.8 in DEPC treated water. The A_{260}/A_{280} ratio is influenced by pH. However, water is not buffered and lower pH results in a lower A_{260}/A_{280} ratio which can reduce the sensitivity to protein contamination. For this reason Tris-EDTA (trishydroxymethylaminomethane-EDTA) solution (pH=8) was used to check the purity of a sample. Pure RNA had an A_{260}/A_{280} ratio of 1.9-2.1.

Integrity of RNA

The integrity and size distribution of total RNA was assessed by gel electrophoresis followed by staining with ethidium bromide. The apparatus had to be free of RNase (rinsing with 0.1 M NaOH followed by DEPC treated water) to avoid any contamination and damage of the isolated RNA.

2.4.2 RNA gel electrophoresis

The quality of RNA preparation was checked by running a 1 % agarose gel in 1X Tris-boric acid-EDTA solution (1X TBE).

TBE (1X, 500 ml):

89 mM Tris base	5.4 g
89 mM boric acid	2.8 g
2 mM EDTA	4 ml (0.25 M)

The required amount of high-quality agarose was dissolved in 1X TBE running buffer and the solution was left to boil in the microwave. Then the agarose mixture was cooled to 60ºC and poured into a gel tray. Prior loading the samples, the gel was prerun in 1X TBE at 80 Volts. After 10 min the gel was ready for loading the samples which contained 2 μg of RNA in water with an equal volume of RNA loading buffer (20 % sucrose, 10 % ficoll, 10 mM Na_2EDTA, 0.25 % bromophenolblue in 1X sterile gel running buffer- TBE). The loaded gel was run for 60 min and then ethidium bromide 1 μg/ ml (in running buffer for 20 min) was used to stain the gel. Under UV illumination the gel was visualised and photographed. The required ribosomal bands 28S and 18S appeared as sharp bands on the stained gel. If the ribosomal bands of a specific sample were not sharp, but appeared as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

However, only the high-quality RNA was used in the next step of the synthesis of the first-strand complementary DNA (cDNA).

2.4.3 cDNA synthesis

Total RNA from 16HBE cells and CHO-hCB_1/hCB_2 cells were used to synthesize the cDNA which was necessary for the amplification in the PCR reaction. The protocol was followed from the products instruction manuals (Invitrogen). The synthesis of the template was performed by mixing 5 μg of total RNA with the reagents in 0.2 ml PCR tubes (kept on ice at all times) in the thermal cycler (Touchgene, Techne, USA). A parallel negative control with water omitting the RNA sample was included. X and Y values represent different volumes depending on the RNA concentration in the sample.

1.)

Oligo$(dT)_{12\text{-}18}$ primers (500 μg/ ml)	2.5 μl
Total RNA 5 μg	X μl
dNTP mix (10 mM each)	2.5 μl
RNase free and sterile water	Y μl
To a final volume of	30 μl

2.)

The mixture was heated to 65ºC for 5 min, then kept at 4ºC for a short time and next components were added:

5X first strand buffer	10 μl
0.1 M DTT	5 μl
RNaseOUT™ (40 U/ μl) inhibitor	2.5 μl

3.)

The tube content was gently mixed and incubated at 42ºC for 2 min. The last component was added shortly before the end of required incubation period:

SuperScript™ II RT (200 U)	2.5 μl
Final volume	50 μl

4.)

Incubation at 42ºC for 50 min

5.)

Inactivation of the reaction by heating at 70ºC for 15 min

2.4.4 PCR

Before starting the PCR analyses, primers were designed for the sequence of human CB_1 and CB_2 receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene. Design of the oligonucleotide primers was performed based on the nucleic acid sequences obtained from GenBank (National Center for Biotechnology Information; NCBI- PubMed, see below). Primers were designed using FastPCR computer software. In order to predict mispairing of primers (due to low annealing temperature of primers) the annealing temperature Tm of the primers was calculated using Oligonucleotide Properties Calculator. Also, the undesirable pairings of primers (hairpin loops or dimers) were checked by the same program.

