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Modulation of trigeminal sensory neuron activity by the dual cannabinoid–vanilloid agonists anandamide, *N*-arachidonoyl-dopamine and arachidonyl-2-chloroethylamide

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1 Peripheral cannabinoids have been shown to suppress nociceptive neurotransmission in a number of behavioral and neurophysiological studies. It is not known, however, whether cannabinoids exert this action through direct interactions with nociceptors in the periphery and/or if other processes are involved. To gain a better understanding of the direct actions of cannabinoid-vanilloid agonists on sensory neurons, we examined the effects of these compounds on trigeminal ganglion (TG) neurons *in vitro*.

2 AEA ($EC_{50} = 11.0\,\mu M$), NADA ($EC_{50} = 857\,nM$) and arachidonyl-2-chloroethylamide ACEA ($EC_{50} = 14.0\,\mu M$) each evoked calcitonin gene-related peptide (CGRP) release from TG neurons. The TRPV1 antagonists iodo-resiniferatoxin (I-RTX) and capsazepine (CPZ) each obtunded AEA-, NADA-, ACEA- and capsaicin (CAP)-evoked CGRP release with individually equivalent IC_{50}'s for each of the compounds (I-RTX IC_{50} range = 2.6–4.0 nM; CPZ IC_{50} range = 523–1140 μM).

3 The pro-inflammatory mediator prostaglandin E_2 significantly increased the maximal effect of AEA-evoked CGRP release without altering the EC_{50}. AEA, ACEA and CAP stimulated cAMP accumulation in TG neurons in a calcium- and TRPV1-dependent fashion. Moreover, the protein kinase inhibitor staurosporine significantly inhibited AEA- and CAP-evoked CGRP release.

4 The pungency of AEA, NADA, ACEA and CAP in the rat eye-wipe assay was also assessed. Interestingly, when applied intraocularly, NADA or CAP each produced nocifensive responses, while AEA or ACEA did not.

5 Finally, the potential inhibitory effects of these cannabinoids on TG nociceptors were evaluated. Neither AEA nor ACEA decreased CAP-evoked CGRP release. Furthermore, neither of the cannabinoid receptor type 1 antagonists SR141716A nor AM251 had any impact on either basal or CAP-evoked CGRP release. AEA also did not inhibit 50 mM K^+-evoked CGRP release and did not influence bradykinin-stimulated inositol phosphate accumulation.

6 We conclude that the major action of AEA, NADA and ACEA on TG neurons is excitatory, while, of these, only NADA is pungent. These findings are discussed in relation to our current understanding of interactions between the cannabinoid and vanilloid systems and nociceptive processing in the periphery.

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Abbreviations: ACEA, arachidonyl-2-chlorethylamide; AEA, anandamide; BK, bradykinin; CAP, capsaicin; CB1, cannabinoid type 1; CB2, cannabinoid type 2; CGRP, calcitonin gene-related peptide; CPZ, capsazepine; DRG, dorsal root ganglion; FAAH, fatty acide amide hydrolase; I-RTX, iodoresiniferatoxin; NADA, *n*-arachidonyl-dopamine; NGF, nerve growth factor; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; SP, substance P; TG, trigeminal ganglion; TRPV1, transient receptor potential vanilloid subfamily type 1; VR1, vanilloid receptor type 1

Introduction

The endogenous cannabinoid system comprises a number of arachidonic acid-derived fatty acids, most of which bind to the two known G protein-coupled cannabinoid receptors, CB1 (Matsuda *et al*., 1990) and CB2 (Munro *et al*., 1993), as well as to the capsaicin (CAP)- and heat-activated ion channel TRPV1 (Caterina *et al*., 1997). Among the known physiological effects of cannabinoid receptor agonism is the production of antinociception. In animals, this cannabinoid-mediated antinociception occurs through mechanisms involving supraspinal and spinal CB1 receptors as well as peripheral CB1 and CB2 receptors (Walker *et al*., 1999; 2001; Hohmann, 2002; Rice *et al*., 2002). The pharmaceutical exploitation of peripheral

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cannabinoid antinociception is particularly promising, because it affords the opportunity to alleviate pain without producing many of the undesirable side effects commonly associated with CNS cannabinoid pharmacology.

In both the dorsal root ganglion (DRG; (Bridges *et al*., 2003) and trigeminal ganglion (TG; (Price *et al*., 2003), CB1 receptors have been localized to large-diameter, myelinated neurons that do not typically mediate nociceptive neurotransmission under normal conditions. Despite these findings, numerous other studies have shown that cannabinoids can act through peripheral CB1 receptors to inhibit nociception (Calignano *et al*., 1998; Richardson *et al*., 1998; Fox *et al*., 2001). Additionally, a growing body of evidence suggests that CB2 receptors, which are not found in sensory neurons, are capable of exerting antinociceptive effects (Malan *et al*., 2001; 2002; Ibrahim *et al*., 2003). However, the cellular site of action for either CB1 or CB2 receptor-dependent antinociception has not been precisely defined.

In addition to being agonist ligands at canonical cannabinoid receptors, multiple endocannabinoids are also activators of TRPV1 (Zygmunt *et al*., 1999; Smart *et al*., 2000). Hence, it has been proposed that these compounds might be endogenous ligands for TRPV1 and, conversely, that TRP channels may be ionotropic cannabinoid receptors (Zygmunt *et al*., 1999); however, this assertion has remained a point of controversy, as such endocannabinoids activate TRPV1 only at low to middle μM concentrations, the physiological achievement of which has yet to be quantitatively demonstrated under basal or activated states. Clearly, TRPV1 both plays a prominent role in normal nociceptive activity and is involved integrally in the development of hyperalgesia associated with inflammation (Caterina *et al*., 2000). Additionally, TRPV1 protein levels are increased by inflammation, and this is dependent on nerve growth factor (NGF; Ji *et al*., 2002), an important mediator of nociceptor sensitization (McMahon, 1996). Thus, certain endocannabinoids may act as dual cannabinoid–vanilloid regulators, particularly under conditions of inflammatory hyperalgesia.

TRPV1-expressing sensory neurons are *de facto* nociceptors, because the prototypical TRPV1 agonist CAP almost uniformly produces pain/nociception in human/animals *in vivo*. Neuropeptide secretion is an important effector mechanism associated with nociceptor stimulation. The neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) are found in a subset of putative nociceptors, many of which express TRPV1 (Guo *et al*., 1999). Thus, stimulation of TRPV1 induces CGRP and SP exocytosis from both the central and peripheral terminals of nociceptive afferents. In the spinal cord, their secretion in the dorsal horn enhances nociceptive sensitivity (Bennett *et al*., 2000; Sun *et al*., 2003), and SP release has been implicated in long-term potentiation associated with central sensitization (Afrah *et al*., 2002; Ikeda *et al*., 2003). In the periphery, on the other hand, both CGRP and SP play important roles in the development and maintenance of neurogenic inflammation through actions on the peri-vasculature (Brain & Williams, 1985; Brain *et al*., 1985; Gamse & Saria, 1986). Taken together, these data support the hypothesis that sensory neuronal secretion of CGRP and SP plays an important role in nociceptive processing and that its measurement is a physiologically relevant index of nociceptor activation.

In the present study, we have utilized a neuronally enriched TG culture system in which to characterize the pharmacological effects of the endogenous cannabinoid compounds anandamide (AEA) and NADA, as well as the synthetic AEA analogue, ACEA, on trigeminal nociceptors through the measurement of CGRP secretion, as well as IP or cAMP accumulation. Furthermore, we assessed the *in vivo* pungency of these compounds in order to relate the *in vitro* pharmacology of these compounds to their physiological relevance to nociception in intact animals.

