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**Interaction between anandamide and sphingosine-1-phosphate in  
mediating vasorelaxation in rat coronary artery.**

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## SUMMARY

**Background:** Anandamide and sphingosine-1-phosphate (S1P) both regulate vascular tone in a variety of vessels. This study aims to examine the mechanisms involved in the regulation of coronary vascular tone by anandamide and S1P, and to determine whether any functional interaction occurs between these receptor systems.

**Experimental approach:** Mechanisms used by anandamide and S1P to regulate rat coronary artery reactivity were investigated using wire myography. Interactions between S1P and the CB<sub>2</sub> receptor were determined using HEK293 cells that stably over-express recombinant CB<sub>2</sub> receptor.

**Key results:** Anandamide and S1P induced relaxation of the rat coronary artery. CB<sub>2</sub> receptor antagonists attenuated anandamide-induced relaxation, while S1P-mediated relaxation was dependent on the vascular endothelium and S1P<sub>3</sub>. Anandamide treatment resulted in an increase in the phosphorylation of sphingosine kinase-1 within the coronary artery. Conversely, anandamide-mediated relaxation was attenuated by inhibition of sphingosine kinase. Moreover S1P<sub>3</sub>, specifically within the vascular endothelium, was required for anandamide-mediated vasorelaxation. In addition to this, S1P-mediated relaxation was also reduced by CB<sub>2</sub> receptor antagonists and sphingosine kinase inhibition. Further evidence that S1P functionally interacts with the CB<sub>2</sub> receptor was also observed in HEK293 cells over-expressing the CB<sub>2</sub> receptor.

**Conclusions and Implications:** In the vascular endothelium of rat coronary artery, anandamide induces relaxation *via* a mechanism requiring sphingosine kinase-1 and S1P/S1P<sub>3</sub>. In addition, we report that S1P may exert some of its effects *via* a CB<sub>2</sub> receptor- and sphingosine kinase-dependent mechanism, where subsequently formed S1P may have privileged access to S1P<sub>3</sub> to induce vascular relaxation.

**Keywords:** Cannabinoids, sphingosine-1-phosphate, sphingosine kinase.

**Abbreviations:** AM251, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; Anandamide, Arachidonylethanolamide; CA, coronary artery; CAY10444, 2-undecyl-thiazolidine-4-carboxylic acid; CB, cannabinoid; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; ERK-1/2, extracellular signal-regulated kinase-1/2; GTP $\gamma$ S, guanosine 5'-[ $\gamma$ -thio]triphosphate; GPCR, G-protein coupled receptor; HA, hemagglutinin; HU210, (6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; JTE-013, 1-[1,3-Dimethyl-4-(2-methylethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicarbazide; JTE907, N-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide; pERK-1/2, phospho-extracellular signal-regulated kinase-1/2; pSK, phospho-sphingosine kinase; SK, sphingosine kinase; SKi, 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole; S1P, sphingosine-1-phosphate; U46619, 9,11-dideoxy-9 $\alpha$ ,11-methanoepoxy prostaglandin F<sub>2 $\alpha$</sub> ; VPC23019, (R)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbonyl)-ethyl] ester; W146, (R)-3-Amino-(3-hexylphenylamino)-4-oxobutylphosphonic acid.

## INTRODUCTION

The psychotropic effects of cannabinoids are well established. There is also growing evidence for the role of endogenous cannabinoids in the regulation of the cardiovascular system, with the release of endogenous cannabinoids in response to stress such as during myocardial infarction reported as being cardioprotective, reducing tissue damage and arrhythmia (Hajrasouliha et al., 2008). Cannabinoids also appear to have a role in modulating vascular tone and the endogenous cannabinoid *N*-arachidonylethanolamide (Anandamide) has been reported to induce relaxation in a variety of vessels, both resistance and conduit, including aorta, mesenteric, hepatic and coronary arteries (CAs) (Herradon et al., 2007, Ho and Randall, 2007, White et al., 2001, Zygmunt et al., 1999). However, no clear consensus has been reached with regards to the mechanisms governing this relaxation.

Cannabinoids can exert their effects *via* activation of two specific G-protein coupled receptors (GPCRs) designated CB<sub>1</sub> and CB<sub>2</sub>, both of which are G<sub>i/o</sub>-coupled and act to inhibit adenylyl cyclase (Howlett et al., 1986, Felder and Glass, 1998). Several studies have reported the importance of these cannabinoid receptors in mediating responses to anandamide, with vascular relaxation mainly attributed to activation of the CB<sub>1</sub> receptor (Romano and Lograno, 2006, O'Sullivan et al., 2004). Conversely, a body of evidence also exists proposing that the effects of anandamide cannot be accredited to either cannabinoid receptor subtype (Grainger and Boachie-Ansah, 2001, White et al., 2001, Herradon et al., 2007). The existence of a novel endothelial receptor referred to as a 'non-CB<sub>1</sub>/nonCB<sub>2</sub>' cannabinoid receptor has been proposed and this receptor has been reported to play a role in mediating relaxation to anandamide (Offertaler et al., 2003, Herradon et al., 2007, Jarai et al.,

1999). In addition, anandamide has been shown to act as a ligand for transient receptor potential vanilloid type 1 (TRPV1) channels (Smart et al., 2000) and in some arteries, such as rat mesenteric the vasodilator effects of anandamide have been attributed to its interaction with this receptor, resulting in release of the potent vasodilator peptide CGRP from sensory nerve endings (Zygmunt et al., 1999, Ho and Hiley, 2003). Furthermore, the role of the vascular endothelium in the vasodilator process is also unclear, as both endothelium-dependent and endothelium-independent mechanisms of anandamide-induced relaxation have been proposed (White et al., 2001, Pratt et al., 1998, Grainger and Boachie-Ansah, 2001, Ho and Hiley, 2003).

At an intracellular level, several mediators have been suggested to facilitate the effect of anandamide. For instance, in bovine ophthalmic arteries and rat aorta, generation of nitric oxide mediates relaxation to anandamide (Romano and Lograno, 2006, Herradon et al., 2007). Studies in a variety of other vessels have also implicated the enzyme cyclooxygenase (COX) in the vasoactive effects of anandamide; some proposing that COX metabolises anandamide following its cellular uptake resulting in the production of vasodilator eicosanoids (Grainger and Boachie-Ansah, 2001, Herradon et al., 2007, Ho and Randall, 2007). Moreover, release of endothelium-derived hyperpolarising factor has also been suggested to play a role in anandamide-induced relaxation (O'Sullivan et al., 2004). In summary, the mechanisms mediating this vasodilator response to anandamide appear to vary significantly depending on the vascular region and species under investigation.

The bioactive lipid mediator sphingosine-1-phosphate (S1P) is also cardioprotective and plays an important role in regulating vascular tone. Synthesised from

sphingosine by the enzyme sphingosine kinase (SK1 and SK2), S1P can exert many of its biological effects *via* five specific GPCRs designated S1P<sub>1-5</sub> (Chun et al., 2002, Pyne et al., 2009). In the vasculature S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> are involved in modulating vascular tone, with the degree and type of vascular response varying between species and vascular beds (Alewijne et al., 2004, Hemmings, 2006, Peters and Alewijne, 2007). For example, mesenteric arteries from the rat have been reported to constrict in response to S1P, whereas rat aorta will dilate when exposed to similar concentrations of S1P (Hemmings et al., 2004, Roviezzo et al., 2006). The effect of S1P on vascular tone appears to depend on the receptor subtypes expressed within that vessel and the distribution of these receptors (Coussin et al., 2002). S1P<sub>1</sub> which is highly expressed on vascular endothelium can mediate vasorelaxation *via* endothelial nitric oxide synthase (eNOS) activation (Igarashi et al., 2001, Igarashi and Michel, 2000). Conversely, S1P is able to increase intracellular calcium levels and activate Rho kinase in smooth muscle cells via S1P<sub>2</sub> leading to vasoconstriction (Peters and Alewijne, 2007, Hemmings, 2006). Therefore, the receptor subtype expression within a vascular bed and the balance between vasodilator and vasoconstrictor signals stimulated by S1P will determine its overall effect on vascular tone.

S1P and cannabinoid receptors both belong to the lysolipid family of GPCRs and exhibit a sequence identity of approximately 20% (Sanchez and Hla, 2004). Furthermore, a link may exist between the cannabinoid and sphingolipid pathways as cannabinoids have been shown to regulate sphingolipid metabolism, with activation of the CB<sub>1</sub> receptor resulting in ceramide accumulation in a variety of cell types (Blazquez et al., 1999, Galve-Roperh et al., 2000). In addition to this,

sphingosine and its analogue, the immunosuppressant FTY720, have also been reported to interact with the CB<sub>1</sub> receptor (Paugh et al., 2006).

Therefore, given the role of both anandamide and S1P in mediating cardioprotection and regulating vascular tone, the present study aims to examine the mechanisms involved in the anandamide- and S1P-mediated regulation of rat coronary vascular tone, and to determine whether any functional interaction/overlapping signalling pathways occurs between these systems.



## MATERIALS AND METHODS

### Materials

Anandamide (in Tocrisolve™100), AM630, JTE907 and AM251 were purchased from Tocris Bioscience (Bristol, UK). Sphingosine-1-phosphate, VPC23019 and W146 were from Avanti Polar Lipid (Alabaster, AL). 4-(4-(4-chlorophenyl)thiazol-2-ylamino)phenol (SKi), U46619 and G418 were from Merck Biosciences Ltd (Nottingham, UK) and CAY10444 was from Axxora (Nottingham, UK). The following chemicals were purchased from Sigma-Aldrich (Poole, UK): Indomethacin, Acetylcholine, Insulin and Phenylmethanesulphonylfluoride (PMSF). Cell culture supplies were obtained from Invitrogen (Paisley, UK). Anti-ERK-2 and anti-phosphorylated ERK-1/2 antibodies were from BD Biosciences (Oxford, UK), anti-phospho-specific sphingosine kinase 1 (Ser-225) from ECM Biosciences (Versailles, KY) and anti-hemagglutinin (HA) antibody from Sigma-Aldrich (Poole, UK). GTPγS and [<sup>35</sup>S]-GTPγS (1250Ci/mmol) were purchased from Bioquote Ltd (York, UK) and Perkin Elmer LAS (Beaconsfield, UK) respectively.

### Tissue Preparation and Myography

All experimental procedures complied with the United Kingdom Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male Sprague Dawley rats (250-400g body weight) were killed with a rising concentration of CO<sub>2</sub> and the heart removed and transferred to cold modified Krebs-Henseleit solution (NaCl 118mM, NaHCO<sub>3</sub> 25mM, KCl 4.7mM, KH<sub>2</sub>PO<sub>4</sub> 1.2mM, MgSO<sub>4</sub> 1mM, CaCl<sub>2</sub> 2.5mM and glucose 11mM). The left anterior descending and right CAs were then carefully dissected free from the heart tissue. 2mm segments were mounted in a wire myograph and maintained at 37°C in Krebs-Henseleit

solution with a constant supply of 95%O<sub>2</sub>/5%CO<sub>2</sub>. Vessels were then normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100mmHg (Mulvany and Halpern, 1977). Tissue was allowed to equilibrate for 30 minutes prior to challenge with 80mM KCl to assess viability. In order to determine vascular endothelial function, vessels were precontracted with the thromboxane A<sub>2</sub> mimetic U46619 (0.3µM) and the response to 1µM acetylcholine (ACh) observed. Relaxation to ACh >50% denoted endothelium intact vessels. Where indicated, vessels were denuded of endothelium by gentle rubbing of the intimal surface with a human hair. Successful denudation was confirmed by a vasodilator response to ACh of <10%.

