ABSTRACT

Aims
To evaluate effects of CP55,940 on normal bladder function in vivo and to examine whether it suppresses urinary frequency induced by nociceptive stimuli in the bladder. Cannabinoid receptor (CBR) activity may be involved in the regulation of bladder function. However, the role of CBR subtypes in micturition has yet to be established. CP55,940 is a synthetic analogue of tetrahydrocannabinol, which is a psychoactive ingredient of the Cannabis plant.

Methods
Cystometry under urethane anaesthesia was performed to evaluate the effect of intravesical CP55,940 with or without administration of CB1 antagonist-AM251 or CB2 antagonist-AM630 on bladder function in female rats. The effects of CP55,940 were also examined in rats with urinary irritation induced by intravesical infusion with acetic acid.

Results
Infusion of CP55,940 significantly (p<0.05) increased the micturition interval (MI) and bladder capacity (BC) by 52% and decreased maximal voiding pressure (MP) by 25%. Pre-treatment with AM251 or AM630 before CP55,940 administration, prevented CP55,940-induced increases in MI, BC and reduction in MP. Acetic acid-induced urinary frequency as evidenced by a reduction in MI, and was suppressed by CP55,940.

Conclusions
CP55,940 decreases bladder activity and urinary frequency induced by nociceptive stimuli, probably by suppression of bladder afferent activity. Effects
of CP55,940 were abolished by both CBR antagonists. This data implicates a role for the endocannabinoid system in bladder mechanoafferent function in rats. In addition, our results show that CP55,940 reverses urinary frequency exemplified in an overactive bladder model suggesting it could be an effective treatment for patients with lower urinary tract symptoms.

Keywords
Cannabinoid receptors, cystometry, urinary bladder, overactive bladder

Brief Summary
Intravesical CP55,940 increased micturition interval and bladder capacity and decreased maximal pressure suggesting a role for the endocannabinoid system in bladder mechanoafferent function in rats.
Introduction

The modulation of urge incontinence by cannabis, in patients with Multiple Sclerosis (MS) presenting with neurogenic detrusor overactivity (DO), has sparked interest in the functional involvement of the endocannabinoid system in the lower urinary tract (1). The endocannabinoid system (ECS) consists of the cannabinoid receptors, their endogenous ligands, and enzymes that regulate the biosynthesis and degradation of the endocannabinoids (2). The presence of the ECS in the urinary bladder has led to speculation that endocannabinoid signalling is involved in signal transduction pathways regulating bladder relaxation and may be involved in pathophysiological processes of the bladder (1). On the basis of this evidence, it was postulated that activation of cannabinoid receptors (CB1 and CB2) might promote relaxation of the urinary bladder during the filling phase. Dysregulation of the ECS in human bladder may be responsible for the pathogenesis of overactive bladder syndrome (OAB) and DO. We have previously localised the components of the endocannabinoid system in rat and human bladders where we showed expression of cannabinoid receptors and the modulating enzymes. Our functional in vitro studies showed that CB1 receptors have both pre- and post-synaptic inhibitory effects on rat bladder contraction, whereas CB2 acts only post-synaptically (3).

Although the processes involved in the pathogenesis of overactive bladder are better understood, the molecular and signal transduction mechanisms involved have not been fully evaluated. The ECS has been suggested as one of these mechanisms (4). Cystometric studies have shown an increase of the micturition threshold in rats receiving systemic cannabinoids in an overactive bladder.
model induced by acetic acid, cyclophosphamide (5) or turpentine oil (6). These effects were stronger when the cannabinoids were administered through a close-arterial route rather than systemically through the tail vein of the rat, supporting the hypothesis of a local regulatory role of the cannabinoid system in the micturition reflex (6). Intravesical infusion of acetic acid has been shown to alter the micturition reflex in rats (7) by increasing the frequency of contractions, and decreasing bladder capacity, with the micturition pressure unaffected or increased (8). We therefore used acetic acid to induce an overactive rat bladder model (6, 9, 10), assessing the effects of intravesical 3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-4-(3-hydroxypropyl)cyclo-hexanol (CP55,940) in a pathophysiological state, in order to determine its potential use as a therapeutic agent. CP55,940 displays similar affinity for both central and peripheral cannabinoid receptors.

The aim of the current study was to characterise the receptor subtype (CB1 or CB2) responsible for mediating normal rat bladder function following intravesical administration of CP55,940. Additionally, the effects of CP55,940 on a pathophysiological overactive model in the rat bladder were also investigated.