Optimal parameters for primer design:

- Primer length: 18-24 bp (the longer the primer, the more inefficient the annealing)
- Primer Tm: 50-70ºC
- Primer G/C content: 50-70 %
- 3'-end sequences:

  1.) Should contain G or C, or CG what prevents ''breathing'' of ends and increases efficiency of priming (GC residues have stronger hydrogen bonds).

  2.) Should not contain 3 or more Cs or Gs which can promote mispriming.

  3.) Should not be complementary, or otherwise primer dimers will be synthesized preferentially to any other product.
- Primer self-complementarity (formation of 2º structures such as hairpins) should be avoided.

Once the primers were designed, they were synthesized by Invitrogen, U.K.

The primers for amplification of the human CB_1 receptor (GenBank accession number *GI: 1657840*):

Sense (5'-3')	TCC TAC CAC TTC ATC GGC AGC
Anti-sense (3'-5')	ACC TGT CGA TGG CTG TGA GG
Fragment length	190 bp

The primers for amplification of the human CB_2 receptor (GenBank accession number *GI: 89161185*):

Sense (5'-3')	GCC TGC TAA GTG CCC TGG AG
Anti-sense (3'-5')	TCG GTC AAT GGC GGT CAG CAG
Fragment length	263 bp

The primers for amplification of the human GAPDH (GenBank accession number *NC_000012*):

Sense (5'-3')	AGC AAT GCC TCC TGC ACC AC
Anti-sense (3'-5')	TCA GGG ATG ACC TTG CCC ACA G
Fragment length	227 bp

PCR conditions

The reactions were performed by mixing the reagents in 0.2 ml PCR tubes according to the following table. The X, Y values required optimisation:

Components	Volume	Final concentration
10X PCR Buffer (minus Mg)	2 µl	1X
10 mM dNTP mixture	0.4 µl	0.2 mM each
50 mM $MgCl_2$	0.6 µl	1.5 mM
Taq DNA polymerase	0.1 µl	1 U
Downstream primer	X µl	
Upstream primer	Y µl	
Template DNA	1 µl	
RNase free water	Z µl	
To a final volume of 20 µl		

Sequence specific sense and anti-sense primers were used for each target DNA sequence to be amplified. Samples were then placed onto the thermal cycler and the following parameters were used for amplification:

Number of cycles	Temperature [ºC]	Function	Time
1	94	Initial denaturation	2 min
35	94	Denaturation	30 sec
	60.1-62.5	Annealing	30 sec
	72	Extension	1 min
1	72	Final extension	10 min
Hold	4	Soak	∞

Temperatures and the concentration of specific sense and anti-sense primers were adjusted for each experiment. Additional PCR conditions for the amplification of required genes were as follows:

Cell line	Gene	Primers		Temperature
		Sense	Anti-sense	[ºC]
16HBE14o-	hCB_1 receptor	3 µl	1 µl	62.5
16HBE14o-	hCB_2 receptor	3 µl	1 µl	61.6
CHO-hCB_1	hCB_1 receptor	3 µl	1 µl	60.1
CHO-hCB_2	hCB_2 receptor	3 µl	1 µl	61.6
16HBE14o-	GAPDH	1 µl	1 µl	62.1

2.4.5 DNA gel electrophoresis

After the amplification, PCR products were analysed by gel electrophoresis on a 2 % agarose gel using 1X Tris-acetate-EDTA (1X TAE; prepared from 50X TAE).

TAE buffer (50X, 500 ml):

2 M Tris base	121 g
5.7 % Glacial acetic acid	28.6 ml
0.05 M EDTA	100 ml (0.25 M)

The required amount of high-quality agarose (1.5 g) was dissolved in 1X TAE running buffer (75 ml) and the solution was left to boil in the microwave. Then the agarose mixture was cooled to 60ºC, 12.5 µl ethidium bromide (5 mg/ ml) was added, and the solution was poured into a gel tray. Prior loading the samples the gel was prerun in 1X TAE at 80 Volts. After 10 min the gel was ready for loading the samples (10 µl) with an equal volume of DNA loading buffer (20 % sucrose, 10 % ficoll, 10 mM EDTA, 0.25 % bromophenol blue). The loaded gel was run for 45-60 min. Under UV illumination the amplified DNA bands were visualised and photographed.