### **Myography Experimental Protocol**

After 30 minutes equilibration, vessels were sub-maximally precontracted with U46619 (0.3 µM). When a stable level of contraction was achieved, concentration-response curves were generated by cumulative addition of agonist. Anandamide and sphingosine-1-phosphate (S1P) (1nM - 30µM) were added in half-log molar concentrations in a cumulative manner with five minutes between each addition. In the case of anandamide, PMSF (200µM) an anandamide breakdown inhibitor was added 10 minutes prior to the first addition. In experiments where the effects of inhibitors on agonist-induced relaxation were to be investigated, the inhibitor of interest was added 10 minutes before precontraction with U46619 and was present during construction of the concentration response curve.

### **Cell Culture**

Human embryonic kidney 293 (HEK 293) cells were maintained at 37°C with 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM) supplemented with essential amino

acids, penicillin (50U/ml), streptomycin (50µg/ml) and 10% (v/v) foetal calf serum (FCS) whereas stable over-expressing HA-tagged CB<sub>2</sub> receptor HEK 293 (CB<sub>2</sub> HEK 293) cells (gift from Prof K Mackie, Indiana University, IN, USA) were maintained in DMEM supplemented with 10% (v/v) FCS and 800µg/ml G418, also known as Geneticin). All HEK 293 cells were quiesced in serum free medium for 24 hours before experimentation. MCF-7 cells were grown in DMEM supplemented with penicillin (50U/ml), streptomycin (50µg/ml), 10% (v/v) FCS, 0.4% G418 and 15µg/ml insulin and maintained at 37°C with 5% CO<sub>2</sub>. MCF-7 cells were quiesced for 48 hours in serum free media before experimentation.

### **[<sup>35</sup>S]GTPγS (guanosine 5'- [γ-<sup>35</sup>S]thio]triphosphate) Binding Assay**

HEK293 or CB<sub>2</sub> HEK293 cells were homogenized in buffer containing 20mM HEPES (pH7.5), 50mM NaCl and 2mM EDTA. Nuclei and cell debris were removed by centrifugation at 2000G for 5 minutes at 4°C and the resulting supernatant centrifuged at 100000G for 1 hour at 4°C. The pellet containing the membrane fraction was then rinsed with and resuspended in buffer containing 50mM HEPES (pH7.5), 100mM NaCl, 1mM MgCl<sub>2</sub> and 2mM EDTA. Samples containing 20µg of membrane protein were then incubated for 15 minutes at 30°C in buffer containing 50mM HEPES (pH7.5), 100mM NaCl, 5mM MgCl<sub>2</sub>, 10µM GDP, 2mM DTT, 0.1nM [<sup>35</sup>S]GTPγS and the indicated concentrations of agonist and/or antagonist. Non specific binding was determined in the presence of unlabelled GTPγS (20µM). Reactions were terminated by rapid filtration through GF/C glass fibre filters using a 24-well Brandel Cell Harvester. Filters were then rinsed with wash buffer containing 20mM Tris/HCl (pH7.4), 120mM NaCl and 25mM MgCl<sub>2</sub> and membrane bound radioactivity quantified by liquid-scintillation counting. Samples were run in duplicate.

## **Immunoblotting**

In experiments where rat coronary artery tissue was used, vessel rings were mounted in a wire myograph and the tension on the vessels normalised as already described prior to stimulation with anandamide for 30 minutes. Vessel rings were then carefully removed from the myograph. Four coronary artery rings were pooled together and homogenised in RIPA buffer (50mM sodium HEPES, pH 7.5, 150mM sodium chloride, 5mM EDTA, 10mM sodium fluoride, 10mM sodium phosphate, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1mM phenylmethylsulphonyl fluoride, 10µg/ml soybean trypsin inhibitor, 10µg/ml benzamidine) to make one sample. Analysis of proteins by SDS-PAGE and immunoblotting was performed as previously described (Long et al., 2006) using anti-ERK2, anti-phosphorylated ERK1/2, anti-HA and anti-phospho-SK1 antibodies. Densitometry was performed on each resulting film exposure to determine normalised levels of the protein of interest using Molecular Analyst software version 2.1.

## **Statistical Analysis**

Relaxation responses are expressed as a percentage loss of the tone induced by U46619.  $EC_{50}$  values were obtained from individual concentration-response curves by fitting the data to a logistics equation (GraphPad PRISM version 4). Data are given as mean  $\pm$  SEM of  $n$ , where  $n$  = the number of arteries from different animals. Statistical comparisons of concentration-response curves were made by two-way ANOVA of the whole data set. Where appropriate one-way ANOVA with Newman-Keuls multiple comparison post tests were employed.  $P$  values less than 0.05 were considered to be statistically significant.

All drug/molecular target nomenclature conform to the British Journal of Pharmacology Guid to Receptors and Channels (Alexander et al.,2009)

## RESULTS

### **Anandamide induces vascular relaxation via the cannabinoid 2 (CB<sub>2</sub>) receptor subtype.**

Addition of anandamide (1nM-30µM) to U46619-pre-contracted intact rat CA resulted in a concentration-dependent, slowly developing relaxation, which reached a maximum of 42.1±5.9%, EC<sub>50</sub> 2.36x10<sup>-8</sup>M, n=13. The relaxant effect of anandamide was not reduced by denudation of the endothelium (maximal relaxation 44.6±4.1%, EC<sub>50</sub> 2.23x10<sup>-8</sup>, n=7, Figure1A).

The pre-treatment of the vessels with the selective CB<sub>1</sub> receptor antagonist AM251 (10µM) did not significantly alter the magnitude of the vasorelaxation induced by anandamide (n=6, Figure 1B, Table 1). However, the CB<sub>2</sub> selective antagonist AM630 (100nM) significantly attenuated the relaxant response to anandamide (n=4, p<0.05, Figure1C),. Initial experiments using AM630 at a higher concentration of 10µM also attenuated anadamide-mediated relaxation reducing the maximal relaxation to 28.5±5.8%, n=6, p<0.05, Table1). Similarly, the CB<sub>2</sub> selective inverse agonist JTE907 (10µM) also markedly reduced relaxation in response to anandamide (maximal relaxation 12.8±2.5%, n=5, Table 1). However, in endothelium denuded vessels AM630 (100nM) had no effect on the ability of anandamide to induce vascular relaxation (n=4, Figure 1D, Table 1).

In addition to the vasodilator effects of anandamide, HU210 a non-selective CB<sub>1/2</sub> agonist also mediates relaxation of the rat CA. This response was not inhibited by

the CB<sub>1</sub> antagonist AM251 (n=5, Figure 1E, Table 1). It was however significantly attenuated by the CB<sub>2</sub> antagonist AM630 (10µM) (n=4, p<0.05, Figure 1F, Table 1).

### **S1P stimulates vascular relaxation in an endothelium-dependent manner.**

To confirm that S1P is a vasorelaxant in rat CA, its effect on CA tone was directly assessed. In endothelium-intact U46619 pre-contracted arteries, S1P caused a concentration-dependent relaxation. In common with anandamide, this was a slow onset response resulting in a maximum relaxation of 47.7±7.3%, n=10 (Figure 2A, Table 2). In vessels where the endothelial layer was removed, relaxation in response to S1P was markedly reduced (maximal relaxation 22.5±10%, n=8, p<0.05, Figure 2A, Table 2). Furthermore, responses to S1P were not significantly inhibited by the S1P<sub>1</sub> antagonist W146 (Figure 2B, Table 2, n=5) or JTE013, an S1P<sub>2</sub> antagonist (Figure 2C, Table 2, n=6). However, the S1P<sub>3</sub> antagonist CAY10444 did significantly attenuate S1P-mediated relaxation (22±6.2%, n=5, p<0.05, Figure 2D, Table 2).

### **Role for cyclooxygenase (COX) in anandamide and S1P-mediated relaxation.**

The non-selective COX inhibitor indomethacin (10µM) was utilised to determine whether COX plays a role downstream of anandamide and S1P in mediating vascular relaxation in the rat CA. Pre-incubation with indomethacin significantly reduced the vasodilator effect of anandamide in endothelium-intact vessels (maximal relaxation 22.7±4.7%, n=7, p<0.05, Figure 3A, Table 1) but did not significantly alter responses to anandamide in denuded vessels (maximal relaxation 56.5±9.1%, n=4, Figure 3B, Table 1). Responses to S1P in intact vessels were also significantly attenuated in the presence of indomethacin (maximal relaxation 11±15.8%, n=4,

p<0.05, Figure 3C, Table 2), indicating the involvement of COX in both anandamide- and S1P-induced relaxation of rat CA.

### **Sphingosine kinase is required for anandamide-induced relaxation.**

Stimulation of rat coronary artery rings with anandamide resulted in a  $50.9 \pm 11.8\%$  increase in the Ser225 phosphorylation of SK1 ( $n=4$ ,  $p<0.05$ , Figure 4A). The phosphorylation of SK1 on Ser225 has been demonstrated before in response to agonist stimulation, and is catalysed by ERK-1/2 (Pitson et al., 2003). Indeed, anandamide stimulation of rat coronary artery rings also increased in the levels of phosphorylated ERK-1/2 ( $33.1 \pm 6.4\%$  increase,  $n=4$ ,  $p<0.05$ , Figure 4A). The SK1 inhibitor, SKi, 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole (French et al., 2003) also had a marked effect on responses to anandamide, almost completely inhibiting relaxation (maximal response  $2.3 \pm 6.2\%$ ,  $n=5$ ,  $p<0.05$ , Figure 4B, Table 1), suggesting that anandamide-mediated vasorelaxation requires SK1/S1P. In contrast to this, incubation with SKi has no effect on anandamide-mediated relaxation in endothelium-denuded vessels ( $n=4$ , Figure 4C, Table 1), highlighting a role for SK1/S1P specifically within the vascular endothelium

### **Anandamide-induced relaxation is mediated by S1P<sub>3</sub> within the vascular endothelium.**

Selective S1P antagonists were utilised to determine if the S1P that would be potentially formed by the anandamide-mediated phosphorylation and activation of SK1 can mediate its effects by acting *via* S1P receptors. The selective S1P<sub>1/3</sub> antagonist, VPC23019, significantly attenuated anandamide-mediated relaxation in the rat coronary artery (maximal response  $16.8 \pm 2.5\%$ ,  $n=8$ ,  $p<0.05$ , Figure 5A, Table 1) but had no effect on the anandamide response in denuded arteries, suggesting any S1P produced in response to anandamide exerts its effect by acting on S1P<sub>1/3</sub> receptors on the vascular endothelium (maximal relaxation  $39.6 \pm 3.3\%$ ,  $n=8$ , Figure 5B, Table 1). To determine the individual effects of S1P<sub>1</sub> and S1P<sub>3</sub> in mediating the



anandamide response selective S1P<sub>1</sub> and S1P<sub>3</sub> were employed. W146, a selective S1P<sub>1</sub> antagonist had no effect on anandamide-induced relaxation (Figure 5C, Table 1). Similar to its effects on exogenously added S1P, the S1P<sub>3</sub> antagonist CAY10444 was found to significantly reduce the relaxation to anandamide in endothelium-intact vessels (maximal relaxation 15.9±2.9%, n=5, Figure 5D, Table 1). However, in endothelium-denuded vessels the inhibitory effect of CAY10444 was no longer observed (maximal relaxation 52±6.1 %, n=6, Figure 5E, Table 1), indicating that anandamide-induced relaxation involves the activation of S1P<sub>3</sub> on the vascular endothelium. Furthermore, combined pretreatment with both AM630 (100nM) and CAY10444 (10µM) while inhibiting the anandamide response does not have an additive effect in attenuating vascular relaxation, suggesting that anandamide does not act directly on S1P<sub>3</sub>.