Methods

Reagents

The cannabinoid/vanilloid agonists AEA (*N*-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide, in water soluble emulsion), arachidonyl-2-chloroethylamide, *N*-(2Chloroethyl)-5Z,11Z,14Z-eicosatetraenamide (ACEA) and *n*-arachidonoyl-dopamine, *N*-[2-(3,4-dihydroxyphenyl)ethyl]-5Z,8Z,11Z,14Z-eicosatetraenamide (NADA) were all from Tocris (Ellisville, MO, U.S.A.). CAP (8-methyl-*N*-vanillyl-trans-6-nonenamide) was from Sigma Aldrich/Fluka (St Louis, MO, U.S.A.). The CB1 antagonists *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride (SR141716A) and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) were from the NIMH chemical synthesis and drug supply program and Tocris, respectively. The TRPV1 antagonists capsazepine, *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazaoine-2-carbothioamide (CPZ) and iodo-resiniferatoxin, 6,7-Deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzeneacetate (I-RTX) were from Tocris. The PLC inhibitor 1-[6[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) was from Tocris. Rolipram (4-[3-(Cyclopentylloxy)-4-methoxyphenyl]-2-pyrrolidinone), indomethacin, thiorphan, phenylmethylsulfonyl fluoride (PMSF) and bradykinin (BK) were from Sigma. The protein kinase inhibitors staurosporine, bisindolylmaleimide I and protein kinase A inhibitor 6-22 amide were from Calbiochem (San Diego, CA, U.S.A.). Full-length rat CGRP and $CGRP_{8-37}$ were from Tocris. Prostaglandin E_2 (PGE_2, 11α-15*S*-dihydroxy-9-oxo-prosta-5Z,13E-dien-1-oic acid) was from Cayman Chemical (Ann Arbor, MI, U.S.A.). NGF (7.0S) was from Harlan (Indianapolis, IN, U.S.A.). [^{125}I]-cAMP tracer and [^{3}H]-myo-inositol were from Perkin-Elmer Life Science Products (Boston, MA, U.S.A.), and the anti-cAMP antibody was from ICN Biomedicals (Costa Mesa, CA, U.S.A.).

Animals

Adult, male, Sprague–Dawley rats weighing 250–300 g were used in this study. All procedures utilizing animals were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio, and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health.

TG culture

TGs were rapidly dissected (within $\sim$30 s) and placed in ice-cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution

(HBSS, Gibco, Carlsbad, CA, U.S.A.). TGs were enzymatically digested for 30 min with 1.5 mg ml^{-1} collagenase, followed by 25 min with 0.1% trypsin type IX supplemented for the last 10 min with 10 units of DNase I (Roche, Indianapolis, IN, U.S.A.). TG homogenates were then centrifuged at 2000 r.p.m. for 2 min, triturated briefly by vortexing and then recentrifuged. They were then resuspended in basal culture media, containing high glucose Dulbecco's modified Eagle's media (DMEM, Gibco), 1 × pen-strep (Gibco), 1 × glutamine (Gibco) and 3 μg ml^{-1} 5-FDU and 7 μg ml^{-1} uridine. TG homogenates were gently triturated with a Pasteur pipette, followed by successive triturations through 19- and 23-gauge needles. TG homogenates were then transferred to a separate container, wherein the volume was adjusted such that a plating density of ~5000 neurons well^{-1} would be achieved.

CGRP release assays

Experiments were performed in 48-well, poly-D-lysine precoated plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). All cultures received 100 ng ml^{-1} NGF. Culture media was changed at 24 and 72 h and included fresh growth factor supplementation, and all CGRP assays were performed on day 5. TG cultures were washed free of culture media by two successive washes with release buffer (HBSS supplemented with 10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10 mM dextrose and 0.1% bovine serum albumin (BSA), pH 7.4). Growth factors were not included in the release buffer. Following washing, TG cultures were exposed to the indicated concentrations of pretreatment agonists or antagonists for 10 min, or to CPZ or I-RTX for 5 min, or to enzyme inhibitors for 15 min (if present in the experimental design, unless otherwise noted), followed by the stimulus drug for 10 min, after which the CGRP containing release buffer was removed and transferred to glass culture tubes. All drugs were diluted from their stock solutions to 10 × concentrations into siliconized glass culture tubes and then 1 : 10 into the culture plate wells with siliconized pipette tips. In some experiments, the neurons were subsequently challenged with 50 mM K^+ buffer (containing 2.5 mM $CaCl_2$, 50 mM KCl, 1.2 mM $MgCl_2$, 90 mM NaCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM dextrose, 15 mM HEPES, 16 μM thiorphan and 0.1% BSA; pH 7.4) for 10 min. CGRP was measured by radioimmunoassay.

CGRP radioimmunoassay

Following culture release assays, individual aliquots of the superfusate (0.5 ml) were incubated with a C-terminally directed anti-CGRP antiserum kindly donated by Dr Michael Iadarola (NIDCR, NIH, Bethesda, MD, U.S.A.). After 24 h, 100 μl of [^{125}I] $CGRP_{28-37}$ (approximately 20,000–25,000 c.p.m.) and 50 μl of goat anti-rabbit antibody conjugated to ferric beads were added. Following another 24 h, the bound peptide was separated from free peptide *via* immunomagnetic separation (PerSeptive Biosystems, Framingham, MA, U.S.A.). All incubations were carried out at 4°C. The minimum detection limit for this assay is approximately 1–2 fmol per tube, with 50% displacement occurring at 20–40 fmol per tube. To account for the possibility of any nonspecific effects on the RIA, all drugs used in the release experiments were included in separate standard curves for the purposes of data analysis. No drugs significantly altered the standard curves; however, 50 mM K^+ significantly right shifted the EC_{50}, necessitating the generation of separate standard curves for these experiments.

IP accumulation

Measurements of IP accumulation were made in TG cultures prepared as described above. Neurons were labeled with 1 μCi ml^{-1} *myo*-[^{3}H]-inositol (10-25 Ci mmol^{-1}) for 24 h at 37°C. After the labeling period, cells were washed three times with HBSS containing calcium and magnesium and supplemented with 20 mM HEPES and 0.1% fatty acid-free BSA. Between washes, the cells were incubated for 5 min in a 37°C water bath (15 min total preincubation time). After the wash procedure, cells were incubated in 0.5 ml of the same media containing 20 mM LiCl and drug. After a 10 min preincubation with AEA or vehicle, neurons were exposed to 10 μM BK for 15 min. Following drug exposure, the media was aspirated quickly, and 1 ml of ice-cold 10 mM formic acid was added to extract the accumulated IP. The [^{3}H]-IP (the mono-, di- and trisphosphates of inositol, collectively referred to as IP) in the supernatant was separated with ion exchange chromatography and quantified by liquid scintillation spectroscopy.

cAMP accumulation

TG neuronal cultures were prepared as described above. Neurons were washed and incubated in release buffer containing 10 μM rolipram for 10 min, after which they were exposed to drug or vehicle for an additional 15 min. For calcium-free buffer experiments, the release buffer was the same, but without Ca^{2+} and Mg^{2+}, and contained 5 mM EGTA. Cellular cAMP was extracted by the immediate addition of 500 μl of ice-cold ethanol. Samples were allowed to sit overnight at –20°C, evaporated the following day in RIA tubes and then resuspended in RIA buffer. cAMP levels were then measured by radioimmunoassay, as described previously (Berg *et al*., 1994).

Behavior

Animals were housed in the university laboratory animal facility, allowed to acclimate for 5 days following their arrival and then brought to the laboratory, where the behavioral experiments were performed. Following an additional several hour acclimation period in the behavior laboratory, drugs were pipetted directly into the left eye of the rat, and the subsequent nocifensive behavior was monitored. This behavior was defined as either holding the eye tightly shut or actively grooming the face and eye area with either the fore- or hindpaws. The number of seconds that this behavior was observed was quantified for 5 min following the administration of drug in all experiments.

Statistics

All data are presented as mean ± s.e.m., unless otherwise noted. Significant differences between groups were assessed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test, unless otherwise stated. Concentration–response curves were analyzed by variable slope nonlinear regression, and differences between curves were assessed using global curve fitting comparing variables (i.e., EC_{50} and E_{max})

by an F-test. All data were analyzed with GraphPad Prism 4.0 for Mac OS X (GraphPad, San Diego, CA, U.S.A.).

Results

Several cannabinoid receptor agonists have also been reported to be agonists of TRPV1 (Zygmunt *et al*., 1999; Huang *et al*., 2002); hence, we examined the ability of the endogenous CB1/TRPV1 agonists AEA and NADA, as well as the synthetic CB1-selective AEA analogue ACEA, to evoke CGRP release from TG neurons. AEA, NADA and ACEA each evoked CGRP release from TG neurons. NADA was the most potent of the compounds with an EC_{50} of 857 nM, while AEA and ACEA had similar potencies of 11.0 and 14.0 μM, respectively (Figure 1a). In these assays, ACEA is a partial agonist at TRPV1 with an α value of $\sim$0.5, whereas AEA, NADA and CAP are each full agonists, and all compounds had Hill slopes (n_H) of approximately 2. The concentration–response relationship of AEA-evoked CGRP release was not influenced by the inclusion of PMSF (10 μM), an amide hydrolase inhibitor that inhibits FAAH (Figure 1b).

