Autocrine Endocannabinoid Signaling through CB₁ Receptors Potentiates OX₁ Orexin Receptor Signaling

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ABSTRACT

It has been proposed that OX₁ orexin receptors and CB₁ cannabinoid receptors can form heteromeric complexes, which affect the trafficking of OX₁ receptors and potentiate OX₁ receptor signaling to extracellular signal-regulated kinase (ERK). We have recently shown that OX₁ receptor activity releases high levels of the endocannabinoid 2-arachidonoylglycerol (2-AG), suggesting an alternative route for OX₁-CB₁ receptor interaction in signaling, for instance, in retrograde synaptic transmission. In the current study, we set out to investigate this possibility utilizing recombinant Chinese hamster ovary K1 cells. 2-AG released from OX₁ receptor-expressing cells acted as a potent paracrine messenger stimulating ERK activity in neighboring CB₁ receptor-expressing cells. When OX₁ and CB₁ receptors were expressed in the same cells, OX₁ stimulation-induced ERK phosphorylation and activity were strongly potentiated. The potentiation but not the OX₁ response as such was fully abolished by specific inhibition of CB₁ receptors or the enzyme responsible for 2-AG generation, diacylglycerol lipase (DAGL). Although the results do not exclude the previously proposed OX₁-CB₁ heteromerization, they nevertheless unequivocally identify DAGL-dependent 2-AG generation as the pivotal determinant of the OX₁-CB₁ synergism and thus suggest a functional rather than a molecular interaction of OX₁ and CB₁ receptors.

Introduction

Neuropeptides orexin-A and -B are best known for their roles in the regulation of sleep/wakefulness and appetite, but many other prominent functions have also been described (reviewed in Aston-Jones et al., 2010; Scammell and Winrow, 2011; Kukkonen, 2013; Perez-Leighton et al., 2012). Orexins mediate their actions via rhodopsin family G-protein-coupled receptors (GPCRs) named OX₁ and OX₂, which can be found in the central nervous system (CNS) but also in other parts of the body (reviewed in Scammell and Winrow, 2011; Kukkonen, 2013). Orexin receptor activation leads to signaling via multiple intracellular pathways (reviewed in Kukkonen, 2013). In neurons, activation of orexin receptors usually leads to depolarization through inhibition of K⁺ channels or activation of nonspecific cation channels (reviewed in Kukkonen, 2013). Intracellular Ca²⁺ concentration is elevated through both Ca²⁺ release and Ca²⁺ influx, and other potently activated targets include phospholipases C (PLC), D, and A₂. Mitogen-activated protein kinase pathways ERK1/2 (extracellular signal-regulated kinase 1/2) and p38 may also be important signal relayers in orexin signaling (Hilairet et al., 2003; Ammoun et al., 2006a, b; Shin et al., 2009; reviewed in Kukkonen, 2013). The main G-protein pathway for orexin receptors is assumed to be G₉, but other G-protein couplings have also been seen (reviewed in Kukkonen, 2013).

Endocannabinoids are locally released lipid metabolites that regulate a wide variety of processes in the CNS, including analgesia, mood, reward, memory, appetite, and energy metabolism (reviewed in Kano et al., 2009). The best known endocannabinoids are 2-arachidonoylglycerol (2-AG) and anandamide (reviewed in Di Marzo and Petrosoin, 2007). The cannabinoid receptors CB₁ and CB₂ also belong to the GPCRs of the rhodopsin subfamily, and they predominantly

ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; AM-251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CNS, central nervous system; DAGL, diacylglycerol lipase; ERK, extracellular signal-regulated kinase; GFP, (enhanced) green fluorescent protein; GPCR, G-protein-coupled receptor; HA, hemagglutinin; HBM, Hapes-buffered medium; HU-210, 3-[1,1'-dimethylheptyl]-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[c,d]pyran-9-methanol; oxtremorine-M, N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyl-1-ammonium iodide; PBS, phosphate-buffered saline; PLC, phospholipase C; SB-334867, 1-(2-methylbenzoazox-6-yl)-3-(L-mataphtyrithrid-4-yl-urea HCl; SR141716, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; THL, tetrahydrodipistatin, N-formyl-L-leucine-(S)-L-serine-(S)-3-hexyl-4-oxo-2-oxetanyl[methyl(dodecyl ester; TK, thymidine kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl)mercapto] butadiene; WB, Western blotting)}
couple to Gαo proteins (reviewed in Pertwee, 1997; Alexander and Kendall, 2007). CB1 receptors are mainly found in the CNS neurons. A central feature of the endocannabinoid system in the CNS is its involvement in retrograde synaptic transmission. In this process, PLC activation and/or Ca2+ elevation in the postsynaptic neuron induces local production and release of endocannabinoids that inhibit neurotransmitter release from the presynaptic neuron via action on presynaptic CB2 receptors (Ohno-Shosaku et al., 2001; reviewed in Kano et al., 2009). Like orexin receptors, CB2 receptors are known to use the ERK pathway in their signaling, in addition to the more often described inhibitory actions on adenylylcyclase or voltage-gated Ca2+ channels or activation of K+ channels (Bouaboula et al., 1995; Felder et al., 2008; Suarez et al., 2011), although the endocannabinoid system is much more widespread in the brain than the orexigenic system (Herkenham et al., 1991; reviewed in Kukkonen et al., 2002; Laitinen, 2004; Freund et al., 2003; Kano et al., 2009). Some studies implicate an interaction between orexigenic and cannabinoidegic systems (reviewed in Kukkonen, 2013). For instance, in rat dorsal raphe nucleus, orexin-B-mediated inhibition of glutamate release can be ascribed to 2-AG release and action on CB2 receptors on presynaptic terminals (Haj-Dahmane and Shen, 2005). Orexin-A-induced analgesic response in rat periaqueductal gray relies on 2-AG-mediated retrograde inhibition of γ-aminobutyric acid release (Ho et al., 2011). Heterologous coexpression of OX1 and CB2 receptors in Chinese hamster ovary (CHO) cells very strongly potentiates OX1 receptor signaling to ERK (Hilairet et al., 2003). This potentiation has been interpreted to be due to heterodimerization of OX1 and CB2 receptors. Further studies have continued along this same line and assessed, by different means, the heterodimerization/oligomerization of these two receptors (Ellis et al., 2006; Ward et al., 2011).

