Δ^9-Tetrahydrocannabinol and Cannabinol Activate Capsaicin-Sensitive Sensory Nerves via a CB_1 and CB_2 Cannabinoid Receptor-Independent Mechanism

Peter M. Zygmunt, David A. Andersson, and Edward D. Högestätt

Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University Hospital, SE-221 85 Lund, Sweden

Although Δ^9-tetrahydrocannabinol (THC) produces analgesia, its effects on nociceptive primary afferents are unknown. These neurons participate not only in pain signaling but also in the local response to tissue injury. Here, we show that THC and cannabinol induce a CB_1/CB_2 cannabinoid receptor-independent release of calcitonin gene-related peptide from capsaicin-sensitive perivascular sensory nerves. Other psychotropic cannabinoids cannot mimic this action. The vanilloid receptor antagonist ruthenium red abolishes the responses to THC and cannabinol. However, the effect of THC on sensory nerves is intact in vanilloid receptor subtype 1 gene knock-out mice. The THC response depends on extracellular calcium but does not involve known voltage-operated calcium channels, glutamate receptors, or protein kinases A and C. These results may indicate the presence of a novel cannabinoid receptor/ion channel in the pain pathway.

Key words: calcitonin gene-related peptide; cannabinoids; cannabinol; cannabis; capsaicin; nociceptors; pain; receptors, sensory; tetrahydrocannabinol

Marijuana contains a mixture of different cannabinoids, of which Δ^9-tetrahydrocannabinol (THC), the major psychoactive ingredient, has been characterized extensively with regard to analgesic and anti-inflammatory effects (Mechoulam and Hanus, 2000; Pertwee, 2001). The presence of CB_1 cannabinoid receptors in the pain pathway may explain the analgesic effects of cannabinoids (Zimmer et al., 1999; Morisset et al., 2001; Pertwee, 2001). However, the well known psychotropic effects of many cannabinoids are attributable to activation of CB_1 receptors and limit their therapeutic value as analgesics (Pertwee, 2001). Interestingly, some cannabinoids, such as cannabidiol, cannabinol, and carboxy derivatives of THC, have analgesic and anti-inflammatory effects despite being weak CB_1 receptor agonists (Srivastava et al., 1998; Burstein, 1999; Malfait et al., 2000). The effect of THC in the hot-plate test is lost in CB_1 receptor gene knock-out mice (Ledent et al., 1999; Zimmer et al., 1999), but the analgesic effect of THC in the tail-flick test is intact (Zimmer et al., 1999). This indicates that THC can induce antinociception also via a CB_1 receptor-independent mechanism.

Although CB_1 receptors are present on a subpopulation of primary sensory neurons, the effects of THC on pain-sensing primary afferents have not been examined. In addition to transmitting nociceptive information to the CNS, these nerves also participate in the local response to tissue injury, including the release of vasodilator neuropeptides (Holzer, 1992; Szallasi and Blumberg, 1999). Thus, primary sensory nerves are able to release neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, in both the periphery and the spinal cord (Holzer, 1992; Szallasi and Blumberg, 1999). In the vasculature, this leads to vasodilatation and increased vascular permeability (Holzer, 1992). Isolated arterial segments provide a sensitive bioassay for studying the effects of drugs acting on such efferent signaling (Hogestatt and Zygmunt, 2002). Initially, using this bioassay, we planned to study whether cannabinoids, including THC and HU-210, inhibit the activity of perivascular sensory nerve. Unexpectedly, we found that THC itself causes activation of capsaicin-sensitive sensory nerves. This effect of THC is not mediated by known cannabinoid receptors and could indicate the existence of a novel target for cannabinoids in the pain pathway.

MATERIALS AND METHODS

Animals. Experiments were performed on hepatic and mesenteric arteries from female Wistar–Hannover rats (250 gm) obtained from M & B (Ry, Denmark) and on mesenteric arteries from male mice (30 gm). Wild-type mice (C57BL/6J) were obtained from M & B, whereas Professor David Julius (University of San Francisco, San Francisco, CA) generously supplied vanilloid receptor subtype 1 gene knock-out ($VR1^{-/-}$) mice and their homozygous controls ($VR1^{+/+}$). The genotype ($VR1^{-/-}$ or $VR1^{+/+}$) was not disclosed until the experiments had been completed.

Recording of tension. The arteries were cut into ring segments and mounted in tissue baths containing physiological salt solution (PSS) of the following composition (in mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5, and (+)-glucose 6.0. The PSS was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, resulting in a pH of 7.4. All experiments were performed at 37°C in the presence of N^G-nitro-L-arginine (300 μM) and indomethacin (10 μM) to eliminate any contribution of nitric oxide and cyclooxygenase products, respectively. Relaxations were studied in vessels contracted with phenylephrine (3 μM). When stable contractions were obtained, agonists were added cumulatively to determine concentration–response relationships. Unless otherwise stated, the effects of test substances on vasorelaxation were recorded after pre-exposure of the vessels to the test substances or vehicle for 30 min. Each vessel segment was exposed to only one treatment. In some experiments, the endothelium was removed by blowing carbogen through the vessel lumen. Lack of relaxation in response to 10 μM acetylcholine confirmed a successful removal of the endothelium.

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Correspondence should be addressed to Peter Zygmunt, Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University Hospital, SE-221 85 Lund, Sweden. E-mail: Peter.Zygmunt@klinfarm.lu.se.

Measurement of CGRP. Segments of rat hepatic or mesenteric arteries were equilibrated for 1 hr in aerated PSS (95% O_2 and 5% CO_2; 37°C; pH 7.4) containing N^G-nitro-L-arginine (300 μM) and indomethacin (10 μM). After a 20 min preincubation period with test drugs in PSS, preparations were transferred to Eppendorff tubes containing the test drugs or vehicle and 0.05% bovine serum albumin in either PSS, nominally calcium-free PSS (10 μM EGTA), or Tris-buffer solution (experiments with lanthanum). The segments were removed after 10 min, and the solution in the test tubes was evaporated. The amount of CGRP in the pellet was determined using a rat ^{125}I-labeled CGRP radioimmunoassay kit (Peninsula Laboratories, Belmont, CA). The Tris-buffer solution was of the following composition (in mM): NaCl 134, Trisma base 5 mM, KCl 4.6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, and (+)-glucose 6.0, pH 7.4.

Calculations and statistics. Relaxations are expressed as percentage reversal of the phenylephrine-induced contraction. The maximal relaxation (E_{max}) and the log molar concentration of drug that elicited half-maximal relaxation (pEC_{50}) were calculated using GraphPad Prism (version 3.00; GraphPad Software Inc., San Diego, CA). When the concentration–response curve did not reach a plateau, and hence E_{max} and pEC_{50} could not be determined, the area under the curve was calculated (GraphPad Prism version 3.00) and used for evaluation of drug effects. Data are presented as mean ± SEM (vertical lines in figures), and *n* indicates the number of experiments performed (number of animals). Statistical analysis was performed using Student's unpaired *t* test (two-tailed) or ANOVA followed by Bonferroni's test (GraphPad Prism version 3.00). Statistical significance was accepted when $p < 0.05$.

