

Effects of abnormal cannabidiol on oxytocin-induced myometrial contractility

Diarmaid D Houlihan, Michael C Denny and John J Morrison

Department of Obstetrics and Gynecology, Clinical Sciences Institute, University College Hospital, National University of Ireland Galway, Newcastle Road, Galway, Ireland

Correspondence should be addressed to J J Morrison; Email: john.morrison@nuigalway.ie

Abstract

The objective of this study was to investigate the effects of abnormal cannabidiol (abn-cbd) on oxytocin-induced myometrial contractility occurring during pregnancy. Isometric tension recordings were performed in isolated myometrial strips from biopsies obtained at elective cesarean section. The effects of cumulative doses of abn-cbd (10^{-9} – 10^{-5} M) on oxytocin-induced myometrial contractions alone, and on those following pre-incubation with SR 144528, AM 251, methylene blue, and iberiotoxin were measured, and dose–response curves were constructed. The pD_2 ($-\log EC_{50}$) values and the maximal inhibitory (MMI) values that were achieved were compared for each tissue type. Abn-cbd exerted a potent relaxant effect on oxytocin-induced myometrial contractions *in vitro*. Pre-incubation with the guanylate cyclase inhibitor, methylene blue, and the BK_{Ca} channel antagonist, iberiotoxin, significantly attenuated this effect (for pD_2 , $P < 0.01$; for MMI, $P < 0.01$). Abn-cbd exerts a potent inhibitory effect on human uterine contractility. This effect is partially mediated through modulation of guanylate cyclase and activation of BK_{Ca} channel activity. These findings have implications for physiologic regulation of myometrial quiescence.

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Introduction

The endocannabinoids comprise a family of eicosanoid and related unsaturated fatty acid derivatives, of which anandamide was the first to be described (Devane *et al.* 1992). Two cannabinoid receptors that belong to the superfamily of G_i/G_o protein-coupled receptors have been cloned (Howlett 2002). The cannabinoid type 1 (CB1) receptor is expressed primarily in the brain and peripheral tissues (Matsuda *et al.* 1990, Howlett 2002), while the cannabinoid type 2 (CB2) receptor appears to be confined to the cellular components of the immune system (Berdyshev 2000). In addition to the well-described neurobehavioral effects of cannabinoids, the endocannabinoids influence important biological and physiological processes (Hillard 2000, Pertwee 2001, Wilson & Nicoll 2002).

The cannabinoids are known to exert profound relaxant effects on smooth muscle systems. The mechanisms underlying these effects are varied and include alteration of sympathetic nervous tone (Ishac *et al.* 1996), modulation of vanilloid receptors located on perivascular nerves (Zygmunt *et al.* 1999), and finally direct interaction with cannabinoid receptors located on smooth muscle cells and on pre-junctional neurons in various mammalian tissues (Gebremedhin *et al.* 1999, Martin *et al.* 2000, Hinds *et al.* 2006). Abnormal cannabidiol (abn-cbd) is a synthetic

regioisomer of cannabidiol, which mediates a relaxant effect in isolated rat mesenteric artery via a mechanism that is distinct from CB1 and CB2 receptor activation (Jarai *et al.* 1999), and is independent of nitric oxide signaling and vanilloid receptor activation (Offertaler *et al.* 2003). The vascular smooth muscle relaxant effect of abn-cbd occurs via a mechanism that involves guanylate cyclase signaling and potassium channel activation, but a distinct receptor mediating this effect has not been identified yet (Begg *et al.* 2003).