Methods

Animals

27 in total, adult female Sprague-Dawley rats (n=5-6 per group weighing 250-300 g, Charles River, UK) were group housed in an environmentally controlled room with a 12-hour light/dark cycle and unlimited access to food and water. All
experiments were carried out in accordance with the UK Home office regulations and the Animals (Scientific Procedures) Act 1986. The study had Home Office Licence Approval (Project Licence 40/3647 Protocol 19B-3)

**General Preparation**

Anaesthesia was induced and maintained with isoflurane (induction dose - 4%; maintenance dose 1.5-3%) until intravenous (i.v.) access was obtained. This took an average of 5-10 min. Once the femoral vein was cannulated with an i.v. infusion set containing an injection port and safety device, urethane (1.2 g / kg i.v.) was slowly administered until a suitable depth of anaesthesia (assessed by an absence of hind limb withdrawal in response to paw pinch and absence of a corneal reflex) was achieved. Supplementary doses of urethane (0.1 g / kg i.v.) were administered as required. Rats were under urethane anaesthesia for the duration of the protocol which on average lasted for 2-2.5 hrs. Body temperature was maintained at 37 °C by placing the animal on a homeothermic blanket system (Harvard, USA) and monitoring changes in temperature using a rectal temperature probe.

**Cystometry**

The ureters were exposed by retroperitoneal incisions and the distal ends were tied and cut to ensure no backflow and hence no leakage of saline from the bladder. The abdomen was opened through a midline incision and a single cuffed polyethylene catheter (PE-90 1.02 mm, Portex, Kent UK) was inserted through a small incision into the bladder dome. The catheter was secured with a suture around the top of the bladder dome and this was followed by closure of
the abdominal incision. The catheter was connected via a three-way stopcock to a pressure transducer (DTX-plus transducer, Digitimer, Singapore) to record bladder pressure and a syringe pump (Pump33, Harvard apparatus) for continuous infusion (0.15 ml/min) to evoke the micturition reflex.

During cystometry, data was captured using the data acquisition software (LabChart v7.3, AD Instruments, UK). The cystometric parameters, such as micturition interval (MI), baseline pressure (BP), maximum voiding pressure (MP) and bladder capacity (BC, calculated as MI x rate of infusion), were measured.

**Experimental protocol**

A stabilisation period of 30-45 min, with continuous intravesical infusion of 0.9% w/v sterile saline (0.15 ml/min) into the bladder was undertaken after completion of surgery. Following the acquisition of stable contractile responses, saline was infused for a further one hour to evaluate bladder activity and this was designated as the 'control period. This step was repeated for each individual rat for every experimental group ensuring there was a control bladder activity trace for each rat.

**Step 1**

The first part of the study was to assess the effect of vehicle (0.2% DMSO) on cystometric parameters and 6 rats were used for this experiment. Each rat underwent a stabilization period with saline described above. Each rat served its own control with the administration of intravesical infusion of saline for 1 hr
and then was administered intravesical vehicle for 1 hour. After completion of
the experiments, rats were administered 5% isoflurane as a schedule 1 method.

Step 2
In order to explore the effects of non-selective CB agonist CP55,940 on
cystometric parameters, 5 rats were administered intravesical CP55,940 (0.005
mg / kg) for 1 hr after the saline stabilization and control period described above.
At completion, rats underwent Schedule 1.

Step 3
In experiments examining effects of the CB1 receptor antagonist AM251, saline
was administered intravesically to a total of 5 rats as a control for 1 hr as
described above. After the 1 hr, AM251 (1 mg / kg), was administered
intravenously while the saline intravesical infusion was running. Five minutes
after the i.v. antagonist was given CP55,940 was administered by intravesical
infusion for 1 hr. At the end of the experiment the 5 rats underwent Schedule 1.

Step 4
This experiment investigated the effects of the CB2 receptor antagonist AM630
in a total of 5 rats. The experimental procedure described in step 3 was
followed using AM630 (3 mg / kg).

Step 5
In the studies examining the effect of CP55,940 on irritated bladders (the acetic
acid-treated group) 6 rats were used and underwent a saline stabilization and
control period described above. To induce bladder hyperactivity in the rats, 0.25% acetic acid (9, 10) was infused intravesically for 30 min to induce bladder irritation as previously described by Hiragata et al. (5). In the same group of rats, intravesical CP55,940 (0.005 mg / kg) infusion was then commenced for 1 hr to evaluate the effects on acetic acid-induced bladder irritation. Rats underwent Schedule 1 after the experiments.