Drugs. Phorbol 12,13-dibutyrate (PDBu), 4α-phorbol 12,13-dibutyrate, and staurosporine (Biomol, Plymouth Meeting, PA); anandamide (Cayman Chemical, Ann Arbor, MI); SR141716A (Sanofi Winthrop, Montpellier, France); cannabinol (−)-Δ^9-tetrahydrocannabinol, 11-OH-Δ^9-tetrahydrocannabinol, and Δ^9-tetrahydrocannabinol-11-oic acid (Sigma, St. Louis, MO); and capsaicin, capsazepine, ryanodine, and AM251 (Tocris, Bristol, UK) were all dissolved in and diluted with ethanol. Distilled water or saline was used as solvent for α-latrotoxin, calcicludine, ω-conotoxin GVIA, and ω-conotoxin MVIIC (Alomone Labs, Jerusalem, Israel); nimodipine (Nimotop; Bayer, Wuppertal, Germany); indomethacin (Confortid; Dumex, Copenhagen, Denmark); L-phenylephrine hydrochloride, acetylcholine hydrochloride, N^G-nitro-L-arginine, caffeine, rat CGRP, human 8-37 CGRP, L-glutamic acid, and ruthenium red (Sigma); CNQX disodium salt, (+)-MK-801, and dantrolene (Tocris); and SIN-1 hydrochloride (Calbiochem, La Jolla, CA).

RESULTS

THC induces a concentration-dependent relaxation in rat isolated hepatic and mesenteric arterial segments (hepatic artery, $pEC_{50} = 6.3 \pm 0.1$; $E_{max} = 96 \pm 1\%$; $n = 17$; mesenteric artery, $pEC_{50} = 6.7 \pm 0.1$; $E_{max} = 97 \pm 1\%$; $n = 6$). The effect of THC does not involve endothelial cells, because THC is equally potent at relaxing hepatic arteries ($pEC_{50} = 6.2 \pm 0.1$; $E_{max} = 96 \pm 2\%$; $n = 5$) and mesenteric arteries (Fig. 1) without endothelium. To investigate whether THC activates capsaicin-sensitive sensory nerves, arteries were pretreated with 10 μM capsaicin for 30 min to cause desensitization and/or neurotransmitter depletion of sensory nerves. The effect of THC was tested after washout of capsaicin for 20 min. As shown in Figure 1*A,B*, THC fails to relax such arteries. Because CGRP is the main vasodilator released from capsaicin-sensitive sensory nerves in rat hepatic and mesenteric arteries (Kawasaki et al., 1988; Zygmunt et al., 1999), we tested the effect of the CGRP receptor antagonist 8-37 CGRP on THC-induced vasorelaxations in these arteries. At 3 μM, 8-37 CGRP abolishes the vasorelaxations elicited by THC (Fig. 1*A,B*). Cannabinol, another naturally occurring cannabinoid, also causes vasorelaxation ($pEC_{50} = 6.2 \pm 0.1$; $E_{max} = 96 \pm 2\%$; $n = 7$), which is abolished in the presence of 8-37 CGRP or in arteries pretreated with capsaicin (Fig. 1*C*). In mesenteric arteries, measurement of CGRP-like immunoreactivity provides direct evidence that THC and cannabinol release CGRP from capsaicin-sensitive sensory nerves. Thus, THC and cannabinol each release CGRP compared with basal CGRP levels (basal, 56.4 ± 2.4 fmol/mg protein; THC, 85.2 ± 7.2 fmol/mg protein; cannabinol, 86.7 ± 7.9 fmol/mg protein; $p < 0.01$; $n = 6$). When arteries had been pretreated with capsaicin for 30 min (followed by washout of capsaicin), THC and cannabinol could no longer evoke release above basal CGRP levels (THC, 57.7 ± 6.5 fmol/mg protein; cannabinol, 45.7 ± 3.5 fmol/mg protein; $n = 6$). Other cannabinoids, such as 11-OH-Δ^9-THC, Δ^9-THC-11-oic acid, and, as shown previously (Zygmunt et al., 1999), HU-210 and CP 55,940, do not produce sensory nerve-mediated vasorelaxation (Fig. 2). The vasodilator effect of THC is not attributable to activation of CB_1 receptors, because antagonists of this receptor do not inhibit the action of THC (Fig. 3*A,D*).

Activation of vanilloid receptors on sensory nerves leads to the release of CGRP and vasodilatation of rat hepatic and mesenteric

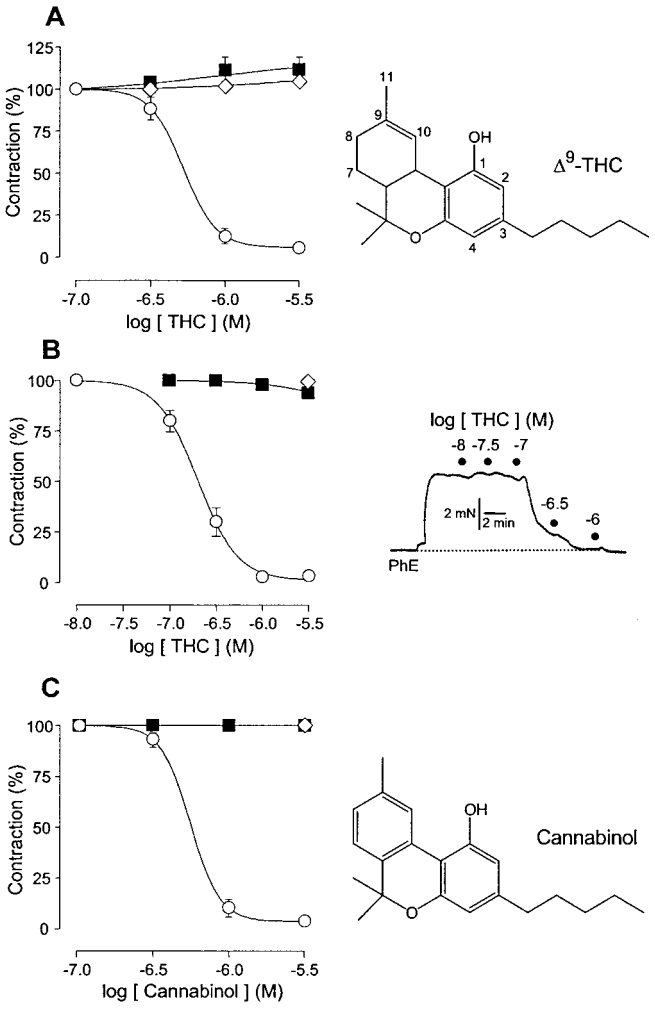

Figure 1. The naturally occurring cannabinoids THC and cannabinol evoke sensory nerve-mediated relaxation of rat hepatic and mesenteric arterial segments contracted with phenylephrine (*PhE*). The concentration-dependent relaxations induced by THC (○) in hepatic ($n = 5$) (*A*) and mesenteric ($n = 6$) (*B*) arteries, and those induced by cannabinol (○) in hepatic arteries ($n = 7$) (*C*) are abolished in arterial segments pretreated with the sensory neurotoxin capsaicin (10 μM; ◇; $n = 5$ and 4 for THC and cannabinol, respectively). The CGRP receptor antagonist 8-37 CGRP (3 μM; ■) also prevents relaxations induced by THC ($n = 5$ and 6 for hepatic and mesenteric arteries, respectively) and cannabinol ($n = 4$). *B*, As shown by the *trace*, THC also relaxes the mesenteric artery without endothelium. The *dotted line* shows the basal tension level before addition of PhE. Data are expressed as mean ± SEM.