We have recently shown that the endocannabinoid 2-AG is strongly produced and released in OX1 receptor signaling, and that this 2-AG is able to act as a potent paracrine messenger via CB2 receptors (Turunen et al., 2012). This, together with the studies in the CNS, suggests that 2-AG production is an important signal in orexin-mediated regulation of synaptic transmission. Therefore, the observed potentiation of OX1 receptor–mediated ERK phosphorylation upon OX1-CB2 receptor coexpression could be due to CB2 receptor activation by 2-AG instead of receptor di-/oligomerization. In the current study, we set out to resolve this issue; the results show that 2-AG produced through OX1 receptor action is a central mediator of the potentiation.

**Materials and Methods**

**Drugs.** 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM-251), 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (SR141716; a.k.a. SR-141716A orrimonabant), and 3-(1,1-dimethylheptyl)-6aR,7,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-

dibenz(b,j)pyran-9- methanol (HUL-210) were from Cayman Europe (Tallinn, Estonia). Human orexin-A and -B were from NeoMPS (Strasbourg, France), N,N,N-trimethyl-4-(2-oxo-1- pyrrolidinyl)-2-butyl-1-ammonium iodide (oxotremorine-M) was from RBI (Natick, MA), and N-formyl-1-leucine-(1S)-1-[(2S,3S)-3-hexyl-4-oxo-2-oxetanylmethyl] dodecyl ester (tetrahydrolipstatin, THL; Orlistat, 1,4-di amino-2,3-dicyano-1,4-bis-(aminophenyl)mercapto)butadiene (U0126), and 1-(2-methylbenzoazao-6-yl)-4,1,5-naphthyridin-4-yl-urea HCl (SR-33467) were from Tocris Bioscience (Bristol, UK). Forskolin and 3-isothyl-1-methylxanthine were from Sigma-Aldrich (St. Louis, MO), and [2,8-3H]-adenine, myo-[2-3H]-inositol (PT6-271), [3H]SR141716, and [125I]orexin-A were from PerkinElmer Life and Analytical Sciences (Waltham, MA).

**Cell Culture.** CHO-hOX1 cells, expressing human OX1 receptors, have been described previously, as have their culture conditions (see, e.g., Turunen et al., 2010, 2012). CHO-hCB1 cells, expressing human CB1a receptor (Grimsey et al., 2010), were a kind gift from Dr. Michelle Glass (University of Auckland, Auckland, New Zealand) via Drs. Jarmo Laitinen and Juha Savinainen (University of Eastern Finland, Kuopio, Finland); the culture conditions for these have been described (Turunen et al., 2012). For Western blotting (WB) experiments, the cells were cultured on six-well plates (9.6-cm2 well bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany); for the luciferase assays, on either 24- or 96-well plates (Greiner Bio-One GmbH) and for the receptor binding experiments, on cell culture dishes (56-cm2 bottom area) or 48-well plates (Greiner Bio-One GmbH). Embryonic HeLa (for baculovirus propagation) and SF9 insect cells (for baculovirus propagation) were cultured in shaker culture as described in Nääreja et al. (2011).

**Plasmids and Transfection.** The plasmid pSG-GalElk1 (fusion of the dimerization domain of the transcription factor Elk-1 and the DNA-binding domain of the yeast transcription factor Gal4) (Kornajenn et al., 1994) was a kind gift from Dr. Peter E. Shaw (Queen’s Medical Centre, Nottingham, UK), pGL3 G5 E4 Δ38 (5 Gal4 binding site controlling firefly luciferase expression) (Kamano et al., 1995) from Dr. Karl-Heinz Klempnauer (Westfälische-Wilhelms-Universität Münster, Germany) via Drs. Peter E. Shaw and Michael J. Courtney (University of Eastern Finland), pcDNA3.1-CB1-GFP (hCB1a receptor with C-terminal (enhanced) green fluorescence protein (GFP)) (D’Antona et al., 2006) from Dr. Debra Kendall (University of Connecticut, Storrs, CT), and pcDNA3.1-3HA-hCB1 [human CB1a receptor with 3 x N-terminal hemagglutinin (HA) tag] from Missouri S&T cDNA Resource Center (Rolla, MO), pRL-TK [Renilla luciferase under herpes simplex virus thymidine kinase (TK) promoter] was from Promega (Madison, WI) and pcDNA1.1-hOX1and pm1-GFP [human M1 muscarinic acetylcholine receptor (m1)-EGFP fusion] were constructed in-house (Holmqvist et al., 2002). Empty plasmids were used to keep DNA amount equal in all transfections.

