

Differential activation of G-protein-mediated signalling by synthetic cannabinoid receptor agonists

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Running Title:

Abstract

Synthetic cannabinoid receptor agonists (SCRAs) are new psychoactive substances associated with acute intoxication and even death. However, the molecular mechanisms through which SCRAs may exert their toxic effects remain unclear - including the potential differential activation of G protein subtypes by CB1, a major target of SCRA. We measured CB1-mediated activation of G α_s and G $\alpha_{i/o}$ proteins by SCRAs by examining stimulation (PTX-treated) as well as inhibition (non-PTX treated) of forskolin-induced cAMP accumulation in HEK cells stably expressing CB1. Real-time measurements of stimulation and inhibition of cAMP levels were made using a BRET biosensor. We found that the maximum concentration of SCRAs tested (10 μ M), increased cAMP levels 12 to 45% above that produced by forskolin alone, while the phytocannabinoid THC did not significantly alter cAMP levels in PTX-treated HEK-CB1 cells. All SCRAs had greater potency to inhibit of forskolin-induced cAMP levels than to stimulate

cAMP levels. The rank order of potencies for SCRA stimulation of cAMP ($G\alpha_s$) was PB-22 > 5F-MDMB-PICA > JWH-018 > AB-FUBINACA > XLR-11. By contrast, the potency of SCRA for inhibition of cAMP ($G\alpha_{i/o}$) was 5F-MDMB-PICA > AB-FUBINACA > PB-22 > JWH-018 > XLR-11. The different rank order of potency of the SCRA to stimulate $G\alpha_s$ -like signalling compared to $G\alpha_{i/o}$ signalling suggests differences in G protein preference between SCRA. Understanding the apparent differences among these drugs may contribute to unravelling their complex effects in humans.

Keywords Synthetic cannabinoid receptor agonist, cannabinoid receptor, G protein, signalling, toxicity

Abbreviations SCRA, synthetic cannabinoid receptor agonists; NPS, novel psychoactive substances; CB1, cannabinoid receptor type 1; HEK-CB1, Human Embryonic Kidney cells stably transfected with HA tagged human CB1 receptors; HA, haemagglutinin; AC, adenylyl cyclase; PTX, pertussis toxin; BRET, bioluminescence resonance energy transfer; CAMYEL, cAMP sensor YFP-Epac-Rluc; PEI, polyethylenimine; GIRK, G protein-gated inwardly rectifying K^+ channel; 2-AG, 2-arachidonoyl glycerol; THC, Δ^9 -tetrahydrocannabinol

INTRODUCTION

The use of synthetic cannabinoid receptor agonist (SCRA) new psychoactive substances (NPS) is associated with significant morbidity and mortality compared to use of Δ^9 -tetrahydrocannabinol (THC), the main psychoactive ingredient of cannabis [1, 2]. SCRA are linked to wide range of toxic effects including seizures, agitation, hypertension, cardiotoxicity, kidney damage, and sometimes death [3, 4]. There has been a rapid increase in the number of structurally diverse SCRA since 2010, with little known about their pharmacology and toxicology at time of identification [5]. The constant evolution of SCRA structures occurs in response to legislative restriction and development of urine drug screens for existing compounds [6, 7, 8]. A time-series of seizures (by tonnage) of NPS reported to United Nations Office on Drug and Crime [9] showed that the SCRA dominated the synthetic NPS market over the period 2011-2017.

Many SCRA are agonists at cannabinoid type-1 and type-2 receptors (CB1 and CB2, respectively [10]; with the psychoactive effects attributed to the activation of CB1 [11]. We have previously described the *in vitro* quantitative measurement of SCRA efficacy at CB1, where all SCRA tested showed between 20-300 fold greater agonist activity at CB1 compared to THC [12]. Cannabinoid receptors mediate downstream signalling predominantly through the $G_{\alpha_i/o}$ protein family [13]; however, under some circumstances, CB1 can also stimulate adenylyl cyclase (AC) through G_{α_s} -proteins [14, 15, 16]. For example, blockade of canonical CB1- G_{α_i} pathway with pertussis toxin (PTX) or sequestration of CB1- G_{α_i} protein in the primary striatal rat neurons on co-expression with D2 results in an augmentation of cyclic adenosine monophosphate (cAMP) levels by cannabinoids, suggesting CB1 couples to G_{α_s} [14, 15]. A recent study characterized the relationship between CB1 receptor expression and signalling, and showed that at very high receptor expression levels, the effect of CB1 activation on cAMP signalling was stimulatory, a phenotype that was reversed by systematic pharmacological

knockdown at the receptor level [17]. The idea that certain SCRA may preferentially activate different CB1 $G\alpha$ subtypes is not unprecedented [18, 19, 20]; in a study by Costain and colleagues [21], AB-CHMINACA elicited an elevation in cAMP levels in both the absence and presence of forskolin in human embryonic kidney (HEK) cells transiently expressing CB1, suggesting an AB-CHMINACA-specific CB1-mediated activation of $G\alpha_s$ signalling.

The mechanism(s) through which SCRA exert different behavioural and physiological effects remains unclear, and which pathways modulated by CB1 activation mediate the specific pharmacological effects of SCRA is also unknown. Similarly, the question of whether these pathways are activated in a quantitatively or qualitatively similar way by SCRA and THC is only beginning to be addressed [22]. Finally, the question of whether SCRA activity at non-cannabinoid receptors is also important for their pharmacological effects is very much open [23, 24, 25]. With more than 250 SCRA identified in the NPS market [9], elucidation of the differential molecular mechanisms by which these compounds can exert distinct pharmacology, including their signalling via CB1, is essential for understanding their adverse effects. This study examined whether SCRA that are representative of structural classes confirmed in patients admitted to emergency departments with presumed SCRA toxicity stimulate $G\alpha_s$ -like cAMP signalling via CB1. We measured the SCRA-mediated stimulation as well as inhibition of forskolin induced cAMP accumulation in HEK cells stably expressing CB1. We have observed SCRA-specific CB1-dependent activation of the two signalling pathways, but THC only coupled to inhibition, not stimulation of cAMP. While AB-CHMINACA, previously identified as having a unique profile among SCRA for elevating cAMP, appeared to signal, in part, through non-CB1 mechanisms.