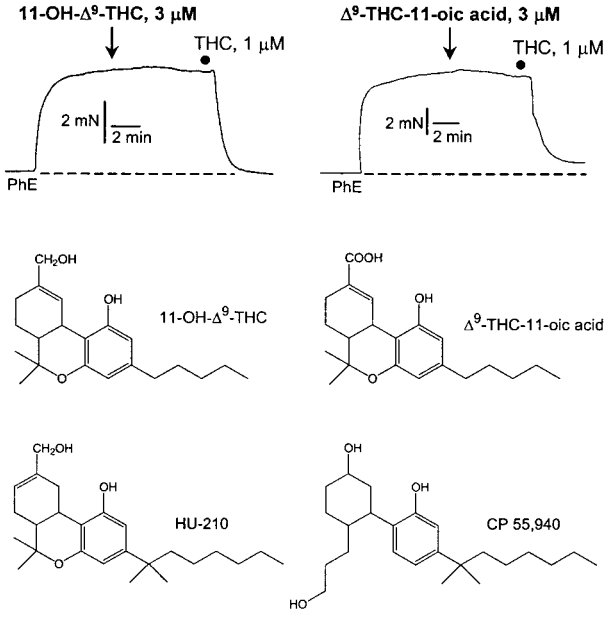

Figure 2. The vasodilator action of THC and cannabinol is not mimicked by C11 hydroxy and carboxy derivatives of THC. In humans, THC is metabolized to 11-OH-Δ^9-THC and Δ^9-THC-11-oic acid (Burstein; 1999), both of which fail to relax phenylephrine (*PhE*)-contracted rat hepatic arteries ($n = 3$). The *dashed line* shows the basal tension level before addition of PhE. The structures of the potent CB_1 and CB_2 receptor agonists HU-210 and CP 55,940 are also shown; these agonists are synthetic derivatives of THC without an intact C11 methyl group. None of these compounds cause sensory nerve-mediated relaxation in the rat hepatic artery (Zygmunt et al., 1999).

arteries (Zygmunt et al., 1999). Therefore, we examined the effects of the vanilloid receptor antagonists capsazepine and ruthenium red (Szallasi and Blumberg, 1999) on relaxations induced by THC and cannabinol in these arteries. Whereas the noncompetitive vanilloid receptor antagonist ruthenium red (1 μM) abolishes the relaxation evoked by THC and cannabinol in hepatic arteries (Fig. 3*B,C*) and causes a substantial inhibition of the THC-induced vasorelaxation in mesenteric arteries ($p < 0.0001$) (Fig. 3*D*), the competitive vanilloid receptor antagonist capsazepine (3 μM) is without effect (Fig. 3*B–D*). In contrast to THC and cannabinol, anandamide induces vasorelaxation in the hepatic artery ($pEC_{50} = 6.7 \pm 0.1$; $E_{max} = 97 \pm 1\%$; $n = 5$) that is inhibited by capsazepine ($p < 0.0001$) (Fig. 3*E*), confirming that capsazepine does indeed inhibit vanilloid receptors in this artery (Zygmunt et al., 1999). Ruthenium red (1 μM) also prevents the release of CGRP in rat hepatic arteries exposed to 10 μM THC (Fig. 3*F*). The neurotoxin α-latrotoxin (1 nM), which causes vasorelaxation via release of CGRP from capsaicin-sensitive sensory nerves in rat hepatic arteries (Zygmunt et al., 1999), produces a complete relaxation in the presence of 1 μM ruthenium red ($E_{max} = 95 \pm 1\%$; $n = 5$), indicating that the nerves are still capable of releasing CGRP in the presence of this inhibitor.

The possibility that THC activates vanilloid receptors in a capsazepine-insensitive manner was tested in mouse isolated mesenteric arteries. THC and the vanilloid receptor agonists capsaicin and anandamide all evoke concentration-dependent relaxations in this preparation (Fig. 4*A*). These agonists are active at submicromolar concentrations, with capsaicin ($pEC_{50} = 7.8 \pm 0.1$; $E_{max} = 91 \pm 4\%$; $n = 4$) being more potent than THC