Transient transfection was used for the Elk-1 activity assay in CHO-hOX1 and CHO-hCB1 cells and for the PLC activity assay and binding experiments, which were to be performed in the same conditions. In addition to the reporter assay plasmids (pSG-GalElk1, pGL3 G5 E4 Δ38, pRL-TK), CB1 receptor (pcDNA3.1-3HA-hCB1 with CHO-hOX1 cells), OX1 receptor (pcDNA3.1-hOX1 with CHO-hCB1 cells), or M1 receptor (pm1-GFP with CHO-hCB1 cells) (or empty plasmid) was used. Cells on 96-well plates were grown to 40–50% confluence and transfected in Ham’s F-12 with 0.312 μg/cm2 DNA and 0.74 μl/cm2 FugeneHD (Roche, Mannheim, Germany). The optimized transfection conditions used for DNA were 0.6% (v/v) pSG-GalElk1-1, 50% pGL3 G5 E4 Δ38, 2.4% pRL-TK, and 47% pcDNA3.1-3HA-hCB1/pcDNA3.1-hOX1/pm1-GFP/empty plasmid. Liposome-based delivery was used in this assay as several plasmids had to be cotransfected, which is more easily accomplished by this technique than using the baculovirus system (below).

**Elk-1 Activity Luciferase Assay.** The cells were transfected as described above, and 5 hours after transfection they were washed and changed to serum-free medium to serum-starve the cells overnight. The following day, the cells were stimulated. When inhibitors were used, they (or the vehicle) were added to the cells 30 minutes prior to
GFP or CB1-GFP constructs; baculovirus expressing only GFP was stimulated and the luciferase activities measured as above. The following morning (48 hours after transfection), the cells were washed with PBS (3 times, 10 minutes each) and incubated with infrared fluorescent secondary antibodies for 1 hour at room temperature, protected from light. Secondary antibodies used were DyLight 900-conjugated goat anti-rabbit IgG (1:10,000; #35571; Li-cor) and DyLight 649-conjugated goat anti-mouse IgG (1:10,000; #35518; Li-cor). After incubation with secondary antibodies, the membranes were washed with PBS (3 times, 10 minutes each) before detecting the bands with Odyssey Infrared Imaging System (Li-cor). The images were analyzed using Nikon NIS-Elements AR (Nikon, Tokyo, Japan).

Within an experiment, each gel included a standard (in duplicate) composed of 100 nM orexin-A in control cells from the same stock material (see Supplemental Fig. 2). The intensity of each gel was additionally normalized according to these lanes to compensate for possibly uneven protein transfer.

**Adenylyl Cyclase Activity.** The measurements were performed essentially as described in Turunen et al. (2012). CHO-hCB1 cells on 24-well plates were pre-labeled with $[^{3}H]$adenine for 2 hours, after which they were washed once with Hepes-buffered medium (HBMM; composition in mM: NaCl, 137; KCl, 5; CaCl$_2$, 1; MgCl$_2$, 1.2; KH$_2$PO$_4$, 0.44; NaHCO$_3$, 4.2; glucose, 10; and Heps, 20; adjusted to pH 7.4 with NaOH) and then preincubated in HBMM containing 500 nM 3-isobutyl-1-methylxanthine (a cyclic nucleotide phosphodiesterase inhibitor) and 0.5 mg/ml stripped bovine serum albumin (BSA, to help to retain specificity) for 30 minutes at 37°C. The stimulants (forskolin, 2-AG) were added and the reactions allowed to proceed for 45 minutes, after which they were interrupted by rapid removal of the medium, addition of ice-cold perchloric acid, and freezing. After thawing, cell debris was spun down and the $[^{3}H]ATP + [^{3}H]ADP and $[^{3}H]AMP fractions isolated from the cell extracts by sequential Dowex-alumina chromatography (see, e.g., Holmqvist et al., 2005). Radioactivity was determined using scintillation counting (Wallac 1414 Liquid Scintillation Counter; PerkinElmer), and the conversion of $[^{3}H]ATP to $[^{3}H]AMP was calculated as a percentage of the total eluted $[^{3}H]ATP + [^{3}H]ADP.

**PLC Activity.** Total inositol phosphate release was measured essentially as described in Jantti et al. (2012). CHO-hCB1 cells were transiently transfected with either OX1 or M1 cDNA and also the other vectors included in the Elk-1 luciferase assay in the same ratios to make the assays comparable. Five hours later, the cells were exposed to $[^{3}H]$inositol to prelabel the membrane phosphoinositides. After 16 hours of labeling, the cells were washed once with HBMM and incubated in HBMM containing 10 mM LiCl (to inhibit inositol monophosphatase) for 10 minutes at 37°C. The cells were then stimulated with orexin-A for 20 minutes. The reactions were stopped by rapid removal of the medium, addition of 0.2 ml of 0.4 M ice-cold perchloric acid, and freezing. Thawed samples were neutralized with 0.1 ml of 0.36 M KOH + 0.3 M KHCO$_3$. The insoluble fragments were spun down and the total inositol phosphate fraction isolated by anion-exchange chromatography. The radioactivity of the inositol phosphate fraction was determined using scintillation counting as above.