### **S1P induced relaxation involves CB<sub>2</sub> receptors and requires SK to mediate its effect.**

To further investigate the relationship between S1P and anandamide mediated responses, we examined whether S1P responses involved cannabinoid receptors. The pre-treatment of the rat CA with the CB<sub>2</sub> antagonist AM630 significantly attenuated S1P-mediated relaxation, reducing the maximal relaxation to 25±7.3%, n=6, p<0.05 (Figure 6A). The CB<sub>2</sub> inverse agonist JTE907 also markedly reduced relaxation in response to S1P (maximum relaxation 21.2±3.8%, n=5, p<0.05, Figure 6B). Furthermore, in MCF-7 cells where ERK-1/2 activation responses to S1P are S1P<sub>3</sub>-mediated (Sukocheva et al., 2006), and can be completely abolished by pre-treatment with CAY10444, neither AM630 nor JTE907 had any effect on the ability of S1P to activate ERK-1/2 (n=4, Figure 6C), demonstrating that neither AM630 nor JTE907 are S1P<sub>3</sub> antagonists. Therefore, we can rule out the possibility that these

antagonists block S1P<sub>3</sub> receptors in the vessels. In addition, similar to anandamide, relaxant responses to S1P were significantly decreased in the rat CA in the presence of the SK1 inhibitor, SKi (22.8±7.7%, n=5, p<0.05, Figure 6D), suggesting that S1P-induced vasorelaxation requires SK1.

### **S1P functionally interacts with CB<sub>2</sub> receptor activation.**

The findings presented in Figure 5 suggest that S1P may activate CB<sub>2</sub> receptors in the rat coronary artery. In order to further establish whether S1P can functionally interact with CB<sub>2</sub> receptors, cell based assays using HEK293 cells which stably over-express CB<sub>2</sub> receptors (CB<sub>2</sub>HEK293s) were carried out. Experiments were also undertaken using parental HEK293s for comparison; these cells also endogenously express S1P receptors (see later for effect of S1P and CB<sub>2</sub> receptor antagonists). Firstly, [<sup>35</sup>S]GTPγS binding assays were undertaken in these cells to establish if S1P can activate CB<sub>2</sub> receptors. HU210, a known CB<sub>1/2</sub> receptor agonist was found to stimulate [<sup>35</sup>S]GTPγS binding in CB<sub>2</sub>HEK293 cells but not parental HEK 293s (n=3, p<0.05, Figure 7A). Similarly, treatment with S1P also resulted in an increase [<sup>35</sup>S]GTPγS binding in CB<sub>2</sub>HEK293s but not parental cells (n=3, p<0.05, Figure 7B). Furthermore, the elevation in [<sup>35</sup>S]GTPγS binding to CB<sub>2</sub>HEK293 membranes stimulated by both HU210 and S1P was significantly attenuated by the CB<sub>2</sub> antagonist AM630 (n=4, p<0.05, Figure 7C), suggesting both HU210 and S1P are agonists of the CB<sub>2</sub> receptor.

The effects of S1P and antagonists on the activation of the ERK-1/2 pathway in parental and CB<sub>2</sub>HEK293 cells was also assessed as a reporter of CB<sub>2</sub> receptor function. Stimulation of both parental HEK293 and CB<sub>2</sub>HEK293s cells with S1P resulted in a marked elevation in the levels of phosphorylated ERK-1/2 (Figure 8A, p<0.01, n=3). The pre-treatment with either of the CB<sub>2</sub> antagonists, AM630 or JTE907 alone had no effect on the ability of S1P to induce ERK-1/2 phosphorylation in either cell type (Figure 8A). However, the S1P response was completely abolished following pre-treatment of HEK293 cells with a combination of CAY10444 (20μM) and W146 (20μM), suggesting that in the parental HEK293 cells, S1P

induces activation of ERK-1/2 *via* S1P<sub>1/3</sub> (Figure 8B). In contrast to this, pre-treatment of CB<sub>2</sub>HEK293 cells with combined CAY10444 (20μM) and W146 (20μM) had no effect on the ability of S1P to induce ERK-1/2 activation (Figures 8B), suggesting that the major effect of S1P in these cells is mediated *via* a distinct receptor. Evidence that this distinct receptor might be CB<sub>2</sub>, was obtained by results showing that the stimulatory effect of S1P on ERK-1/2 activation was no longer observed in CB<sub>2</sub> HEK293 cells that were treated with AM630 in addition to CAY10444 and W146 (Figure 7B). Similar results were also obtained using JTE907 in the presence of S1P antagonists (data not shown). The pre-treatment with combined W146 and CAY10444 and AM630 or JTE907 did not significantly alter basal levels of phosphorylated ERK in either cell type.

## DISCUSSION

Anandamide and S1P both play an important role in regulating vascular tone. Here we show that both these lipid mediators stimulate relaxation in isolated rat CAs and that a functional interaction between these receptor systems exists within the vascular endothelium to regulate vasorelaxation.

Various studies have reported the role of the CB<sub>1</sub> receptor in mediating relaxation to anandamide (Romano and Lograno, 2006, O'Sullivan et al., 2004). However, the present study found that AM251, a selective CB<sub>1</sub> antagonist had no effect on anandamide-induced vasorelaxation. Blocking CB<sub>2</sub> receptors, on the other hand, with either AM630 or JTE907, significantly attenuated the effects of anandamide. Similar effects were also observed on relaxation to the synthetic cannabinoid agonist HU210 (. These findings suggest the CB<sub>2</sub> receptor is important in mediating the effects of cannabinoid agonists on rat CA reactivity. Moreover, the inhibitory effect of AM630 on anandamide-mediated relaxation is abolished in endothelium-denuded vessels, specifically implicating the vascular endothelium in CB<sub>2</sub> receptor-mediated vascular relaxation. The CB<sub>2</sub> receptor has mainly been suggested to play a role in cardioprotection. For instance, remote ischaemic preconditioning is thought to involve the release of endogenous cannabinoids, which exert their cardioprotective effect by acting on the CB<sub>2</sub> receptor (Hajrasouliha et al., 2008).

Similar to anandamide, we found that S1P also induced relaxation of the rat CA and that this effect was significantly attenuated by removal of the vascular endothelium. Furthermore, this vasodilator effect of S1P appears to be mediated by activation of S1P<sub>3</sub>, as CAY10444 was able to significantly attenuate S1P-induced vascular relaxation. A large body of evidence exists implicating S1P<sub>3</sub> in the vasodilator

effects of S1P, mainly via activation of eNOS (Nofer et al., 2004). However, in this instance, pre-treatment of the coronary vessels with L-NAME had no effect on S1P-stimulated relaxation suggesting NO is not involved in the vasoactive effects of S1P in this vessel (data not shown).

Both anandamide and S1P induce relaxation of a similar magnitude in the rat CA, which is partially sensitive to the COX inhibitor indomethacin, suggesting a common downstream pathway involving a vasodilator prostanoid. Only the endothelium-dependent component of anandamide-induced relaxation is inhibited by indomethacin. Furthermore, the effects of S1P are also dependent on the endothelial layer, suggesting any common pathway shared by anandamide and S1P may occur specifically within the vascular endothelium.

Recently cannabinoids have been implicated in the regulation of sphingolipid metabolism (Giuliano et al., 2006). In this study we investigated the potential role of SK1, the enzyme responsible for synthesising S1P, in mediating the effects of anandamide. There are two mammalian SK isoforms, SK1 and SK2, which are ubiquitously expressed. SK1 can be phosphorylated by ERK-1/2 (Pitson et al., 2003), which results in the phosphorylation of the enzyme on Ser225. This phosphorylation is required for translocation of SK1 to the plasma-membrane, where it produces S1P for 'inside-out' signaling at S1P receptors. This involves the release of S1P into the extracellular milieu and/or partition into lipid micro-environments in close proximity to the S1P receptor, therefore allowing privileged and efficient binding of S1P to the S1P receptor. Indeed, various stimuli shown to activate SK1, induce its translocation to the plasma membrane (Sarkar et al., 2005, Melendez and Khaw, 2002). Here we demonstrate that stimulation of the rat CA with anandamide

stimulates Ser225 phosphorylation of SK1, suggesting a potential role for SK1 in 'inside-out' signalling. The pre-treatment of rat CAs with the SK inhibitor SKi also completely abolished the ability of anandamide to induce relaxation, thereby suggesting a role for SK1 in mediating the effects of anandamide. SKi also had similar effects on HU210-induced relaxation (data not shown). Additionally, the inhibitory effect of SKi on anandamide-mediated relaxation was dependent on the vascular endothelium. Moreover, CAY10444, an S1P<sub>3</sub> antagonist, significantly attenuated anandamide-induced relaxation. This effect was also abolished by removal of the vascular endothelium. These findings suggest that SK1 is required downstream of cannabinoids and that S1P that is likely to be formed then acts at S1P<sub>3</sub> to induce vasorelaxation, a process that appears to occur specifically within the endothelial layer. To support this model others have demonstrated that the generation of intracellular S1P by the activation of SK1 is involved in mediating the effects of various agonists and phorbol esters (Taha et al., 2006, Alemany et al., 2007). Furthermore, S1P can be released from cells to exert its effects extracellularly *via* S1P receptors (Kobayashi et al., 2006). Endothelial cells have been reported to release both S1P and active SK to form S1P outside the cell (Ancellin et al., 2002, Venkataraman et al., 2006). Finally, in cardiac myocytes a similar mechanism of action has been proposed for the adipose-derived plasma protein adiponectin (APN) whereby APN was found to activate SK to generate S1P which then exerted its effects *via* activation of the S1P<sub>1/3</sub> (Ikeda et al., 2008).

The role of the CB<sub>2</sub>, S1P/SK1 and S1P<sub>3</sub> in anandamide-induced relaxation appears confined to the endothelial layer. However, in denuded vessels anandamide still induces relaxation to a magnitude similar to that observed in endothelium intact vessels. This suggests that a compensatory mechanism exists within the vascular

smooth muscle allowing anandamide to mediate vasorelaxation in the event of endothelial damage. Others have also reported the existence of distinct mechanisms that regulate the effects of anandamide in vascular endothelium and smooth muscle (O'Sullivan et al., 2004, Romano and Lograno, 2006, Mukhopadhyay et al., 2002). Although not investigated in the current study in rat CA, one potential compensatory mechanism that may mediate anandamide-induced relaxation in the absence of the vascular endothelium is the activation of large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\text{BK}_{\text{ca}}$ ).  $\text{BK}_{\text{ca}}$  have previously been reported to be activated by anandamide within vascular smooth muscle, inducing relaxation (White et al., 2001).

We conclude from the current findings that two distinct mechanisms of anandamide-induced relaxation exist, one dependent on the vascular endothelium involving the  $\text{CB}_2$  receptor,  $\text{S1P/S1P}_3$  and COX, in addition to another compensatory mechanism that acts independently of the endothelial layer.