Endocannabinoids may play an important role in the modulation of parturition and pregnancy (Park *et al.* 2004). Our group has demonstrated previously that endogenous and exogenous cannabinoids, Δ^9 -tetrahydrocannabinol and anandamide, mediate a direct, relaxant effect on myometrial contractility *in vitro* through an action at the CB1 receptor, which is sensitive to blockade by the CB1 receptor antagonist SR 141716, but not to that by the CB2 receptor antagonist SR 144728 (Denny *et al.* 2004). There are no further studies investigating other putative mechanisms of cannabinoid-induced myometrial relaxation. The aims of this study were to investigate the direct effects of the synthetic cannabinoid, abn-cbd, on human uterine contractility during pregnancy, and to investigate non-CB1/non-CB2 mechanisms through which this compound produces myometrial relaxation.

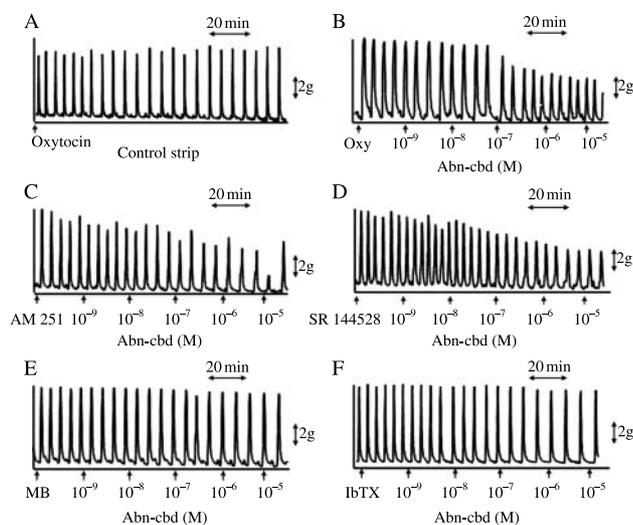


Figure 1 Effects of abn-cbd on myometrial contractions. (A) Representative recordings of oxytocin-induced contractions of pregnant human myometrium. (B) The effects of cumulative additions of abn-cbd (1 nM–10 μM) at 20-min intervals to this tissue type. The myometrial response to incremental doses of abn-cbd following pre-incubation with (C) AM 251, (D) SR 144528, (E) methylene blue (MB), and (F) iberiotoxin (IbTX).

Results

Abn-cbd exerted a potent concentration-dependent inhibitory effect on oxytocin-induced myometrial contractions *in vitro*. The calculated pD_2 for abn-cbd was 5.81 ± 0.34 , while the maximal inhibitory (MMI) response value was $60.27 \pm 4.70\%$. Representative recordings demonstrating the effects of abn-cbd on oxytocin-induced myometrial contractions and the effects of vehicle control on similar strips are shown in Fig. 1A and B.

The effects of pre-treatment with the following agents: AM 251 (Fig. 1C), SR 144528 (Fig. 1D), methylene blue (Fig. 1E), and IbTX (Fig. 1F) on uterine contractions, following the addition of cumulative doses of abn-cbd, are demonstrated in Fig. 1. Comparison of pD_2 and MMI values for the relaxant effects of abn-cbd on all myometrial contraction types revealed a significant difference across the groups (for pD_2 , $P < 0.01$; for MMI, $P < 0.01$). The MMI value and pD_2 value for each respective group are given in Table 1. Pre-incubation

with the CB1 antagonist, AM 251, or the CB2 antagonist, SR 144528, did not produce an effect on the dose–response curve following the sequential addition of abn-cbd as illustrated in Figs 2 and 3. *Post hoc* analysis showed that the addition of these compounds did not yield a significant difference in the pD_2 or MMI values across these groups ($n = 6$, Table 1).

Conversely, pre-incubation of the tissue baths with both the guanylate cyclase inhibitor, methylene blue, and the selective BK_{Ca} channel antagonist, IbTX, produced a parallel rightward shift of the dose–response compared with vehicle controls. *Post hoc* analysis of these data showed a significant difference between the pD_2 and the MMI values for abn-cbd in the presence and absence of these compounds ($n = 6$, $P < 0.01$, Table 1). Figures 4 and 5 illustrate the respective dose–response curves. There were no differences in the contractile activity between control strips and those pre-incubated with each of the test drugs.