METHODS

CB1 receptor transfection and cell culture

HEK 293 FlpIn cells with homogeneous G protein-gated inwardly rectifying K⁺ (GIRK4) channel expression (the construction of these cells by Grimsey and colleagues will be described elsewhere) were co-transfected with pcDNA5/FRT construct encoding haemagglutinin (HA)-tagged human CB1 receptor cDNA and pOG44 (Flp recombinase plasmid) using transfection reagent Fugene HD (Promega) as previously described for AtT-20 pituitary tumour cells [26]. Cells stably expressing the CB1 receptor were cultured in Dulbecco's Modified Eagle Media (Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (Thermo Fischer Scientific, Waltham, MA, USA), 400 µg ml⁻¹ G418 (GIRK4 selection antibiotic) and 100 µg ml⁻¹ hygromycin (CB1 selection antibiotic) up to passage 5 (selection phase). Hygromycin concentration was reduced to 80 µg ml⁻¹ beyond passage 5 (maintenance phase). Cells were grown in 75 cm² flask at 37 °C/5 % CO₂ and passaged at 80% confluency as required. Assays were carried out on cells up to 25 passages.

Assay for cAMP measurement

Intracellular cAMP levels were measured using pcDNA3L-His-CAMYEL plasmid, which encodes the cAMP sensor YFP-Epac-RLuc (CAMYEL) as outlined in [27, 28]. Cells were detached from the flask using trypsin/EDTA (Sigma-Aldrich), and resuspended in DMEM supplemented with 10% FBS, 100 units ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Cells were seeded in 10 cm dishes at a density of 7,000,000 such that they would be 60-70% confluent the next day. On the following day, the cells were transiently transfected with 5 µg of pcDNA3L-His-CAMYEL plasmid using the linear polyethylenimine (PEI, m.w. 25 kDa) (Polysciences, Warrington, PA, USA). The PEI/DNA complex mixture was sequentially added to the cells at the ratio of 1:6, and cells were incubated in 5% CO₂ at 37 °C. Approximately 24 hours after transfection, the cells were then detached from the dish and the pellet was resuspended in Leibovitz's (L-15 -Thermo Fischer Scientific, Waltham, MA, USA) media

supplemented with 1% FBS, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 15 mM glucose. In the experiments with pertussis toxin (PTX) to irreversibly uncouple Gα_i proteins, the cells were resuspended in the media containing 200 ng ml⁻¹ PTX. The PTX-treated and control (non-PTX treated) cells were plated at a density of 100,000 cells per well in poly D-lysine (Sigma-Aldrich) coated, white wall, clear bottomed 96-well microplates. Cells were incubated overnight at 37 °C in ambient CO₂.

The day after plating, forskolin (FSK, an activator of AC) was prepared in HBSS composed of (mM) NaCl 145, HEPES 22, Na₂HPO₄ 0.338, NaHCO₃ 4.17, KH₂PO₄ 0.441, MgSO₄ 0.407, MgCl₂ 0.493, CaCl₂ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 15), and supplemented with 0.1% BSA. All the drugs used for the series of real-time measurements of stimulation and inhibition of cAMP levels were made in 3 µM of forskolin immediately before the assay. The concentration of DMSO (0.10-0.13%) was kept constant for all experiments, however this limited the maximum drug concentration that could be tested. Coelenterazine H substrate (NanoLight Technologies, Pinetop, AZ, USA) was added to a final concentration of 5 µM to the cells, and incubated for 5 min after which 10 µl of (10X) drug was added to each well to obtain the desired concentration. A vehicle (HBSS plus DMSO alone) was included in each column of 96-well microplate and routinely subtracted from the measurements. The PTX-treated and control cells were compared side by side. Luminescence was measured using PHERAstar plate reader (BMG Labtech, Germany) at 37 °C. The cells signalling was measured at an emission wavelength of 475 nm and 535 nm simultaneously, and the readings were made every 40 s for approximately 20 min. A concentration response curve (CRC) for CP55940 and WIN55212-2 inhibition of cAMP accumulation were performed for each experimental replicate as a reference standard (Figure 1).

Data Analysis

Raw data are presented as inverse bioluminescence resonance energy transfer (BRET) ratio of emission at 475 nm/535 nm, such that an increase in ratio corresponds with increase in cAMP production. Area under curve (AUC) analysis was performed in GraphPad PRISM (Graph Pad Software Inc., San Diego, CA). Data were normalized to forskolin (set as 100%) over a 20 min period for each experiment. Concentration response curves were obtained by fitting four-parameter non-linear regression curves in PRISM to derive EC₅₀ and E_{MAX}. All final datasets passed the Shapiro-Wilk test for normality. Unless otherwise stated, the data represent mean \pm SEM of at least 5 independent experiments, each conducted in duplicate. The differences between groups were tested using unpaired Student's t-test (PRISM). Statistical significance is defined as $P < 0.05$.

Materials

CP55940, WIN55212-2, 2-arachidonoylglycerol (2-AG), CUMYL-4CN-BINACA, and SR141716 were purchased from Cayman Chemical (Ann Arbor, MI, USA), Δ^9 -tetrahydrocannabinol (THC) was from THC Pharm GmbH (Frankfurt, Germany) and was a kind gift from the Lambert Initiative for Cannabis Therapeutics (University of Sydney). PTX was from HelloBio (Bristol, UK), and forskolin was from Ascent Scientific Ltd. All the SCRAAs, unless otherwise stated, were synthesized by Dr. Samuel D. Banister in the lab of Professor Michael Kassiou at Sydney University (Sydney, NSW, Australia). Chemical structure of SCRAAs can be found elsewhere [12]. All the SCRAAs were prepared in DMSO and stored in aliquots of 30 mM in -30 °C until needed.