**Drugs and solutions**

CP55,940 was purchased from Tocris, UK, and was dissolved in DMSO. The dose of 0.005 mg / kg of CP55,940 was based on the evidence from a previous study by Gratzke et al. (11), who demonstrated a significant increase in the micturition interval following intravesical infusion of the drug. AM251, the selective CB1 receptor antagonist, and AM630, the selective CB2 antagonist, were purchased from Tocris, UK and dissolved in DMSO. The dose of 1 mg / kg used for AM251 was selected as an acceptable dose based on current knowledge that AM251 ‘loses’ CB1 receptor selectivity at higher concentrations through actions on PPARs, GPR18 and GPR55 receptors. The dose for AM630 (3 mg / kg) and the timescale for administration (5 min) was chosen based on previous published studies (5, 12).

**Statistical Analysis**

For data comparison, the cystometric parameters were measured for 1 hr from the application of the drug. In a 1 hr trace there were an average of 42 micturition cycles that were analysed. A software macro was designed to analyse all the micturition cycles in the control 1 hr trace and in the 1 hr
experimental trace. All data values are expressed as mean ± standard error of the mean. Student’s t-test was used to compare the cystometric parameters between control with saline and after application of CP55,940 in the same group and also used to compare CP55,940 and acetic acid-exposed rats. Kruskal-Wallis with Dunn’s multiple comparisons test was used to compare cystometric parameters between CP55,940 and CB receptor antagonists. p<0.05 was considered significant. GraphPad Prism v6.0 was used for statistical analysis and for the generation of graphs.

Results

Rats with normal bladders

Representative cystometric traces taken from Sprague-Dawley rats are presented in Figure 1 (A-D) showing the effects of vehicle, CP55,940, CB antagonists and acetic acid and saline control. Figure 1A illustrates the cystometric parameters measured (MI, MP, BP).

CP55,940 (0.005 mg / kg) increased MI and BC by 52 ± 5.5% (each p<0.05, Figure 2, Table 1) compared to the saline treated control. Although there was a 25 ± 2.9% decrease in MP recorded after intravesical CP55,940 administration (p<0.05, Table 1), there was no reproducible change in baseline pressure compared to intravesical saline infusion. Addition of the DMSO (vehicle) had no effect on any of the cystometric parameters when compared to the saline-treated control (Table 1).

Intravenous pre-treatment with AM251 or AM630 5 min prior to intravesical
infusion of CP55,940, inhibited the previously observed increases in MI and BC, as well as the decreases in MP (Figure 3, Table 1). Representative cystometry traces showing the effects of the addition of either AM251 or AM630 prior to infusion of CP55,940 are displayed in Figure 1B and C, respectively. This postulates a functional role of CB1 and CB2 receptors mediating bladder function.

Acetic acid induced hyperactivity

Changes in cystometric parameters were compared to the saline control to ensure that acetic acid had indeed induced bladder hyperactivity. The effects of CP55,940 on cystometric parameters were compared to changes induced by acetic acid. Intravesical infusion of acetic acid induced bladder irritation (Figure 1D), as shown by a significant 41 ± 6.3% reduction in MI (p<0.01) compared with values obtained from the saline control. For the other cystometric parameters, acetic acid did not cause any significant changes in MP, BP or BC when compared to saline control. Addition of intravesical CP55,940 in the same rats increased MI by 78 ± 16.7% (p<0.05) and reduced MP by 22 ± 5.8% (p<0.05, Table 2).

Discussion

The results of this study demonstrate that intravesical infusion of CP55,940 suppressed bladder activity, as well as urinary frequency induced by bladder irritation. The inhibitory effects of CP55,940 appeared to be mediated by both CB1 and CB2 receptor subtypes. Since, MI and BC cystometric parameters can
be considered indicators for afferent function of the micturition reflex and MP and BP are indicators of motor activity (11), then the main function of CP55,940 seemed to be mediated by modulation of both afferent and motor activity as it not only induced an increase in MI and BC but also affected MP. In a similar study, which utilised intravesical bolus dosing of CP55,940, an increase in MI and BC was reported, with no changes in MP (11). Furthermore, the authors did not report the CB receptor subtype involved in mediating these responses (11). One reason for the difference between the results presented in our study and those reported previously could be differences in methodologies and/or anaesthetic agents used. In this study, isoflurane and i.v. urethane were used to anaesthetise the rats, whereas Gratzke et al. (2009) used intraperitoneal ketamine and xylazine and the cystometry was performed on conscious rats. It has been reported that ketamine inhibits the micturition reflex (13,14). Castroman and Ness (2002) reported that ketamine inhibits the reflexive responses of the bladder to the acute stimulus of distension in a dose-dependent manner (13). In addition to the effects of CP55,940 on afferent activity, its effect on motor activity, is in accordance with our previously published in vitro studies, where activation of both CB receptors reduced contractions of the detrusor muscle (3). Taken together, it may be speculated that CP55,940 activates CB receptors directly on the muscle resulting to inhibition of contractility.