2.5 Western blotting

2.5.1 Protein extraction from 16HBE cells and CHO-hCB_1/CB_2 cells

Western blot analysis has been performed in the laboratory of Prof. Anwar Baydoun (University of Hertfordshire, U.K.). This technique was applied to determine the possible expression of CB_1 and CB_2 receptors as proteins in the 16HBE cell line. In addition, CHO-hCB_1/CB_2 cells were used as parallel positive controls.

In T-75 flasks 16HBE cells (90 % confluency) and CHO-CB_1/CB_2 cells (the confluency up to 60%) were grown to required confluency, removed from growth medium and washed twice with ice-cold PBS. 500 µl of ice cold lysis buffer was added to each flask, ensuring a complete covering of the flask surface. The stock lysis buffer contained Tris, NaCl, EDTA, Na-deoxycholate (detergent) and Igepal (surfactant). In order to slow down proteolysis, dephosphorylation and denaturation, protease inhibitors were added fresh to the ice cold stock lysis buffer: Na_3VO_4 (inhibitor of all tyrosine protein phosphatases), NaF (inhibitor of serine/threonine protein phosphatases), aprotinin (protease inhibitor), leupeptin (lysosomal protease inhibitor) and PMSF (protease inhibitor).

Stock lysis buffer (50 ml, pH 7.4):

50 mM Tris base	0.3 g
150 mM NaCl	0.4 g
1 mM EDTA	0.25 ml (200 mM)
0.25 % Na Deoxycholate	5 ml (2.5 %)
1 % Igepal	0.5 ml

Lysis buffer (10 ml):

Stock lysis buffer	7.8 ml
1 mM Na_3VO_4	1 ml
1 mM NaF	1 ml
10 μg/ ml Leupeptin	2 μl
10 μg/ ml Aprotinin	70 μl
1 mM PMSF	50 μl

The flask was left on ice for 15 min and after that the content of the flask (lysis buffer with cells) was transferred to an eppendorf tube and immediately heated to 95ºC for 5 min. The lysate was sonicated for 15 sec to reduce the viscosity of the sample. After centrifugation (13,000 g for 15 min at 4ºC) the supernatant was transferred into new eppendorf tubes and stored at - 20ºC.

2.5.2 Determination of the protein concentration levels

The total concentration of protein in each sample was quantified using a bicinchoninic acid (BCA) protein assay (Pierce, U.K.). The biuret reaction is the basis for a simple and rapid colorimetric detection. This method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium and the chelation of Cu^{+} with BCA, a highly sensitive and selective colorimetric detection reagent. The purple coloured reaction product exhibits a strong absorbance at 562 nm. The intensity of the colour was measured by Multiskan Ascent spectrophotometer (Labsystems, U.K.).

In order to quantitatively determine how much protein is represented by a particular absorbance reading it is necessary to construct a standard curve, a graph of absorbance as a function of protein concentration (calculated in Microsoft Office Excel). The absorbance readings are obtained from a series of samples containing known amounts of protein. The standard curve for the assay can be used to convert the absorbance readings for the experimental samples into a protein amount or

concentration. BSA (working concentration, 1 mg/ ml) was used as a protein standard which was diluted with the lysis buffer into final BSA concentrations (table 2.5).

The assay was performed in a 96-well plate. 10 µl of BSA standard (0-1 µg/ µl) and 10 µl of selected samples were added both in triplicates into wells. In addition, 100 µl of the BCA reagent was added into each well. The plate was covered and incubated (shaking at 750 RPM) for 1 hour at normal temperature. The absorbance was measured at 620 nm. In order to load 35 µg of the protein, the appropriate volume was calculated in Microsoft Office Excel.

