Intracellular signaling systems of G protein-coupled receptors are well-established, but their role in paracrine regulation of adjacent cells is generally considered as a tissue specific mechanism. We have shown previously that AT1 receptor (AT1R) stimulation leads to diacylglycerol lipase-mediated transactivation of coexpressed CB1Rs in CHO cells. In the present study we detected a paracrine effect of the endocannabinoid release from CHO, COS7 and HEK293 cells during the stimulation of AT1 angiotensin receptors by determining CB1 cannabinoid receptor activity with bioluminescence resonance energy transfer- (BRET-) based sensors of G protein activation expressed in separate cells. The angiotensin II-induced, paracrine activation of CB1 receptors was visualized by detecting translocation of GFP-tagged β-arrestin2. Mass spectrometry analyses have demonstrated angiotensin II-induced stimulation of 2-arachidonoylglycerol production, whereas no increase of anandamide levels was observed. Stimulation of G_q/11-coupled M_1, M_3, M_5 muscarinic, V_1 vasopressin, α_1a adrenergic, B_2 bradykinin receptors, but not G_i/o-coupled M_2 and M_4 muscarinic receptors, also led to paracrine transactivation of CB1 receptors. These data suggest that, in addition to their retrograde neurotransmitter role, endocannabinoids have much broader paracrine mediator functions during activation of G_q/11-coupled receptors.

Hormones, neurotransmitters and other chemical mediators acting on G protein-coupled receptors (GPCRs) exert their effects on the target cells by stimulating G protein dependent and independent intracellular signaling pathways (1-4). Activation of G_q/11 protein-coupled receptors causes phospholipase C activation, which produces inositol-trisphosphate and diacylglycerol from PtdIns(4,5)P_2, leading to Ca^{2+}-signal generation and protein kinase C activation. However, the concerted response of tissues to chemical mediators frequently also involves the activation of cells adjacent to the target cells, due to the release of paracrine mediators. A well-known example is NO, which can be released from activated endothelial cells to cause relaxation of adjacent vascular smooth muscle cells. Lipid mediators can also act as intercellular messengers. For example, endocannabinoids released from postsynaptic neurons after depolarization act as retrograde
transmitters by binding to and stimulating presynaptic cannabinoid receptors, which leads to inhibition of GABA release (an event termed depolarization induced suppression of inhibition, DSI) (5-7).

Cannabinoid receptors were first identified based on their ability to selectively recognize marijuana analogs. To date, two cannabinoid receptors have been identified by molecular cloning, CB1 and CB2 receptors (CB1R and CB2R, respectively) (5;8;9), although additional GPCRs have also been proposed to function as cannabinoid receptors (10;11). Cannabinoid receptors also recognize certain lipids present in animal tissues termed endocannabinoids, such as arachidonylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG) and 2-arachidonoylglyceryl ether (noladin ether) (7;12-16). In adult and foetal neural tissues, the two major endocannabinoids, AEA and 2-AG, are produced on demand, usually after depolarization of postsynaptic cells or following stimulation of Gq-coupled metabotropic glutamate or muscarinic acetylcholine receptors (7;12;17-20). Enzymes responsible for 2-AG production and metabolism in tissues are localized to well defined structures at synapses, near the axon terminals of CB1R expressing cells (5;7). In contrast, in peripheral tissues baseline levels of endocannabinoid production usually manifest as “endocannabinoid tone”, with poorly understood localization of the various components of the endocannabinoid system. 2-AG levels in brain homogenates and in many peripheral tissues are near its $K_d$ for the CB1R (19), suggesting that function of endocannabinoids may not be limited to localized synaptic signaling.

There is mounting evidence that endocannabinoids play important roles in peripheral cardiovascular, inflammatory, intestinal and metabolic regulation (21-24). 2-AG is produced by diacylglycerol-lipase (DAGL) after cleavage of the fatty-acid in the sn-1 position of diacylglycerol (DAG) (19;25). Phospholipase C activation by G$_{q/11}$ protein-coupled receptors produces DAG, which can serve as substrate for DAGL. Plasma membrane phosphoinositides are enriched in arachidonic acid in the sn-2 position (26) and DAGL is expressed ubiquitously (27), which suggests that phospholipase C-mediated cleavage of polyphosphoinositides may routinely lead to the formation of 2-AG. In accordance with this hypothesis, we have recently shown that angiotensin II- (Ang II)-mediated activation of the G$_{q/11}$-coupled AT$_1$ angiotensin receptor (AT$_1$R) leads to DAGL-dependent activation of CB$_1$Rs expressed in Chinese hamster ovary (CHO) cells (28).