Discussion

Our study demonstrates that the compound abn-cbd exerts a potent inhibitory effect on human uterine contractility *in vitro*. The magnitude of this inhibition is comparable to that of other established physiological and pharmacological uterorelaxant agents including human chorionic gonadotropin, cyclooxygenase inhibitors, β -adrenergic agonists, and oxytocin antagonists (Buscher *et al.* 2001, Denny *et al.* 2001, Slattery *et al.* 2001, Doheny *et al.* 2003). Abn-cbd has a potency of myometrial relaxation that is similar to the naturally occurring cannabinoids, anandamide (pD_2 value 5.58 ± 0.42 and MMI value 75.8 ± 2.20) and Δ^9 -tetrahydrocannabinol (pD_2 value 5.19 ± 0.91 and MMI value 75.1 ± 1.15 ; Denny *et al.* 2004). The findings from this study show that signaling via the CB1 or CB2 receptor is not a major component of the abn-cbd-mediated myometrial relaxation. Pre-incubation with methylene blue caused a significant reduction in the inhibitory effect of abn-cbd on myometrial contractility. At tissue bath concentrations used in this study, methylene blue is a selective antagonist of soluble guanylate cyclase and does not influence spontaneous uterine contractile activity (Houlihan *et al.* 2002,

Table 1 pD_2 and maximal inhibitory (MMI) values following the sequential addition of cumulative doses of abnormal cannabidiol (abn-cbd) to all tissue types.

Agonist	Antagonist	Site of action	n	$pD_2 \pm S.E.M.$	MMI $\pm S.E.M.$ %
Abn-cbd	None	None	6	5.81 ± 0.34	60.27 ± 4.71
Abn-cbd	AM 251	CB1 receptor antagonist	6	5.61 ± 0.46	56.32 ± 7.40
Abn-cbd	SR 144528	CB2 receptor antagonist	6	5.63 ± 0.33	55.67 ± 6.72
Abn-cbd	Methylene blue	Guanylate cyclase inhibitor	6	$4.48 \pm 0.17^*$	$30.40 \pm 3.78^\dagger$
Abn-cbd	Iberiotoxin	BK_{Ca} channel antagonist	6	$4.52 \pm 0.21^*$	$31.17 \pm 4.51^\dagger$

* $P < 0.01$ versus pD_2 values for abn-cbd and all other groups. $^\dagger P < 0.01$ versus maximal inhibition values for abn-cbd and all groups. Analysis of variance followed by Fisher's LSD (protected *t*-test) test.

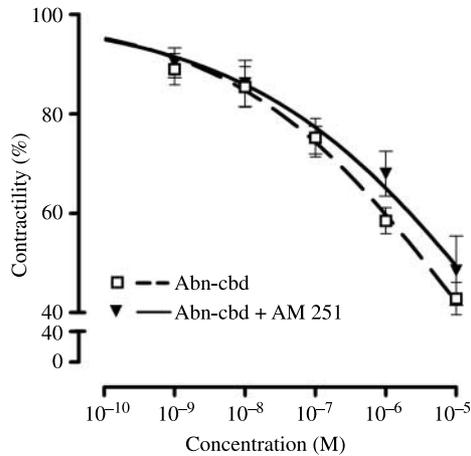


Figure 2 Dose–response curves for abn-cbd in the presence and absence of the selective CB1 receptor antagonist AM 251. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of abn-cbd (1 nM–10 μ M) at 20-min intervals on tissue contractility following the addition of oxytocin only (open squares) and following pre-incubation with AM 251 (shaded triangle). Percentage contractility is shown on the y-axis and the concentration of abn-cbd is shown on the x-axis. The symbols that are used represent the mean values within each group. Vertical error bars represent the s.e.m.