Volume BSA solution	**Volume Lysis buffer**	**Final BSA concentration**
0 µl	1 ml	0 µg/ µl
25 µl	975 µl	0.025 µg/ µl
50 µl	950 µl	0.05 µg/ µl
100 µl	900 µl	0.1 µg/ µl
200 µl	800 µl	0.2 µg/ µl
300 µl	700 µl	0.3 µg/ µl
400 µl	600 µl	0.4 µg/ µl
500 µl	500 µl	0.5 µg/ µl
750 µl	250 µl	0.75 µg/ µl
1 ml	0 µl	1 µg/ µl

Table 2.5 **Preparation of diluted BSA standard.**

2.5.3 SDS-PAGE electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate individual proteins (possibly cannabinoid receptors in 16HBE and CHO-hCB_1/CB_2 cell lines) by their size (molecular weight) under the influence of an electrical field in the Mini-Protein II gel apparatus. SDS is an ionic detergent, it denatures proteins by ‘’wrapping around’’ the polypeptide backbone and confers a negative charge to the polypeptide- protein molecules which helps in the migration of protein molecules towards the positive pole. The resolving gel (10 % SDS PAGE gel) was prepared and poured between the glass plates of the gel apparatus and a thin film of butan-2-ol was applied to prevent the inhibition of gel polymerisation by oxygen. The gel was left to polymerise at room temperature for 1 hour. After the alcohol removal, the stacking gel (5 %) was poured on the top of the resolving gel. Loading wells were created by inserting a 0.75 mm comb into the top of the stacking gel which was left to polymerise at room temperature for 45 min.

Resolving gel (20 ml- for 4 gels):

Ultra Pure Water	8.00
30 % Acrylamide	6.67
1.5 M Tris-HCl buffer (Resolving), pH 8.8	5.00
10 % SDS	0.20
10 % Ammonium persulphate	0.10
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.02

Stacking gel (10 ml- for 4 gels):

Ultra Pure Water	5.67
30 % Acrylamide	1.67
0.5 M Tris-HCl buffer (Stacking), pH 6.8	2.50
10 % SDS	0.10
10 % Ammonium persulphate	0.05
TEMED	0.01

To enable visualization of the migration of proteins, the sample lysates were mixed with an equal volume of the loading buffer.

Loading buffer (50 ml):

120 mM Tris-HCl,	12 ml (0.5 M, pH 6.8)
4 % SDS	20 ml (10 %)
10 % Glycerol	5 ml
0.006 % Bromophenol Blue	3 µl
2 % Mercaptoethanol	1 ml

Prior to loading a gel (together 4 gels), sample lysates and sample markers were heated to 95ºC for 5 min and quickly centrifuged. In the first gel, the molecular weight marker (enables the determination of the protein size, 10-200 kDa) was loaded into the first lane, 35 µg of CHO-hCB_1 lysate (positive control) in the second lane, and 35 µg of 16HBE lysate in the third lane. The second gel was the same apart of the second lane where 35 µg of CHO-hCB_2 lysate (positive control) was loaded. In parallel, 2 gels were run with the same loading pattern and considered as negative controls (with blocking peptides). The gel apparatus was filled with the tank buffer (protein gel running buffer, 1X) and electrophoresed at 200 V until the dye reached the bottom of the resolving gel (~ 60 min).

Tank buffer (10X, 1000 ml, pH 8.3):

250 mM Tris base	30.3 g
2 M Glycine	144 g
1 % SDS	100 ml (10 %)

2.5.4 Immunoblotting of the protein

The blotting process is a method for the protein transfer from the gel to the solid support membrane, PVDF membrane (Immun-Blot® polyvinylidene difluoride; Amersham, U.K.), by applying a current using the transfer cell.

Towards the end of gel electrophoresis, PVDF membranes were cut to match the size of gels, soaked in 100 % methanol (removes the hydrophobicity of the membrane and allows protein adsorbtion) for 5 min, then washed with distilled water and kept in the transfer buffer 1X. Filter papers (12 sheets) were also cut to fit the gels exactly and soaked in the transfer buffer 1X.