Here our aim has been to examine the possibility that 2-AG serves as a common paracrine signal generated via activation of G$_{q/11}$ protein-coupled, Ca$^{2+}$-mobilizing receptors. Accordingly, we co-expressed CB$_1$Rs and BRET-based sensors of G protein activation in CHO cells, and used these cells to detect endocannabinoid release from adjacent cells that express AT$_1$R or other Ca$^{2+}$-mobilizing GPCRs. We have further shown that activation of AT$_1$R by Ang II increases 2-AG levels in CHO cells. These findings suggest that 2-AG is commonly released following activation of Ca$^{2+}$-mobilizing GPCRs and serves as a paracrine signal to activate CB$_1$R in neighboring cells.

**Materials and Methods**

*Materials*-Rat $\alpha_o$-CFP G protein subunit was provided by N. Gautam (29). Human $V_1$ vasopressin, $\alpha_1$ adrenergic, $B_2$ bradykinin and $M_1$ muscarinic acetylcholine receptor, $\beta_1$ and $\gamma_{11}$ G protein subunits were obtained from cDNA.org. The cDNA of the rat vascular CB$_1$R was provided by Zsolt Lenkei (Centre National de la Recherche Scientifique, Paris) (30). $\beta$-arrestin2-EGFP (β-ar2-GFP) was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC) (31).
Plasmid constructs and transfection- RFP-tagged CB1R was constructed by subcloning the CB1R cDNA into an mRFP containing vector (provided by Dr. R. Tsien, University of California, San Diego, CA). EYFP-β1 was generated by subcloning human β1 subunit into the mammalian expression vector pEYFP-C1 (Clontech, Palo Alto, CA). αo-Rluc was constructed by replacing the CFP coding region in αo-CFP with Renilla luciferase. Rat HA-AT1R receptor and AT1-EYFP were constructed as described earlier (32). AT1R-Cerulean was constructed by replacing the cDNA of EYFP with Cerulean coding region (provided by Dr. R. Tsien, University of California, San Diego, CA). Chinese Hamster Ovary (CHO) cells were transfected with Lipofectamine 2000 according to manufacturer's suggestions using 2 or 16-24 µg DNA and 2 or 16 µl Lipofectamine 2000 in 6 well plates or 100 mm tissue culture plates respectively. CHO cells were maintained in Ham’s F12 supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 IU/ml penicillin. COS-7 kidney fibroblast cells and HEK293 human embryonic fibroblast cells were grown in complete DMEM containing glucose, glutamine, sodium bicarbonate and supplemented with 10% FBS, 100 µg/ml streptomycin, 100 IU/ml penicillin.

Site-directed mutagenesis-Mutations in the rat CB1R (D214A and R215A) were performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene®, La Jolla, CA) according to manufacturer's suggestions, and verified using automated sequencing.

Confocal laser-scanning microscopy-CHO cells were grown on glass coverslips and transfected with labeled CB1R and AT1R and GFP-labeled β-arrestin2, as described above, 48 hours prior to measurement. In transactivation experiments, the cells were detached with Versene one day after transfection; the indicated cells were mixed and placed back to coverslips. Cerulean, EGFP and RFP were excited with the 458- and 488-nm lines of the argon laser and the 543-nm line of the helium/neon laser, respectively; and emitted fluorescence was detected in multitrack mode with 480-520 nm, 500-530 nm band pass and 560 nm long pass filters, respectively. Since GFP caused a weak cross-talk in this setup in the 480-520 nm band pass channel used for Cerulean, the full images showing Cerulean were corrected for the cross-talk of the GFP signals.

BRET assay of G protein activation-Energy transfer between G protein subunits was measured using αo G protein subunit fused with Renilla luciferase (αo-Rluc) and β1 subunit labeled with enhanced yellow fluorescent protein (EYFP-β1). Medium was changed to FBS-supplemented Ham’s F12 6 hours following transfection and incubated overnight. Before the experiments the cells were detached with Versene and centrifuged. Cells were suspended in a modified Krebs-Ringer buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 CaCl2, 0.7 MgSO4, 10 glucose, 10 sodium Hepes, pH 7.4 (containing 1 g/l albumin in paracrine transactivation studies) and transferred to white 96-well plates. The cell density was between 100,000 and 200,000 cells/well. Coelenterazine h was added to a final concentration of 5 µM and readings were collected using a Mithras LB 940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). BRET ratio was defined as (emission at 530 nm) / (emission at 485), and the normalized BRET ratio was calculated as the BRET ratio for the co-expressed EYFP-tagged and Rluc-tagged molecules minus the BRET ratio for the co-expressed non-tagged and Rluc-tagged molecules. Data for G protein activity are shown as the percent changes in normalized BRET ratios compared to the mean of the four control BRET ratio points before the first stimulation (BRET ratio, % of control).