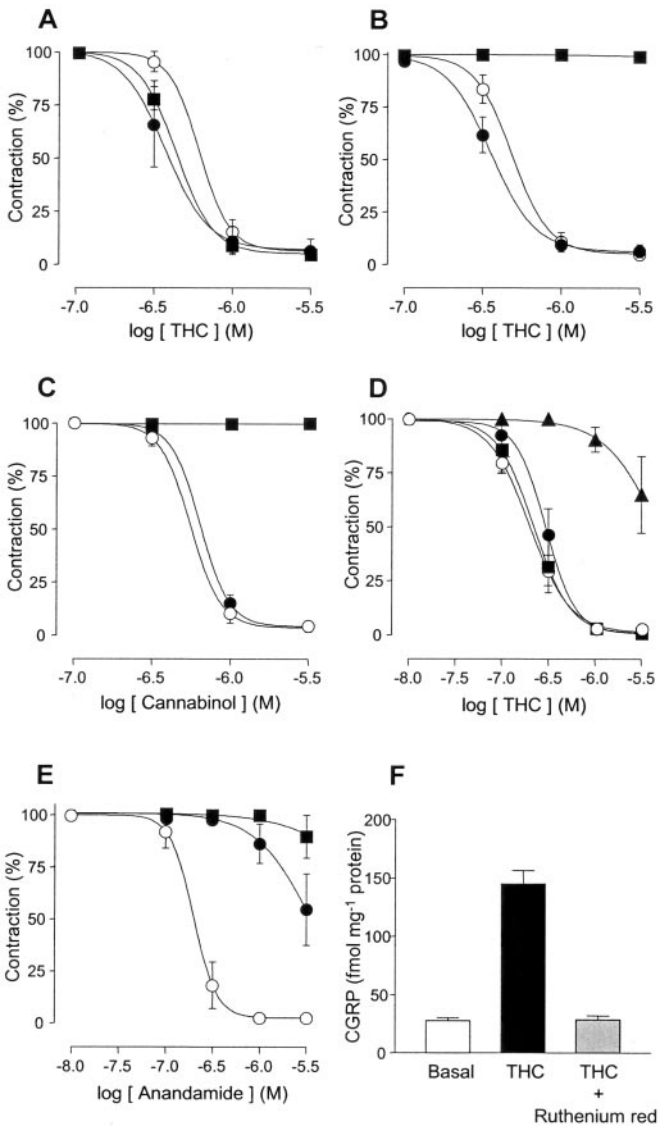

Figure 3. Effects of CB_1 and vanilloid receptor antagonists on sensory nerve-mediated relaxation induced by THC, cannabinol, and anandamide in rat hepatic and mesenteric arteries. *A*, The THC-induced vasorelaxation in hepatic arteries (○; $n = 8$) is not inhibited by the CB_1 receptor antagonists SR141716A (300 nM; ■; $n = 5$) and AM251 (30 nM; ●; $n = 4$). Vasorelaxations evoked by THC (○; $n = 10$) (*B*) and cannabinol (○; $n = 7$) (*C*) are not inhibited by the competitive vanilloid receptor antagonist capsazepine (3 μM; ●; $n = 8$ and 4 for THC and cannabinol, respectively) but are abolished by the noncompetitive vanilloid receptor antagonist ruthenium red (1 μM; ■; $n = 8$ and 4 for THC and cannabinol, respectively). *D*, In mesenteric arteries, THC-induced relaxations (○; $n = 6$; same as in Fig. 1*B*) are also unaffected by SR141716A (300 nM; ●; $n = 4$) and capsazepine (3 μM; ■; $n = 4$) and are inhibited by 1 μM ruthenium red (▲; $n = 5$). *E*, Anandamide-induced vasorelaxations in the absence (○; $n = 5$) and presence (●; $n = 5$) of 3 μM capsazepine or 1 μM ruthenium red (■; $n = 4$). *F*, THC (10 μM) releases CGRP from rat hepatic arteries in the absence ($n = 6$; $p < 0.001$) but not in the presence ($n = 6$) of 1 μM ruthenium red compared with basal CGRP release ($n = 5$). Data are expressed as mean ± SEM.

($pEC_{50} = 6.6 \pm 0.1$; $E_{max} = 89 \pm 3\%$; $n = 4$) and anandamide ($pEC_{50} = 6.4 \pm 0.1$; $E_{max} = 86 \pm 3\%$; $n = 4$). THC (10 μM) and anandamide (10 μM) cannot relax arteries pre-exposed to 10 μM capsaicin or in the presence of 3 μM 8-37 CGRP ($n = 3$–4) (Fig. 4*A*). As shown in Figure 4*B*, THC causes relaxation in mesenteric arteries from $VR1^{-/-}$ mice and their control littermates

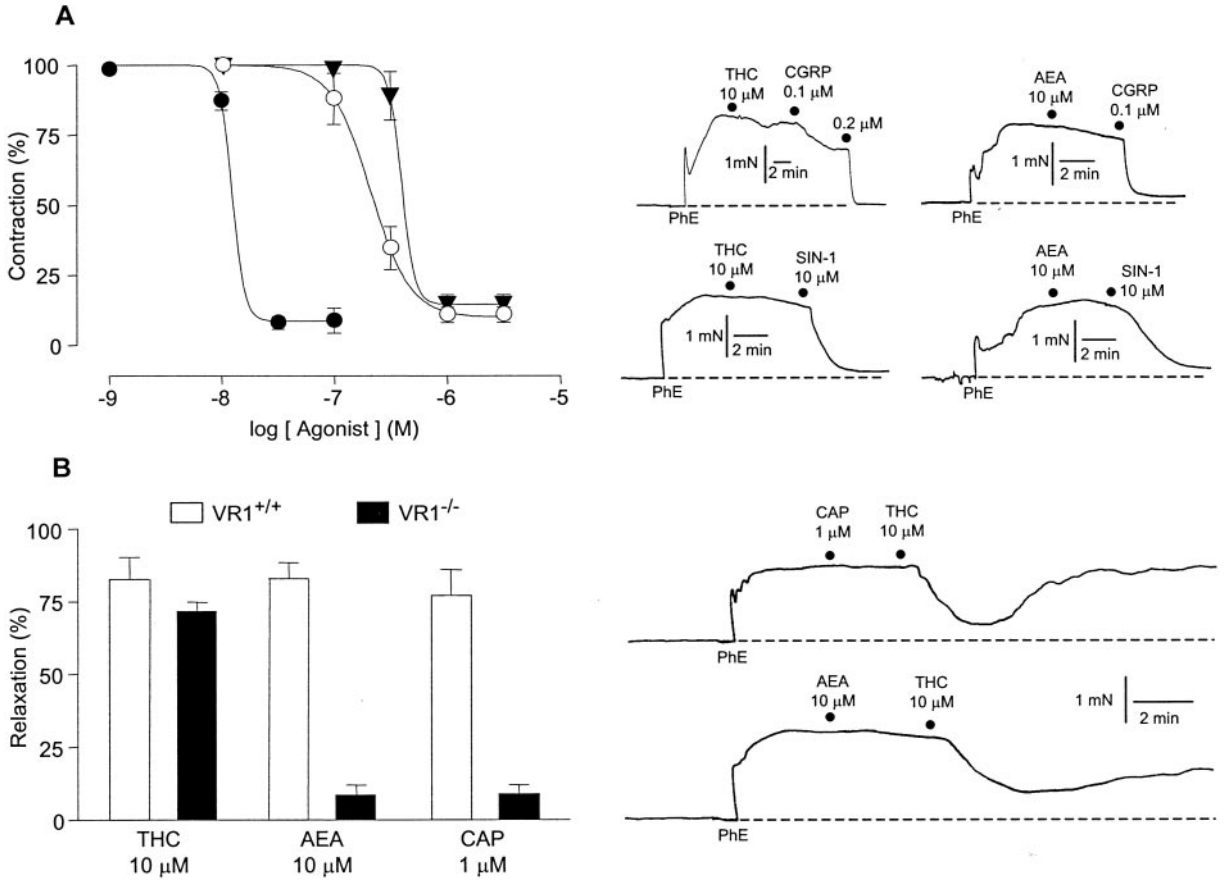

Figure 4. THC elicits sensory nerve-mediated relaxation in mouse isolated mesenteric arteries via a vanilloid receptor-independent mechanism. *A*, Capsaicin (●), anandamide (▼), and THC (○) evoke concentration-dependent relaxations of mesenteric arterial segments from wild-type mice contracted with phenylephrine (*PhE*; $n = 4$). *Traces*, all from separate arterial segments, show that THC and anandamide (*AEA*) fail to relax arteries pretreated with capsaicin (10 μM; *top traces*) or in the presence of 8-37 CGRP (3 μM; *bottom traces*) ($n = 3$–4). This lack of effect of THC and anandamide is not attributable to the inability of arteries to respond to vasodilators, because CGRP and SIN-1 (a nitric oxide donor) cause complete relaxations. *B*, THC induces relaxations of the same magnitude in arteries from VR1 gene knock-out mice ($VR1^{-/-}$; $n = 5$) and their control littermates ($VR1^{+/+}$; $n = 7$). *AEA* ($n = 6$) and capsaicin (*CAP*; $n = 4$) are equally as effective as THC at relaxing arteries from $VR1^{+/+}$ mice, but they produce only minor relaxations in arteries from $VR1^{-/-}$ mice ($n = 6$ and 7 for *AEA* and *CAP*, respectively). As shown by the *traces*, THC also relaxes arteries that do not respond to either *CAP* or *AEA*, indicating that sensory nerves are functional in $VR1^{-/-}$ mice ($E_{max} = 72 \pm 3\%$; $n = 13$). Data are expressed as mean ± SEM. The *dashed lines* in *traces* show the basal tension level before addition of PhE.

($VR1^{+/+}$), whereas the relaxant effects of anandamide and capsaicin are almost absent in arteries from $VR1^{-/-}$ mice.