We also examined the ability of AEA and ACEA to inhibit evoked CGRP release from TG neurons. AEA treatment (6–600 nM) did not inhibit CAP-evoked CGRP release at E_{max} (100 nM) or EC_{50} (30 nM) concentrations of CAP (Figure 2a and b, respectively). Additionally, AEA did not inhibit 50 mM K^+-evoked CGRP release (Figure 2c). The relatively more potent and selective CB1 agonist ACEA also did not inhibit 50 nM CAP-evoked CGRP release from TG neurons (Figure 2d). To test the hypothesis that evoked neurosecretion from cultured TG neurons might be subject to tonic cannabinoid inhibitory tone, we evaluated CAP-evoked CGRP release in the presence of a CB1 antagonist. Neither 200 nM SR141716A (Figure 2e) nor 200 nM AM251 (Figure 2f) augmented basal or evoked CGRP release at multiple CAP concentrations (30–300 nM). On the other hand, SR141716A (10 or 30 μM) inhibited CAP-evoked CGRP release (Figure 2g), while it evoked CGRP release on its own at concentrations above 50 μM (data not shown). To assess whether the endocannabinoids under study might exert regulatory effects on the generation of second messengers rather than exocytosis, we tested the ability of AEA to inhibit BK-stimulated IP accumulation. In fact, as shown in Figure 2h, AEA neither stimulated IP accumulation by itself, even at concentrations that evoked CGRP release from TG neurons (10 μM), nor inhibited 10 μM BK-induced IP accumulation at any concentrations tested (0.1 nM–10 μM).

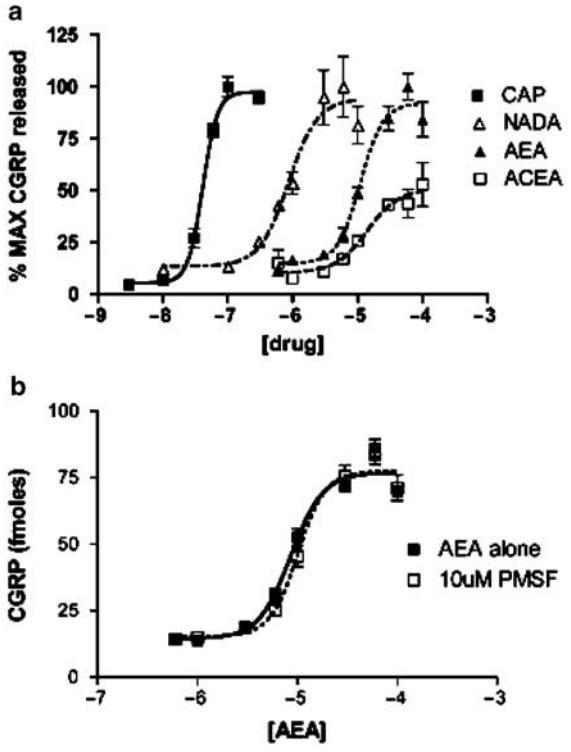

Figure 1 Cannabinoid agonists evoke CGRP release from TG neurons: (a) TG neuronal cultures were exposed to the indicated concentrations of AEA, NADA, ACEA or CAP for 10 min ($n=3$). (b) TG neuronal cultures were exposed to 10 μM PMSF or vehicle, for 15 min prior to a 10 min exposure of AEA at the indicated concentrations ($n=6$). Supernatants were assayed for CGRP by RIA as described in Methods.

The inflammatory mediator PGE_2 is known to sensitize CAP responsiveness in sensory neurons through the activation of the cAMP/PKA pathway (Lopshire & Nicol, 1998). Therefore, we tested the ability of PGE_2 to augment AEA- or ACEA-evoked CGRP release. As shown in Figure 3a, while PGE_2 (1 μM) alone did not significantly increase CGRP release from TG neurons, in accordance with previous findings (Evans *et al*., 1996), it significantly potentiated 30 μM AEA-evoked CGRP release from a 5.8-fold increase over baseline to a 7.4-fold increase. Similarly, 1 μM PGE_2 potentiated 30 μM ACEA-evoked CGRP release (from 2.2- to 4.09-fold over baseline) and 100 nM CAP-evoked CGRP release (from 5.5- to 8.2-fold over baseline), as shown in Figure 3b and c, respectively. Interestingly, 1 μM PGE_2 did not change the EC_{50} for AEA-evoked CGRP release, while it significantly elevated the E_{max} of the concentration–response curve from 116.6 ± 4.77 to 177.2 ± 3.835 (Figure 3d).

We next tested the ability of AEA, ACEA or CAP to stimulate cAMP accumulation in TG neurons. Each of 30 μM AEA, 30 μM ACEA and 100 nM CAP significantly stimulated cAMP accumulation in TG neurons (Figure 4a). Furthermore, this stimulation was calcium-dependent, as the exclusion of calcium from the extracellular solution eliminated the stimulatory effects of AEA and CAP (Figure 4b); in contrast, 1 μM PGE_2 was still able to stimulate cAMP accumulation in the absence of extracellular calcium (data not shown). Both CAP- and AEA-stimulated cAMP accumulation were completely blocked by the TRPV1 antagonist I-RTX (Figure 4c and d, respectively). To exclude the possibility that endogenous CGRP release was leading to cAMP accumulation, we preincubated TG neurons with the CGRP receptor antagonist $CGRP_{8-37}$. In fact, $CGRP_{8-37}$ (3 μM) did not inhibit CAP- or AEA-stimulated cAMP accumulation (Figure 4e). We also examined the ability of exogenous CGRP to stimulate cAMP accumulation in TG neurons. Based on previous CAP-evoked CGRP release assays, we estimated that the concentration of CGRP per well would be $\sim$0.3 nM; therefore, we exposed TG neurons for 15 min to increasing concentrations of CGRP, ranging from 0.3 to 100 nM. Exogenous CGRP at concentrations less than 10 nM had no effect on cAMP accumulation; however, 10–100 nM CGRP significantly stimulated cAMP accumulation in TG neurons (Figure 4f). CGRP-stimulated