Measurement of 2-AG and anandamide levels-CHO cells transfected with AT1R were maintained in Ham’s F12 medium in 10 cm tissue culture plates as described above. Aliquots of 4x10^6 cells in 10 cm tissue culture plates containing 3.5 ml of modified Krebs-
Ringer buffer were incubated with vehicle or 100 nM angiotensin II for the indicated times, following which the cells + medium were extracted in 2 volumes of ice-cold chloroform:methanol (2:1, v/v) containing 7 ng of 4H2-anandamide as internal standard. The chloroform phase was separated and re-extracted twice and finally dried under a stream of nitrogen. The dried residue was reconstructed in 100 µl chloroform, deproteinated with 2 ml ice-cold acetone, centrifuged, and the clear supernatant was evaporated to dryness. Samples were resuspended in 50 µl of methanol for analysis of endocannabinoid content by liquid chromatography/in line mass spectrometry, as described (33).

Statistical Analysis-All data are presented as means ± S.E. Differences between groups were analyzed by one- or two-way repeated measures analysis of variance combined with Holm-Sidak test using the software SigmaStat for Windows 3.5 (Systat Software Inc., Richmond, CA). The value of p less than 0.05 was considered significant.

Results

Stimulation of AT1Rs expressed in CHO, HEK and COS cells leads to paracrine transactivation of CB1Rs. We have previously shown that CB1R can be transactivated with angiotensin II (Ang II) in cells co-expressing CB1R and AT1R (28). We therefore postulated that if the transactivation of CB1R was caused by a released endocannabinoid, it would also occur if AT1R and CB1R were expressed in separate, adjacent cells. To examine this possibility, we monitored the activation of CB1Rs expressed in CHO cells to detect Ang II-induced endocannabinoid release by other cells mixed to the cells expressing CB1R. CB1R activity was quantified by detecting bioluminescence resonance energy transfer (BRET) between Go protein subunits (Renilla luciferase-tagged αo and EYFP-tagged β1, αo-Rluc and EYFP-β1, respectively) as described previously (28). In this experimental setting, activation of CB1R leads to a decrease of the BRET signal resulting from the dissociation of activated Go protein subunits, whereas inactivation by an antagonist increases the BRET signal because it leads to the association of G protein subunits (28;29). CHO cells were transfected either with expression plasmids of AT1R, or were co-transfected with plasmids of CB1R and tagged G protein subunits. Twenty four hours later, the cells were mixed and CB1R activity was measured by detecting BRET interaction between G protein subunits, as a way to monitor endocannabinoid release. Stimulation of the cells with Ang II led to a decrease in BRET signal indicating activation of CB1R (Fig. 1A, filled circles). Administration of the CB1R inverse agonist AM251 caused an increase in the BRET signal, reflecting the inhibition of the basal activity of the receptor. AM251 also blocked the Ang II-induced transactivation (Fig. 1A, open circles). A slight baseline shift of the BRET signal occurred during treatment, which might have been caused by the small increase in volume; and the kinetics of the response was too fast to analyze its initial phase with manual stimulation. Therefore, an automated injector was used in separate experiments to analyze the initial phase of the Ang II-induced response in higher time-resolution (Fig. 1B and C). The initial baseline shift occurred promptly both in control and stimulated cells. In Ang II stimulated cells (filled circles) after a lag time of ca. 15-20 seconds the BRET signal decreased rapidly (Fig, 1B), which was not observed in cells treated with medium (control cells, open squares). AM251 fully prevented the effect of Ang II stimulation on the BRET signal (Fig. 1C).

When AT1R-transfected cells were mixed with cells expressing the Go sensor, but no CB1R, Go protein activation did not occur, which provides additional evidence that it was mediated through CB1R (Fig. 1D). Similarly, when cells
expressing CB1R, but not AT1R, were stimulated with Ang II, the BRET signal remained unaffected (Fig. 1E). In other experiments, the original 1:1 ratio of AT1R or CB1R-expressing cells was increased to 5:1 or decreased to 0.1:1, keeping the number of CB1R expressing cells constant at 100,000 cells/well, which resulted in a corresponding increase or decrease, respectively, in Go protein activation, approaching a plateau between 1:1 and 5:1 cell ratios (Fig. 1F). These findings suggest that Go protein activation in CB1R-expressing cells was mediated by Ang II-induced endocannabinoid release from CHO cells expressing AT1R. Transactivation of CB1R was also inhibited by pretreatment of the cells expressing AT1R with the DAGL inhibitor tetrahydrolipstatin (THL, 1 μM) (Fig. 2A open triangles), suggesting that DAGL plays a role in the process. Statistical analysis of the data is shown in Fig. 2B.