In addition to this, S1P may elicit its effects *via* a process that requires the  $\text{CB}_2$  receptor, as both AM630 and JTE907 significantly reduced the vasorelaxant response to S1P in the rat CA. The effect is not due to the blockade of  $\text{S1P}_3$  as neither AM630 nor JTE907 attenuated the ability of S1P to induce ERK-1/2 activation in MCF-7 cells, a breast cancer cell line where the effects of S1P are *via*  $\text{S1P}_3$  activation (Sukocheva et al., 2006). Moreover, AM630 and JTE907 did not modify the activation of ERK-1/2 by S1P in parental HEK293s, which express functional  $\text{S1P}_1$  and  $\text{S1P}_3$ . The finding that S1P induced relaxation is blocked by  $\text{CB}_2$  receptor antagonists suggests that S1P might use the  $\text{CB}_2$  receptor in order to induce vascular relaxation. This is supported by our studies using  $\text{CB}_2$  HEK293 cells where S1P was shown to stimulate GTP $\gamma$ S binding, and that this was blocked by



AM630. Furthermore, ERK-1/2 activation in response to S1P in these cells was still observed following pre-treatment with a combination of S1P<sub>1</sub> and S1P<sub>3</sub> antagonists sufficient to significantly reduce S1P-induced ERK-1/2 activation in the parental HEK293 cells. In addition, the ability of S1P to stimulate ERK activation in CB<sub>2</sub> HEK293s when S1P<sub>1/3</sub> are blocked is completely ablated when these cell are concurrently treated with CB<sub>2</sub> antagonists. Given that CB<sub>2</sub> antagonists on their own have no inhibitory effect of S1P-mediated ERK activation unless S1P<sub>1/3</sub> are blocked, these findings suggest that S1P may activate CB<sub>2</sub> receptors in HEK293 cells to initiate downstream signalling cascades when the functionality of S1P receptors is experimentally impaired. Others have also highlighted a link between lysolipids and cannabinoid receptors. For instance, sphingosine has been shown to interact with cannabinoid receptors; this study reported that sphingosine and its analogue FTY720 were able to bind to CB<sub>1</sub> receptors where they acted as antagonists (Paugh et al., 2006). Moreover, both cannabinoids and lysophosphatidylinositol (LPI), an acidic lysophospholipid similar to S1P, have been proposed to act as agonists at the orphan GPCR, GRP55 (Oka et al., 2007).

We also observed that relaxation to S1P in the rat CA is significantly reduced by SKI, suggesting a role for SK1 downstream of CB<sub>2</sub> receptors. Thus, in rat CA, stimulation of CB<sub>2</sub> receptors in response to S1P may provide an alternative mechanism by which S1P mediates vascular relaxation through the generation of intracellular S1P, which is then released locally to access S1P<sub>3</sub>. This mechanism may deliver S1P more efficiently to S1P<sub>3</sub> compared with exogenous S1P, suggesting privileged access to S1P receptors, possibly *via* efficient disposition of S1P in a lipid microdomain in close proximity to S1P<sub>3</sub>.

In summary, both anandamide and S1P induced vascular relaxation in the rat CA, with the involvement of both CB<sub>2</sub> and S1P<sub>3</sub>. Within the vascular endothelium both lipid mediators require COX to exert their vasodilator effects, potentially by prostaglandin production. Phosphorylation of ERK-1/2 is known to activate cytosolic phospholipase A<sub>2</sub> resulting in an increase in arachidonate production and subsequent prostanoid production *via* COX. Therefore, ERK activation within the coronary artery in response to anandamide may result in the production of vasodilator prostaglandins. For the first time we demonstrate that SK1 has an important role in anandamide-induced relaxation and suggest that activation of SK1 by anandamide may result in the generation of S1P which can then exert its effects via activation of S1P<sub>3</sub> on the vascular endothelium. We also provide evidence that S1P might use the CB<sub>2</sub> receptor to stimulate localised production of S1P in the vicinity of S1P<sub>3</sub>, the activation of which mediates coronary vascular relaxation.

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## **CONFLICT OF INTEREST**

None declared.

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**Figure 1.** Anandamide induces vascular relaxation via the CB<sub>2</sub> receptor. Concentration-response curves for the vasorelaxant effect of anandamide (1nM-30μM) in **A** endothelium-intact (n=13) and endothelium-denuded (n=7) rat CA and concentration-response curves for the relaxation of U46619-induced tone in endothelium-intact rat CA for anandamide (1nM-30μM) in the presence of **B** AM251, a selective CB<sub>1</sub> antagonist (10μM) (n=6), **C** AM630, a selective CB<sub>2</sub> antagonist (100nM) (n=5) and **D** AM630 (100nM), (n=4) in endothelium-denuded rat CA. **D** concentration-response curves showing the vasorelaxant effect of HU210 alone (1nM-30μM) and in the presence of AM251 (10μM) (n=6) and **E** in the presence of AM630 (10μM) (n=4). Data are expressed as a percentage of U46619-induced tone and shown as mean ± SEM for *n* arteries from different animals.. \* *P*<0.05 versus anandamide alone as determined by two-way ANOVA.

**Figure 2.** S1P stimulates vascular relaxation in an endothelium-dependent manner. **A** Concentration-response curves showing the vasorelaxant effect of S1P (1nM-30 $\mu$ M) on U46619-precontracted endothelium-intact (n=10) and endothelium-denuded (n=8) isolated rat CA and concentration-response curves showing the effect of S1P (1nM-30 $\mu$ M, n=10) in the presence of **B** W146, a selective S1P<sub>1</sub> antagonist (10 $\mu$ M) (n=5), **C** JTE013, a selective S1P<sub>2</sub> antagonist (10 $\mu$ M) (n=6) and **D** CAY10444, a selective S1P<sub>3</sub> antagonist (10 $\mu$ M) (n=5) in endothelium-intact U46619-precontracted rat CA. Data are expressed as a % of U46619-induced tone and shown as mean  $\pm$  SEM for *n* arteries from different animals . \* *P*<0.05 versus S1P alone as determined by two-way ANOVA.

**Figure 3.** Role for COX in anandamide and S1P-mediated relaxation. Concentration-response curves for the vasorelaxant effect of anandamide alone (1nM-30µM, n=13) and in the presence of indomethacin 10µM in **A** endothelium-intact (n=7) and in **B** endothelium-denuded (n=6) U46619-precontracted rat CA. **C** concentration-response curves showing the vasorelaxant effect of S1P alone (1nM-30µM, n=10) and in the presence of indomethacin 10µM (n=4) in endothelium-intact U46619-precontracted rat CA. Data are expressed as a percentage of U46619-induced tone and shown as mean ± SEM for *n* arteries from different animals . \* *P*<0.05 versus anandamide or S1P alone as determined by two-way ANOVA.

**Figure 4.** Sphingosine kinase is required for anandamide-induced relaxation in the rat coronary artery. **A** Representative immunoblot showing the effect of anandamide (10 $\mu$ M) on pSK1, pERK-1/2 and ERK2 levels in rat coronary artery rings. Graphs shows quantitation of densitometry results expressed as mean  $\pm$  SEM for 4 separate samples. \*  $P < 0.05$  versus vehicle as determined by students unpaired two-tailed t-test. **B** Concentration-response curves for the relaxation to anandamide alone (1nM - 30 $\mu$ M, n=13) and in the presence of SKi, a selective SK1 inhibitor 10 $\mu$ M (n=5) in U46619 precontracted endothelium-intact rat CA and **C** endothelium denuded rat CA (n=4). Data are expressed as a % of U46619-induced tone and shown as mean  $\pm$  SEM for  $n$  arteries from different animals. \*  $P < 0.05$  versus anandamide alone as determined by two-way ANOVA.

**Figure 5. Anandamide-induced relaxation is mediated by S1P<sub>3</sub> within the vascular endothelium.** Concentration-response curves for the relaxation to anandamide alone (1nM - 30μM, n=13) and in the presence of VPC23019, a S1P<sub>1/3</sub> antagonist (10μM) in **A** endothelium-intact (n=8) and **B** endothelium-denuded (n=8) U46619 precontracted rat CA. Concentration-response curves for the relaxation to anandamide alone (1nM - 30μM, n=13) and in the presence of **C** W146, a selective S1P<sub>1</sub> receptor antagonist (10μM) (n=5) and **D** CAY10444, a selective S1P<sub>3</sub> antagonist (10μM) (n=5) in U46619 precontracted endothelium-intact rat CA and **E** in the presence of CAY10444 10μM in endothelium-denuded U46619 precontracted arteries (n=6). **F** Concentration response curves for anandamide alone (n=6) and in the presence of a combination of AM630 (100nM) and CAY10444 (10μM) (n=4) in endothelium-intact rat CA. Data are expressed as a % of U46619-induced tone and shown as mean ± SEM for *n* arteries from different animals . \* *P*<0.05 versus anandamide alone as determined by two-way ANOVA.

**Figure 6.** S1P-induces relaxation *via* the CB<sub>2</sub> receptor and requires SK to mediate its effect. Concentration-response curves showing the vasorelaxant effect of S1P alone (1nM-30μM, n=10) and in the presence of **A** AM630, a selective CB<sub>2</sub> receptor antagonist (10μM) (n=6) and **B** JTE907, a selective CB<sub>2</sub> receptor antagonist (10μM) (n=5) in endothelium-intact U46619-precontracted rat CA. **C** representative immunoblots showing the effect of CAY10444 10μM (a selective S1P<sub>3</sub> antagonist), AM630 10μM and JTE907 10μM (both selective CB<sub>2</sub> receptor antagonists) on S1P-mediated (1μM) ERK-1/2 phosphorylation in MCF-7 cells. Graph shows quantitation of densitometry results expressed as mean ± SEM for 4 separate experiments. **D** concentration-response curves showing the effect of S1P alone (1nM-30μM, n=10) and in the presence of Ski, a selective SK1 inhibitor (10μM) (n=5) in endothelium-intact U46619-precontracted rat CA. Myography data are expressed as a percentage of U46619-induced tone and shown as mean ± SEM for *n* arteries from different animals. \* *p*<0.05 *versus* S1P alone as determined by two-way ANOVA. \*\* *p*<0.01 *versus* unstimulated cells and # *p*<0.05 *versus* S1P stimulated cells as determined by one-way ANOVA with Newman-Keuls post test.