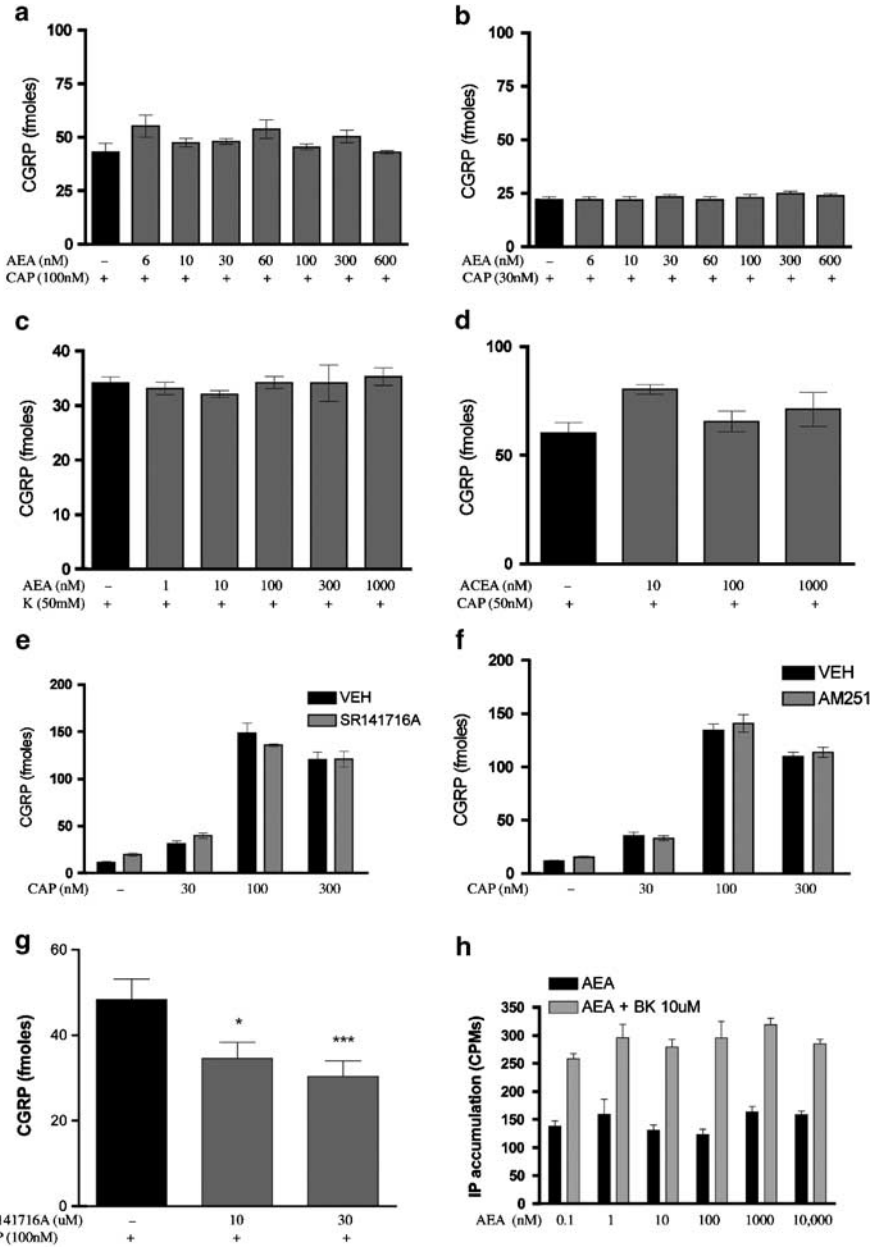

Figure 2 Lack of evidence for inhibitory effects of cannabinoids on TG nociceptors: TG neuronal cultures were pretreated for 10 min with the indicated concentrations of AEA and then exposed to either 100 nM (a) or 30 nM (b) CAP in the continued presence of AEA for 10 min. (c) TG neuronal cultures were pretreated with the indicated concentrations of AEA for 10 min and then exposed to 50 mM K^+ in the continued presence of AEA for 10 min. (d) TG neuronal cultures were pretreated for 10 min with ACEA and then exposed to 50 nM CAP in the continued presence of ACEA for 10 min. To assess basal cannabinoid-mediated inhibitory tone in TG neurons, TG neuronal cultures were pretreated for 10 min to 200 nM of the CB1 antagonists SR141716A (e) or AM251 (f) and then exposed to the indicated concentrations of CAP for 10 min in the continued presence of antagonist. (g) TG neuronal cultures were pretreated for 10 min to the indicated concentration of SR141716A and then exposed to 100 nM CAP in the continued presence of SR141716A ($n=6$, all CGRP-release experiments, $^*P<0.05$, $^{***}P<0.001$). Supernatants were assayed for CGRP by RIA as described in Methods. (h) Finally, IP accumulation was assessed over the indicated range of AEA concentrations in the absence and presence of 10 μM BK ($n=4$).

cAMP accumulation (100 nM) was completely blocked by the CGRP receptor antagonist $CGRP_{8-37}$ (3 μM).

As cAMP accumulation can stimulate protein kinase A (PKA) activity, we examined the effect of the nonselective PKA/PKC inhibitor staurosporine on CAP- and AEA-evoked CGRP release. Pretreatment with staurosporine (1 μM) for 5 min significantly inhibited CAP- (50 and 100 nM) and AEA- (30 μM) evoked CGRP release (Figure 5a). Since staurosporine is a nonselective protein kinase inhibitor, we examined CAP-evoked CGRP release in the presence of specific inhibitors of PKA and PKC. Pretreatment with the cell-permeable PKA inhibitor peptide myristolated PKI 6–22 amide (10 μM) for 15 min significantly right-shifted the EC_{50} for CAP-evoked CGRP release from 39.3 ± 6.5 to 61.1 ± 9.8 nM, without significantly altering the E_{max} (Figure 5b). Also, pretreatment with the specific PKC inhibitor

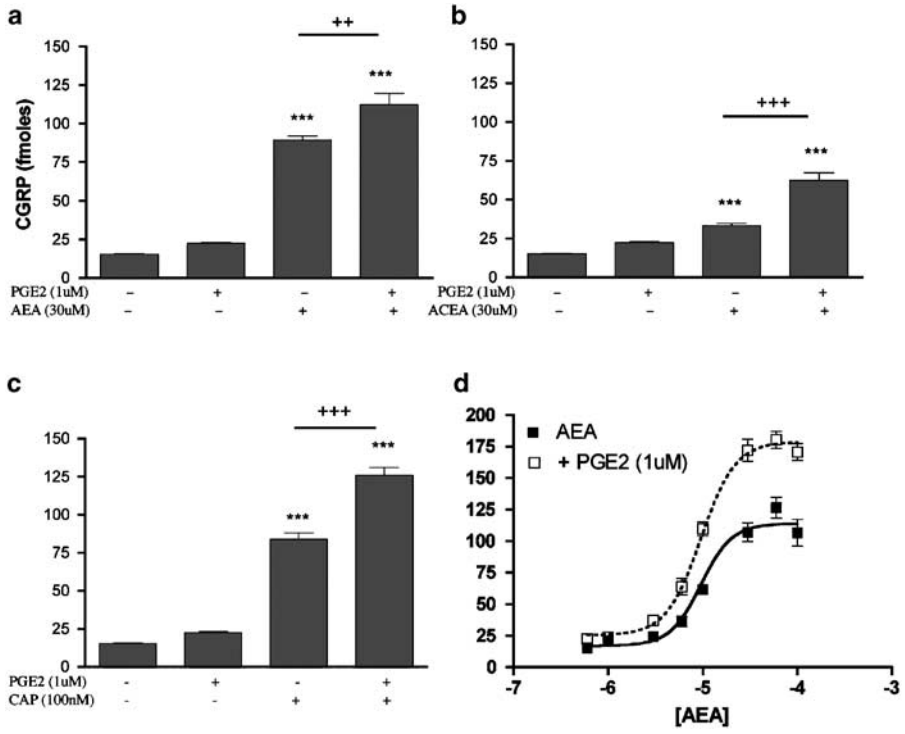

Figure 3 PGE_2 augments the effects of AEA and ACEA: TG neuronal cultures were pretreated with 1 μM PGE_2 or vehicle for 15 min and then exposed to the indicated concentration of AEA (a), ACEA (b) or CAP (c) for 10 min in the continued presence of PGE_2 (***$P<0.001$ vs BL; $^{++}P<0.01$ vs AEA alone, $^{+++}P<0.001$ vs ACEA or CAP alone). (d) Concentration–response relationships for 10 min AEA exposure were determined following a 15 min pretreatment and cotreatment with 1 μM PGE_2 ($n=6$). Supernatants were assayed for CGRP by RIA as described in Methods.

bisindoylmaleimide I (1 μM) for 15 min significantly right-shifted the EC_{50} for CAP-evoked CGRP release from 40.7 ± 3.7 to 80.0 ± 7.6 nM and significantly reduced the E_{max} (Figure 5c).

CAP initially causes desensitization, then excitotoxicity and eventually death of sensory neurons, thereby progressively rendering these neurons nonresponsive to a number of chemical stimuli (Jancso *et al*., 1967). Therefore, we compared the effects of AEA, ACEA or CAP pretreatment on a subsequent CAP response. TG neurons were plated, grown for 24 h and exposed to one of multiple sub- or supramaximal concentrations of AEA, ACEA or CAP for 4 h. On day 5, TG neuronal cultures were exposed to 100 nM CAP, followed by 50 mM K^+ treatment, and all samples were assessed for CGRP release. Submaximal concentrations of AEA (30 μM) or CAP (100 nM) did not influence the subsequent response to 100 nM CAP on day 5 (Figure 6). On the other hand, supramaximal concentrations of AEA (100 μM) or CAP (1 μM) significantly reduced the subsequent 100 nM CAP-evoked CGRP release ($P<0.01$), and no subsequent increase in release with 50 mM K^+ was observed (data not shown). In contrast to the effects of AEA and CAP pretreatment, pretreatment with ACEA at either 30 or 100 μM significantly sensitized the subsequent response to 100 nM CAP.

As the endocannabinoids AEA and NADA are known to be TRPV1 agonists, we tested the hypothesis that AEA, NADA and ACEA each act through TRPV1 by utilizing either of the two TRPV1 antagonists CPZ or I-RTX. I-RTX (Figure 7a) and CPZ (Figure 7b) each concentration-dependently inhibited E_{max} concentrations of AEA-, NADA-, ACEA and CAP-evoked CGRP release with roughly equivalent IC_{50} values (Table 1). I-RTX completely reversed the effects of NADA and CAP; however, neither antagonist was able to return AEA- and ACEA-evoked CGRP release completely to baseline levels, nor did CPZ return NADA-evoked CGRP release to baseline. Accordingly, 10–100 μM AEA, NADA, ACEA and CAP each evoked CGRP release in the presence of 200 nM I-RTX (Figure 7c) to only 20–30% of maximal levels (i.e., 70–80% inhibition). Neither 1 μM PMSF nor 1 μM indomethacin inhibited AEA- or ACEA-evoked CGRP release in the presence of 10 μM CPZ (Figure 7d).