Transfer buffer (10X, 1000 ml, pH 8.3):

480 mM Tris base	58.2 g
390 mM Glycine	29.3 g
0.0375 % SDS	3.75 ml (10 %)

Transfer buffer (1X, 1000 ml):

Transfer buffer	100 ml (10X)
Methanol	200 ml
Distilled water	700 ml

Following the SDS-PAGE, gels were taken out from the glass plates and stacking gels were separated from resolving gels with a gel cutter. The transfer ‘’sandwich’’ was assembled with the following layers in order from the cathode to the anode: 1) filter papers (3 sheets) soaked in the transfer buffer, 2) resolving gel, 3) PVDF membrane 4) filter papers (3 sheets) soaked in transfer buffer. The transfer sandwich was placed in the transfer apparatus, Trans-Blot® SD Semi-dry electrophoretic transfer cell (Bio-Rad, U.S.A.) and was subjected to a current of 0.8 mA/ cm^2 for 2 hours.

The membranes were removed from the transfer cell and immediately placed into the blocking buffer (wash buffer supplemented with 5 % dried milk) for 1 hour at room temperature and kept on a rotating plate at moderate speed. Without blocking, there would not be prevention of non-specific protein interactions between the membrane and the antibody protein.

Wash buffer (10X, 1000 ml, pH 7.5):

0.2 M Tris base	24.2 g
1.5 M NaCl	87.7 g

Blocking buffer (100 ml):

Wash buffer	10 ml (10X)
0.1 %Tween 20	0.1 ml
5 % Fat free milk	5 g

The quality of the transfer was checked by subsequent staining of gels in the staining solution containing 0.1 % Coomasie Brilliant Blue in 45 % methanol, 45 % water and 10 % glacial acid. Gels were kept overnight at room temperature in the solution and the next day it was possible to visualize any remaining protein bands on gels.

After blocking, each membrane was washed 3 times by agitation in wash buffer (5 min per wash). When the membrane was washed, it could be probed with primary antibody which binds to the protein of interest. The membrane was incubated in either anti-CB_1/anti-CB_2 antibody alone or in the presence of their blocking peptides (they neutralise the antibodies, once the antibody is bound to the blocking peptide, it is no longer available to the epitop of the protein. The antibodies and their blocking peptides were kindly provided by Dr. Ken Mackie (University of Washington, Seattle, WA, U.S.A.). Anti-CB_1 antibody was diluted 1:1000, anti-CB_2 antibody was diluted 1:500 in blocking buffer. Blocking peptides (5 μg/ ml) used as negative controls were mixed with the correspondent primary antibodies in blocking buffer. The incubation

was for 1 hour at room temperature with gentle agitation. The incubation time with primary antibodies alone or in the presence of correspondent blocking peptides was over the weekend (up to 72 hours) at 4ºC at a rotating plate.

The probing with primary antibodies was followed by membrane washing (3 times for 5 min) in the wash buffer to remove any unbound primary antibody. The membranes were then incubated with secondary antibodies (horseradish peroxidase-conjugated secondary antibody which binds the first antibody), diluted 1:3000 in blocking buffer, for 90 min at room temperature on a rotating plate. Finally, the membranes were washed in the wash buffer (3 times for 5 min) to remove any unbound secondary antibodies.

2.5.5 Detection of chemiluminiscence and film development

The membranes were placed onto cling film. Equal volumes of ECL (Enhanced ChemiLuminiscence) detection solutions A and B (Amersham, U.K.) were mixed and added to the protein side of the membranes. After 1 min incubation excess of the detection reagent was removed, the membranes were wrapped in cling film with air bubbles excluded. The autoradiography film (Hyperfilm^{TM}-ECL, Amersham, U.K.) was placed on the top of the membranes. After 15 min of exposure time, the film was left to develop in Dektol developer (Kodak, U.K.), washed in tap water, fixed in Unifix fixer (Kodak, U.K.), and again washed under tap water.