Transactivation of CHO cells expressing CB1Rs was also detected when these cells were mixed with equal number of HEK293 or COS7 cells expressing AT1Rs, and stimulated with Ang II. These data demonstrate that the AT1R-mediated paracrine transactivation of CB1Rs is not restricted to CHO cells and may be a common mechanism in different cell types (Fig. 3). Slightly increased transactivation of CB1Rs was observed, when these AT1R-expressing cells were added in a 5 fold excess, suggesting that the response was almost maximal under these conditions (Supplementary Fig. 1.)

AT1R activation leads to β-arrestin translocation to CB1R in adjacent cells. To provide additional evidence that Ang II-induced activation of AT1R leads to the formation and release of endocannabinoids, β-arrestin translocation to transactivated CB1Rs was measured in CHO cells. β-Arrestin coupling to GPCRs has often been used as an indicator of receptor activation (31;32;34). When RFP-tagged CB1R (CB1R-RFP) was co-expressed with GFP-tagged β-arrestin2 (β-arr2GFP), CB1R-RFP localized both to the cell membrane and to intracellular vesicles (Fig. 4A), which is consistent with the previously reported constitutive internalization of this receptor, which may be caused by constitutive receptor activity, endocannabinoid formation or other mechanisms (28;30;35). Following stimulation with the synthetic cannabinoid agonist WIN 55,212-2, β-arr2-GFP translocated to the activated CB1Rs at the cell surface, and was detected in punctate structures at the plasma membrane (Fig. 4A). The conserved DRY motif in the second intracellular loop of GPCRs has been previously implicated in β-arrestin binding of some GPCRs (36-38). In other GPCRs, mutations in the conserved DRY region caused enhanced β-arrestin binding (39;40). The enhanced β-arrestin binding of these receptors may be caused by their reduced G protein coupling (41-43). We have generated mutations in the conserved DRY motif within the second intracellular loop of CB1R (D214A and R215A) (CB1R[DRY/AAY]). To study the interaction of the mutant CB1R with β-arrestin, RFP-tagged CB1R(DRY/AAY) (CB1R(DRY/AAY)-RFP) was co-expressed with β-arr2-GFP in CHO cells. In non-stimulated cells, β-arr2-GFP was distributed diffusely in the cytoplasm, but basal activity of this mutant CB1R caused its appearance in punctate structures at the plasma membrane (Fig. 4B, left). Stimulation with WIN55,212-2 caused robust translocation of β-arr2-GFP to the plasma membrane, with only very faint fluorescence remaining in the cytoplasm (Fig. 4B, middle). These data demonstrate that co-expression of CB1R(DRY/AAY)-RFP with β-arr2-GFP is a sensitive tool for visualization of both basal and agonist-induced CB1R activity.

To test if endocannabinoid release could be detected by β-arr2-GFP translocation to CB1R, CB1R(DRY/AAY)-RFP and β-arr2-GFP were co-expressed in CHO cells, and a separate pool of cells were transfected independently with AT1R-Cerulean. Twenty-four hours after transfection, the cells were mixed and placed on
coverslips and another day later they were visualized using confocal microscopy. As shown in Fig. 5, after Ang II-induced stimulation of the mixed CHO cell population expressing CB1R(DRY/AAY)-RFP and β-arr2-GFP or AT1R-Cerulean, translocation of β-arr2-GFP to the plasma membrane was observed in cells expressing CB1R(DRY/AAY)-RFP. This response was observed in 67 ± 7% of cells (n=3, 86 cells total) with neighboring AT1R-Cerulean expressing cells; whereas in parallel experiments, when cells expressing CB1R(DRY/AAY)-RFP and β-arr2-GFP were stimulated with Ang II in the absence of AT1R expressing cells, no translocation of β-arr2-GFP was detected (Fig. 4C). These data provide additional evidence that a paracrine endocannabinoid mediator is released as a result of AT1R activation.

2-AG formation is enhanced by stimulation of AT1Rs. To verify whether the Ang II-induced increase in BRET signal detected by confocal microscopy was, in fact, due to endocannabinoid release, we measured 2-AG and AEA levels in control and Ang II-stimulated AT1R expressing cells. As shown in Fig. 6, stimulation of cells with Ang II caused a statistically significant sustained elevation of 2-AG levels with similar kinetics to that of CB1R transactivation measured with BRET (Fig. 1A). Anandamide levels were very low and did not show statistically significant changes in response to Ang II stimulation (Supplementary Fig. 2). These data are consistent with our hypothesis that Gq/11-mediated DAG formation serves as a source of endocannabinoid release, and suggest that the endocannabinoid involved in Ang II-induced paracrine CB1R transactivation is 2-AG.