RESULTS

Real-time cAMP BRET measurement of the G_{as} mediated signalling of SCRAAs

Using the CAMYEL assay, we measured the effect of seventeen cannabinoids (10 μ M each) on the forskolin-stimulated cellular cAMP levels in HEK-CB1 cells following pre-treatment

with PTX. All the SCRAAs produced an increase in cAMP levels above that produced by forskolin alone (100%). Examples of raw traces are shown for some SCRAAs (Figure 2), note that the stimulation of cAMP by SCRAAs in presence of forskolin and PTX plateaued approximately after 12 min, and maintained at that level for the entire course of the assay (20 min). The effects of SCRAAs tested ranged from 12 to 45% increase in signal relative to forskolin alone. Most of the SCRAAs had approximately 1.5 times higher effect than CP55940 (19%) or WIN55212-2 (18%), except for JWH-018, UR-144, AM-2201, and CUMYL-4CN-BINACA, which showed similar or lower effect (Figure 2). AB-FUBINACA had up to 2.5 times higher effect than CP55940. In PTX treated cells, the endocannabinoid 2-AG (10 μ M) produced an increase in forskolin-stimulated cAMP levels approximately twice that of CP55940, while the phytocannabinoid THC did not significantly alter cAMP levels in the presence of forskolin (compared to forskolin alone Figure 2, $P > 0.05$).

Differential SCRAAs-induced stimulation and inhibition of cAMP signalling in HEK-CB1

To assess whether there was any evidence of preferential coupling to $G\alpha_{i/o}$ over $G\alpha_s$ among SCRAAs, we assessed the pharmacological activity (EC_{50} and E_{MAX}) of a selection of the most prevalent SCRAAs (JWH-018, PB-22, AB-FUBINACA, XLR-11, and 5F-MDMB-PICA), to stimulate and inhibit cAMP in HEK-CB1 cells. All SCRAAs tested activated CB1 via $G\alpha_{i/o}$ (inhibitory, non-PTX treated), and $G\alpha_s$ (stimulatory, PTX-treated) in a concentration dependent manner (Figure 3). As previously reported [29], treatment with CP55940 and WIN55212-2 produced an immediate concentration dependent inhibition of forskolin-mediated cAMP production (pEC_{50} CP55940 8.2 ± 0.3 , pEC_{50} WIN55212-2 7.4 ± 0.2). All SCRAAs had greater potency (0.62-16 nM) for inhibition of forskolin-induced cAMP levels in non-PTX treated HEK cells compared to their potency to stimulate cAMP levels. The activation of CB1- $G\alpha_s$ by SCRAAs showed a wide variation in E_{MAX} values, with significant differences in efficacies proceeding AB-FUBINACA (most efficacious) $>$ PB-22 \approx XLR-11 \approx 5F-MDMB-PICA $>$

JWH-018, whereas all the SCRA were similarly effective at inhibiting cAMP production (Table 1). The first SCRA to be identified in spice, JWH-018, caused partial (13% increase over forskolin alone) activation of $G\alpha_s$ pathway, but produced greater inhibition of the forskolin-induced cAMP response (64% of forskolin response). Whereas other SCRA tested in the present study induce moderate activation of $G\alpha_s$ pathway (26-44% relative to forskolin) compared to their activity at $G\alpha_{i/o}$ inhibitory pathway. The rank order of potencies for SCRA for inhibition of cAMP ($G\alpha_{i/o}$) is 5F-MDMB-PICA > AB-FUBINACA > PB-22 > JWH-018 > XLR-11. By contrast, the potency of SCRA for stimulation of cAMP ($G\alpha_s$) is PB-22 > 5F-MDMB-PICA > JWH-018 > AB-FUBINACA > XLR-11. The most efficacious SCRA at $G\alpha_s$ -pathway (AB-FUBINACA) was roughly 350 times less potent at $G\alpha_s$ than the $G\alpha_{i/o}$ -pathway, while JWH-018 was only 14 times less potent. XLR-11 had much lower potency compared to all the other SCRA for both $G\alpha_s$ -and $G\alpha_{i/o}$ -pathway.

We then tested if the SCRA-induced observed stimulatory effects were mediated through CB1 receptors. Pre-treatment of HEK-CB1 with SR141716A (3 μ M, 5 min), a potent and selective CB1 antagonist [30], largely prevented the subsequent SCRA (10 μ M)-mediated stimulation of forskolin-induced cAMP response compared to the vehicle-treated cells (Figure 4, $P < 0.05$). Consistent with $G\alpha_s$ CB1-specific responses of SCRA, pre-treatment with SR141716A also blocked the inhibitory cAMP signalling induced by SCRA (Supplementary Figure 1, $P < 0.05$).

AB-CHMINACA has previously been reported to stimulate $G\alpha_s$ -like cAMP signalling pathway in a concentration dependent manner in HEK-CB1 cells [21]. Following PTX treatment, AB-CHMINACA increased cAMP levels above that of forskolin alone (Figure 5) in a concentration-dependent manner, with an increase of $86 \pm 21\%$ at 30 μ M. However, in cells pre-treated with SR141716A (3 μ M, 5 min), the stimulatory effects of AB-CHMINACA (10 μ M) was only partially inhibited, in contrast to other SCRA tested in the present study. To

confirm that this response was at least in part non-CB1-mediated, AB-CHMINACA was tested in HEK 293 wild-type (WT) cells; in these cells, AB-CHMINACA (10 μ M) also produced a small increase in forskolin-stimulated cAMP accumulation (Figure 5, $29 \pm 10\%$), suggesting that some of these stimulatory effects were occurring via mechanism(s) unrelated to CB1 receptor activity.

DISCUSSION

In this study, we set out to systematically characterize the ability of several SCRA to activate $G\alpha_s$ and $G\alpha_{i/o}$ proteins by examining stimulation as well as inhibition of forskolin-induced cAMP accumulation in HEK cells stably expressing CB1. Assays of cAMP signalling revealed that the maximum concentration of SCRA tested (10 μ M), increased cAMP levels 12 to 45% above that produced by forskolin alone, while THC failed to increase cAMP levels, an observation consistent with the findings of Finlay et al. [17]. To further investigate the differential response of SCRA-induced activation and inhibition of cAMP production, we constructed the concentration response curves for the most prevalent group of SCRA (JWH-018, PB-22, AB-FUBINACA, XLR-11, and 5F-MDMB-PICA); the rank order of potency of these SCRA to stimulate $G\alpha_s$ -like cAMP signalling pathway was different from their activity at $G\alpha_{i/o}$ -pathway (inhibition of cAMP), suggesting that some of these drugs differentially regulate G protein coupling to CB1.