Finally, to determine the extent to which AEA, NADA, ACEA and CAP are pungent (i.e., noxious), we examined the behavioral effects of all four compounds in the rat eye-wipe assay (Hammond & Ruda, 1991). These experiments necessitated controlling for different vehicles due to the manner in which the compounds are supplied from their sources; hence, comparisons were made to vehicle based on the ethanol (3.3% for CAP, and 20% for ACEA and NADA) or Tocrisolve (10% for AEA) concentration used at the highest dose of drug. CAP elicited immediate nocifensive behavior at all concentrations tested, peaking at 73.8 ± 4.87 s at 0.1% CAP (Figure 8a). Interestingly, AEA did not cause nocifensive behavior at any concentration tested (0.1–1% in 60 μl, equivalent to a total dose of 175–1750 nmol). Additionally, the inclusion of the CB1 antagonist SR141716A (0.1%) or the FAAH inhibitor PMSF (100 μM) did not unmask a nociceptive effect of AEA in this assay (Figure 8b). Likewise, ACEA (0.1%) was incapable of eliciting nocifensive behavior, while NADA (0.1%), on the other hand, did (Figure 8c). It is noteworthy that, while

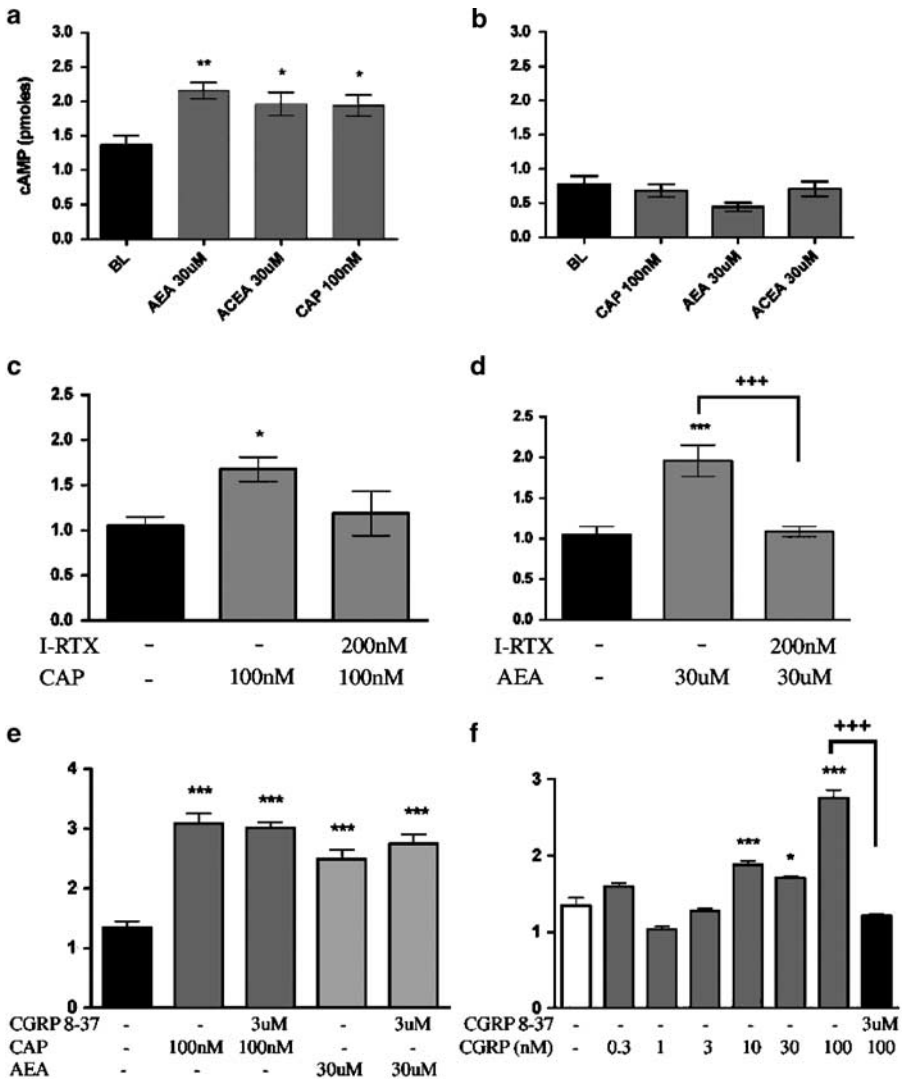

Figure 4 AEA, ACEA and CAP each stimulate cAMP accumulation in an extracellular calcium- and TRPV1-dependent fashion: (a) TG neuronal cultures were exposed to the indicated concentration of drugs in the presence of 10 μM rolipram for 15 min, and then assayed for cAMP accumulation (*$P<0.05$, **$P<0.01$ vs BL). (b) Using the same paradigm, the calcium dependence of the observed cAMP accumulation was tested in calcium-free buffer. CAP- and AEA-evoked cAMP accumulation (c and d, respectively) were tested in the presence of I-RTX (*$P<0.05$, ***$P<0.001$ vs BL; $^{+++}P<0.001$ vs AEA alone; $n=6$). (e) TG neuronal cultures were pretreated with 3 μM $CGRP_{8-37}$ for 10 min and then exposed to the indicated concentration of AEA or CAP in the continued presence of $CGRP_{8-37}$ (***$P<0.001$ vs BL; $n=9$). (f) TG neuronal cultures were pretreated with 3 μM $CGRP_{8-37}$ for 10 min and then were exposed to the indicated concentration of exogenous CGRP for 15 min in the continued presence of $CGRP_{8-37}$ (*$P<0.05$, ***$P<0.001$ vs BL; $^{+++}P<0.001$ vs 100 nM CGRP; $n=6$). Neuronal extracts were assayed for cAMP as described in Methods.

the nocifensive response of animals exposed to CAP was immediate, there was an initial lag period of approximately 90 s in the response to NADA, and this was followed by an intense period of facial wiping, including a holding of the eye in a closed position, which lasted for approximately 25 s. Additionally, when the eye was pre-exposed to 0.1% AEA either 30 s or 5 min prior to 0.1% CAP application, the subsequent response was unaltered (Figure 8d).

Discussion

The present work was undertaken to elucidate the effects of certain cannabinoids on nociceptive TG neurons using an *in vitro* culture system. This neuronally enriched, primary culture system affords the ability of separating out the potential contributions of cannabinoid receptors located on extra-neuronal cells, not present in this preparation, allowing us to focus on the direct effects of cannabinoids on the nociceptive neurons only. The present results indicate that the predominant effects of the endocannabinoids AEA and NADA, as well as the synthetic AEA analogue ACEA, on TG nociceptors are excitatory and, at least in part, mediated by TRPV1.

In agreement with the demonstration that CB1 receptors are almost exclusively localized to myelinated neurons in both the TG (Price *et al*., 2003) and DRG (Bridges *et al*., 2003), we were unable to demonstrate an inhibitory effect of AEA or ACEA on TG nociceptors at concentrations relevant to CB1 agonism. Neither AEA nor ACEA inhibited CAP-evoked CGRP release at multiple concentrations of CAP, indicating that CB1

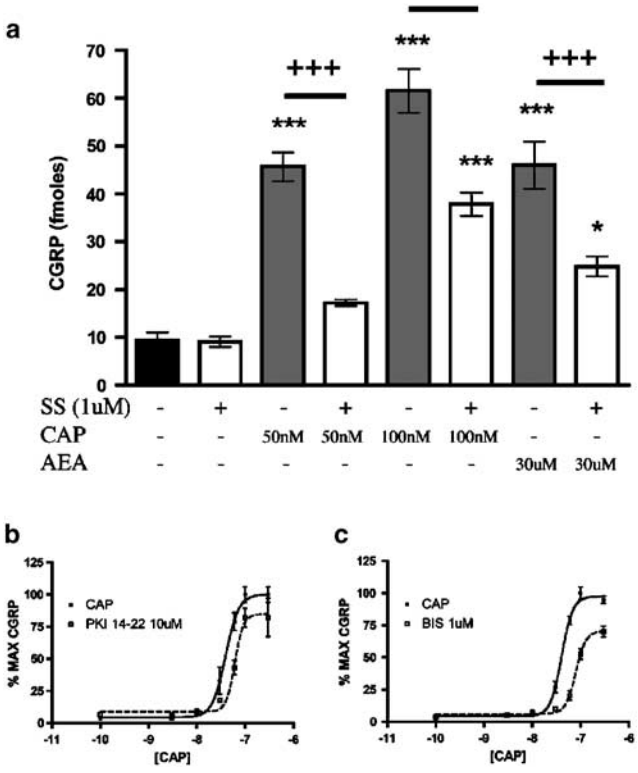

Figure 5 Effect of PKA and PKC inhibitors on CAP- or AEA-evoked CGRP release: (a) TG neuronal cultures were pretreated for 5 min with 1 μM staurosporine (SS) or vehicle and then exposed to the indicated concentration of CAP or AEA in the continued presence of SS or vehicle ($^{*}P<0.05$, $^{***}P<0.001$ vs BL; $^{+++}P<0.001$ vs CAP or AEA alone). Concentration–response relationships for 10 min CAP exposure were determined following a 15 min pretreatment and cotreatment with 10 μM myristolated PKI 14–22 amide (b) or 10 μM bisindolylmaleimide (BIS; c). The EC_{50} of CAP was significantly increased in the presence of PKI 14–22 ($F_{3,32}=9.06$; $P<0.001$), while the EC_{50} of CAP was significantly increased and its E_{max} was significantly decreased in the presence of BIS ($F_{3,33}=116.0$; $P<0.0001$). Supernatants were assayed for CGRP by RIA as described in Methods.