**Receptor Binding.** Receptor expression levels were determined by radioligand binding. $[^{32}P]$Orexin-A was used for OX1 receptors. The cells were plated on cell culture dishes and transfected (if relevant for the assay) the following day. The cDNA mixtures contained the different vectors in the same ratios as for the Elk-1 luciferase assay to make the assays comparable. After yet another 24 hours, the cells were detached (PBS + 0.02% EDTA), spun down, and resuspended in HBMM containing 0.1% (v/v) BSA. The binding assay was performed in this buffer in low-protein-binding tubes (Eppendorf Nordic Aps, Horsholm, Denmark) with $[^{32}P]$Orexin-A at room temperature. The nonspecific binding was determined with 10 µM
SB-334867 (preincubated for 10 minutes before orexin-A). The reaction was allowed to proceed for 10 minutes, after which the cells were rapidly spun down (+2°C, 14,000 g, 30 seconds). The pellet was washed superficially (no resuspension) with ice-cold HBM and resuspended in HBM, and the radioactivity counted (Wallac Wizard 1480 Gamma Counter; PerkinElmer).

\[{^{[125]I}}\text{Orexin-A has several complicating properties (Kukkonen, 2012, 2013), and the assay conditions were thus carefully optimized. Due to its high filter binding, the separation of bound and free radioligand is not possible by filtration (Holmqvist et al., 2001; Kukkonen, 2012; J. Putula and J. P. Kukkonen, unpublished), and centrifugation of cells in suspension was thus rather applied. BSA and low-protein-binding tubes and pipette tips were used to reduce binding of orexin-A to plastic surfaces. Orexin-A (as well as \n\[{^{[125]I}}\text{orexin-A}) is an agonist and stimulates receptor internalization. Cell-surface-bound orexin-A was dissociated upon exposure to low-pH, urea-containing buffer (Chang et al., 1993) and the internalized radioligand seen after the NaOH incubation following this wash. At 10 minutes, very little internalization was seen. Thus, binding under these conditions reflects cell-surface rather than total receptor expression.

CB1 receptor expression was measured in cells attached on polyethyleneimine-coated (25 μg/ml for 1 hour at 37°C; Sigma-Aldrich) 48-well plates (Greiner). The cells were transfected as above and used 24 hours later. The cells were incubated in HBM containing 0.1% (w/v) BSA + \[{^{[3H]}\text{SR141716}} \text{(÷ 10 μM HU-210 to determine nonspecific binding) for 60 minutes at room temperature. The plates were placed on ice, the medium rapidly removed, the wells washed rapidly twice with ice-cold HBM, the cells detached with NaOH, and their radioactivity measured by scintillation counting (PerkinElmer). Both the radioligand and all available nonlabeled ligands to determine nonspecific binding are cell-permeable and can thus not separate cell-surface and intracellular binding. \[{^{[3H]}\text{SR141716}}\text{ binding reported is thus a measure of the total CB1 receptor pool.}

For both \[{^{[125]I}}\text{orexin-A and \n\[{^{[3H]}\text{SR141716}} binding, the cells were preincubated with THL for 30 minutes before the addition of the radioligand, also to mimic the conditions in the Elk-1 assay.

Data Analysis. All data are presented as mean ± S.E.M.; n refers to the number of batches of cells (and equals the number of independent experiments). Each experiment was performed at least three times, and the averaged data presented are thus from at least three independent experiments. WB experiments were performed with two, Elk-1-luciferase measurements with three, and cAMP measurements with four data points in parallel. Student’s paired or nonpaired two-tailed t test with Bonferroni correction was used in all pairwise comparisons.

\[\text{Results}

The aim of the study was to investigate the molecular mechanism behind the previous finding of strong potentiation of \(\text{OX}_1\) orexin receptor–induced ERK activity by \(\text{CB}_1\) cannabinoid receptor coexpression. For the studies, we used two stable cell lines expressing human \(\text{OX}_1\) and \(\text{CB}_1\) receptors, CHO-h\(\text{OX}_1\) and CHO-h\(\text{CB}_1\) cells, respectively. These cells were additionally transiently transfected or transduced to express \(\text{CB}_1\) and \(\text{OX}_1\) (or \(\text{M}_1\)) receptors, respectively (Supplemental Fig. 1). ERK activity was assessed by WB against the phosphorylated (active) ERK and downstream activity of the ERK target, transcription factor Elk-1.

\[\text{CHO-hCB}_1\text{ Cells with Transient Expression of \(\text{OX}_1\) Receptors Show \(\text{CB}_1\) Receptor Signaling–Dependent Potentiation of \(\text{OX}_1\) Signaling to ERK. In CHO-h\(\text{CB}_1\) cells, \(\text{CB}_1\) receptor stimulation with 2-AG or HU-210 produced an \(\text{ERK1/2}\) phosphorylation response, as assessed by WB with antibodies against phosphorylated (active) ERK (Fig. 1A). There was no ERK phosphorylation in response to orexin stimulation in CHO-h\(\text{CB}_1\) cells (Fig. 1A), but when \(\text{OX}_1\) receptors were transiently expressed in these cells utilizing baculovirus, orexin-A produced a marked response (Fig. 1B). The same was seen in the Elk-1 activity assay (Fig. 1C).