Ruthenium red is also an inhibitor of the ryanodine receptor channel present on intracellular calcium stores (Ma, 1993). The possibility that such an action of ruthenium red is responsible for its inhibition of THC-induced relaxation was therefore explored. In rat isolated hepatic arteries, neither 10 μM ryanodine nor 10 μM dantrolene, both of which inhibit the ryanodine receptor channel and caffeine-sensitive calcium stores at this concentration (Usachev et al., 1993; Chavis et al., 1996; Zhao et al., 2001), affects the THC-induced relaxation (THC, $pEC_{50} = 6.2 \pm 0.1$; $E_{max} = 98 \pm 1\%$; THC plus ryanodine, $pEC_{50} = 6.2 \pm 0.1$; $E_{max} = 99 \pm 1\%$; THC plus dantrolene, $pEC_{50} = 6.2 \pm 0.1$; $E_{max} = 98 \pm 1\%$; $n = 4$).

Subsequently, we examined the effect of extracellular calcium on the CGRP release evoked by THC in rat isolated mesenteric arteries. Both 10 μM THC and 10 mM caffeine release CGRP from rat mesenteric arteries when the extracellular calcium level is normal (Fig. 5*A*). In the absence of extracellular calcium, THC can no longer release CGRP. However, the ability of caffeine to release CGRP is unaffected under the same conditions (Fig. 5*A*), indicating that the intracellular calcium stores remain functionally intact in low extracellular calcium. The effect of 1 mM lanthanum, which is a nonselective calcium influx inhibitor, on THC-induced CGRP release was also examined. THC is unable to release CGRP in the presence of lanthanum, whereas caffeine responses are not significantly inhibited (Fig. 5*B*).

Influx of calcium through voltage-operated calcium channels (VOCCs) present on sensory nerves leads to neurotransmitter release (Geppetti et al., 1990; Evans et al., 1996; Lundberg, 1996; White, 1996). Therefore, we tested a mixture of L-, N-, and P/Q-type VOCC inhibitors on the vasorelaxation and release of CGRP evoked by THC. Neither relaxation nor CGRP release is inhibited by either calcicludine (L-, N-, and P-type VOCC inhibitor with IC_{50} values of 1–80 nM) (Schweitz et al., 1994) or nimodipine (L-type VOCC inhibitor with an IC_{50} value of ~1 nM) (Godfraind et al., 1986) in combination with ω-conotoxin GVIA and ω-conotoxin MVIIC (N- and P/Q-type VOCC inhibitors with IC_{50} values of 1–100 nM) (Zygmunt and Hogestatt, 1993; Olivera et al., 1994; Hirota et al., 2000). Thus, the vasorelaxation induced by THC in rat hepatic arteries is unaffected by calcicludine plus ω-conotoxin GVIA plus ω-conotoxin MVIIC,

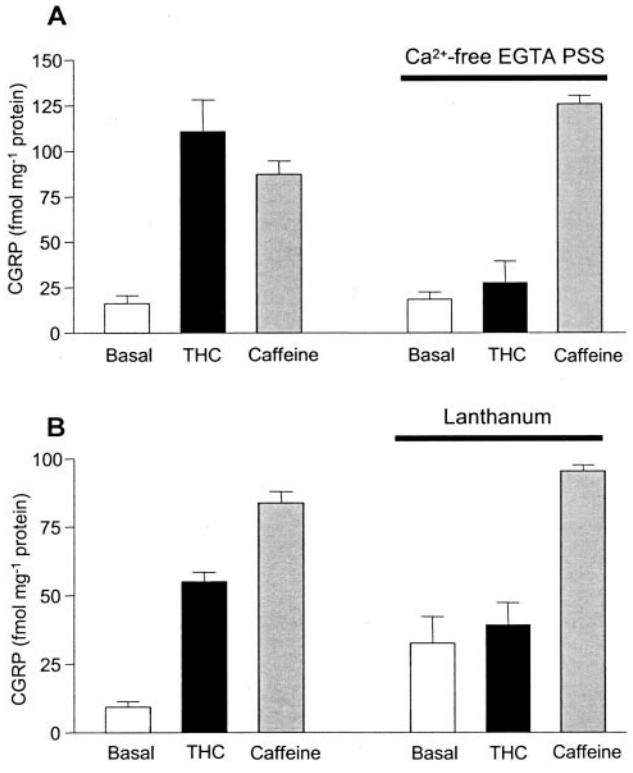

Figure 5. THC-induced release of CGRP from sensory nerves in rat mesenteric arteries is dependent on calcium influx. *A*, THC (10 μM; $n = 5$) and caffeine (10 mM; $n = 5$) release CGRP from rat mesenteric arteries in PSS ($p < 0.001$ compared with basal CGRP release; $n = 4$). When calcium in the PSS is replaced by 10 μM EGTA, caffeine ($n = 4$) but not THC ($n = 5$) still evokes a release of CGRP ($p < 0.001$ compared with basal CGRP release; $n = 4$). *B*, THC (10 μM; $n = 5$) and caffeine (10 mM; $n = 5$) also release CGRP in Tris-buffer solution ($p < 0.001$ compared with basal CGRP release; $n = 5$). In the presence of 1 mM lanthanum, caffeine ($n = 5$) but not THC ($n = 5$) is able to release CGRP ($p < 0.001$ compared with basal CGRP release; $n = 5$). Data are expressed as mean ± SEM.

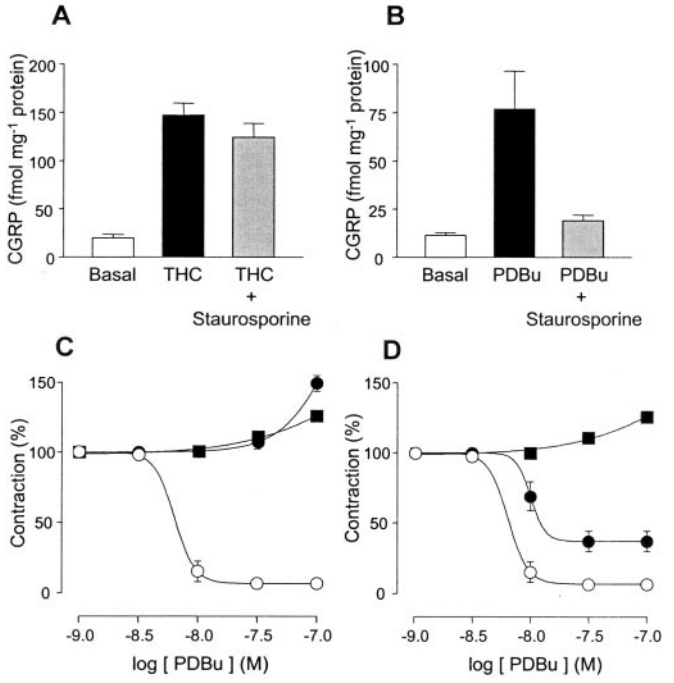

Figure 6. The effect of THC on perivascular sensory nerves does not involve protein kinases A and C. *A*, THC (10 μM) evokes CGRP release in rat hepatic arteries in both the absence and presence of the nonselective protein kinase inhibitor staurosporine (3 μM; $p < 0.001$ compared with basal CGRP release; $n = 6$). *B*, The protein kinase C activator PDBu releases CGRP from rat hepatic arteries in the absence ($p < 0.01$; $n = 6$) but not in the presence of 100 nM staurosporine ($n = 5$) compared with basal CGRP release ($n = 6$). *C*, PDBu elicits concentration-dependent relaxations in rat hepatic arteries contracted with phenylephrine. However, PDBu cannot relax arteries pretreated with 10 μM capsaicin (●; $n = 5$) or in the presence of 3 μM 8-37 CGRP (■; $n = 5$). *D*, PDBu-induced vasorelaxations are also prevented by 1 μM ruthenium red (■; $n = 5$) and partially inhibited by 3 μM capsazepine (●; $n = 6$). For clarity, the same controls (○) are shown in *C* and *D* ($n = 7$). Data are expressed as mean ± SEM.