CB1R transactivation by AT1R receptor stimulation is not dependent on intracellular Ca2+ signal generation. 2-AG production in neural cells can be stimulated either by the Ca2+ signal caused by activation of ionotropic receptors, by stimulation of Gq activating (metabotropic) cholinergic or glutamatergic receptors, or by the coincidence of two signals (44). 2-AG production can be stimulated in cultured cell lines overexpressing DAGLs by inducing a Ca2+ signal with ionomycin (27;44). Therefore we asked if transactivation of CB1R by AT1R is dependent on Ca2+. In control cells, G protein activation occurred after Ang II stimulation and a Ca2+ signal was detected (Fig. 7A and D). In cells preincubated with BAPTA-AM (60 μM) and preloaded with FURA-2, Ang II-induced Ca2+ elevation was not detected even though transactivation of CB1R still occurred (Fig. 7B and D). Moreover, administration of ionomycin (1μM) was not able to fully mimic the effect of AT1R stimulation, although a small, reproducible, but in this set of experiments statistically not significant Gq protein activation was detectable (Fig. 7C and D). The averaged BRET data are presented in Fig. 7D. Since under these conditions ionomycin causes larger elevations of cytoplasmic [Ca2+] than those caused by Ang II (Fig. 7A and C, insert), these results suggest that transactivation was not primarily mediated by intracellular Ca2+ elevation.

Gq/11-coupled GPCRs cause paracrine transactivation of CB1R. It has been demonstrated previously that activation of Gq-coupled muscarinic or metabotropic glutamate receptors induces endocannabinoid release in neurons (17-19). To test if endocannabinoid release common occurs after stimulation of Gq-coupled receptors, we expressed M1, M3 and M5 cholinergic (M1R, M3R and M5R, respectively), V1 vasopressin (V1R), α1A adrenergic (α1AAR), and B2 bradykinin (B2R) receptors in CHO cells, and tested if paracrine transactivation of CB1R occurs. We also tested the effect of stimulation of Gq/o-coupled M2 and M4 muscarinic cholinergic receptors (M2R and M4R, respectively). Gq11- or Gq/o-activating receptors were expressed in one set of cells, and CB1R and Gq sensor were expressed in separate
population of cells. The two populations of cells were mixed and stimulated with the appropriate ligands. As shown in Fig. 8, CB1R was transactivated when G_{q/11} protein activating M_1R, M_2R, M_3R, α_1AAR, V_1R or B_2R expressing cells were stimulated with the appropriate ligands, but no transactivation was detected when G_{i/o} protein-coupled M_2R or M_4R were stimulated. The degree of transactivation in the former groups was similar to that measured with cells expressing AT_{1R} (Fig. 2B). Transactivation did not occur in the absence of either CB1R or the G_{q/11} activating receptor (Supplementary Fig. 3.). These findings demonstrate that paracrine CB1R transactivation by endocannabinoids is not specific to AT_{1R}, and can be also initiated in cells that express other G_{q/11} activating GPCRs.

Discussion

Seven transmembrane GPCRs constitute the largest group of membrane receptors (45). They respond to a large variety of stimuli and transduce various signals across the plasma membrane by coupling to heterotrimeric G proteins (46;47). There are about 1000 GPCRs in the human genome and about half of the medications used in current clinical practice modify the biological activity of GPCRs (48;49). Stimulation of GPCRs leads to activation of heterotrimeric G proteins (50) composed of α, β and γ subunits, and GPCR activation causes dissociation or conformational rearrangement of the α subunit from the βγ complex (51). This is followed by intracellular responses depending on the composition of the heterotrimer. In case of G_{q/11}-coupled receptors, such as the AT_{1R}, early events include cleavage of PIP_2 by phospholipase Cβ and formation of second messengers such as IP_3 and DAG (2;52). IP_3 initiates Ca^{2+} release from intracellular stores, whereas DAG activates protein kinase C (53). Since DAG is a common signaling molecule in different cell types and DAGL is found in almost every tissue (27), we hypothesized that 2-AG formation can be a common early signaling event in most cells. In order to detect the formation and release of endocannabinoids as a general consequence of G_{q/11} protein activation, we expressed AT_{1R} in CHO, HEK293 and COS7 cell lines, which do not express cannabinoid receptors constitutively.