SCRA-mediated inhibition of cAMP has been extensively studied in cell models expressing cannabinoid receptors [21, 24]. Indeed, in some studies of CB1 signalling outputs, SCRA have demonstrated $G\alpha_s$ -like phenotype [14, 15, 16, 17]. Initial experiments were conducted to determine whether traditional, endogenous, and synthetic cannabinoids stimulate $G\alpha_s$ mediated stimulation of cAMP synthesis. Our results are consistent with the previous reports, showing the greater maximal effect of 2-AG at $G\alpha_s$ -like CB1 signalling compared to CP55940 and

WIN55212-2 [17]. We found that 3 of the 16 SCRA tested, AB-FUBINACA, PB-22, and AB-PINACA, activated $G\alpha_s$ -like CB1 signalling to more than 30% above the forskolin response. In a previous study using AB-CHMINACA, Costain and colleagues [21] showed similar increases in cAMP levels to that seen in this study without the need for FSK or PTX pre-treatment. Costain et al. [21], performed their assays on HEK293T cells transiently transfected with CB1. Transient transfection of CB1 may have led to a higher level of receptor expression than in our cells, and high levels of CB1 receptor expression is sufficient to result in a switch in cAMP signalling from $G\alpha_i$ -mediated (inhibitory) to $G\alpha_s$ -mediated (stimulatory) [17]. Furthermore, the HEK-293 “T” subclone used in the previous study harbors considerable genomic differences to the parental HEK 293 cell line used in the present study [31, 32], which may also contribute to altered cAMP responses (via different adenylyl cyclase isoforms). However, our data, together with that of Costain et al. [21] suggest potentially different receptor/effector coupling pathways in the presence of some SCRA (AB-FUBINACA, PB-22, and AB-PINACA, AB-CHMINACA) compared to other CB1 ligands.

We further sought to investigate SCRA differential activation of distinctive G protein subsets—inhibition and stimulation of forskolin-mediated cAMP signalling. The relative ability of SCRA to induce inhibition of cAMP production via $G\alpha_{i/o}$ is very similar to that observed in previous studies in assays of membrane potential and [35 S]GTP γ S binding [12, 25, 33, 34]. The similar E_{MAX} observed for the SCRA-mediated activation of $G\alpha_{i/o}$ -CB1 signalling probably reflects receptor reserve for inhibition of cAMP accumulation in these cells, wherein maximal responses are elicited at less than maximal receptor occupancy because the system maximum is already achieved [12]. SCRA-induced stimulation of cAMP showed significant differences in E_{MAX} (Table 1), suggesting lower levels of receptor reserve for SCRA coupled to $G\alpha_s$ protein. This may (at least for the drugs with a lower E_{MAX}) reflect an accurate representation of intrinsic efficacy of the ligands at this pathway [35]. The observed dynamic range of E_{MAX}

for cannabinoids is consistent with CB1 having low coupling efficiency to both $G\alpha_s$ -pathway and β -arrestin-2 (as observed previously; [32], compared to that of $G\alpha_i$ -pathway [17, 36, 37]. Future studies could examine the structure of SCRA-bound CB1- $G\alpha_s$ complexes, which might assist in explaining the observed cAMP signalling profiles. This is particularly interesting given that the interaction of SCRA MDMB-FUBINACA with the “toggle twin switch” in the CB1 binding pocket coupled to $G\alpha_i$ was recently studied [38]. The rigid C-shape geometry of MDMB-FUBINACA along with the strong pi-pi interaction of its indazole ring with “toggle twin switch” residues, might help distinguish the high efficacy agonist activity of SCRA from partial agonists like THC lacking “toggle twin switch” interaction [38]. Promiscuous coupling to both $G\alpha_i$ and $G\alpha_s$ has been reported for multiple GPCRs (e.g. β_2 -adrenergic receptor) [39], while some receptors couple pre-dominantly to one G protein subtype (e.g. μ -opioid receptor coupling to the $G\alpha_{i/o}$ family, [40]). The potential of cannabinoids to differentially activate one signalling cascade over another (functional selectivity, [41]) may aid the development of new therapeutic compounds with reduced psychoactive effects; a research domain that has attracted much recent interest [42].

Considering the adverse effects associated with SCRA use, it is important to continue characterizing the pharmacological profile of these compounds in order to understand the mechanisms driving their toxicity [43, 44]. Although this study does not identify which pathway contributes to the toxic effects observed following SCRA consumption, our data do provide valuable insights into SCRA-mediated stimulation and inhibition of cAMP signalling *in vitro*. Previous studies have shown that JWH-018-, AM-2201-, 5F-AB-PINACA-, and CUMYL-4CN-BINACA-induced seizures are CB1-mediated in mice, which might explain some of the toxicity experienced by recreational users of these drugs [43, 44, 45, 46, 47, 48, 49]. Our data shows that SCRA-induced cAMP increase was abolished after SR141716A treatment, supporting the hypothesis that SCRA's $G\alpha_s$ -like effects were mediated through CB1

receptor. All the SCRA tested in this study exhibited greater potency at $G\alpha_i$ - than $G\alpha_s$ -like pathways, and the efficacies of these SCRA have previously been measured in response to $G\alpha_i$ -mediated activation of GIRK channel in AtT20-CB1 cells [12]. The rank order of SCRA efficacy based on selectivity for $G\alpha_i$ -GIRK signalling was found to be 5F-MDMB-PICA > XLR-11 > AB-FUBINACA > PB-22 \approx JWH-018 [12]. 5F-MDMB-PICA showed the highest efficacy for modulation of K channel activity via $G\alpha_i$ -pathway in the former study, in contrast to the intermediate efficacy of 5F-MDMB-PICA to stimulate the $G\alpha_s$ -like cAMP signalling pathway in the present study. AB-FUBINACA exhibited greater efficacy for the $G\alpha_s$ -pathway compared to its $G\alpha_i$ -mediated activity profile in the membrane potential assay [12]. Evaluating the differences in G protein preference between SCRA may be an important part of understanding the apparent differences in effect between these drugs in humans.