Modzelewska & Kostrzevska 2005). The addition of IbTX, a potent inhibitor of BK_{Ca} channel activity, also significantly attenuated the myometrial relaxant effect produced by the sequential addition of incremental doses of abn-cbd. It is therefore possible that abn-cbd-mediated uterine relaxation is largely effected via cyclic GMP or via BK_{Ca} channel activation, and there

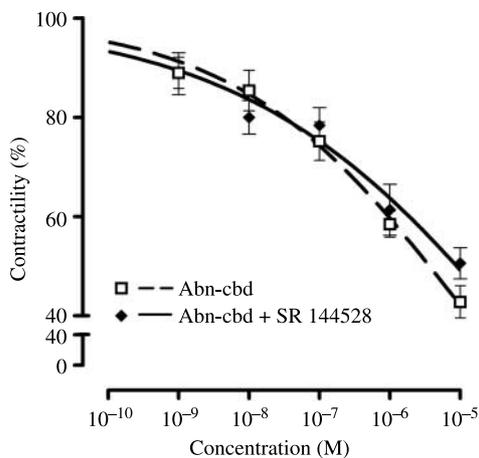


Figure 3 Dose–response curves for abn-cbd in the presence and absence of the selective CB2 receptor antagonist SR 144528. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of abn-cbd (1 nM–10 μ M) at 20-min intervals on tissue contractility following the addition of oxytocin only (open squares) and following pre-incubation with SR 144528 (shaded diamond). Percentage contractility is shown on the y-axis and the concentration of abn-cbd is shown on the x-axis. The symbols that are used represent the mean values within each group. Vertical error bars represent the s.e.m.

remains the possibility, not proven in this study, that an alternative or novel cannabinoid receptor may be involved.

The role of the cannabinoids in human pregnancy and parturition remains incompletely understood, and it is the subject of further study. Serial measurement of the endocannabinoid, anandamide, during human pregnancy suggests that serum concentrations of this compound increase during pregnancy with peak concentrations detected just before the onset of labor (Habayeb *et al.* 2004). CB1 receptor inactivation in mice induces preterm labor by altering normal progesterone and estrogen levels, in addition to interfering with the corticotropin-releasing hormone–corticosterone axis during late pregnancy (Wang *et al.* 2008). We have demonstrated previously the expression of CB1 and CB2 receptors on human pregnant myometrium, and have also shown that endocannabinoids and naturally occurring cannabinoids exert a potent relaxant effect on human pregnant myometrium. This effect is mediated predominantly by the activation of the CB1 receptor, and occurs independently of the CB2 receptor (Dennedy *et al.* 2004). Myometrial cells are richly endowed with BK_{Ca} channels, and endogenous regulation of these channels is thought to play an important role in the maintenance of uterine quiescence during pregnancy (Khan *et al.* 1997). The cGMP/guanylate cyclase, a second messenger system in human myometrium, is gestationally regulated and directly modulates BK_{Ca} channel activity during early pregnancy (Leviton 1994, Zhou *et al.* 2000).

There are three further points that are to be considered in the interpretation of the findings of this study. First, all tissue specimens for our study were excised from the upper region of the lower uterine segment. As there are no data quantifying or describing the distribution of cannabinoid receptors, atypical or otherwise, in human uterine tissue, it is not possible to conclude that similar sensitivity to abn-cbd would be demonstrated in myometrial biopsy taken from the uterine fundus. There are significant ethical constraints to excising biopsies from the uterine fundus for research purposes. However, there is reasonable evidence indicating that many functional effects and contractile properties of isolated myometrium from the upper and lower segments of pregnant women are similar (Luckas & Wray 2000). Secondly, our studies were all carried out in myometrial tissue obtained at term. While there is no reason to suspect altered sensitivity to these compounds in preterm myometrial tissue, further studies investigating gestation- or parturition-linked changes in cannabinoid function in human myometrium are required. The primary purpose of this study was to examine the effects of the compound abn-cbd on human myometrial contractions, and the findings have revealed that it exerts a potent relaxant effect, the mechanism of which remains partially explained.