**Figure 7.** S1P stimulates CB<sub>2</sub> receptor activation.

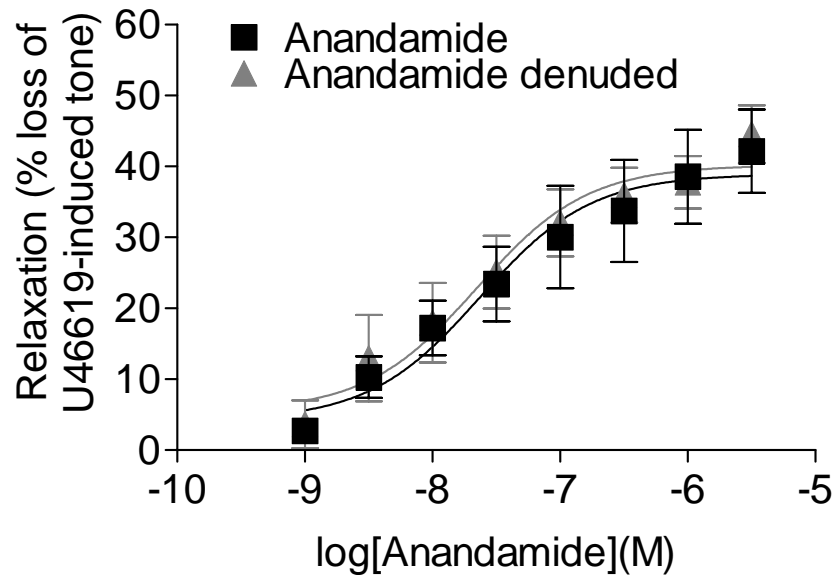
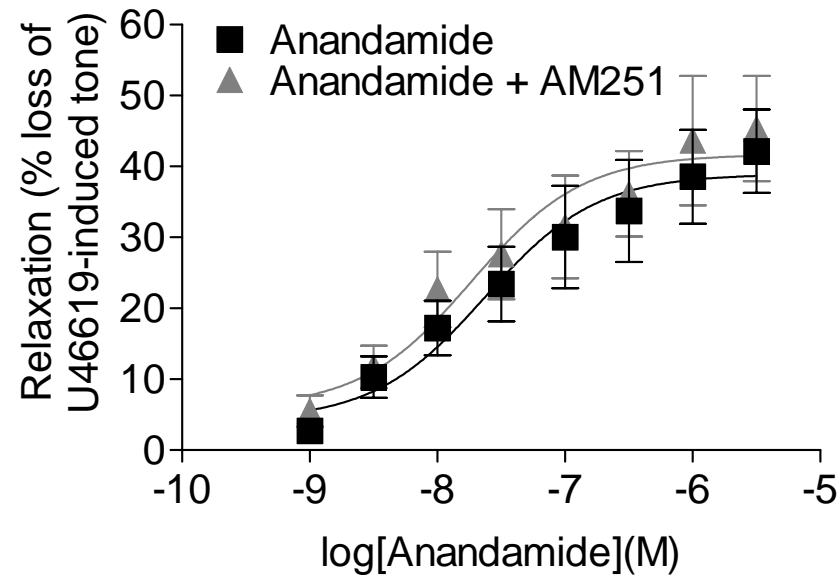
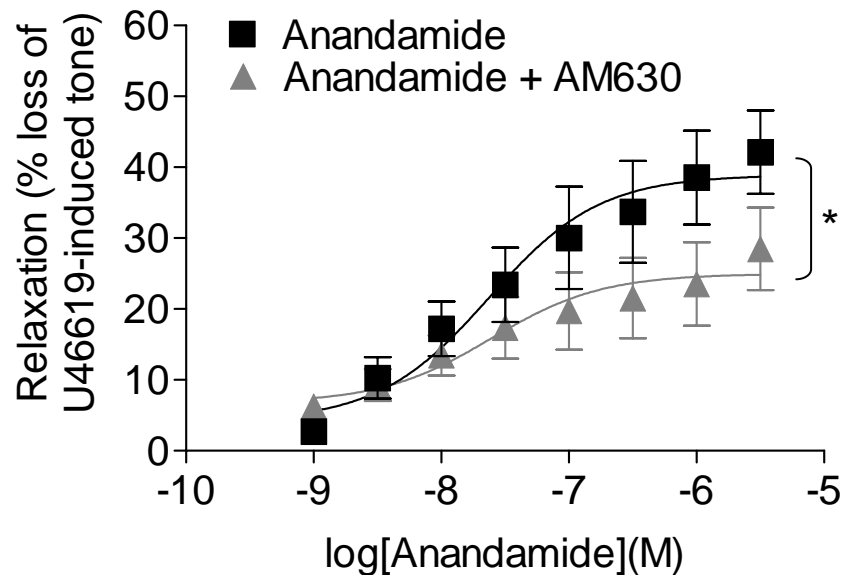
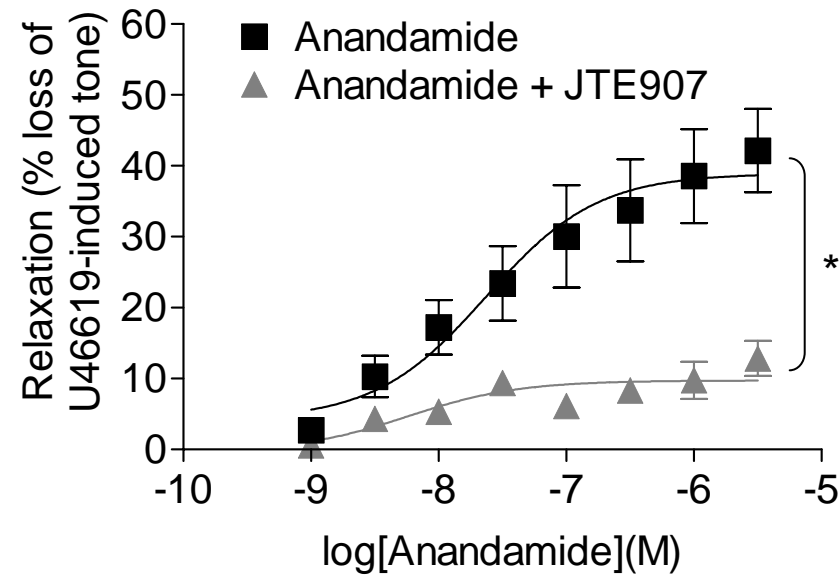
The effects of **A** HU210, a CB<sub>1/2</sub> agonist (1pM-10nM) (n=3) and **B** S1P (1nM - 10μM) (n=3) on [<sup>35</sup>S]-GTPγS binding to HEK293 and CB<sub>2</sub>HEK293 cell membranes. Data expressed as a % of maximal HU210-induced [<sup>35</sup>S]-GTPγS binding. Results shown as mean ± SEM for 3 separate experiments. \* *p*<0.05 *versus* HEK293 membranes as determined by two-way ANOVA. **C** the effects of AM630, a selective CB<sub>2</sub> receptor antagonist (10μM) on HU210 (10μM) and S1P (10μM) mediated [<sup>35</sup>S]-GTPγS binding to CB<sub>2</sub>HEK293 cell membranes. Data expressed as a % of maximal HU210-induced [<sup>35</sup>S]-GTPγS binding. Results shown as mean ± SEM for 4 separate experiments. \*\*\**p*<0.001 *versus* vehicle, \*\* *p*<0.01 *versus* vehicle, # *p*<0.05 *versus* HU210 and \* *p*<0.05 *versus* S1P as determined by one-way ANOVA with Newman-Keuls post test.

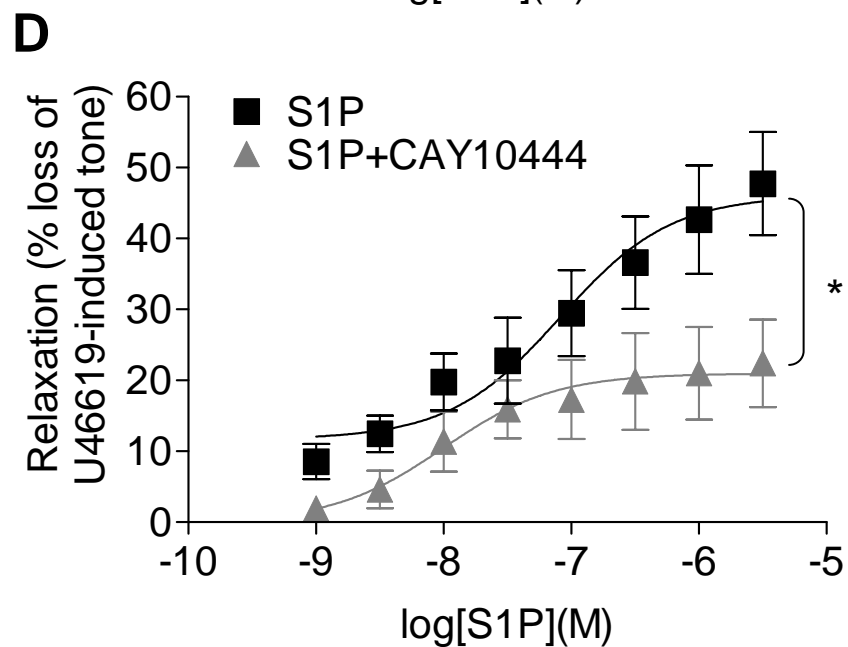
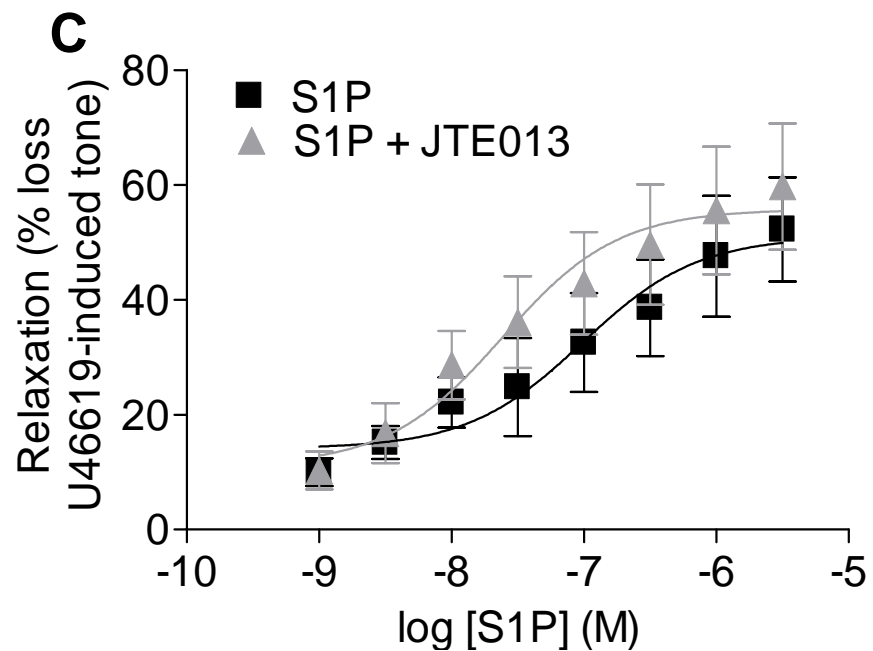
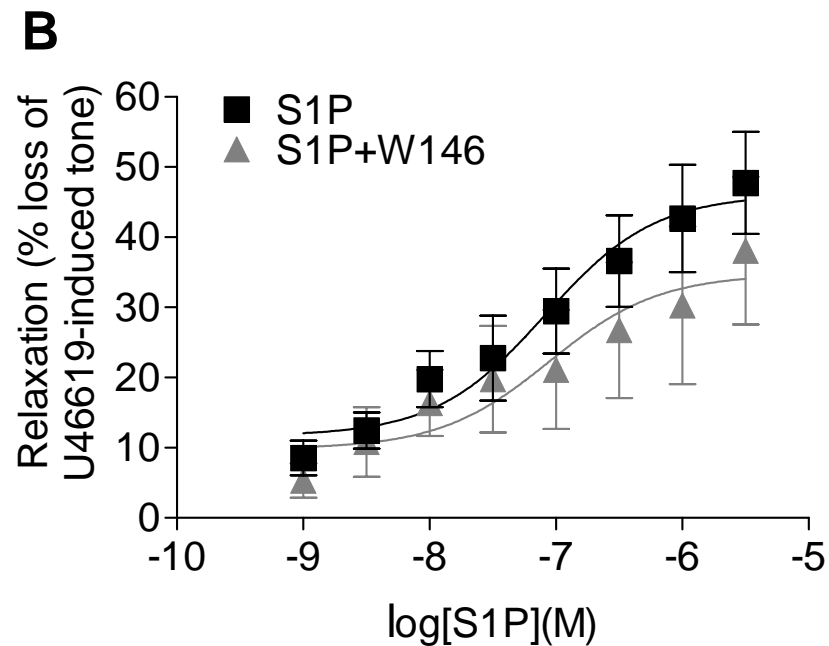
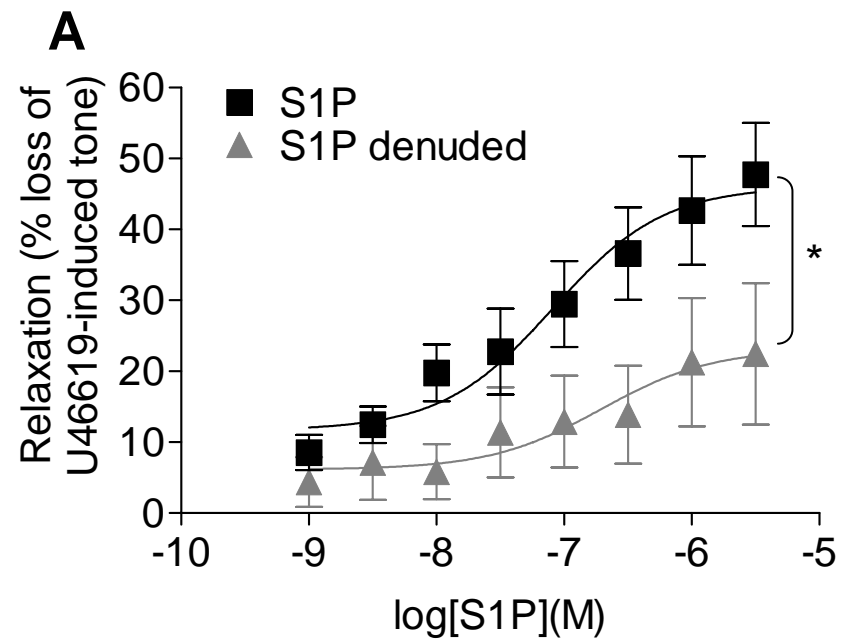
**Figure 8.** S1P stimulated ERK-1/2 activation involves CB<sub>2</sub> receptor. **A** representative immunoblots showing the effect of AM630 (10μM) and JTE907 (10μM) (both selective CB<sub>2</sub> receptor antagonists) and **B** representative immunoblots showing the effect of combined pretreatment with W146, a selective S1P<sub>1</sub> antagonist (20 μM), CAY10444 (CAY), a selective S1P<sub>3</sub> antagonist (20μM) and AM630 (10μM) on S1P-mediated (5μM) ERK1/2 activation in lysates prepared from parental HEK293 cells and CB<sub>2</sub> HEK293s. Graph shows quantitation of densitometry data with results expressed as mean ± SEM for 3 separate experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  versus unstimulated cells. #  $p < 0.05$  versus S1P stimulated cells as determined by one-way ANOVA with Newman-Keuls post test.