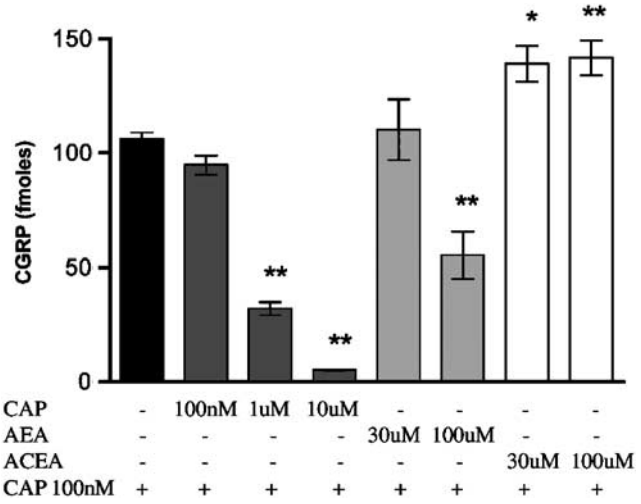

Figure 6 Change in CAP sensitivity by TRPV1 agonist pretreatment: TG neuronal cultures were exposed 1 day post plating to the indicated concentrations top 3 rows of agonist for 4 h, after which the neurons were incubated in fresh media. On day 5, all cultures were exposed to 100 nM CAP ($^{*}P<0.5$, $^{**}P<0.01$ vs VEH; $n=6$). Supernatants were assayed for CGRP by RIA as described in Methods.

receptors either are not found on CAP-sensitive TG nociceptors, as would be indicated by colocalization studies in TG and DRG, or are unable to alter the neuropeptide secretogogue effects of CAP. Although cannabinoids, acting through their cognate receptors, are known to inhibit calcium channels, it has been demonstrated that, in cultured sensory neurons, CAP-evoked peptide release is not dependent on voltage-gated calcium channels (Evans *et al*., 1996). Therefore, we tested the ability of AEA to inhibit K^+-evoked CGRP release. In agreement with previous calcium-imaging studies in DRG (Khasabova *et al*., 2002), we saw no effect of AEA on K^+-evoked CGRP release, supporting the hypothesis that sensory neuronal CB1 receptors do not inhibit the activation of voltage-gated calcium channels in this population.

Furthermore, and in contrast to a previous study in cultured DRG neurons (Ahluwalia *et al*., 2003b), we found no evidence for endocannabinoid tone in TG neurons, as neither of the CB1 antagonists SR141716A nor AM251 was able to unmask an endogenous cannabinoid effect on basal or evoked CGRP release. Somewhat surprisingly, SR141716A, at μM concentrations, attenuated CAP-evoked release, and, taken together with our observations that concentrations of SR141716A above 50 μM were capable of evoking CGRP release, provide further evidence that SR141716A might act as a TRPV1 antagonist/partial agonist at high concentrations (Jennings *et al*., 2003). In any case, taken together with the paradoxical findings by Ahluwalia *et al*. (2003a) that AEA inhibits CAP-evoked CGRP release in DRG cultures, the present findings suggest a potential difference in cannabinoid-mediated modulation of neuropeptide secretion between DRG and TG neurons. Indeed, such evidence has already been provided for another cannabinoid-responsive TRP channel (ANKTM1), which is expressed at the mRNA level in 20% of newborn rat TG neurons, but only in 4% of mouse DRG neurons (Jordt *et al*., 2004). The potential existence of other ionotropic cannabinoid receptors, which are preferentially expressed in the DRG compared with the TG, warrants further study.

As it is possible that CB1 receptors might be capable of inhibiting other signaling pathways in TG neurons, we examined the effects of AEA on BK-stimulated IP accumulation. Indeed, at a concentration that evokes CGRP release (i.e., 10 μM), neither did AEA inhibit BK-stimulated IP accumulation nor did it have any effect on its own. Taken together with the demonstration that WIN55,212-2 does not inhibit PGE_2-stimulated cAMP accumulation in TG neurons (Price *et al*., unpublished observations) or forskolin-stimulated cAMP accumulation in DRG neurons (Ross *et al*., 2001), the data indicate that cannabinoids are not capable of inhibiting signaling pathways associated with the major inflammatory mediators that are known to sensitize nociceptors.

These lines of evidence, however, call attention to other pathways through which cannabinoids might act to alleviate nociception. In particular, the role of CB1 receptors on myelinated sensory nerve fibers warrants further attention, as recent studies have more keenly elucidated the involvement of Aβ fibers in mediating hyperalgesia and allodynia (Garcia-Nicas *et al*., 2001; Kim *et al*., 2001). Clearly, cannabinoids are capable of inhibiting nociceptive behavior in a number