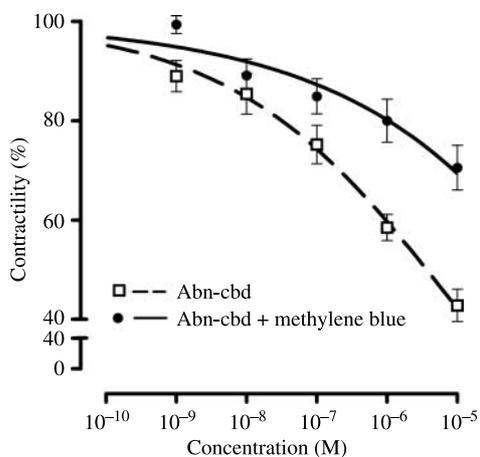


Figure 4 Dose–response curves for abn-cbd in the presence and absence of the selective guanylate cyclase inhibitor methylene blue. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of abn-cbd (1 nM–10 μ M) at 20-min intervals on tissue contractility following the addition of oxytocin only (open squares) and following pre-incubation with methylene blue (shaded circle). Percentage contractility is shown on the y-axis and the concentration of abn-cbd is shown on the x-axis. The symbols that are used represent the mean values within each group. Vertical error bars represent the s.e.m.

Materials and Methods

Tissue collection

Women were recruited for the study from those attending the Department of Obstetrics and Gynecology, University College Hospital, Galway, Ireland. Biopsies of human myometrial

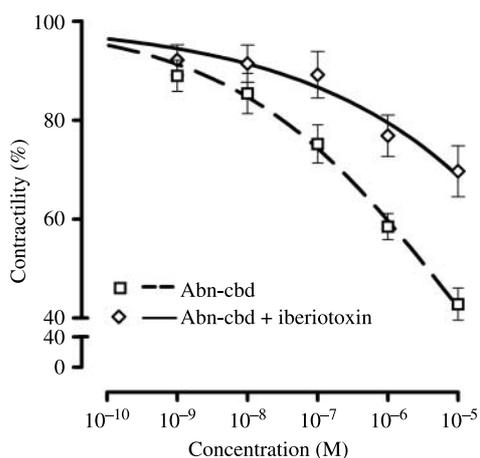


Figure 5 Dose–response curves for abn-cbd in the presence and absence of the selective BK_{Ca} channel antagonist iberiotoxin. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of abn-cbd (1 nM–10 μ M) at 20-min intervals on tissue contractility following the addition of oxytocin only (open squares) and following pre-incubation with iberiotoxin (open diamonds). Percentage contractility is shown on the y-axis and the concentration of abn-cbd is shown on the x-axis. The symbols that are used represent the mean values within each group. Vertical error bars represent the s.e.m.

tissue in pregnancy were obtained at elective cesarean section performed at term (mean maternal age 33.4 years, $n=12$). The reasons for cesarean section included breech presentation, previous cesarean section, and clinically diagnosed cephalopelvic disproportion. The median parity value of the women at the time of delivery was 2 (range 0–3). All women were given regional anesthesia for cesarean delivery. The biopsies were excised from the midline portion of the upper lip of the incision in the lower uterine segment. Ethical committee approval for the study was obtained from the Research Ethics Committee at University College Hospital, Galway, and recruitment was done by written informed consent. Following collection, the tissue was placed in Krebs–Henseleit physiological salt solution of the following composition: potassium chloride, 4.7 mmol/l; sodium chloride, 118 mmol/l; magnesium sulfate, 1.2 mmol/l; calcium chloride, 1.2 mmol/l; potassium phosphate, 1.2 mmol/l; sodium bicarbonate, 25 mmol/l; and glucose, 11 mmol/l (Sigma–Aldrich). Tissue was stored at 4 °C and used within 12 h of collection.