each at a concentration of 100 nM (THC, $pEC_{50} = 6.1 \pm 0.1$; $E_{max} = 98 \pm 1\%$; THC plus VOCC inhibitors, $pEC_{50} = 6.1 \pm 0.1$; $E_{max} = 96 \pm 3\%$; $n = 4$). Furthermore, in rat mesenteric arteries, THC induces a significant CGRP release ($p < 0.01$) that is not different in the absence or presence of nimodipine plus ω-conotoxin GVIA plus ω-conotoxin MVIIC, each at a concentration of 100 nM (basal, 16.3 ± 4.3 fmol/mg protein; THC, 111 ± 17 fmol/mg protein; THC plus VOCC inhibitors, 117 ± 18 fmol/mg protein; $n = 5$).

Activation of glutamate receptors, which are present on sensory nerves (Li et al., 1997; Carlton and Coggeshall, 1999), is another possibility by which THC may cause calcium influx and subsequent neurotransmitter release. However, 3 μM MK-801 and 300 μM CNQX, inhibitors of ionotropic glutamate receptors (Castellano et al., 2001; Lerma et al., 2001), do not suppress the relaxation evoked by THC in rat mesenteric arteries (THC, $pEC_{50} = 6.5 \pm 0.1$; $E_{max} = 97 \pm 1\%$; THC plus MK-801, $pEC_{50} = 6.7 \pm 0.2$; $E_{max} = 98 \pm 2\%$; THC plus CNQX, $pEC_{50} = 7.2 \pm 0.1$; $E_{max} = 100 \pm 0\%$; $n = 4$). In fact, THC is more potent in the presence than in the absence of CNQX ($p < 0.05$). Glutamate (1 mM) does not relax mesenteric arteries, although the arterial segments respond to subsequent application of THC ($n = 3$).

The possibility that protein kinases mediate the THC-induced release of CGRP was also explored. We tested the effect of the nonselective protein kinase inhibitor staurosporine, which acts on both protein kinases A and C (Ruegg and Burgess, 1989), on the ability of THC and the protein kinase C activator PDBu to release CGRP in rat hepatic arteries. THC (10 μM) induces a significant and almost identical CGRP release in the absence and presence of 3 μM staurosporine (Fig. 6*A*). At 100 nM, staurosporine completely inhibits the CGRP release induced by 1 μM PDBu (Fig. 6*B*). In rat hepatic arteries, PDBu induces concentration-dependent relaxations, which are abolished by 3 μM 8-37 CGRP or by pretreatment with 10 μM capsaicin (Fig. 6*C*). The vasorelaxations are also completely inhibited by 1 μM ruthenium red (Fig. 6*D*). Capsazepine (3 μM) reduces the potency of PDBu (PDBu, $pEC_{50} = 8.2 \pm 0.1$; PDBu plus capsazepine, $pEC_{50} = 8.0 \pm 0.1$; $n = 6–7$; $p = 0.055$) and the maximal vasorelaxation induced by PDBu (PDBu, $E_{max} = 97 \pm 3\%$; PDBu plus capsazepine, $E_{max} = 63 \pm 7\%$; $n = 6–7$; $p = 0.0017$) (Fig. 6*D*). No vasorelaxation is obtained with 4α-PDBu (1–100 nM; $n = 6$), which does not activate protein kinase C (Blumberg, 1980).

DISCUSSION

This study describes a novel effect of THC and cannabinol on capsaicin-sensitive primary sensory nerves. The effect of these cannabinoids, which are active at submicromolar concentrations, is not mediated by known cannabinoid receptors, because CB_1

receptor antagonists are without inhibitory effect and, as shown previously, the CB_1/CB_2 receptor agonists HU-210, CP 55,940, and WIN 55,2128-372 cannot elicit capsaicin-sensitive vasorelaxation (Plane et al., 1997; Zygmunt et al., 1999). The presence of an intact C11 methyl group seems to be crucial for activity, because oxidation or lack of this methyl group results in inactive compounds, such as 11-OH-Δ^9-THC, Δ^9-THC-11-oic acid, HU-210, and CP 55,940. The ability of THC and cannabinol to activate sensory nerves is not related to their psychotropic activity, because the psychotropic cannabinoids 11-OH-Δ^9-THC, HU-210, CP 55,940, and WIN 55,2128-372 do not evoke capsaicin-sensitive vasorelaxations. Furthermore, cannabinol, which is a weak CB_1 receptor agonist and has little or no psychotropic activity (Pertwee, 1988; Rhee et al., 1997), is as potent as THC at eliciting vasorelaxation in the present study. This structure–activity relationship is also not consistent with the theory of alterations in membrane fluidity being the key activation mechanism (Pertwee, 1988).

We found that the effect of THC on sensory nerves is dependent on extracellular calcium and inhibited by the noncompetitive vanilloid receptor blocker ruthenium red (Amann and Maggi, 1991; Caterina et al., 1997). Interestingly, the endogenous cannabinoid anandamide induces calcium influx in sensory neurons via activation of vanilloid receptors (Zygmunt et al., 1999; Smart et al., 2000). A recent study shows that cannabidiol, a naturally occurring nonpsychotropic cannabinoid having anti-inflammatory properties (Srivastava et al., 1998; Malfait et al., 2000), activates vanilloid receptors on sensory neurons (Bisogno et al., 2001). However, our experiments with VR1 gene knock-out mice clearly show that the molecular target for THC is distinct from the VR1. The vanilloid receptor-like (VRL-1) channel is also expressed in sensory ganglia and displays a pharmacology similar to that of the putative THC-activated receptor/ion channel (Caterina et al., 1999). However, THC does not induce calcium transients in human embryonic kidney 293 cells expressing VRL-1 (P. M. Zygmunt and D. Julius, unpublished observations), and it is unclear whether VRL-1 is present on capsaicin-sensitive sensory neurons (Caterina et al., 1999). VR1 and VRL-1 belong to the family of transient receptor potential (TRP) ion channels, all of which are permeable to monovalent cations and calcium ions (Clapham et al., 2001). In addition to VR1 (TRPV1) and VRL-1 (TRPV2), TRPV4, TRPV5, and TRPV6 are sensitive to ruthenium red (Clapham et al., 2001; Hoenderop et al., 2001). Interestingly, TRPV4 and the recently cloned menthol receptor (TRPM8) are present on sensory nerves (Clapham et al., 2001; McKemy et al., 2002; Peier et al., 2002). Therefore, it would not be surprising if a member of the TRP ion channel family mediates the CB_1/CB_2 receptor-independent effect of THC and cannabinol.