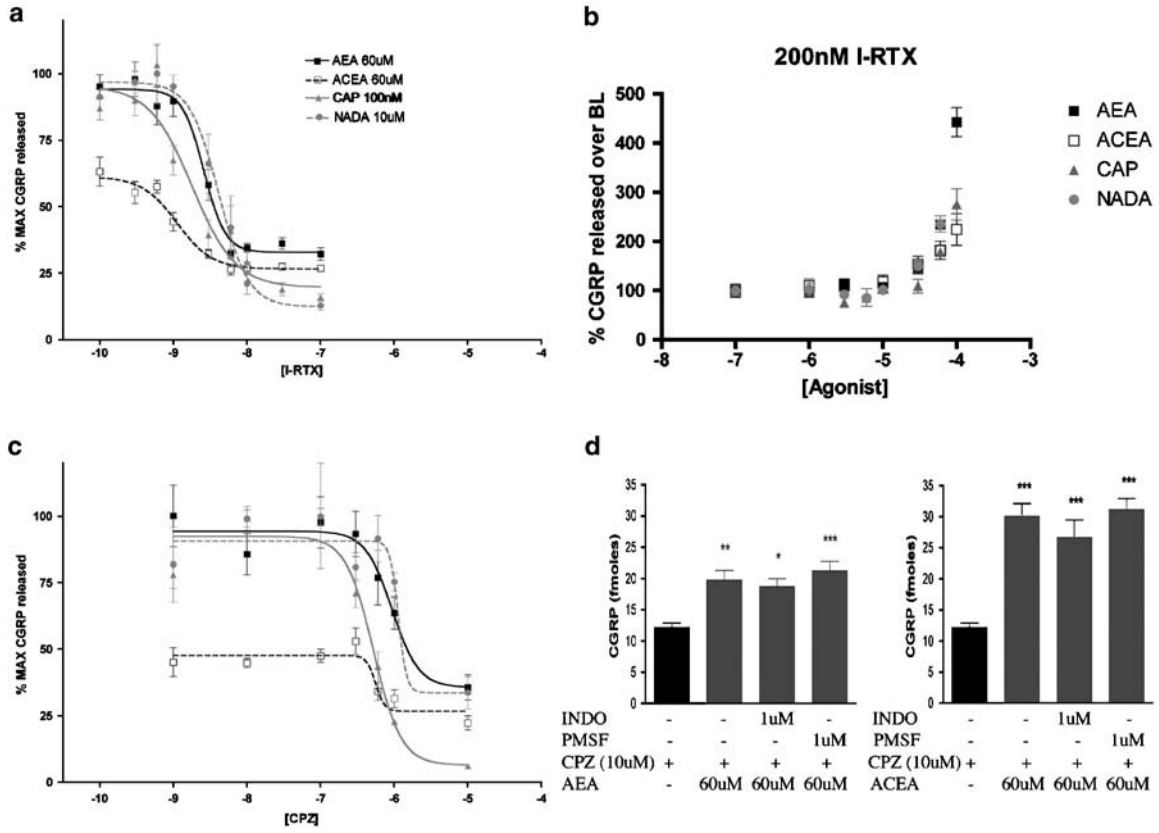

Figure 7 CPZ and I-RTX each inhibit AEA-, NADA-, ACEA- and CAP-evoked CGRP release although CGRP release persists at high concentrations: Concentration–response relationships for AEA-, NADA-, ACEA-and CAP-evoked CGRP release in the presence of increasing concentrations of I-RTX (a) or CPZ (b; $n=12$: AEA, ACEA and CAP, $n=6$: NADA). (c) Concentration–response relationships were also constructed for AEA, NADA, ACEA and CAP in the presence of 200 nM I-RTX to determine TRPV1-independent effects ($n=3$). Under these conditions, the slopes for all compounds were significantly non-zero ($P<0.0001$). (d) In the continuous presence of 10 μM CPZ, TG neuronal cultures were pretreated with 1 μM indomethacin (INDO), phenylmethylsulfonyl fluoride (PMSF) or vehicle for 10 min and then exposed to 60 μM AEA (left subpanel) or ACEA (right subpanel) for 10 min in the continued presence of INDO, PMSF or vehicle (*$P<0.01$, **$P<0.01$; ***$P<0.001$ vs BL; $n=6$). Supernatants were assayed for CGRP by RIA as described in Methods.

Table 1

Antagonist	*AEA*	*NADA*	*ACEA*	*CAP*
I-RTX	-8.59 ± 0.05[a]	-8.38 ± 0.07	-8.95 ± 0.08	-8.74 ± 0.10
CPZ	-6.03 ± 0.10	-5.95 ± 0.29	-6.03 ± 0.21	-6.28 ± 0.08

[a]Mean $\pm$ s.e.m. Log IC_{50}'s for I-RTX and CPZ in the presence of E_{max} doses of each of the four agonists AEA, NADA, ACEA and CAP.

of assays involving either inflammation or nerve injury (Calignano *et al*., 1998; Richardson *et al*., 1998; Farquhar-Smith & Rice, 2001; Fox *et al*., 2001). Likewise, peripheral CB1 receptors have been demonstrated to inhibit nociception in rodents and primates (Richardson *et al*., 1998; Ko & Woods, 1999; Calignano *et al*., 2001; Rukwied *et al*., 2003). Moreover, peripheral CB2 receptors, likely on extraneuronal cell types, have been shown to mediate antinociception (Calignano *et al*., 1998; 2001; Malan *et al*., 2001; Farquhar-Smith *et al*., 2002; Ibrahim *et al*., 2003).

Related to these findings, it has recently been demonstrated that keratinocytes express CB1 (Maccarrone *et al*., 2003) as well as TRPV1 (Denda *et al*., 2001; Inoue *et al*., 2002), and activation of the latter leads to pro-inflammatory cytokine release (Southall *et al*., 2003), collectively suggesting an additional potential site for cannabinoid action in the periphery. Insofar as acute nociception is likely mediated exclusively by the direct actions of noxious stimuli on nociceptors, we propose that the preponderance of evidence presented here and elsewhere indicates that CB1-mediated effects should be examined in terms of their inhibitory actions on Aβ fibers as well as extraneuronal cell types. Collectively, these studies serve to expand the pharmacological and cellular repertoire of cannabinoid-responsive analgesic targets.

Although we were unable to demonstrate an inhibitory effect of cannabinoids in the assays described herein, we repeatedly observed excitatory effects of AEA, ACEA and NADA on TG neurons. All three compounds evoked CGRP release in a concentration-dependent fashion, with a rank order potency of CAP > NADA > AEA = ACEA. We utilized two TRPV1 antagonists to demonstrate that AEA, ACEA, NADA and CAP all act through the TRPV1 receptor to evoke CGRP release. In addition, we have provided the initial demonstration that ACEA is a partial agonist at TRPV1 with an α value of $\sim$0.5. That AEA, NADA, ACEA and CAP were each antagonized by CPZ and I-RTX with respectively similar IC_{50} values indicates that each acts through TRPV1. I-RTX was the more potent antagonist in these assays, with an almost

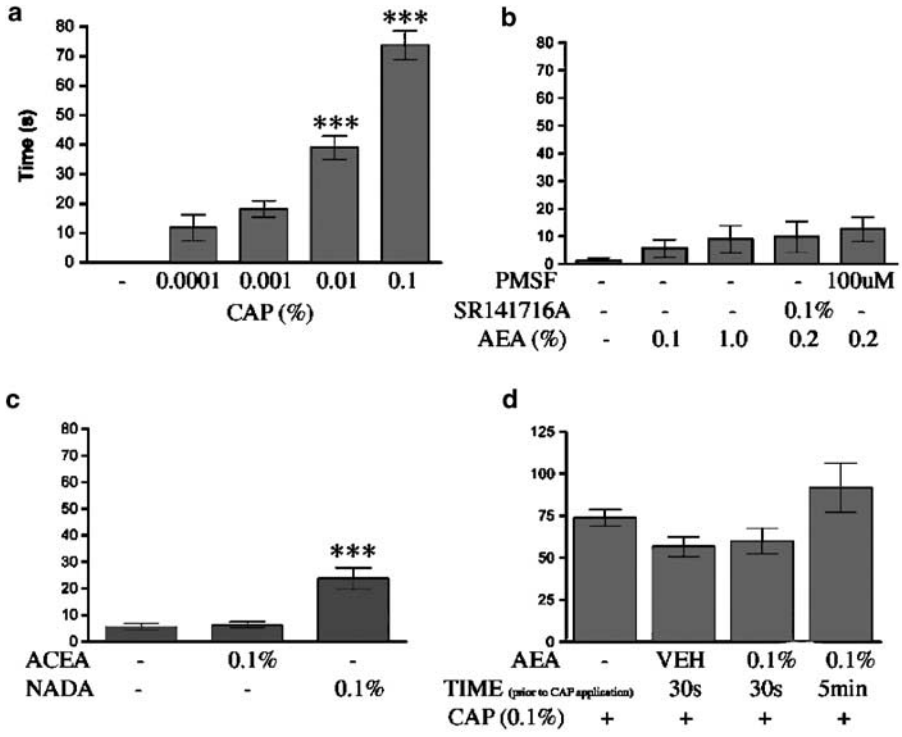

Figure 8 Assessment of pungency for AEA, NADA, ACEA and CAP in the rat eye-wipe test: CAP (a, ***$P<0.001$; $n=4$), AEA or AEA with SR141716A or PMSF (b, $n=6$) or ACEA or NADA (c, ***$P<0.001$ vs Veh; $n=6$) were applied directly to the eye of freely behaving rats at the indicated concentrations, and nocifensive behaviors (defined in Methods) were subsequently measured for 5 min. (d) Following pretreatment with 0.1% AEA or vehicle, nocifensive behaviors in response to 0.1% CAP were measured ($n=6$).

100-fold lower IC_{50} than CPZ, consistent with previous studies (Seabrook *et al*., 2002; Jennings *et al*., 2003). Surprisingly, neither CPZ nor I-RTX was able to return AEA- or ACEA-evoked CGRP release to baseline levels, and in fact, AEA, ACEA, NADA and CAP were each capable of inducing CGRP release in a concentration-dependent (ranging from 10–100 μM) fashion in the presence of a supramaximal concentration of I-RTX. This finding indicates that AEA, ACEA, NADA and CAP act in part through an additional, unknown mechanism to increase CGRP outflow from TG neurons. This effect was not blocked by PMSF or indomethacin, indicating that AEA and ACEA themselves, rather than FAAH- or cycloxygenase-generated metabolites of these compounds, are mediating this effect.