Our study showed that SCRA have significantly different pharmacological profiles (maximal activities and potencies) for the activation of CB1-G protein-stimulation and -inhibition of forskolin-mediated cAMP signalling. Although it is speculated that the adverse effects of SCRA are mediated by CB1 [49, 50], based on the results presented here we wonder how the differential responses of SCRA are related to the physiological effects resulting from the activation of each intracellular pathway, and if these may be correlated with the *in vivo* toxicity of SCRA. The unique toxicological profile of SCRA may result from a combination of factors; pharmacokinetic differences, activity at both cannabinoid and non-cannabinoid targets, pharmacological activity of metabolites and thermolytic degradants [25, 37, 51, 52]. These findings may provide a starting point to help predict the pharmacological characteristics of SCRA that demonstrate differential activation of $G\alpha_i$ versus $G\alpha_s$ coupling to CB1.

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Author contributions

S.S. designed and performed experiments, analysed the data and wrote the manuscript. S.D.B. synthesized SCRAAs supervised by M.K. M.S. and C.B. advised on the CAMYEL assay and data analysis. M.C. provided critical feedback and helped shape the research, analysis, and manuscript. All authors reviewed and edited the manuscript.

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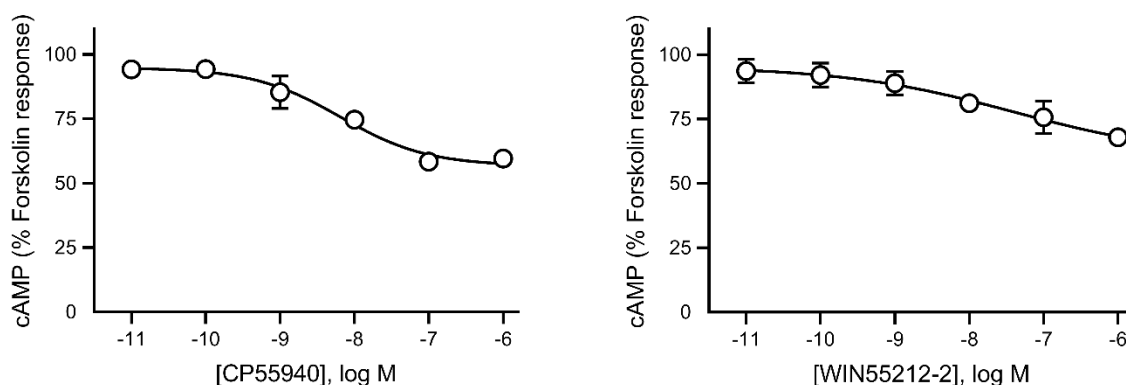
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Table 1

Comparison of pharmacological activity (EC_{50} and E_{MAX}) of SCRA-induced stimulation (G_s (+PTX)) and inhibition (G_i (-PTX)) of cAMP signalling in HEK-CB1 cells. The selectivity is expressed as the ratio of G_s (+PTX) EC_{50} to G_i (-PTX) EC_{50} .

Compound	G_i (-PTX)		G_s (+PTX)		G_i (-PTX) selectivity
	p $EC_{50} \pm$ SEM (EC_{50} , nM)	E_{max} (% of FSK) \pm SEM	p $EC_{50} \pm$ SEM (EC_{50} , nM)	E_{max} (% of FSK) \pm SEM	
CP55940	8.2 ± 0.3 (6.4)	57 ± 5	-	-	-
WIN55212-2	7.4 ± 2 (40)	60 ± 9	-	-	-
JWH-018	7.8 ± 0.3 (16)	64 ± 4	6.7 ± 0.7 (221)	113 ± 4	14
XLR-11	7.2 ± 0.2 (63)	62 ± 3	5.2 ± 0.8 (6490)	127 ± 16	103
PB-22	8.6 ± 0.2 (2.5)	64 ± 3	7.2 ± 0.5 (69)	131 ± 5	28
AB- FUBINACA	9.0 ± 0.2 (1.1)	61 ± 2	6.4 ± 0.5 (383)	144 ± 12	348
5F-MDMB- PICA	9.2 ± 0.2 (0.62)	59 ± 4	7.1 ± 0.4 (85)	126 ± 5	137

Figure 1



Concentration response curve (CRC) for CP55940 and WIN55212-2. Treatment with CP55940 or WIN55212-2 produced a concentration-dependent inhibition of forskolin-mediated cAMP production in HEK 293-CB1. Area under the curve analysis for CP55,940 or WIN55212-2 in the presence of 3 μ M forskolin. Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean \pm SEM for at least 5 independent experiments performed in duplicate.

Figure 2

G α_s mediated signalling of SCRAs. **A.** Representative data for real-time measurement of stimulation of cAMP levels by 10 μ M of cannabinoids (THC, 2-arachidinoylglycerol, and AB-FUBINACA) in HEK cells expressing CB1 receptors, an increase in inverse BRET ratio (emission at 475/535 nm) corresponds to an increase in cAMP. **B.** A bar chart summarising the cAMP signalling peaks for seventeen cannabinoids showing an increase in cAMP levels above that of forskolin alone (100%). Graphs show mean \pm SEM for at least 5 independent experiments performed in duplicate.