The observed effects of CP55,940 on bladder function, demonstrated in this study, are in agreement with the effects of cannabinoid agonists reported in human clinical trials. In a large randomised controlled trial, patients with
neurogenic DO who were randomised to the THC and cannabidiol (CBD) treatment groups, reported a significant reduction in urge incontinence episodes compared to placebo (1). Similarly, in another study, patients with neurogenic DO given a cannabis extract containing THC and CBD, demonstrated a reduction in urinary urgency, number of incontinence episodes, frequency and nocturia. In addition, there was an increase in the maximum cystometric capacity in these patients (15), which is in accordance with the cystometric observations described in this study, where CP55,940 increased MI and BC.

With the knowledge that cannabinoid agonists are negatively coupled to calcium channels, and their inhibition of adenylate cyclase, the mixed picture of cystometric effects described in this study and those reported in the literature is understandable. The increased micturition interval and the decrease in maximal pressure of the contractions could be due to inhibition of adenylate cyclase, which may be involved in relaxation of detrusor smooth muscle during urine collection. The alteration of calcium fluxes may decrease neurotransmitter release required for detrusor contractions, and that decrease could be manifested as an increase in the volume requirement (demonstrated as an increase in bladder capacity). More experiments must be performed to clarify these preliminary results.

It appears from our study, that the activation of both CB1 and CB2 receptors have inhibitory effects on the bladder, as demonstrated by both CB antagonists blocking the actions of CP55,940. This is supported by a study that suggested a CB2-mediated effect in the rat urinary bladder (15), where cannabinor (a
selective high-affinity CB2 receptor full agonist) was used and resulted in a 44% increase in MI. In another study, using the non-selective CB receptor agonist WIN55212, an increase in the threshold for micturition was reported in anaesthetised rats, and this effect was counteracted by the CB1 receptor antagonist SR141716A, suggesting a CB1-mediated effect (6). Similarly, yet another study using a synthetic analogue of THC, IP-751 at 10 mg / kg, reported an increase in MI by 63% and threshold pressure by 53%. These effects were blocked by the CB1 antagonist AM251 but not the CB2 antagonist AM630 (5). In the present study both AM251 and AM630 inhibited the effects of CP55,940 suggesting a role for both CB receptors in urinary bladder function. A reason for this discrepancy between the results presented in this study and that of Hiragata, could be that in the present study CP55,940 was administered intravesically while Hiragata administered IP-751 systemically. The intravesical route allows the instillation of the cannabinoid drug directly to the bladder therefore bypassing any systemic effects. Therefore the effects of IP-751 on the central nervous system cannot be excluded. Another possibility for these differences could be related to the receptor subtype that is activated when using different agonists suggesting differences in receptor activation between the urothelium and detrusor muscle. It has been reported that CB1 and CB2 receptor expression is different in the urothelium and detrusor (16) and so different agonists may differentially activate the endocannabinoid receptor response in each of these specific tissues resulting in different downstream events. In addition, there may be an element of CB receptor turnover that depends on the type of agonist used, which could explain this discrepancy. This
can be assumed since full reversal of CP55,940 effects seen on some cystometric parameters occurred with both CB1 and CB2 antagonists.

The mechanism by which cannabinoid receptors modulate the micturition reflex is by their presence in the afferent nerve fibre endings located in the suburothelial layer, which is supported by in vitro studies using CB1 or CB2 receptor agonists. In vitro studies using CB1 receptor agonists have been shown to reduce neuronal activity (17) and attenuate bladder contractility as a result of electrical field stimulation in isolated mouse bladder strips (18). In rats, anandamide, WIN 55212-2 (a synthetic CB non-selective agonist), and IP-751 (also known as Ajulemic acid), suppressed normal bladder activity and urinary frequency induced by bladder irritation, suggesting that the inhibitory effects are in part mediated by CB1 receptors (5, 6, 19). In conscious rats, cannabinor, a selective CB2 receptor agonist, increased the micturition intervals and volumes by 52% (p<0.05) and 96% (p<0.01), respectively, and increased the threshold and flow pressures by 73% (p<0.01) and 49% (p<0.001), respectively (15). To support the findings described and the results presented in this study, ATP-evoked calcitonin gene-related peptide release (CGRP; a neurotransmitter released by sensory nerve endings) was reported to be inhibited by the activation of both CB1 and CB2 receptors after an alteration in the afferent cystometric parameters from the infusion of a CB receptor agonist (20). However, it is possible that some of the effect could be centrally mediated and whether these actions are related to CB receptors in the central nervous system, at peripheral sites in the lower urinary tract, or both needs to be clarified.
Furthermore, it is not known which of the two CB receptor subtypes is primarily responsible for the regulation of micturition in different species.