VOCCs represent an important calcium influx pathway in sensory neurons, and bradykinin and prostaglandin E_2 (PGE_2) cause the release of sensory neuropeptides via activation of N-type VOCCs (Geppetti et al., 1990; Evans et al., 1996; Lundberg, 1996; White, 1996). However, inhibitors of common neuronal VOCCs are without effect on both CGRP release and vasorelaxation evoked by THC, excluding the involvement of neuronal VOCCs of the N-, L-, and P/Q type in the action of THC. Ionotropic glutamate receptors not only are present on primary sensory neurons (Carlton and Coggeshall, 1999) but also may mediate release of CGRP from such nerves (Jackson and Hargreaves, 1999). However, inhibitors of glutamate receptors did not suppress the action of THC in the present study. Instead, one of these inhibitors (CNQX), acting on non-NMDA glutamate receptors (Lerma et al., 2001), potentiated the THC-induced relaxation. Additional studies are needed to clarify the mechanism behind this effect, but one possibility could be that tonic glutamate receptor activity suppresses the THC signal pathway in sensory neurons. Indeed, activation of non-NMDA receptors can lead to a decrease in calcium influx and neurotransmitter release (Lerma et al., 2001). Metabotropic glutamate receptors are also present on sensory neurons (Li et al., 1997). Activation of these receptors releases calcium from caffeine- and ryanodine-sensitive intracellular calcium stores, which can lead to activation of protein kinase C (Chavis et al., 1996; Conn and Pin, 1997). Caffeine-induced calcium release from these stores triggers the release of CGRP (present study) and is inhibited by ryanodine or dantrolene (each at 10 μM) in rat dorsal root ganglion neurons (Usachev et al., 1993). However, the involvement of metabotropic glutamate receptors in THC-induced responses is unlikely, because glutamate could not mimic the action of THC. Also, THC, but not caffeine, was unable to release CGRP in the absence of extracellular calcium, and inhibition of the ryanodine receptor channel by ryanodine or dantrolene was without effect on THC-induced vasorelaxation. Together, these findings show that although ruthenium red is an inhibitor of VOCCs (Hamilton and Lundy, 1995; Cibulsky and Sather, 1999) and the ryanodine receptor channel (Ma, 1993), inhibition of these channels cannot explain the ability of ruthenium red to block the response to THC and cannabinol in the present study.

Protein kinases A and C are believed to play an important role in pain signaling (Malmberg et al., 1997; Cesare et al., 1999). Phorbol esters, such as PDBu and phorbol 12-myristate 13-acetate, activate protein kinase C and release substance P and CGRP from rat dorsal root ganglion neurons and skin sensory nerves (Ruegg and Burgess, 1989; Barber and Vasko, 1996; Kessler et al., 1999). In agreement with these studies, we found that PDBu triggers the release of CGRP from sensory nerves, leading to vasorelaxation. This PDBu-induced CGRP release is prevented by the protein kinase C inhibitor staurosporine. In contrast, THC does not act via protein kinase C, because its CGRP-releasing effect was unaffected by staurosporine even at a concentration 30 times higher than that used to inhibit the effect of PDBu. Protein kinase C can also sensitize sensory neurons and vanilloid receptors to inflammatory mediators (Cesare et al., 1999; Premkumar and Ahern, 2000; Vellani et al., 2001). Interestingly, we found that the competitive vanilloid receptor antagonist capsazepine produces only a small inhibition of the PDBu-induced relaxation, whereas ruthenium red completely blocks the response. This could indicate that PDBu, via a protein kinase C-dependent mechanism, activates the same ruthenium red-sensitive pathway as THC, which raises the possibility that the putative cannabinoid receptor/ion channel is affected by inflammatory mediators and phospholipase C activation. It is unlikely that the capsazepine-sensitive component of the PDBu-induced relaxation is attributable to a direct effect of PDBu on vanilloid receptors, because PDBu does not bind to VR1 (Chuang et al., 2001), and its release of CGRP was abolished by staurosporine in the present study. Staurosporine binds to and inhibits a variety of kinases, including protein kinase A (Ruegg and Burgess, 1989; Herbert et al., 1990), which has been proposed as a mediator of PGE_2- and anandamide-induced enhancement of sensory neuropeptide release, possibly via phosphorylation of the vanilloid receptor (Hingtgen et al., 1995; Lopshire and Nicol, 1998; Cesare et al., 1999; De Petrocellis et al., 2001). However, the lack of effect

of staurosporine on THC-induced CGRP release also excludes a role for cAMP-activated protein kinase A in this response.

The present study shows that THC and cannabinol cause release of sensory neuropeptides and vasorelaxation. Although they act on a molecular target distinct from VR1, these drugs have an effect on primary sensory nerves similar to those of capsaicin and other vanilloid receptor agonists (Szallasi and Blumberg, 1999; Zygmunt et al., 1999). These latter drugs are known to produce paradoxical analgesia via calcium influx and desensitization of sensory nerves (Szallasi and Blumberg, 1999; Urban et al., 2000). Whether such a mechanism contributes to the analgesic effects of THC remains to be determined. In conclusion, we have described a previously unknown action of THC and cannabinol on primary sensory nerves. Our findings are compatible with the existence of a novel cannabinoid receptor/ion channel, possibly belonging to the TRP ion channel family, which could be targeted by future analgesic and anti-inflammatory drugs devoid of psychotropic effects.

REFERENCES

Amann R, Maggi CA (1991) Ruthenium red as a capsaicin antagonist. Life Sci 49:849–856.

Barber LA, Vasko MR (1996) Activation of protein kinase C augments peptide release from rat sensory neurons. J Neurochem 67:72–80.

Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 134:845–852.

Blumberg PM (1980) In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: part 1. Crit Rev Toxicol 8:153–197.

Burstein SH (1999) The cannabinoid acids: nonpsychoactive derivatives with therapeutic potential. Pharmacol Ther 82:87–96.

Carlton SM, Coggeshall RE (1999) Inflammation-induced changes in peripheral glutamate receptor populations. Brain Res 820:63–70.

Castellano C, Cestari V, Ciamei A (2001) NMDA receptors and learning and memory processes. Curr Drug Targets 2:273–283.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824.

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398:436–441.

Cesare P, Moriondo A, Vellani V, McNaughton PA (1999) Ion channels gated by heat. Proc Natl Acad Sci USA 96:7658–7663.

Chavis P, Fagni L, Lansman JB, Bockaert J (1996) Functional coupling between ryanodine receptors and L-type calcium channels in neurons. Nature 382:719–722.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411:957–962.

Cibulsky SM, Sather WA (1999) Block by ruthenium red of cloned neuronal voltage-gated calcium channels. J Pharmacol Exp Ther 289:1447–1453.

Clapham DE, Runnels LW, Strubing C (2001) The TRP ion channel family. Nat Rev Neurosci 2:387–396.

Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37:205–237.