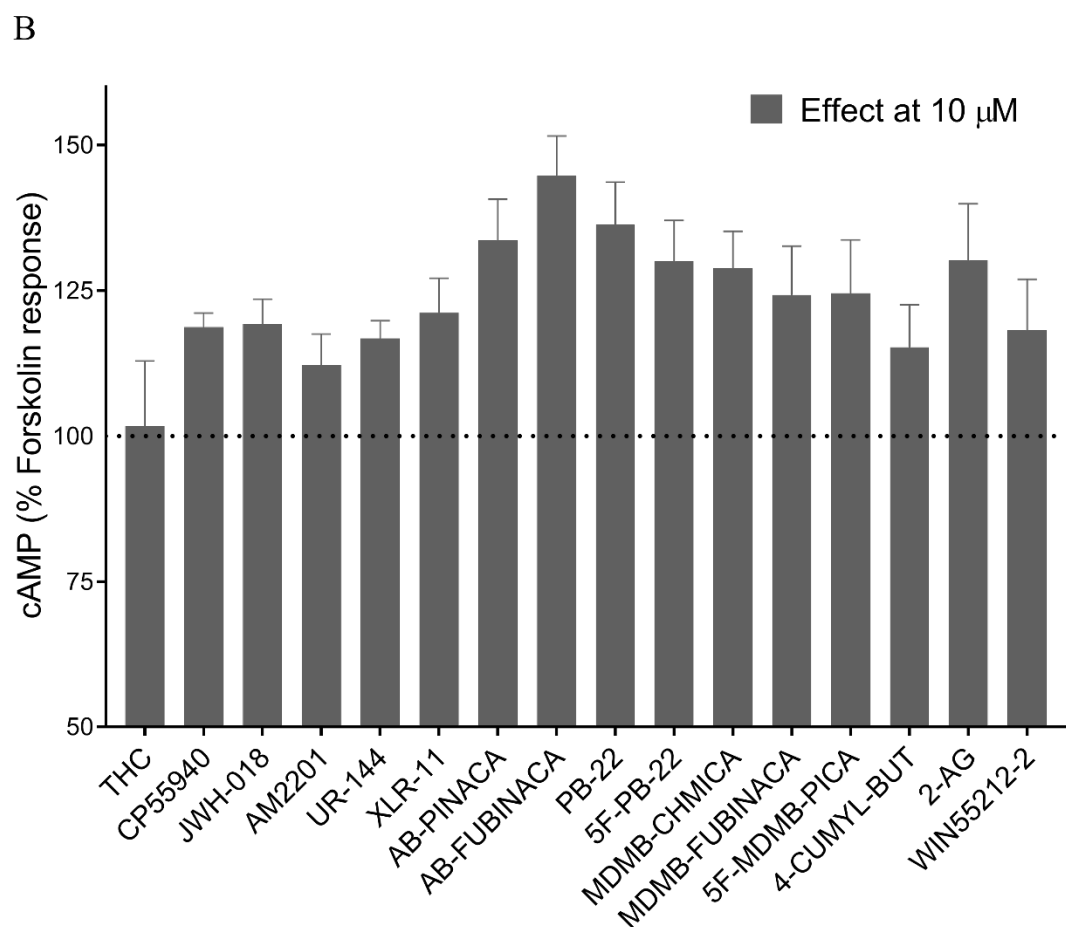
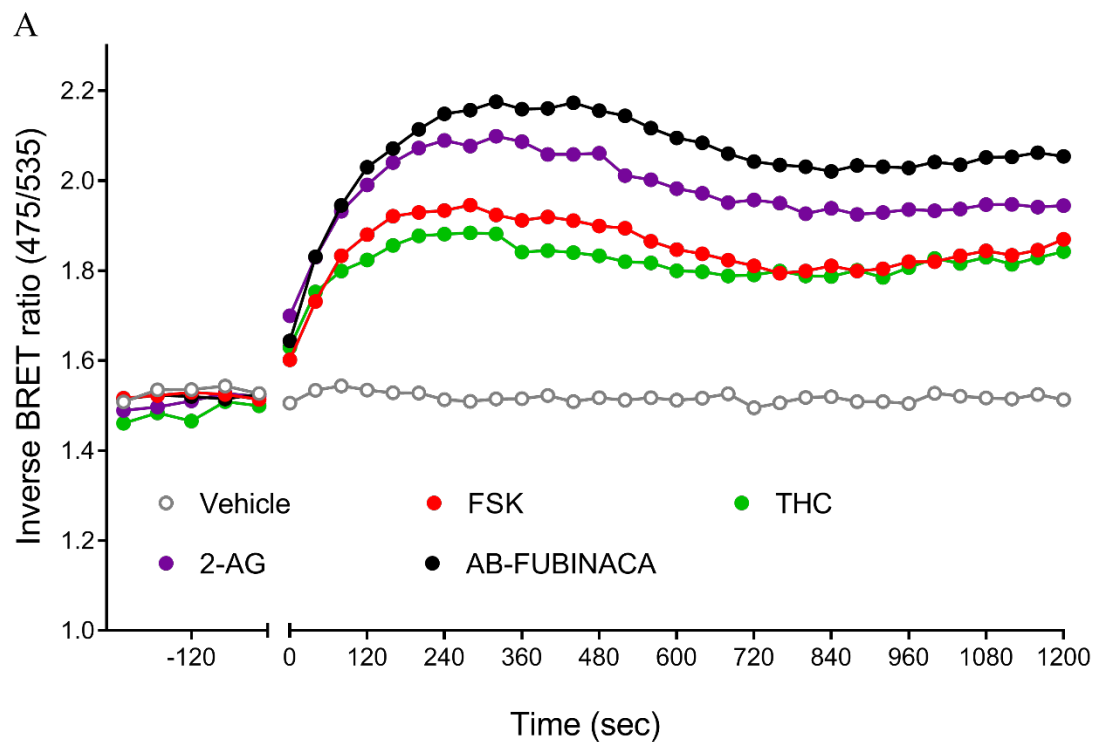


Figure 3

Concentration response curves for SCRA-induced stimulation and inhibition of cAMP signalling. Concentration response relationship for five SCRA (PB-22, 5F-MDMB-PICA, AB-FUBINACA, XLR-11, and JWH-018) for two signalling outputs of CB1 – stimulation and inhibition of cAMP levels following overnight treatment in the absence (- PTX, black), or presence (+ PTX, red) of PTX. Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean \pm SEM for at least 5 independent experiments performed in duplicate. For some points, the error bars are shorter than the height of the symbol.