Orexin-A produced a concentration-dependent stimulation of ERK phosphorylation in transiently \(\text{OX}_1\)-expressing CHO-h\(\text{CB}_1\) cells (Fig. 2, A and B; Supplemental Fig. 2; Supplemental Table 1). When \(\text{CB}_1\) receptors were blocked with the CB1

\[\text{Fig. 1.} \text{ pERK and Elk-1 activity responses in CHO-hCB}_1\text{ cells expressing \(\text{OX}_1\) receptors upon transduction or transfection. ERK phosphorylation in response to \(\text{CB}_1\) receptor stimulation with HU-210 or 2-AG or orexin receptor stimulation with orexin-A in native CHO-h\(\text{CB}_1\) cells (A) and in cells transduced with \(\text{OX}_1\)-GFP baculovirus (B). The results come from infrared fluorescence, and thus the figures appear inverted as compared with film images. Also, the dynamic range is much wider than on the film or can be shown here; a compromise in terms of the intensity had to be made to present the data here. (C) Responses in CHO-h\(\text{CB}_1\) cells transduced with \(\text{OX}_1\) cDNA with respect to the Elk-1 activity assay (n = 5). **P < 0.01 vs basal.}
receptor antagonist/inverse agonist SR141716, the maximum response to orexin-A was strongly reduced (Fig. 2, A–C; Supplemental Fig. 2). An equal inhibition was seen with the diacylglycerol lipase (DAGL) inhibitor THL (Fig. 2, A–C; Supplemental Fig. 2). The potency of orexin-A was also reduced 100- to 150-fold by SR141716 and THL (Fig. 2, A, B, and D; Supplemental Fig. 2; Supplemental Tables 1 and 2).

Elk-1 was activated by orexin-A with somewhat lower potency than ERK phosphorylation in CHO-hCB1 cells transiently expressing OX1 receptors (Fig. 3; Supplemental Table 1). The DAGL inhibitor THL and another CB1 receptor antagonist, AM-251, produced a 5- to 8-fold reduction in the potency of orexin-A (Fig. 3, A and E; Supplemental Tables 1 and 2), but there was no difference in the basal (Fig. 3C) or the maximum response (Fig. 3D).

M1 Muscarinic Receptor Response Is More Weakly Potentiated Than the OX1 Receptor Response. We expressed another strongly Gq-PLC-coupled receptor, human M1 muscarinic acetylcholine receptor, in CHO-hCB1 cells and assessed Elk-1 activity as for the OX1 receptor. Stimulation with the potent agonist oxotremorine-M produced a clear concentration-dependent Elk-1 activation (Fig. 3C). Inclusion of THL and AM-251 slightly reduced the maximum response as well as slightly shifted the concentration-response curve (Fig. 3, B, D, and E). The shift in the concentration-response curve by THL and AM-251 was significantly smaller for M1 than for the OX1 receptors (Fig. 3D). This indicates that the M1 response is more weakly potentiated by CB1 receptor signaling than the OX1 response, despite the fact that both receptors produced essentially equal PLC activation (Fig. 3F). AM-251 clearly reduced the basal Elk-1 activity in M1-expressing cells, unlike in OX1-expressing cells (Fig. 3C).

CB1 Expression on CHO-hOX1 Background Potentiates the ERK Pathway. Orexin-A produced a concentration-dependent stimulation of Elk-1 activity in CHO-hOX1 cells (Fig. 4A; Supplemental Table 1). No response to the CB1 cannabinoid receptor stimulus (HU-210) was seen (not shown). Upon transient expression of CB1 receptors, HU-210 was able to induce Elk-1 activity (not shown; see below for Fig. 6B). Transient expression of CB1 receptors increased the potency of orexin-A 6-fold; this was fully blocked by THL or AM-251 (Fig. 4, A and D; Supplemental Tables 1 and 2). CB1 expression did not affect the maximum response (Fig. 4C). Interestingly, the basal Elk-1 activity was elevated by CB1 receptor coexpression (Fig. 4B). This was clearly more efficiently inhibited by AM-251 than THL, and may thus relate to constitutive activity of CB1 receptors (see, e.g., Turunen et al., 2012).
DAGL Inhibitor THL and CB₁ Receptor Antagonist/Inverse Agonist AM-251 Do Not Show Nonspecific Effects. As a control, we tested the drugs used for nonspecific effects. THL did not have any direct blocking effect on CB₁ receptor signaling, and similarly, THL and AM-251 were devoid of an effect on OX₁ orexin receptor or M₁ muscarinic receptor signaling (Fig. 5), which is in agreement with previous findings (see Discussion).

The Impact of the Receptor Stoichiometry on the CB₁ Receptor Potentiation of OX₁ Signaling. Potentiation of
ERK signaling was obtained upon combination of OX1 and CB1 receptors on either cell background (CHO-hCB1 and CHO-hOX1, respectively), but the potentiation was somewhat more modest when CB1 receptors were transiently expressed on the CHO-hOX1 background (compare Figs. 3 and 4). Interestingly, the pEC50 value of orexin-A for ERK phosphorylation or Elk-1 activation in both stable CHO cell clones was around 7.4–7.8 when CB1 receptors were not present or when they were inhibited (THL or CB1 antagonist; Supplemental Table 1).