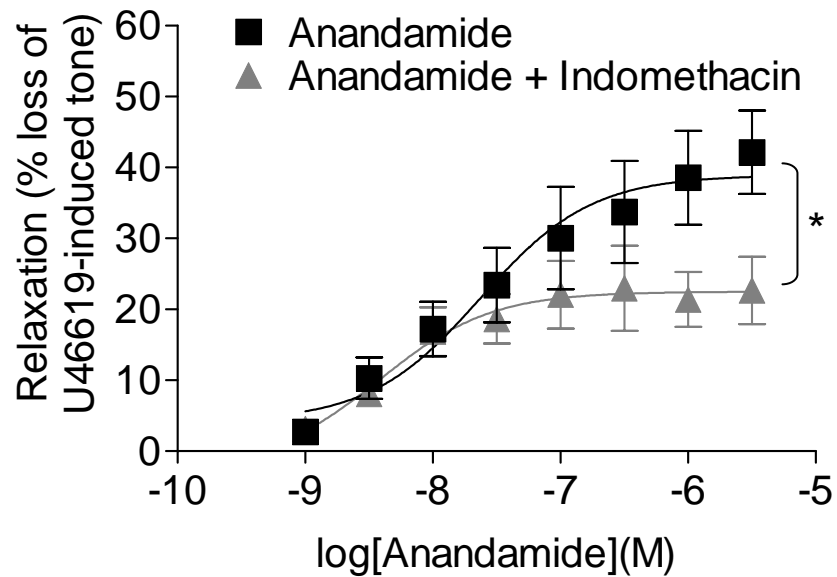
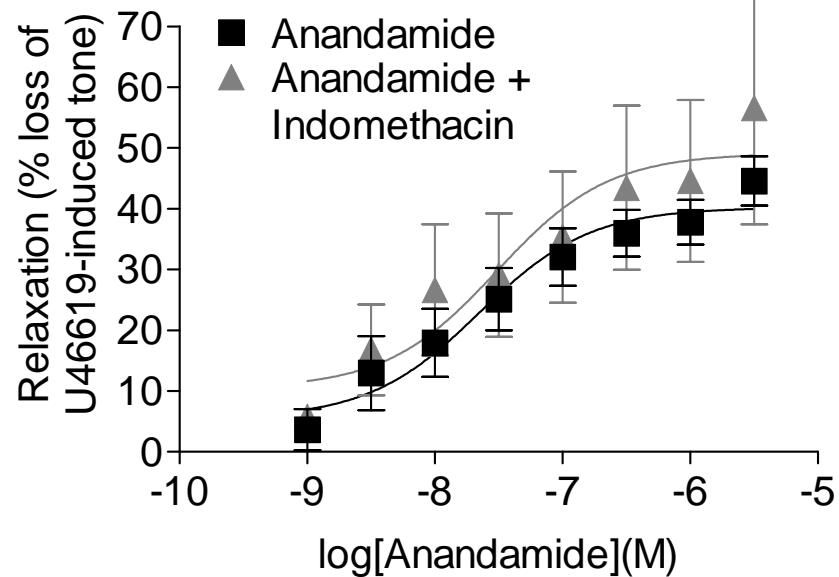
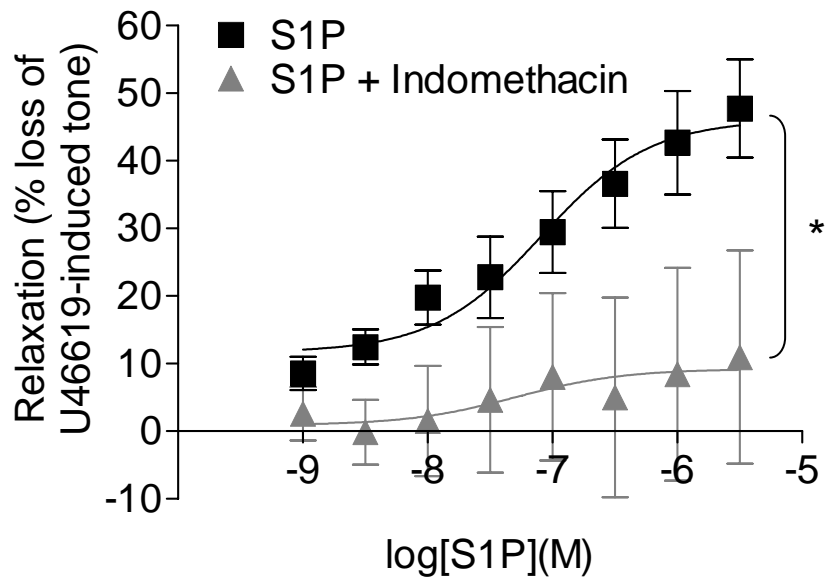


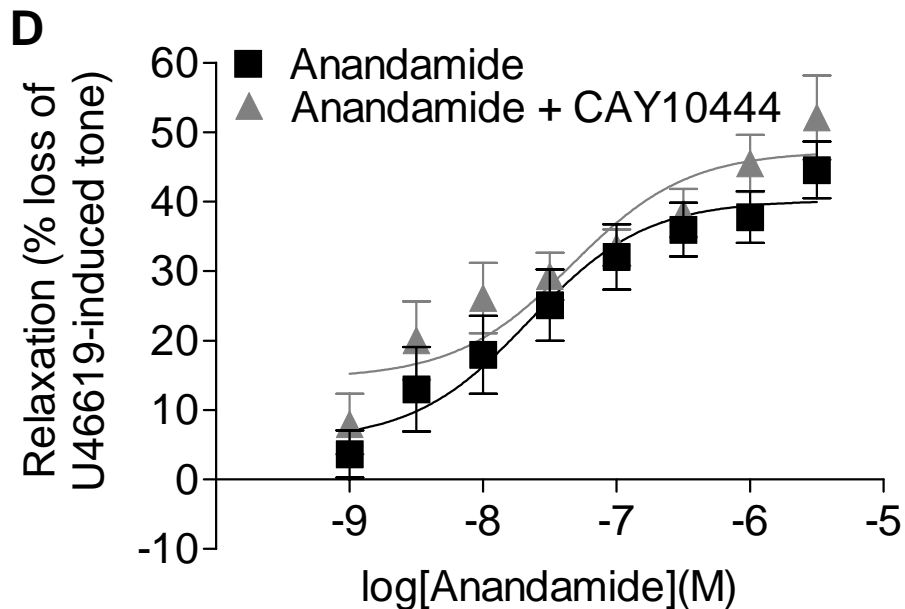
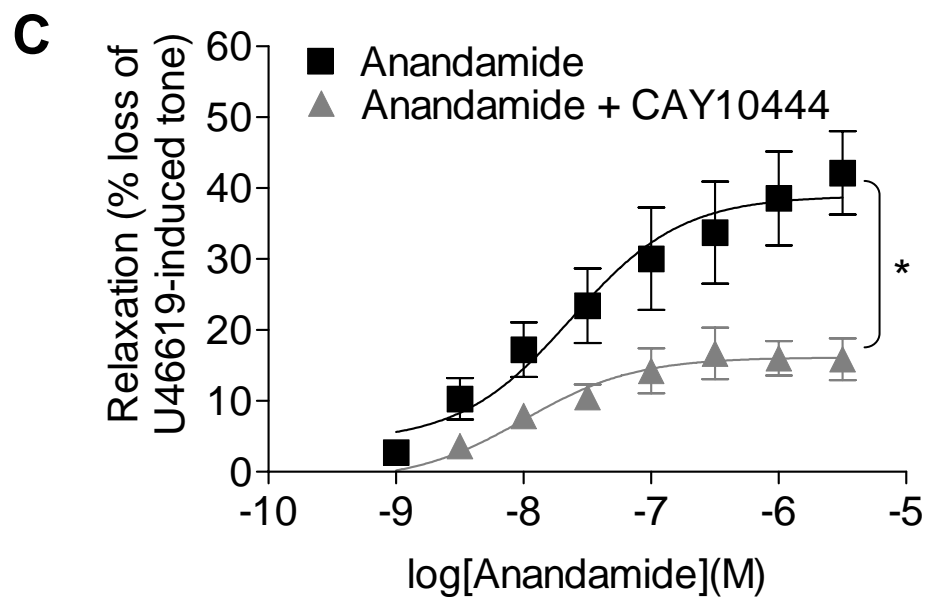
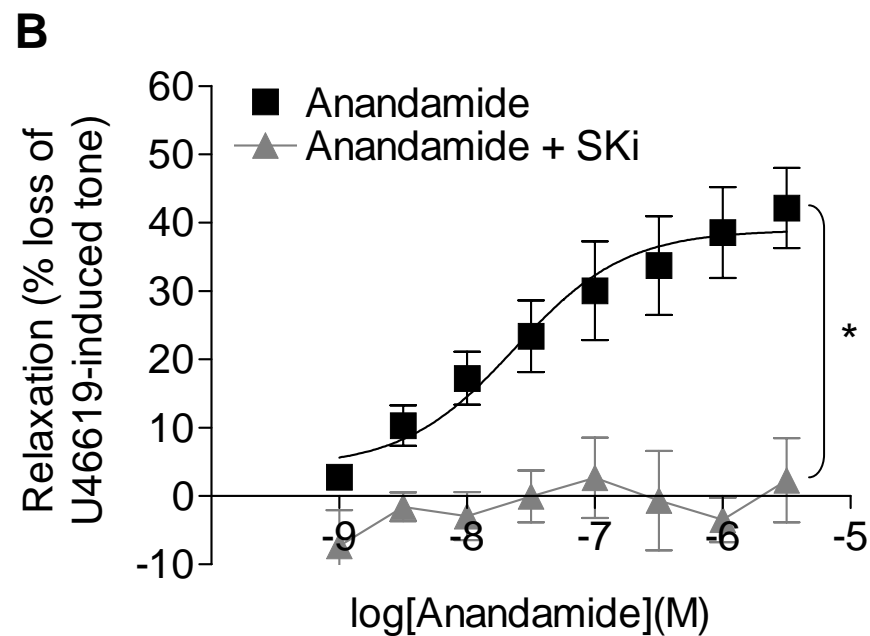
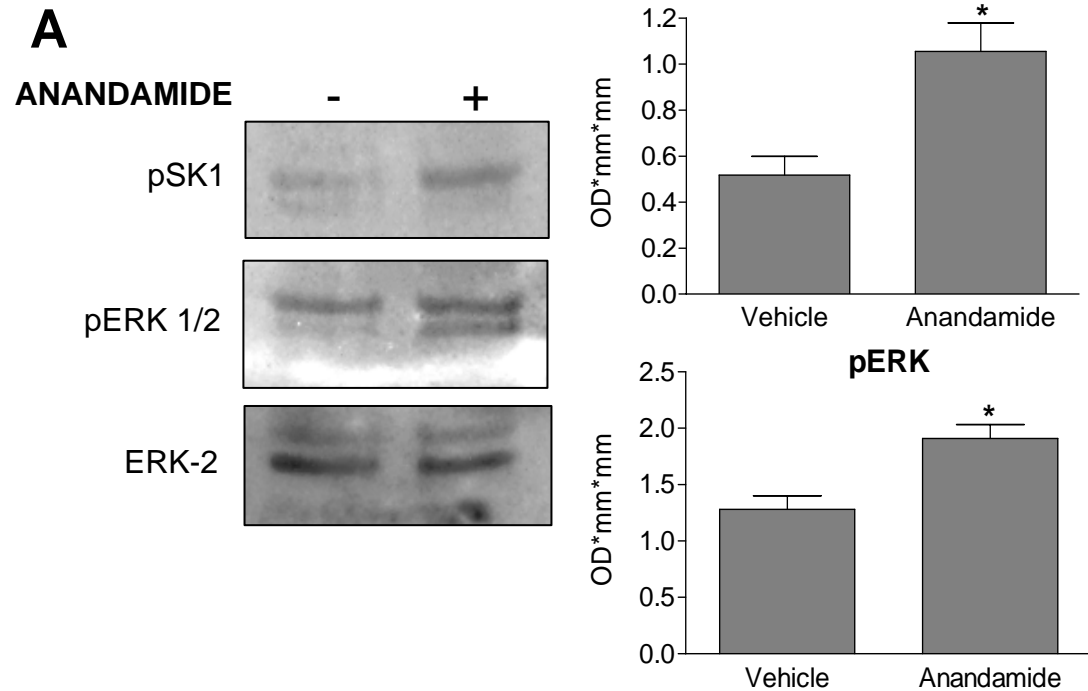
Table 1. Effects of antagonist and enzyme inhibitors on anandamide and HU210-mediated relaxation in rat CA. Data are expressed as mean  $\pm$  SEM for  $n$  arteries from different animals. \*  $p < 0.05$  versus anandamide alone, #  $p < 0.05$  versus HU210 alone as determined by two-way ANOVA.