CHO cells expressing CB1R and a G_{i/o} BRET sensor were used to monitor endocannabinoid release (28;29;51). Mixing these cells with cells expressing other GPCRs allowed us to detect the endocannabinoid release caused by these GPCRs. An advantage of using these sensors is that the signal is less affected by the rapid degradation of endocannabinoids. Our findings clearly indicate that activation of AT_{1Rs} leads to endocannabinoid formation and release, with the subsequent activation of CB1Rs. Our data also indicate that activation of G_{q/11}-coupled receptors can cause paracrine activation of adjacent cells, since in our experiments the stimulated G_{q/11}-coupled receptors and the CB1R were expressed in different cells. We have also demonstrated that Ang II increased the level of 2-AG in CHO cells expressing AT_{1R}. In control cells, 2-AG was also detected, which is consistent with the possible role of this molecule in the basal activity of CB1R (28). These data support our hypothesis that the Ang II-induced DAGL-dependent transactivation of CB1Rs is mediated by 2-AG. Therefore, 2-AG is the most likely candidate to mediate the observed paracrine effects in our cells. The release of endocannabinoids from cells may occur via unidentified transporter molecules, which is consistent with their proposed paracrine mediator role (54). However, we could not detect transactivation when the supernatant from the stimulated cell population was transferred to cells expressing CB1Rs (Turu et al., unpublished observation). It is possible that endocannabinoids being lipophilic molecules, they remain membrane-associated and require direct contact with adjacent CB1R-expressing cells for receptor activation.
We have also tested other G_{q/11} activating GPCRs, including muscarinic acetylcholine receptors, which are known to cause 2-AG release in neural tissues, as well as α_{1} adrenergic receptors, V_{1} vasopressin receptors and B_{2} bradykinin receptors. Our data suggest that 2-AG release is a common consequence of activation of various G_{q/11}-coupled receptors. Since 2-AG can be released by cells (55), its release can cause paracrine regulation of adjacent cells. Based on our data we propose that activation of G_{q/11}-coupled receptors results in a coordinated intracellular (InsP_{3} and DAG) and intercellular (2-AG) signaling, and the phospholipase C-catalyzed hydrolysis of polyphosphoinositides serves as a source of both types of messengers.

Blockade or stimulation of cannabinoid receptors has wide-ranging effects in a number of organ systems including brain, cardiovascular system, adipose tissue, liver, immune system and the eye (12;21;22). The present results indicate that endocannabinoid activation of CB_{1}R can also be triggered indirectly via G_{q/11}-coupled receptors, which means that the primary response to agonists of these receptors can be modulated by endocannabinoids. Furthermore, some of the physiological effects observed following CB1 receptor blockade may be due to removal of such a modulation of tonically active G_{q/11}-coupled receptor signaling. Furthermore, these data also suggest that endocannabinoid production is not restricted to small, well defined regions/tissues in the organism, but is a general property of many, if not all, tissues. Although CB_{1}R is expressed mainly in the central nervous system, it is also found in many peripheral tissues, and stimulation or blocking of these receptors has marked effects on their function (12;21;22).

In conclusion, our data show that 2-AG formation and release is a general paracrine signaling mechanism of G_{q/11}-coupled GPCRs. Based on these results we propose that, in addition to IP_{3}- and DAG-mediated intracellular signaling, 2-AG formed from the DAG generated during activation of G_{q/11} proteins is released and acts as a paracrine signal for cannabinoid receptors on adjacent cells, which would modulate the primary response to Ca^{2+}-mobilizing hormones in a region-specific manner. Since 2-arachidonoylglycerol has been implicated in a variety of physiological functions and the enzymes responsible for its biosynthesis are present in most tissues, we are proposing that the previously recognized retrograde transmitter role of endocannabinoids is part of a much broader paracrine signaling role of these mediators.
References

1. DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007) *Annu. Rev. Physiol.* **69**, 483-510

2. Hunyady, L. and Catt, K. J. (2006) *Mol. Endocrinol.* **20**, 953-970

3. Lefkowitz, R. J. (2004) *Trends Pharmacol. Sci.* **25**, 413-422

4. Shenoy, S. K. and Lefkowitz, R. J. (2005) *Sci. STKE.* **2005**, cm14

5. Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., Mechoulam, R., and Pertwee, R. G. (2002) *Pharmacol. Rev.* **54**, 161-202

6. Wilson, R. I., Kunos, G., and Nicoll, R. A. (2001) *Neuron* **31**, 453-462

7. Freund, T. F., Katona, I., and Piomelli, D. (2003) *Physiol Rev.* **83**, 1017-1066

8. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) *Nature* **365**, 61-65

9. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) *Nature* **346**, 561-564

10. Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N. O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., and Greasley, P. J. (2007) *Br. J. Pharmacol.* **152**, 1092-1101

11. Brown, A. J. (2007) *Br. J. Pharmacol.* **152**, 567-575

12. Pacher, P., Batkai, S., and Kunos, G. (2006) *Pharmacol. Rev.* **58**, 389-462

13. Stella, N., Schweitzer, P., and Piomelli, D. (1997) *Nature* **388**, 773-778

14. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258**, 1946-1949

15. Hanus, L., Abu-Lafi, S., Fride, E., Breuer, A., Vogel, Z., Shalev, D. E., Kustanovich, I., and Mechoulam, R. (2001) *Proc. Natl. Acad. Sci. U. S. A* **98**, 3662-3665

16. Harkany, T., Guzman, M., Galve-Roperh, I., Berghuis, P., Devi, L. A., and Mackie, K. (2007) *Trends Pharmacol. Sci.* **28**, 83-92