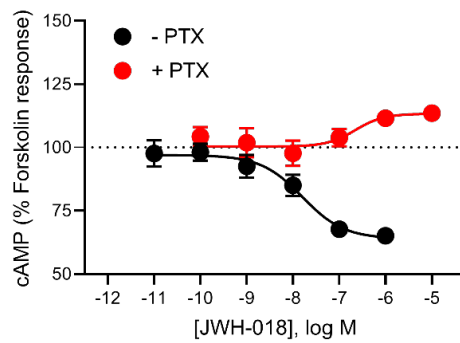
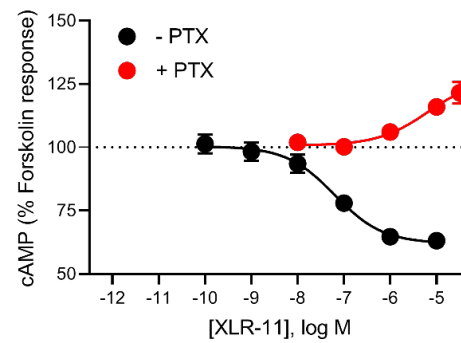
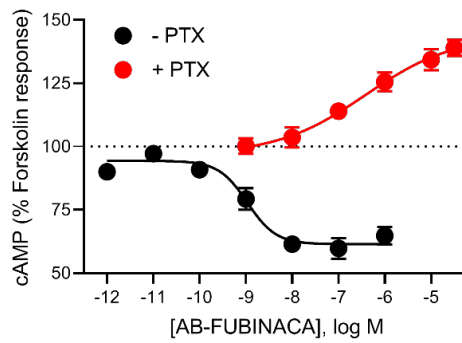
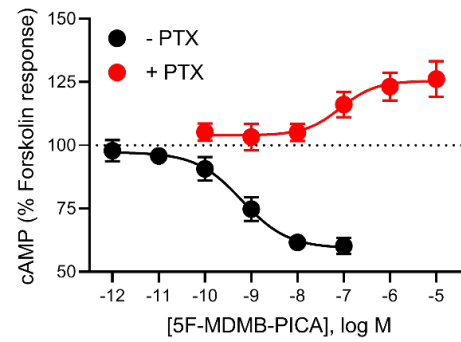
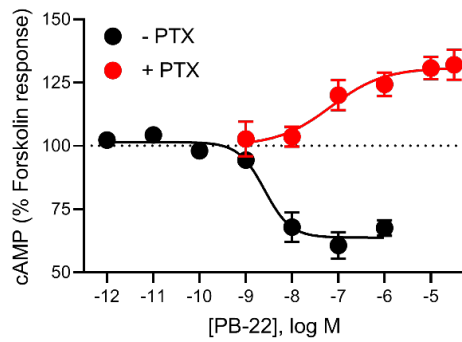


Figure 4

Effect of CB1 antagonist on the SCRA-mediated cAMP signalling peaks in HEK-CB1

cells. A. Traces from a representative experiment showing that SCRA (JWH-018, 5F-MDMB-PICA, and AB-FUBINACA) induced observed stimulatory effects were inhibited by SR141716A (CB1 antagonist, 3 μ M) pre-treatment. **B.** Scatter dot plot representing SCRA-mediated stimulation of forskolin-induced cAMP response in presence and absence of SR141716A 3 μ M on HEK 293 cells expressing CB1. Within each set SCRA (10 μ M) were compared to SCRA + SR141716 (Unpaired Student's t-test, $P < 0.05$ marked with *). Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean \pm SEM for at least 5 independent experiments performed in duplicate.