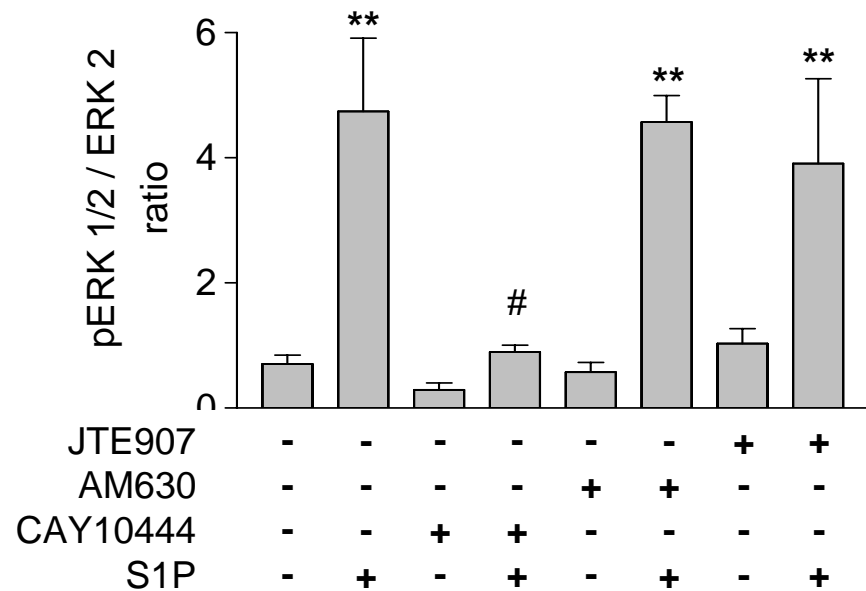
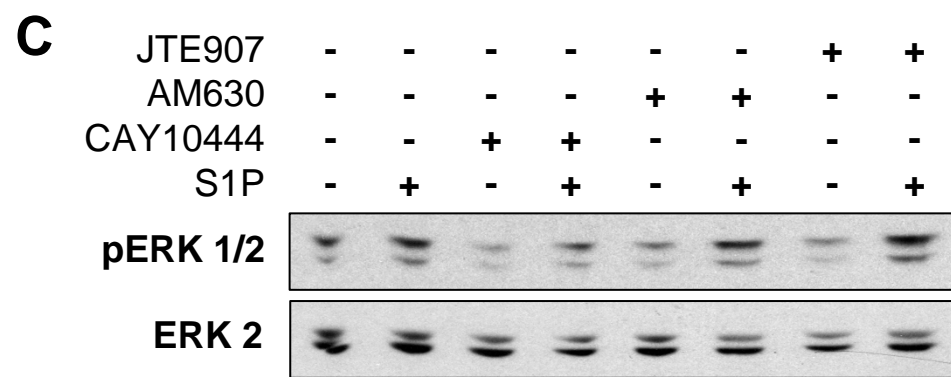
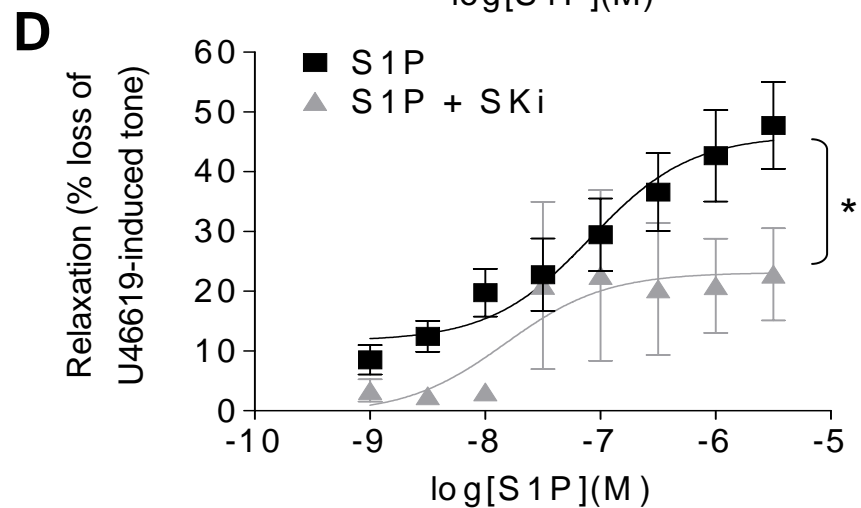
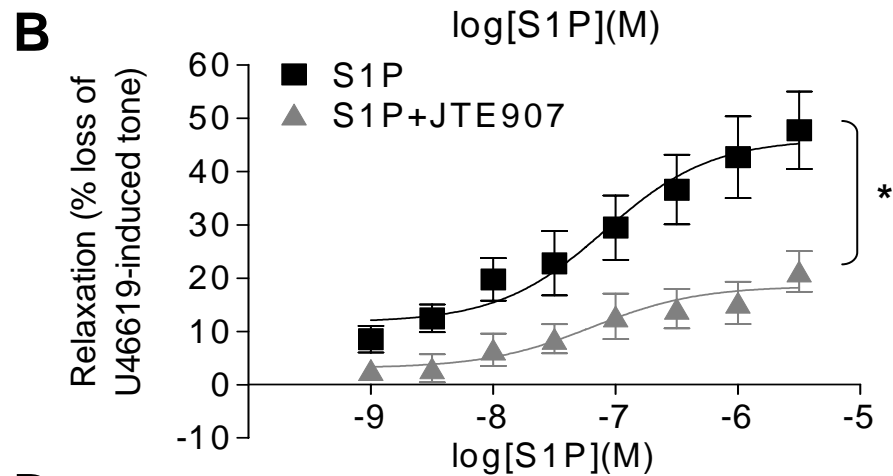
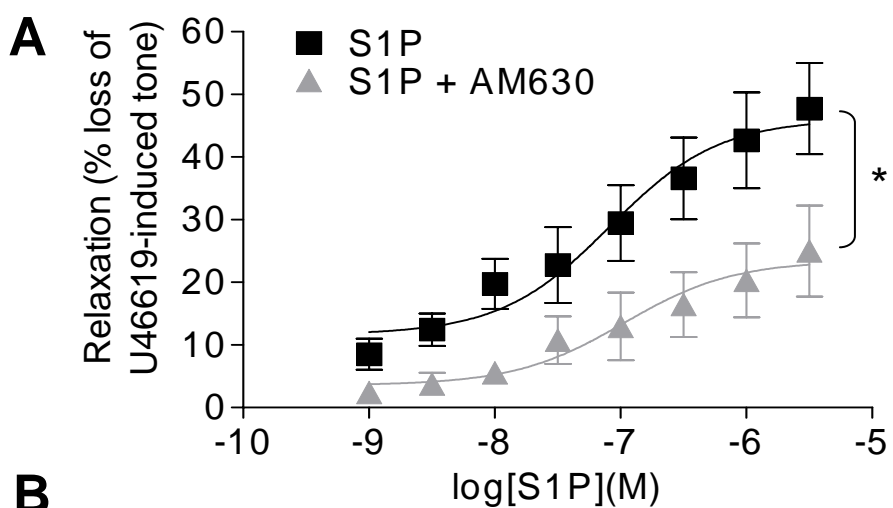
Table 2. Effects of antagonist and enzyme inhibitors on S1P-mediated relaxation in rat CA. Data are expressed as mean  $\pm$  SEM for  $n$  arteries from different animals. \*  $p < 0.05$  versus S1P alone as determined by two-way ANOVA.