Nociceptor sensitization at the level of TRPV1 has been shown to involve the cAMP/PKA pathaway (Hingtgen *et al*., 1995; Lopshire & Nicol, 1998; Bhave *et al*., 2002). This likely occurs through direct phosphorylation of the channel, causing it to remain in the plasma membrane, thereby making it available for ligand binding and, hence, channel activation (Bhave *et al*., 2002). Accordingly, and in agreement with previous findings for CAP, we observed increased AEA-, ACEA- and CAP-evoked CGRP release in the presence of PGE_2, which is known to stimulate cAMP accumulation and PKA activity in sensory neurons (Lopshire & Nicol, 1998; Gu *et al*., 2003). In TRPV1-expressing HEK-293 cells, as well as in DRG slices, forskolin-stimulated PKA activation has been shown to augment AEA responsiveness and left-shift its potency (De Petrocellis *et al*., 2001). Here, there was no shift in the potency of AEA; rather, the presence of PGE_2 merely increased the E_{max}, indicating that, at least in this system, sensitization of nociceptors through the PKA pathway does not lead to a lowering of the concentration of AEA needed to excite TG neurons.

Despite studies to the contrary in DRG neurons (Wood *et al*., 1989; Dymshitz & Vasko, 1994), it has been demonstrated that CAP stimulates cAMP accumulation in dorsal spinal cord slices in a calcium-dependent and SP-independent fashion (Northam & Jones, 1984) and in TG neurons in a CPZ-sensitive fashion (Liu *et al*., 2001; Agopyan *et al*., 2003). In the present studies, we have reported the novel finding that AEA and ACEA each stimulate cAMP accumulation in TG neurons as well. Importantly, the ability of AEA and CAP to stimulate cAMP accumulation was dependent on extracellular calcium, indicating that the opening of calcium-permeant channels is involved in this signaling event. TRPV1-positive sensory neurons express multiple calcium-dependent adenylyl cyclases (Distler *et al*., 2003), indicating that TG neurons likely contain the requisite molecules for calcium-mediated cAMP accumulation. Furthermore, this cAMP-stimulating effect of AEA appears to be mediated by TRPV1, as it was antagonized by I-RTX. Additionally, CAP- and AEA-stimulated cAMP accumulation does not appear to depend on CGRP release, as it was not blocked by a CGRP receptor antagonist. Almost certainly, this can be explained by the fact that the maximal amount of CGRP released by any of the secretagogues gives rise to a concentration (i.e., $\sim$0.3 nM) that is subthreshold for inducing cAMP accumulation. The otherwise intriguing finding here, that at higher concentrations (i.e., 10–100 nM) exogenously applied CGRP increased cAMP accumulation in

the cultures in a $CGRP_{8-37}$-sensitive manner, is consistent with the hypothesis that CGRP is acting in an autocrine fashion to regulate its own release and/or to mediate interneuronal signaling at the level of the trigeminal ganglion *in vivo*, as previously suggested (Ulrich-Lai *et al*., 2001). While we cannot exclude the possibility of other soluble factors in CAP- or AEA-stimulated cAMP accumulation, our CGRP receptor antagonist findings, taken together with the demonstration by Northam & Jones (1984), that CAP-stimulated cAMP accumulation in dorsal horn slices is SP receptor-independent, indicate that two of the major neuropeptides released by nociceptors (i.e., CGRP and SP) do not contribute to TRPV1-mediated cAMP accumulation and strengthen the argument for a direct influence of TRPV1-gated calcium influx.

CAP- and AEA-stimulated cAMP accumulation is especially interesting in terms of the ability of PKA to phosphorylate and regulate the activity of the TRPV1 channel (Bhave *et al*., 2002), and implies that TRPV1 might be capable of controlling its own sensitization/desensitization state through a positive feedback loop, wherein cAMP accumulation leads to PKA activation and PKA phosphorylation of TRPV1 maintains the localization of the channel in the plasma membrane (Bhave *et al*., 2002). In support of this hypothesis, the nonselective protein kinase inhibitor staurosporine reduced AEA- and CAP-evoked CGRP release. Additionally, selective PKA (PKI 14–22) and PKC (bisindolylmaleimide) inhibitors significantly right-shifted the EC_{50} for CAP-evoked CGRP release, suggesting that both cAMP/PKA and PKC signaling pathways play an acute role in modulating CAP-evoked neuropeptide release. Although the selective inhibitor of PKC both right-shifted the EC_{50} and reduced the E_{max} while the selective PKA inhibitor right-shifted the EC_{50} and had no effect on the E_{max}, these data demonstrate that the cAMP/PKA pathway, and calcium-mediated second-messenger generation, in general, might constitute a system through which TRPV1 can regulate its own activity.

An important property of CAP is its ability to cause the selective denervation of certain small-diameter sensory neurons (Jancso *et al*., 1967), a finding which has subsequently been extended to AEA (Szoke *et al*., 2002a). We have demonstrated here that long-term exposure of supramaximal concentrations of CAP or AEA causes a loss of responsiveness to a subsequent CAP challenge. Since we observed no subsequent increase in CGRP exocytosis when TG neurons were exposed to 50 mM K^+, it is evident that the CAP or AEA pre-exposure is leading either to a loss of peptide content or to an overall decrease in the number of neurons containing CGRP at 4 days following the pre-exposure. Both CAP and AEA produce mitochondrial dysfunction (Szoke *et al*., 2002a, b) in sensory neurons, possibly causing entry into apoptosis, supporting the hypothesis that a loss of peptidergic neurons from the TG neuronal cultures is responsible for the lack of responsiveness observed here. Interestingly, the partial agonist ACEA sensitized the subsequent CAP challenge, even at supramaximal concentrations. This implies that partial agonists of TRPV1 are incapable of promoting some, but not all, of the same cellular events (e.g., a loss of CAP responsiveness) as full agonists.

Finally, the pungency of AEA, ACEA and NADA were tested and compared to CAP in the rat eye-wipe assay (Hammond & Ruda, 1991; Urban *et al*., 2000). While CAP clearly elicited a nocifensive response at all concentrations tested, AEA and ACEA were not capable of doing so. Additionally, the inclusion of a CB1 antagonist or FAAH inhibitor with AEA did not unmask a nocifensive response to AEA. Since the FAAH inhibitor PMSF was without effect on AEA, it is unlikely that the lack of pungency observed with AEA is due to pharmacokinetic issues (e.g., rapid degradation), as the major degradatory enzyme for AEA is FAAH. We cannot rule out, however, that the nonpungency of AEA or ACEA in this assay is due to pharmacodynamic issues (e.g., lack of efficacy on the vanilloid receptors in the eye). That being the case, the structurally similar, dual cannabinoid–vanilloid agonist NADA was capable of eliciting a nocifensive response, indicating nominally that NADA is capable of gaining access to free nerve endings in the eye. Indeed, this is consistent with previous findings (Huang *et al*., 2002), although the effect occurred over a different time course than that for CAP. Furthermore, the close proximity of C-fiber endings to the surface of the cornea (Maciver & Tanelian, 1993) and the recent demonstration of the ability to load fluorescent dye into C-fibers through the corneal surface (Gover *et al*., 2003) indicate that both AEA and ACEA would likely have access to nerve endings of the cornea. CAP is approximately 200-fold more potent than AEA, based on the relative potencies determined in TG neuron CGRP release assays. Hence, notwithstanding potential pharmacokinetic differences, if the pharmacodynamic relationships hold true in this behavioral assay, then a nocifensive response would have been expected at the concentrations of AEA tested here. The lack of observed nocifensive behavior with AEA (or ACEA) indicates that, while these compounds are clearly TRPV1 agonists, they are nonpungent (i.e., non-noxious). Although the mechanism whereby TRPV1 agonists can be nonpungent remains a mystery, there are multiple other examples of such compounds such as scutigeral and phorbol 12-phenylacetate 13 acetate 20-homovanillate (Liu *et al*., 1998; Szallasi *et al*., 1999; Urban *et al*., 2000; Iida *et al*., 2003). While the nonpungency of AEA and ACEA clearly needs to be extended to human studies of nociception, our data indicate that AEA or ACEA might be similarly utilized without concerns over pungency at the relatively higher doses needed for transdermal application.

In conclusion, we have demonstrated that AEA, NADA and ACEA each act through excitatory mechanisms in TG nociceptors. We conclude that most of these actions are mediated by TRPV1, as either CPZ or I-RTX, respectively, blocked AEA-, NADA-, ACEA- and CAP-evoked CGRP release equipotently. Interestingly, while CAP and NADA were found to be pungent, AEA and ACEA did not evoke a nocifensive response when administered intraocularly. In assays to assess cannabinoid-mediated inhibition of TG neuronal responses, we were unable to observe any inhibitory effects mediated by AEA or ACEA, consistent with the lack of CB1 localization to the neuronal profiles examined in the assays performed here. These findings highlight the necessity for further study of the cannabinoid system as it relates to nociception, with particular attention to the role of CB1 receptors in myelinated sensory neurons and extra-neuronal cannabinoid-responsive cells in the periphery.

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