Tissue bath experiments

Longitudinal myometrial strips were dissected, measuring $\sim 2 \times 2 \times 10$ mm, and mounted under 2 g of tension in organ tissue baths for isometric recording as described previously (Houlihan *et al.* 2002). The tissue baths contained 20 ml of Krebs–Henseleit physiological salt solution, which was maintained at 37 °C and at a pH of 7.4 and gassed continuously with a mixture of 95% O₂/5% CO₂. The strips were allowed to equilibrate for at least 1 h prior to the addition of oxytocin. The Krebs–Henseleit solution was changed every 15 min during the equilibration period. Following equilibration, contractions were stimulated by the addition of the uterotonic agent oxytocin to achieve a tissue bath concentration of 0.5 nM for a period of 30 min. Pre-incubation was then performed for a further 30 min with the following compounds: SR 144528 (a selective CB2 receptor antagonist), AM 251 (a selective CB1 receptor antagonist), methylene blue (a guanylate cyclase inhibitor), and iberiotoxin (IBTX, a BK_{Ca} channel antagonist). The final bath concentration of SR 144528, AM 251, and methylene blue was 1 μ M, while that of iberiotoxin was 100 nM in accordance with previously published data (Dennedy *et al.* 2001, Houlihan *et al.* 2002, Bonz *et al.* 2003, Doheny *et al.* 2003). This was followed by the addition of abn-cbd in a cumulative manner at bath concentrations of 1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M (i.e. 10⁻⁹–10⁻⁵ M) at 20-min intervals. Control experiments that tested for tachyphylaxis, with a single dose for a similar time period, revealed no difference between isolated exposure and cumulative exposure during this time.

Measurement of the contractile activity was performed by calculation of the integral of the selected area using the PowerLab hardware unit and Chart v3.6 software (AD Instruments, Oxfordshire, UK). The integrated tension for the 20-min period prior to the addition of the compound abn-cbd was calculated, and this value served as a control value because no significant reduction in myometrial contractility was observed in control strips over the duration of the experiment. The effects of cumulative doses of abn-cbd in the

presence and absence of the various antagonists outlined above were then assessed by calculating the integral of contractile activity for 20 min following the administration of each drug concentration and by expressing these values as a percentage of the integrated contractile activity prior to any drug addition (i.e. percentage contractility).

Drugs and solutions

Stock solutions of oxytocin (Sigma–Aldrich) (1 mM), AM 251 (Tocris, Bristol, UK) (10 mM), and abn-cbd (Tocris) (1 mM) were prepared in ethanol. A stock solution of methylene blue (Sigma–Aldrich) (10 mM) and IbTX (10 μM; Tocris) was prepared using physiological saline (0.9% NaCl). The compound SR 144528 was donated by Sanofi Recherche, Montpellier, France. A stock solution (1 mM) was prepared using 0.9% NaCl. Serial dilutions of each drug were prepared daily as appropriate, and were maintained at room temperature. Fresh Krebs–Henseleit physiological salt solution was prepared daily.

Statistical analysis

Using the calculated integrals of contractile activity at each bath concentration, dose–response curves were analyzed by fitting the logistic equation: $Y = Y_{\max} \times D^n / (EC_{50}^n + D^n)$, where Y is the response (percentage contractility), Y_{\max} is the maximal relaxation achieved, D is the dose of agonist (abn-cbd), n is the slope function, and EC_{50} is the agonist dose giving the half maximal response. Curve fitting was performed with the software package Prism (Graphpad Software, San Diego, CA, USA). For multiple comparisons, an ANOVA test was used, and *post hoc* comparisons were carried out using Fisher's LSD (protected *t*-test) test to compare pD_2 ($-\log EC_{50}$) values and MMI response values between two groups. The statistical package GBSTAT version 6.5 was used for statistical calculations. A *P* value < 0.05 was accepted as statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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