2.6 FLIPR

FLIPR (Fluorescence imaging plate reader) is a system which provides fast, simple, reliable fluorescence based assay for detecting changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). The method has been performed in collaboration with Dr. Malcolm Begg (GlaxoSmithKline, Stevenage). The technique was chosen to determine whether cannabinoids can affect cytosolic Ca^{2+} concentration in the 16HBE cell line. ATP was used as a positive control and additionally, capsaicin as a vanilloid was tested. The following cannabinoid ligands were monitored: AEA, VIR (endocannabinoids), CBD (phytocannabinoid), CP55940, WIN55212-2 (non-selective CB_1/CB_2 agonists), and JWH133 (CB_2 agonist). In addition, the assay provided a test for evaluation of the functional potency at the CB_1 receptors identified in 16HBE cells.

One day before the assay was performed, 16HBE cells expressing CB_1 receptors were plated in 96-well, black walled, assay plates, at a density of 10 000 cells per well. These cells were then returned to the cell-culture incubator maintained at 37ºC, 5 % CO_2, 95 % O_2. On the day of the assessment, a 96-well plate was prepared with 11 different concentrations (10 μM-0.1 nM) of ATP, 6 cannabinoid ligands and capsaicin. All drugs were dissolved in DMSO excluding ATP. The vehicle of ATP was distilled water. Additionally, all drugs were diluted in FLIPR buffer (1X) containing Probenecid (prevents Ca^{2+} leak out of cells) and Brilliant Black (quenches excess of the Ca^{2+} reporter dye, Fluo4). On the day of the experimentation, 2.5 mM Probenecid and 500 μM Brilliant Black were added fresh to the assay buffer.

FLIPR buffer (10X, 1000 ml):

1.5 M NaCl	84.74 g
50 mM KCl	3.73 g
100 mM HEPES	23.83 g
100 mM D-Glucose	18.02 g
8 mM $CaCl_2$	8 ml (1 M stock)

Confluent 16HBE cells on black 96-well plate with a clear bottom were washed 2 times with 100 µl of warm PBS, before being incubated for 45 min at 37ºC with the assay buffer (100 µl/ well) containing the cytoplasmic Ca^{2+} indicator, Fluo4 (2 mM). The plates were then placed into a FLIPR^{TM} apparatus (Molecular Devices, U.K.) to monitor cell fluorescence (λ_{ex}=488 and λ_{EM}=540 nm) before and after the addition of ligands of interest.

2.6.1 Analysis of data from FLIPR experiments

If applicable, values are expressed as means± sem.

2.6.2 Drugs used in the study

Name	*Main cellular function*	*Supplier*	*Solvent*
Anandamide (AEA) N—(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide	Endogenous cannabinoid, CB_1/CB_2 and $TRPV_1$ receptor agonist	Tocris-Cookson (U.K.)	Ethanol
ATP Na_2 (ATP) Adenosine 5'-triphosphate disodium salt	Purinergic receptor agonist/activator of K^+_{Ca}	Sigma (U.K.)	Distilled water
(-)-Cannabidiol (CBD) 2-[(1R,6R)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol	Weak CB_1 receptor antagonist and $TRPV_1$ receptor agonist	Tocris-Cookson (U.K.)	Ethanol
CP55940 (-)-cis-3-[2-OH-4-(1,1-dimethylheptyl0phenyl]-trans-4-3(3-hydroxypropyl)cyklohexanol	CB_1/CB_2 receptor agonist	Pfizer (U.K.)	Ethanol
Capsaicin (CPS) 8-Methyl-N-vanillyl-trans-6-nonenamide	$TRPV_1$ receptor agonist	Sigma (U.K.)	Ethanol
JWH133 (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro -6,6,9-trimethyl-6H-dibenzo[b,d]pyran	Potent CB_2 receptor agonist	Tocris-Cookson (U.K.)	Ethanol
Virodhamine (VIR) O-(2-aminoethyl)-5Z,8Z,11Z,14Z-eicosatetraenoate	Endogenous cannabinoid, partial agonist/antagonist at the CB_1 receptor (EC_{50}=1.9 μM), full agonist at the CB_2 receptor (EC_{50}=1.4 μM)	Tocris-Cookson (U.K.)	Ethanol
WIN55212-2 mesylate (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone mesylate	CB_1/CB_2 receptor agonist	Tocris-Cookson (U.K.)	Ethanol