When the responses to OX1 and CB1 receptor stimulation were compared between the cell types, it was clearly seen that the maximal CB1 and OX1 receptor activation equally strongly stimulated Elk-1 activity in CHO-hCB1 cells (Fig. 6, A and C), whereas OX1 receptors much more strongly stimulated Elk-1 activity in CHO-hOX1 cells (Fig. 6, B and C). The efficacy of the synergistic signaling of OX1 and CB1 receptors could be imagined to relate to the receptor expression levels and stoichiometry. High OX1 expression might give a more robust 2-AG production, which is able to stimulate CB1 receptors even not in immediate vicinity of OX1 receptors. Efficient CB1 signaling, as indicated by the receptor’s ability to connect to the ERK cascade when stimulated directly (HU-210), should suggest good ability to potentiate the orexin response. We thus measured the apparent expression levels of OX1 and CB1 receptors in the different cell systems utilizing the same expression of constructs as for the Elk-1 assay, to make the results as comparable as possible. The protocol applied (see Receptor Binding) visualizes, for OX1 receptors, the cell-surface expression. The binding was for CHO-hOX1 cells 460 ± 30 fmol/mg protein (n = 3) (Fig. 6D). Transient expression of CB1 receptor in these cells did not significantly alter the OX1 expression. CHO-hCB1 cells transiently transfected with OX1 receptor cDNA expressed very low levels of OX1 receptors (22 ± 6 fmol/mg protein; n = 5) (Fig. 6D). It should be pointed out that [125I]orexin-A binding only indicates the so-called agonist high-affinity sites and not the total receptor pool (Kukkonen, 2004). In contrast, the total CB1 receptor pool (both cell surface and internal, both agonist affinity states) is visualized by [3H]SR141716 binding (Fig. 6E). This binding amounted to 3.2 ± 0.3 pmol/mg protein in CHO-hCB1 cells. Transient expression of OX1 in these cells slightly elevated

Fig. 4. Elk-1 activity in CHO-hOX1 cells expressing CB1 receptors upon transient transfection. (A) Representative concentration-response curves. (B–D) Average responses (n = 3). The data were normalized to the noninhibited CB1-OX1 control in each batch of cells before averaging. The first comparisons are to the corresponding controls. *P < 0.05, **P < 0.01, ***P < 0.001 vs. controls. In (D), the second comparison is to the control CHO-hOX1 cells (no CB1 expression). NS, not significant (P > 0.05).
CB1 expression (Fig. 6E). Transient CB1 expression level in CHO-hOX1 cells was significantly lower than the stable expression in CHO-hCB1 cells (Fig. 6E). THL did not affect OX1 or CB1 expression levels (Fig. 6, D and F). Although the receptor expression levels (Fig. 6C) affect the responses obtained by stimulation of OX1 and CB1 receptors (Fig. 6F), the potentiation works equally well at very low OX1 receptor expression levels (CHO-hCB1OX1 cells) and thus also over a wide range of OX1-CB1 receptor stoichiometries (Fig. 6F).

OX1 Receptor Signaling Relays Endocannabinoid Signals via CB1 Receptor to ERK Also in Paracrine Fashion. The results thus suggest that OX1 receptor stimulation in CHO-hOX1 cells induces significant endocannabinoid 2-AG production by activating DAGL (Turunen et al., 2012). In the current study, we show that this 2-AG released from CHO-hOX1 cells upon exposure to orexin-A was an effective stimulant of the ERK cascade in nearby CHO-hCB1 cells (Fig. 7). The response obtained was sensitive to THL and AM-251 (Fig. 7), as expected (Turunen et al., 2012).

Discussion

We have recently shown that OX1 receptor stimulation in CHO cells induces significant endocannabinoid 2-AG production by activating DAGL (Turunen et al., 2012). In the current study, we show that this 2-AG is able to act both as auto- and paracrine messenger on CB1 cannabinoid receptors. If the receptors are expressed in the same cells, OX1 receptor signaling to the ERK cascade is strongly potentiated by autocrine CB1 signaling. The potentiation is fully reversed by
blocking CB1 receptors with SR141716 or AM-251 or inhibition of DAGL with THL. This shows that the potentiation requires OX1 receptor–induced release of 2-AG, which acts via CB1 receptors.

Our findings are centrally based on the inhibitors THL, SR141716, and AM-251. THL was originally used as triacylglyceride lipase inhibitor, but was later found to be an even more potent inhibitor of DAGLα and β (Bisogno et al., 2003; Szabo et al., 2006). Most importantly, we have recently shown that THL at 1 μM fully blocks 2-AG production in CHO cells (Turunen et al., 2012). THL displays weak affinity for CB1 receptors too (Ki ~ 4 μM) (Szabo et al., 2006), but this is too low to cause any significant inhibition of CB1 receptors here (see also Fig. 5). We also tried overexpression of monoacylglycerol lipase, but did not obtain strong enough reduction in 2-AG levels (not shown). Whether this is due to low expression or lack of an activation signal remains elusive. SR141716 has mainly been used as a CB1 receptor antagonist/inverse agonist in the previous studies on OX1-CB1 interaction (Hilairet et al., 2003; Ellis et al., 2006; Ward et al., 2011), and we thus used it in the ERK phosphorylation assay. As an additional level of control, we used another well known CB1 receptor antagonist/inverse agonist, AM-251, in the Elk-1 activity assay. Both ligands behaved in a similar manner and showed no nonspecific effects, despite the fact that AM-251 is actually an inverse agonist. DAGL block by THL and CB1 receptor block with AM-251 or SR141716 produce an essentially equal inhibition of the potentiation of ERK signaling, and thus the putative constitutive activity of CB1 receptors seems largely redundant for the agonist-stimulated ERK activity.