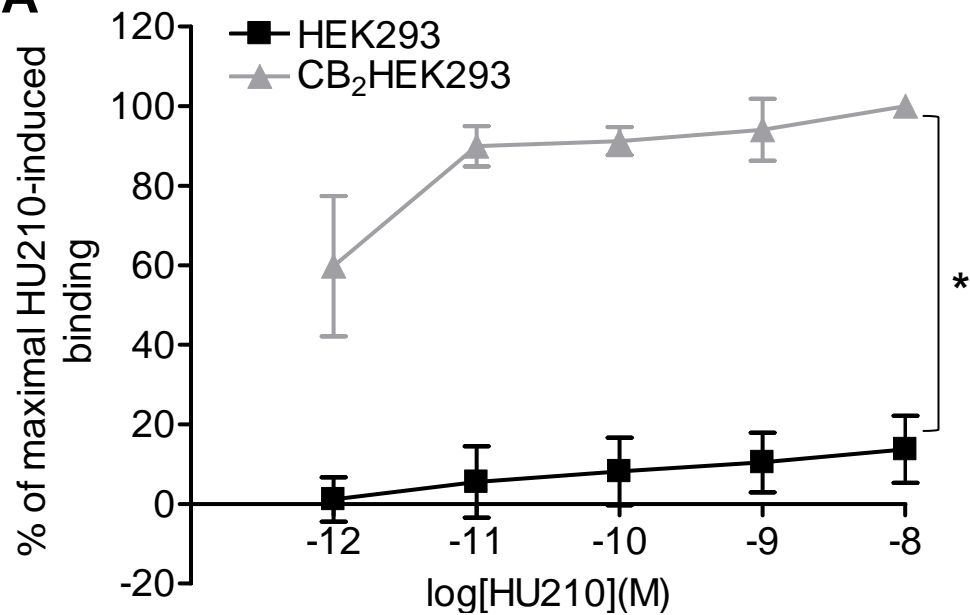
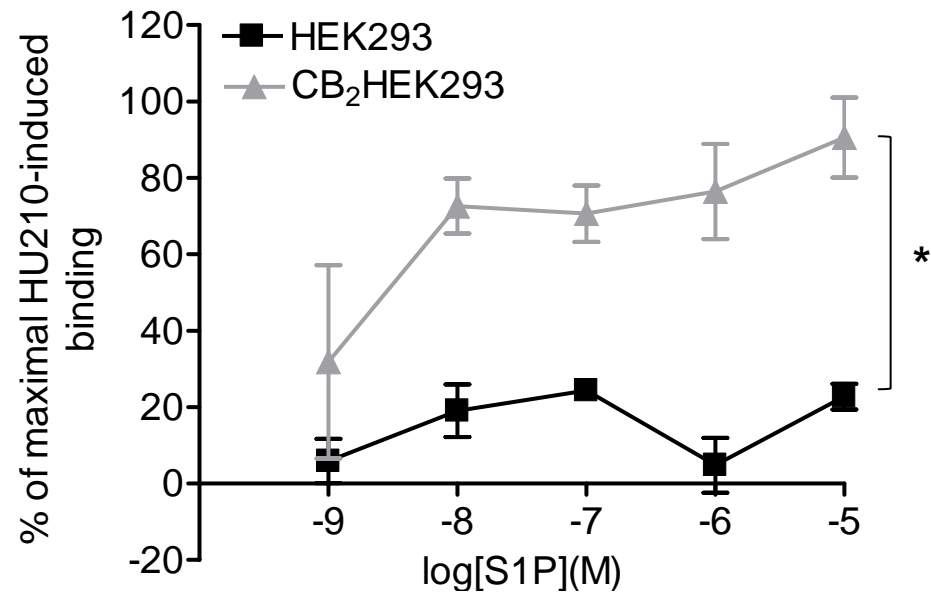
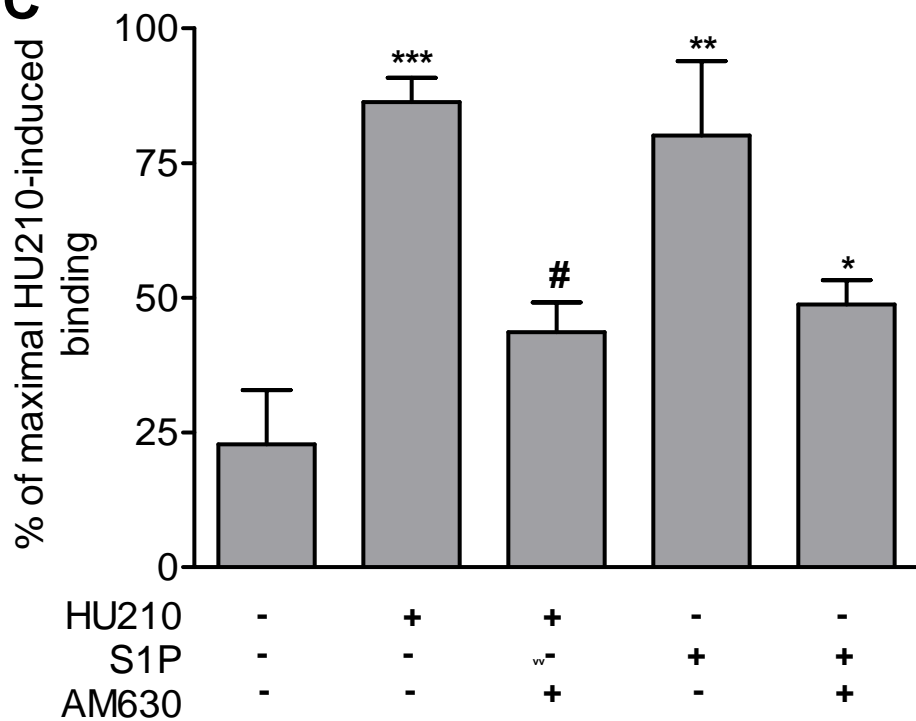
**A****B****C****D**



**A****B****C**



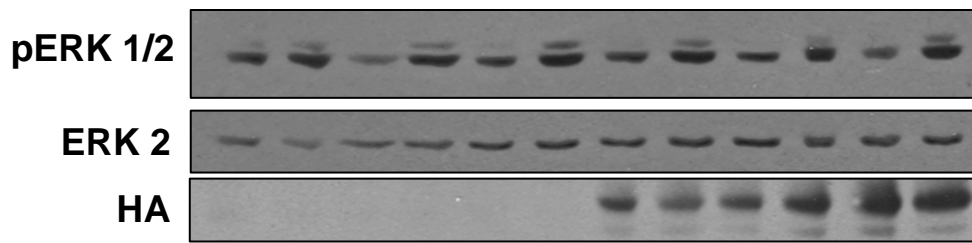


**A****B****C**

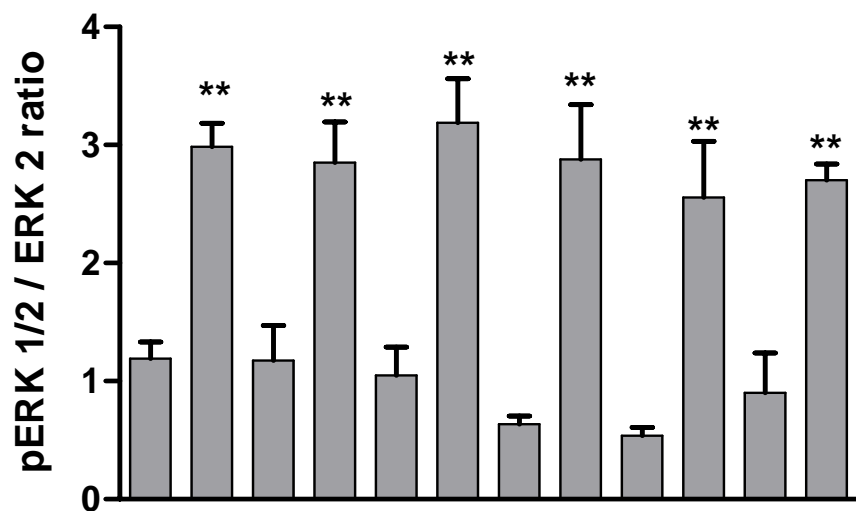


**A**

S1P	-	+	-	+	-	+	-	+	-	+	-	+
AM630	-	-	+	+	-	-	-	-	+	+	-	-
JTE907	-	-	-	-	+	+	-	-	-	-	+	+



HEK293s

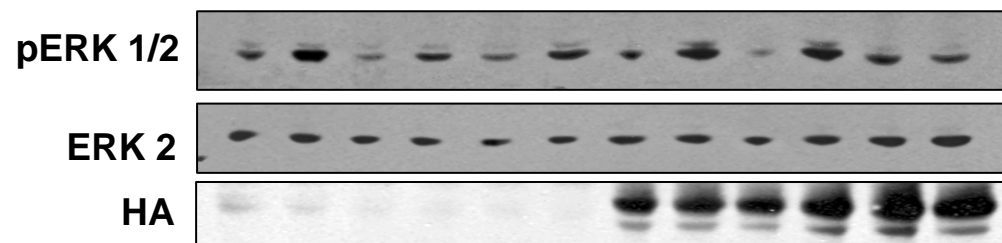
CB<sub>2</sub> HEK293s

S1P	-	+	-	+	-	+	-	+	-	+	-	+
AM630	-	-	+	+	-	-	-	-	+	+	-	-
JTE907	-	-	-	-	+	+	-	-	-	-	+	+

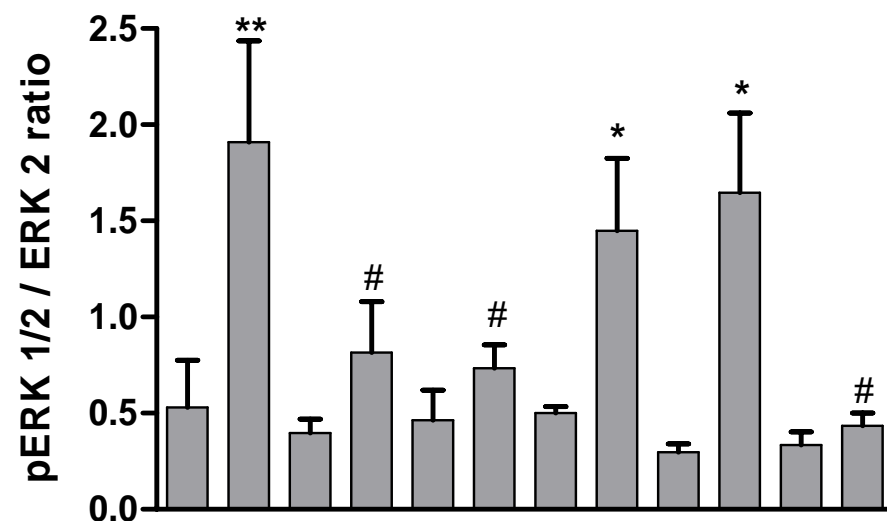
HEK293s

CB<sub>2</sub> HEK293s**B**

S1P	-	+	-	+	-	+	-	+	-	+	-	+
CAY/ W146	-	-	+	+	+	+	-	-	+	+	+	+
AM630	-	-	-	-	+	+	-	-	-	-	+	+



HEK293s

CB<sub>2</sub> HEK293s

S1P	-	+	-	+	-	+	-	+	-	+	-	+
CAY/ W146	-	-	+	+	+	+	-	-	+	+	+	+
AM630	-	-	-	-	+	+	-	-	-	-	+	+

HEK293s

CB<sub>2</sub> HEK293s