Cannabinoid receptor expression appears to be altered in pathological conditions, as has been shown in a clinical study where CB1 receptor immunoreactive nerve fibres were increased in the suburothelium of patients with painful bladder syndrome and detrusor overactivity (4). In addition, CB1 mRNA transcripts were increased in patients with chronic bladder pain syndromes (21) and CB2 transcripts and protein expression was increased in rats with bladder inflammation induced by acrolein (22). In vivo studies have shown an increase in micturition interval by cannabinoid agonists in rat bladders with induced bladder hyperactivity (5,19,23,24).

The results in this study and those in the literature support a regulatory role for the CB1 and CB2 receptor subtypes in both normal bladder function and bladder dysfunction. Furthermore, it is not known which of the two CB receptor isoforms is of primary importance in the regulation of micturition. In this study, both CB1 and CB2 antagonists inhibited the effects of CP55,940 equally. As AM251 and AM630 are known to cross the blood-brain barrier (25,26), it can be speculated that CB1 and CB2-receptor-related changes in micturition could result from CNS or peripheral nervous site of action (or both). It should be kept in mind that genes can compensate for each other, especially as CB1 and CB2 share intracellular pathways (G\textsubscript{i/o} protein). Therefore, discrepancies in reports of CB receptor subtype involvement in regulation of micturition may very well
suggest their multiple functions in different regulatory units of lower urinary tract control, reached via systemic or local administration of drugs.

Conclusions

CP55,940 decreased rat bladder activity and urinary frequency induced by bladder nociceptive stimuli, probably by suppression of bladder afferent activity. These effects are modulated by activation of both CB1 and CB2 receptors and these data implicate a role for the endocannabinoid system in bladder mechanoafferent functions in rats. In addition, the results of this study show that intravesical CP55,940 reverses urinary frequency exemplified in an overactive bladder model, suggesting it could potentially be an effective treatment for patients with lower urinary tract symptoms.
Acknowledgements

This study was funded by a Wellbeing of Women Training Research Fellowship awarded to Dr E Bakali.

Conflicts of Interest

The authors declare no conflicts of interest
References


Figure Legends

**Figure 1.** Representative cystometry pressure recordings obtained from female Sprague-Dawley rats. Scale bars indicate 60 second intervals. **A.** Shows a section of the trace from infusion with intravesical saline as control and a section showing the addition of intravesical CP55,940 (0.005 mg / Kg) infusion. The trace is annotated to demonstrate maximal voiding pressure (MP), micturition interval (MI), and basal pressure (BP). **B.** Representative trace showing control and then the addition of DMSO (vehicle). **C.** A cystometry trace showing infusion with saline (control) and then CP55,940 infusion after administration of intravenous AM251 (1 mg / Kg). **D.** A cystometry trace showing infusion with saline (control) and then intravesical infusion of then CP55,940 infusion after administration of intravenous AM630 (3 mg / Kg). **E.** Representative sections of a cystometry recording showing saline (control), the effects of intravesical 0.25% acetic acid infusion and the effects of intravesical CP55,940 on the irritated bladder.

**Figure 2.** Results of cystometry using female Sprague-Dawley rats (n=5). Panels **A** and **B** show a significant increase in MI and BC, respectively, on addition of CP55,940. BC was calculated by rate of infusion x MI. Panel **C** shows that CP55,940 significantly reduced MP but did not affect BP. Control was performed with a saline infusion (0.15 ml/hr) for 1 hour and then CP55,940 (0.005mg/Kg) infusion for an additional hour. Data are presented as the mean ± SEM for 5 separate experiments; **p<0.01 Student’s paired t-test.
Figure 3. The cystometry results using Sprague-Dawley rats are shown as a percentage of the saline control. Micturition interval (MI), bladder capacity (BC) and maximum voiding pressure (MP) for vehicle, CP55,940 with or without addition of AM251 or AM630 (n=5 for each group). Values are given as mean ± SEM. For comparisons between CP55,940 and AM251 and AM630, Kruskal-Wallis with Dunn's multiple comparisons test was used. * p<0.05, ** p<0.01.