The two assays used to measure ERK activation produced qualitatively equal potentiation—i.e., CB1 receptor coexpression with OX1 receptors always potentiated OX1 signaling to
ERK—but the level of potentiation was lower for the Elk-1 activity assay and the maximum response was not affected in CHO-hCB1 cells. The assays are run for very different times, 10 minutes and 5 hours for pERK and Elk-1, respectively. One explanation for the result is that the temporal profile of ERK activation is different in the absence and presence of CB1 receptor signaling, e.g., that the CB1 receptor contribution ceases faster than the OX1 receptor component. Another explanation readily at hand is that Elk-1 response saturates at submaximal ERK activation levels. For ERK signaling there is the complicating factor of possible different cytosolic and nuclear ERK activity.

Potentiation of the ERK signaling of OX1 receptors by CB1 receptor coexpression has been previously observed in CHO cells (Hilairet et al., 2003). The potentiation was found to be blocked by the CB1 antagonist SR141716 and pertussis toxin. The authors inferred, based on colocalization studies, that OX1 and CB1 receptors form heteromeric complexes with enhanced signaling capabilities (Hilairet et al., 2003). In the current study, we have assessed the ERK signaling cascade and can show that 2-AG, produced by OX1 receptor activity, signals to ERK via CB1 receptors (Fig. 7) and also mediates the potentiation when the receptors are expressed in the same cells. Also, the other findings of Hilairet et al. (2003)—that CB1 receptor stimulation-induced ERK phosphorylation is not affected by OX1 receptor coexpression and OX1-mediated PLC activation is not potentiated by CB1 coexpression—are likely explained by this signaling scheme. Since there is no PLC coupling of CB1 receptors, PLC activity cannot be potentiated, and OX1 receptor coexpression does not potentiate CB1 receptor coupling to ERK, since CB1 receptor activation does not produce any ligand for OX1 receptors.

The concept of OX1-CB1 receptor heterodimerization was further refined by Milligan and coworkers in their work with HEK-293 cells (Ellis et al., 2006). In these cells, potentiation of ERK phosphorylation is very weak (Ellis et al., 2006). We have not investigated OX1-CB1 signaling in HEK-293 cells, but it is possible that the weak potentiation of the ERK response could be due to much lower or less potent 2-AG production upon OX1 receptor activation in this cell type as compared with CHO cells, as is also indicated by our recent findings (Turunen et al., 2012); however, we have not had access to the same clone of HEK-293 cells. This group has also presented convincing Förster (fluorescence) resonance energy transfer and immunoprecipitation data on OX1-CB1 complex formation (Ward et al., 2011). Thus, there is little doubt that these receptors have the capacity to combine into heteromers like many other GPCRs (reviewed in Birdsall, 2010; Rozenfeld and Devi, 2011). Previously, CB1 receptors have been reported to interact with many other GPCRs via either dimerization or functional interaction (Glass and Felder, 1997; Rios et al., 2006; Harkany et al., 2007; Marcellino et al., 2008; Turu et al., 2009; Rozenfeld et al., 2011). We have no means of readily inhibiting the receptor heteromerization process, and we therefore cannot assess the significance of this for the potentiation of ERK signaling. However, we currently feel that the most important interaction between OX1 and CB1 receptors takes place via OX1 receptor–stimulated 2-AG production, i.e., a functional interaction, which, theoretically, would not demand receptor complex formation. However, it is possible that functional interaction via endocannabinoids and receptor heteromerization collaborate. If receptor heteromers were placed in signaling complexes including PLC, DAGL, and the putative 2-AG extrusion machinery, CB1 receptors would be exposed to highest possible 2-AG levels. This would be an attractive hypothesis, but at least in CHO-hOX1 cells 2-AG production is high enough to even allow diffusion in the extracellular space (Fig. 7) (Turunen et al., 2012), as also should be the case for the retrograde synaptic transmission.

Potentiation of ERK signaling by CB1 receptors also works well at very low orexin receptor expression levels. The high OX1 receptor level in CHO-hOX1 cells may thus be to a large part superfluous with respect to 2-AG generation for autocrine CB1 receptor stimulation. Orexin receptor coupling to the PLC cascade is very efficient, similar to what we have previously found for muscarinic receptors (Kukkonen et al., 1996). OX1 receptors also potently coupled to the ERK–Elk-1 cascade even at low expression levels. CB1 receptors were efficiently expressed both stably and transiently; some receptor reserve may also exist for CB1 receptors, as indicated by the similar Elk-1 response in these different cell types. The cell-surface receptor levels are likely significantly lower than measured here, as CB1 receptors often show a high degree of constitutive internalization (Ellis et al., 2006). We could not, unfortunately, directly assess this, as the binding assay does not allow separation of cell-surface and intracellular receptors. However, local receptor levels in synaptic areas are also high.