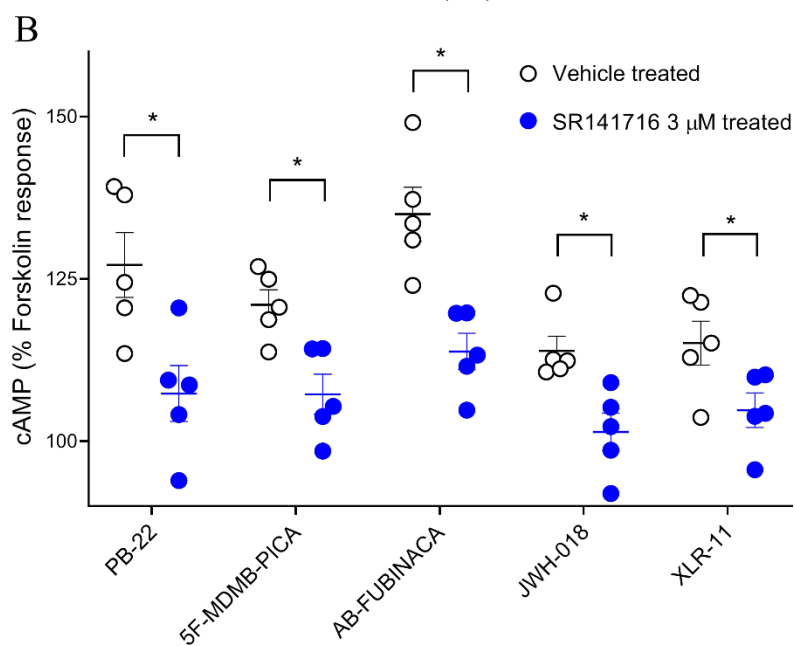
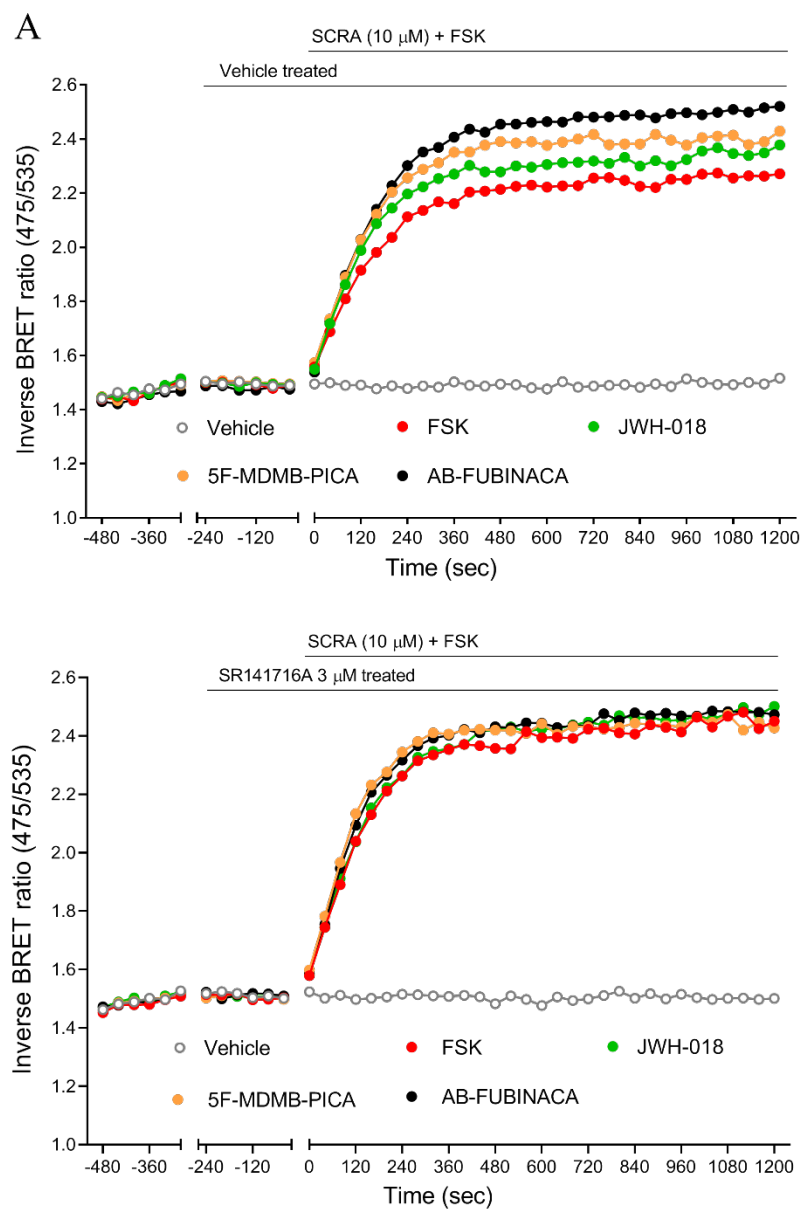
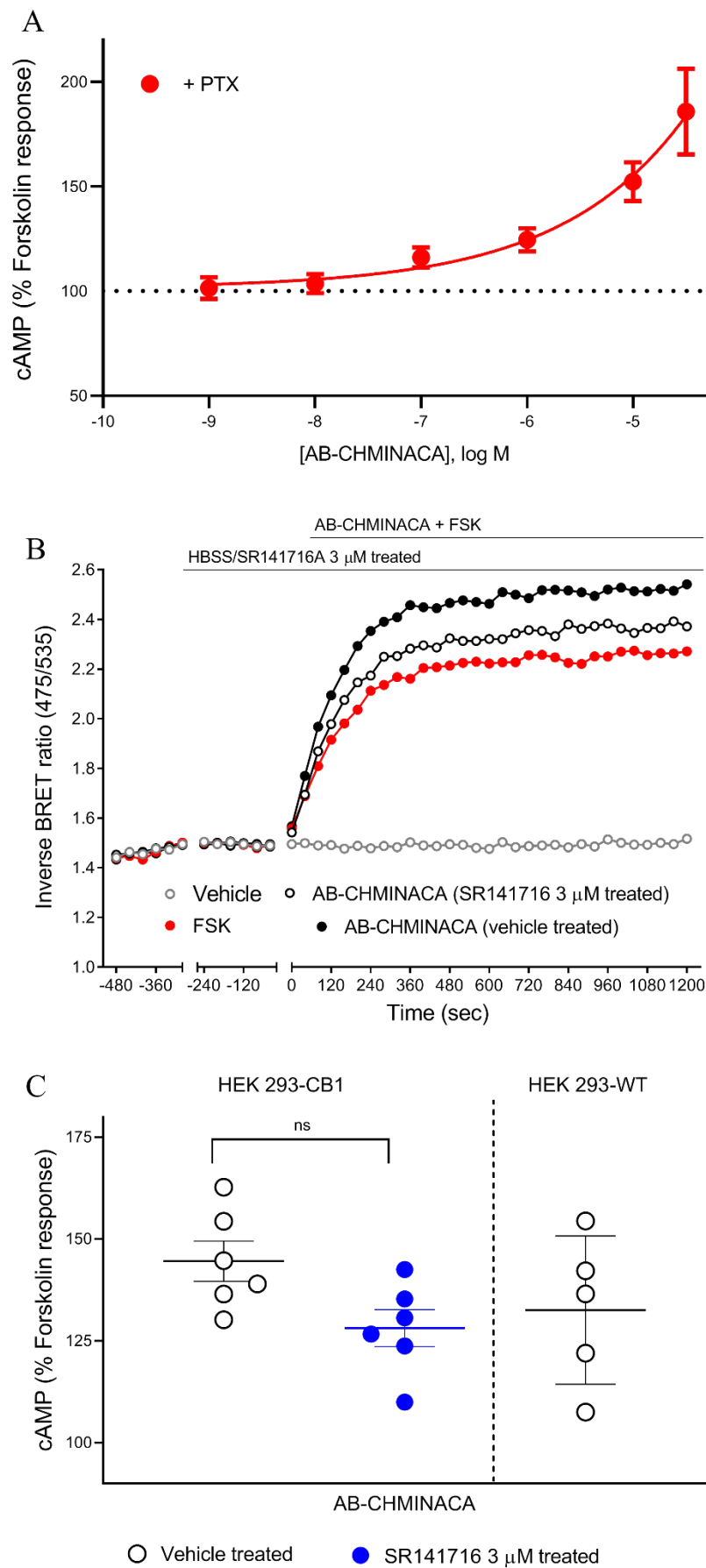


Figure 5

AB-CHMINACA does not modulate cAMP levels via CB1 receptors in HEK 293 cells. A.

Treatment with AB-CHMINACA produced a concentration-dependent increase in forskolin-mediated cAMP production in HEK 293-CB1 in presence of PTX. **B.** Traces from a representative experiment showing that AB-CHMINACA (10 μ M) induced observed stimulatory effects were only partially inhibited by SR141716A 3 μ M. **C.** Scatter dot plot comparing AB-CHMINACA-mediated stimulation of forskolin-induced cAMP response in presence and absence of SR141716 3 μ M in HEK 293-CB1 cells, and the data was not significantly different. AB-CHMINACA (10 μ M) also modestly augmented forskolin-stimulated cAMP levels in HEK-WT cells (not containing CB1 receptors). Graphs show mean \pm SEM for at least 5 independent experiments performed in duplicate.



Supplementary Figure 1

Effect of CB1 antagonist on the SCRA induced inhibition of cAMP signalling. A. Traces from a representative experiment showing that SCRA (JWH-018, 5F-MDMB-PICA, and AB-FUBINACA) induced inhibitory effects were completely blocked by SR141716A (CB1 antagonist, 3 μ M) pre-treatment. **B.** Scatter dot plot representing SCRA-mediated inhibition of forskolin-induced cAMP response in presence and absence of SR141716A 3 μ M on HEK 293 cells expressing CB1. Within each set SCRA (100 nM) were compared to SCRA + SR141716. Data were normalized to forskolin (100%) and vehicle (0%), and plotted as mean \pm SEM for at least 5 independent experiments performed in duplicate.