17. Kim, J., Isokawa, M., Ledent, C., and Alger, B. E. (2002) *J. Neurosci.* **22**, 10182-10191

18. Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A., and Kano, M. (2001) *Neuron* **31**, 463-475

19. Sugiura, T., Kishimoto, S., Oka, S., and Gokoh, M. (2006) *Prog. Lipid Res.* **45**, 405-446
20. Berghuis, P., Rajnicek, A. M., Morozov, Y. M., Ross, R. A., Mulder, J., Urban, G. M.,
Monory, K., Marsicano, G., Matteoli, M., Canty, A., Irving, A. J., Katona, I., Yanagawa, Y.,
Rakic, P., Lutz, B., Mackie, K., and Harkany, T. (2007) *Science* **316**, 1212-1216

21. Kogan, N. M. and Mechoulam, R. (2007) *Dialogues Clin. Neurosci.* **9**, 413-430

22. Kunos, G. (2007) *Am. J. Med.* **120**, S18-S24

23. Pacher, P., Batkai, S., and Kunos, G. (2005) *Handb. Exp. Pharmacol.* 599-625

24. Jeong, W. I., Osei-Hyiaman, D., Park, O., Liu, J., Batkai, S., Mukhopadhyay, P., Horiguchi,
N., Harvey-White, J., Marsicano, G., Lutz, B., Gao, B., and Kunos, G. (2008) *Cell Metab.* **7**, 227-235

25. Basavarajappa, B. S. (2007) *Protein Pept. Lett.* **14**, 237-246

26. Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81-47

27. Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M. G., Ligresti, A., Matias, I.,
Schiano-Moriello, A., Paul, P., Williams, E. J., Gangadharan, U., Hobbs, C., Di Marzo, V.,
and Doherty, P. (2003) *J. Cell Biol.* **163**, 463-468

28. Turu, G., Simon, A., Gyombolai, P., Szigonya, L., Bagdy, G., Lenkei, Z., and Hunyady, L.
(2007) *J. Biol. Chem.* **282**, 7753-7757

29. Azpiazu, I. and Gautam, N. (2004) *J. Biol. Chem.* **279**, 27709-27718

30. Leterrier, C., Bonnard, D., Carrel, D., Rossier, J., and Lenkei, Z. (2004) *J. Biol. Chem.* **279**, 36013-36021

31. Barak, L. S., Ferguson, S. S. G., Zhang, J., and Caron, M. G. (1997) *J. Biol. Chem.* **272**, 27497-27500

32. Turu, G., Szigonya, L., Gáborik, Z., Buday, L., Spät, A., Clark, A. J. L., and Hunyady, L.
(2006) *FEBS Lett.* **580**, 41-45

33. Wang, L., Liu, J., Harvey-White, J., Zimmer, A., and Kunos, G. (2003) *Proc. Natl. Acad. Sci. U. S. A* **100**, 1393-1398