De Petrocellis L, Harrison S, Bisogno T, Tognetto M, Brandi I, Smith GD, Creminon C, Davis JB, Geppetti P, Di Marzo V (2001) The vanilloid receptor (VR1)-mediated effects of anandamide are potently enhanced by the cAMP-dependent protein kinase. J Neurochem 77:1660–1663.

Evans AR, Nicol GD, Vasko MR (1996) Differential regulation of evoked peptide release by voltage-sensitive calcium channels in rat sensory neurons. Brain Res 712:265–273.

Geppetti P, Tramontana M, Santicioli P, Del Bianco E, Giuliani S, Maggi CA (1990) Bradykinin-induced release of calcitonin gene-related peptide from capsaicin-sensitive nerves in guinea-pig atria: mechanism of action and calcium requirements. Neuroscience 38:687–692.

Godfraind T, Miller R, Wibo M (1986) Calcium antagonism and calcium entry blockade. Pharmacol Rev 38:321–416.

Hamilton MG, Lundy PM (1995) Effect of ruthenium red on voltage-sensitive Ca^{++} channels. J Pharmacol Exp Ther 273:940–947.

Herbert JM, Seban E, Maffrand JP (1990) Characterization of specific binding sites for [^{3}H]-staurosporine on various protein kinases. Biochem Biophys Res Commun 171:189–195.

Hingtgen CM, Waite KJ, Vasko MR (1995) Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3′,5′-cyclic monophosphate transduction cascade. J Neurosci 15:5411–5419.

Hirota K, Kudo M, Kudo T, Matsuki A, Lambert DG (2000) Inhibitory effects of intravenous anaesthetic agents on K^+-evoked norepinephrine and dopamine release from rat striatal slices: possible involvement of P/Q-type voltage-sensitive Ca^{2+} channels. Br J Anaesth 85:874–880.

Hoenderop JG, Vennekens R, Muller D, Prenen J, Droogmans G, Bindels RJ, Nilius B (2001) Function and expression of the epithelial Ca^{2+} channel family: comparison of mammalian ECaC1 and 2. J Physiol (Lond) 537:747–761.

Hogestatt ED, Zygmunt PM (2002) Cardiovascular pharmacology of anandamide. Prostaglandins Leukot Essent Fatty Acids 66:355–363.

Holzer P (1992) Peptidergic sensory neurons in the control of vascular functions: mechanisms and significance in the cutaneous and splanchnic vascular beds. Rev Physiol Biochem Pharmacol 121:49–146.

Jackson DL, Hargreaves KM (1999) Activation of excitatory amino acid receptors in bovine dental pulp evokes the release of iCGRP. J Dent Res 78:54–60.

Kawasaki H, Takasaki K, Saito A, Goto K (1988) Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. Nature 335:164–167.

Kessler F, Habelt C, Averbeck B, Reeh PW, Kress M (1999) Heat-induced release of CGRP from isolated rat skin and effects of bradykinin and the protein kinase C activator PMA. Pain 83:289–295.

Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, Bohme GA, Imperato A, Pedrazzini T, Roques BP, Vassart G, Fratta W, Parmentier M (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 283:401–404.

Lerma J, Paternain AV, Rodriguez-Moreno A, Lopez-Garcia JC (2001) Molecular physiology of kainate receptors. Physiol Rev 81:971–998.

Li H, Ohishi H, Kinoshita A, Shigemoto R, Nomura S, Mizuno N (1997) Localization of a metabotropic glutamate receptor, mGluR7, in axon terminals of presumed nociceptive, primary afferent fibers in the superficial layers of the spinal dorsal horn: an electron microscope study in the rat. Neurosci Lett 223:153–156.

Lopshire JC, Nicol GD (1998) The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. J Neurosci 18:6081–6092.

Lundberg JM (1996) Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 48:113–178.

Ma J (1993) Block by ruthenium red of the ryanodine-activated calcium release channel of skeletal muscle. J Gen Physiol 102:1031–1056.

Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreakos E, Mechoulam R, Feldmann M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. Proc Natl Acad Sci USA 97:9561–9566.

Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. Science 278:279–283.

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52–58.

Mechoulam R, Hanus L (2000) A historical overview of chemical research on cannabinoids. Chem Phys Lipids 108:1–13.

Morisset V, Ahluwalia J, Nagy I, Urban L (2001) Possible mechanisms of cannabinoid-induced antinociception in the spinal cord. Eur J Pharmacol 429:93–100.

Olivera BM, Miljanich GP, Ramachandran J, Adams ME (1994) Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. Annu Rev Biochem 63:823–867.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Early TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002) A TRP channel that senses cold stimuli and menthol. Cell 108:705–715.

Pertwee RG (1988) The central neuropharmacology of psychotropic cannabinoids. Pharmacol Ther 36:189–261.

Pertwee RG (2001) Cannabinoid receptors and pain. Prog Neurobiol 63:569–611.

Plane F, Holland M, Waldron GJ, Garland CJ, Boyle JP (1997) Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries. Br J Pharmacol 121:1509–1511.

Premkumar LS, Ahern GP (2000) Induction of vanilloid receptor channel activity by protein kinase C. Nature 408:985–990.

Rhee MH, Vogel Z, Barg J, Bayewitch M, Levy R, Hanus L, Breuer A, Mechoulam R (1997) Cannabinol derivatives: binding to cannabinoid receptors and inhibition of adenylylcyclase. J Med Chem 40:3228–3233.

Ruegg UT, Burgess GM (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol Sci 10:218–220.

Schweitz H, Heurteaux C, Bois P, Moinier D, Romey G, Lazdunski M (1994) Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, is a potent blocker of high-threshold Ca^{2+} channels with a high affinity for L-type channels in cerebellar granule neurons. Proc Natl Acad Sci USA 91:878–882.

Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, Davis JB (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br J Pharmacol 129:227–230.

Srivastava MD, Srivastava BI, Brouhard B (1998) Delta9 tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. Immunopharmacology 40:179–185.

Szallasi A, Blumberg PM (1999) Vanilloid (capsaicin) receptors and mechanisms. Pharmacol Rev 51:159–212.

Urban L, Campbell EA, Panesar M, Patel S, Chaudhry N, Kane S, Buchheit K, Sandells B, James IF (2000) In vivo pharmacology of SDZ 249–665, a novel, non-pungent capsaicin analogue. Pain 89:65–74.

Usachev Y, Shmigol A, Pronchuk N, Kostyuk P, Verkhratsky A (1993) Caffeine-induced calcium release from internal stores in cultured rat sensory neurons. Neuroscience 57:845–859.

Vellani V, Mapplebeck S, Moriondo A, Davis JB, McNaughton PA (2001) Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. J Physiol (Lond) 534:813–825.

White DM (1996) Mechanism of prostaglandin E2-induced substance P release from cultured sensory neurons. Neuroscience 70:561–565.

Zhao F, Li P, Chen SR, Louis CF, Fruen BR (2001) Dantrolene inhibition of ryanodine receptor Ca^{2+} release channels: molecular mechanism and isoform selectivity. J Biol Chem 276:13810–13816.

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc Natl Acad Sci USA 96:5780–5785.

Zygmunt PM, Hogestatt ED (1993) Calcium channels at the adrenergic neuroeffector junction in the rabbit ear artery. Naunyn Schmiedebergs Arch Pharmacol 347:617–623.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D, Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400:452–457.