Fig. 7. Elk-1 data from CHO-hOX1 cell–CHO-hCB1 cell communication assay (see Elk-1 Activity Luciferase Assay). Elk-1-driven luciferase activity was measured in CHO-hCB1 cells interspersed among CHO-hOX1 cells stimulated with orexin-A. Inhibition of the luciferase activity with THL and AM-251 indicates that the Elk-1 activity is induced by the cascade orexin-A → OX1 receptors (on CHO-hOX1 cells) → 2-AG (extracellular) → CB1 receptors (on CHO-hCB1 cells) (n = 4) (Turunen et al., 2012). ***P < 0.001 vs. basal. ns, not significant (P > 0.05).
If the potentiation of ERK signaling by OX1-CB1 “collaboration” solely takes place via functional interaction of the signal pathways, what then are the molecular mechanisms? Orexin receptors are suggested to be able to interact with at least G_{i/o} G_{a} and G_{b} family G proteins (Randleva et al., 2001; Holmqvist et al., 2005; Karteris et al., 2005). However, both G_{i/o} and G_{b} couplings appear rather weak, at least in CHO cells (Holmqvist et al., 2005). In contrast, CB1 receptors strongly couple to G_{i/o} proteins, although other couplings have also been suggested (reviewed in Pertwee, 1997; Alexander and Kendall, 2007; see also Fig. 5). It is thus likely that the two receptors preferentially use different pathways to ERK, and these pathways could act synergistically to activate ERK. Interestingly, the signaling of M1 muscarinic receptors was only weakly potentiated by CB1 receptor signaling, although both M1 and OX1 receptors equally strongly coupled to the PLC cascade in transient expression. Whether this is due to differences in complexing with CB1 receptors or efficacy of 2-AG production remains unknown. However, it would be tempting to speculate that the coupling of OX1 receptors to 2-AG production remains unknown. However, it would be tempting to speculate that the coupling of OX1 receptors to DAGL stimulation than that obtained with muscarinic receptors (Magga et al., 2006; Johansson et al., 2007).

In conclusion, we have here shown that the critical determinant of the synergistic OX1-CB1 signaling to ERK is the communication cascade OX1 → DAGL → 2-AG → CB1 receptors, which allows both OX1 and CB1 receptors to simultaneously signal to ERK. Whether receptor heteromerization takes part in the process cannot be resolved, but the data indicate that this may not be needed. However, it is of interest, in future studies, to focus on the possible effects of 2-AG on receptor dimerization, trafficking, and other properties described to be affected upon OX1-CB1 coexpression.

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Supplemental Fig. 1. Cell systems and receptor constructs utilized in the studies. Cells are on the left and DNA constructs for transient expression on the right. A, CHO-hCB₁ cells transduced with OX₁-GFP baculovirus for ERK phosphorylation WB studies; B, CHO-hCB₁ cells transfected to express OX₁ receptors for Elk-1 activity measurements and binding; C, CHO-hCB₁ cells transfected to express M₁ receptors for Elk-1 activity measurements, binding and PLC assay; D, CHO-hOX₁ cells transfected to express CB₁ receptors for Elk-1 activity measurements and binding. The orange receptor is CB₁, the blue one OX₁ and the purple one M₁.
Supplemental Fig. 2. WBs from a representative experiment made with CHO-hCB₁ cells transduced with OX₁-GFP baculovirus. Please observe that the results come from infrared fluorescence and thus the figures appear inverted as compared to film images. Also, the dynamic range is much wider than on the film or can be shown here; a compromise what concerns the intensity had to be made in order to present the data here.
### SUPPLEMENTAL TABLE 1

pEC$_{50}$-values for orexin-A presented as averages of values obtained in each batch of cells; $N$ = 3–5. The comparison for CHO-hCB$_1$ + OX$_1$ cells is to the ctrl (no inhibitors); *, p < 0.05; **, p < 0.01; ***, p < 0.001. The first comparison for all CHO-hOX$_1$ + CB$_1$ cells is to the CHO-hOX$_1$ (no CB$_1$); ns (not significant), p > 0.05; ***, p < 0.001. –, no response; n.d., not determined.

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<th>pERK1</th>
<th>pERK2</th>
<th>Elk-1</th>
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<td>9.6 ± 0.4</td>
<td>8.3 ± 0.2</td>
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<td>+OX$_1$: THL</td>
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<td>+OX$_1$: SR141716 or AM-251</td>
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<td>−OX$_1$: ctrl</td>
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<td>n.d.</td>
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<td>n.d.</td>
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<td>7.7 ± 0.2</td>
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SUPPLEMENTAL TABLE 2

Relative pEC\textsubscript{50}-values for orexin-A. The values obtained in each batch of cells were normalized to the control (CHO-hCB\textsubscript{1} + OX\textsubscript{1} or CHO-hOX\textsubscript{1} + CB\textsubscript{1}) in each batch of cells and then averaged. The normalization is pEC\textsubscript{50-ctrl} − pEC\textsubscript{50-condition}; thus, a right-shift in the concentration-response curve gives a positive value. \( N = 3–5 \). The comparison for CHO-hCB\textsubscript{1} + OX\textsubscript{1} cells is to the ctrl (no inhibitors); *, \( p < 0.05 \); **, \( p < 0.01 \). The first comparison is to the control CHO-hOX\textsubscript{1} + CB\textsubscript{1} cells (no inhibitors); *, \( p < 0.05 \); **, \( p < 0.01 \). The second comparison is to the control CHO-hOX\textsubscript{1} cells (no CB\textsubscript{1}); NS (not significant), \( p > 0.05 \). –, no response.

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<th>+OX\textsubscript{1}: ctrl</th>
<th>+OX\textsubscript{1}: THL</th>
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