34. Ferguson, S. S. G. and Caron, M. G. (2004) *Methods Mol. Biol.* **237**, 121-126

35. McDonald, N. A., Henstridge, C. M., Connolly, C. N., and Irving, A. J. (2007) *Mol.
Pharmacol.* **71**, 976-984

36. Bennett, T. A., Maestas, D. C., and Prossnitz, E. R. (2000) *J. Biol. Chem.* **275**, 24590-24594

37. Huttenrauch, F., Nitzki, A., Lin, F. T., Honing, S., and Oppermann, M. (2002) *J. Biol. Chem.* **277**, 30769-30777
38. Mhaouty-Kodja, S., Barak, L. S., Scheer, A., Abuin, L., Diviani, D., Caron, M. G., and Cotecchia, S. (1999) Mol. Pharmacol. 55, 339-347
39. Barak, L. S., Oakley, R. H., Laporte, S. A., and Caron, M. G. (2001) Proc. Natl. Acad. Sci. U. S. A 98, 93-98
40. Lagane, B., Ballet, S., Planchenault, T., Balabanian, K., Le Poul, E., Blanpain, C., Percherancier, Y., Staropoli, I., Vassart, G., Oppermann, M., Parmentier, M., and Bachelerie, F. (2005) Mol. Pharmacol. 67, 1966-1976
41. Gáborik, Z., Jagadeesh, G., Zhang, M., Spät, A., Catt, K. J., and Hunyady, L. (2003) Endocrinology 144, 2220-2228
42. Savarese, T. M. and Fraser, C. M. (1992) Biochem. J. 283 (Pt 1), 1-19
43. Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., and Lefkowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A 100, 10782-10787
44. Hashimotodani, Y., Ohno-Shosaku, T., Tsubokawa, H., Ogata, H., Emoto, K., Maejima, T., Araishi, K., Shin, H. S., and Kano, M. (2005) Neuron 45, 257-268
45. Fredriksson, R., Lagerstrom, M. C., and Schioth, H. B. (2005) Ann. N. Y. Acad. Sci. 1040, 89-94
46. Bockaert, J. and Pin, J. P. (1999) EMBO J. 18, 1723-1729
47. Luttrell, L. M. (2006) Methods Mol. Biol. 332, 3-49
48. Tyndall, J. D. and Sandilya, R. (2005) Med. Chem. 1, 405-421
49. Jacoby, E., Bouhelal, R., Gerspacher, M., and Seuwen, K. (2006) ChemMedChem. 1, 761-782
50. Oldham, W. M. and Hamm, H. E. (2008) Nat. Rev. Mol. Cell Biol. 9, 60-71
51. Gales, C., Van Durm, J. J., Schaak, S., Pontier, S., Percherancier, Y., Audet, M., Paris, H., and Bouvier, M. (2006) Nat. Struct. Mol. Biol. 13, 778-786
52. Lefkowitz, R. J. (2004) Trends Pharmacol. Sci. 25, 413-422
53. Spät, A. and Hunyady, L. (2004) Physiol. Rev. 84, 489-539
54. Mechoulam, R. and Deutsch, D. G. (2005) Proc. Natl. Acad. Sci. U. S. A 102, 17541-17542
55. Bisogno, T., Sepe, N., Melck, D., Maurelli, S., De Petrocellis, L., and Di, M., V (1997) Biochem. J. 322 (Pt 2), 671-677
Footnotes

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Figure Legends

Figure 1: Effects of AT1R and CB1R ligands on \( G_\alpha \) protein subunit association measured by BRET. CHO cells expressing AT1R were mixed with equal quantities of cells expressing CB1R and \( G_\alpha \) protein subunits and CB1R activity was measured by BRET. A. BRET changes are shown in CHO cells treated with vehicle (veh) – vehicle (empty squares), Ang II – WIN (100 nM, filled circles) and AM251 –Ang II (10 μM, empty circles) (n=5). B and C. BRET values are shown in higher time resolution in vehicle- (B) and AM251-pretreated cells (C) (n=3). D and E. BRET change is shown in vehicle - AM (open squares) and Ang II – WIN55 stimulated (filled circles) cells when cells expressing AT1R were mixed with cells expressing \( G_\alpha \) probe but no CB1R (D) and when cells expressing only CB1R and \( G_\alpha \) probe were stimulated (E). F. Constant numbers of cells expressing CB1R were mixed with increasing number of cells expressing AT1R and change in CB1R activity was expressed as compared to vehicle treated cells. Relative activity was calculated from BRET ratio values compared to those following WIN55 (1 μM) treatment (n=3). Arrows show the time points for the indicated single (B, C) or sequential (A, D, E) treatments.

Figure 2: DAG lipase inhibitor (THL) prevents paracrine transactivation of CB1R by AT1R.

A. Cells expressing AT1R were pretreated with 1 μM THL for 15 minutes before the start of the experiment, mixed with CB1R expressing cells and were immediately moved to plates to start the experiment. Cells were treated sequentially with vehicle and AM251 (10 μM, filled squares), or with Ang II (100 nM) and WIN55 (1 μM, open triangles) (n=3). The time of treatments are indicated by arrows. B. Quantification of data on panel A on Fig. 1 and panel A on Fig. 2: mean values of five time points after first stimulation compared to average levels of 5 measurements before stimulation (100 %) (n=3; *, p<0.05).

Figure 3: Paracrine transactivation of CB1R by AT1R activation in HEK293 and COS7 cells. HEK293 (A) and COS7 (B) cells expressing AT1R were mixed with CHO cells expressing CB1R and \( G_\alpha \) protein subunits and CB1R activity was measured by BRET. Cells were stimulated with Ang II (100 nM), and BRET signal decreased (filled circles) compared to control cells (empty squares). AM251 treatment (10 μM) increased the BRET signal and prevented the Ang II induced decrease (empty circles). C. Quantification of data on panels A, B: mean values of five time points after first stimulation compared to average levels of 5 measurements before stimulation (100 %) (n=3; *, p<0.01). The arrows show the time of the indicated treatments.

Figure 4: Confocal analysis of \( \beta\)-arr2-GFP coupling to CB1R in CHO cells. A. CB1R-RFP and \( \beta\)-arr2-GFP localization in control (upper panels) and WIN55 stimulated cells (lower panels).
B. β-arr2-GFP localization in control and stimulated cells expressing CB₁R(DRY/AAY)-RFP. Cells were treated with WIN55 (1 μM) and AM251 (10 μM). C. β-arr2-GFP localization in control (left), Ang II (middle) and WIN55 (right) stimulated cells coexpressing CB₁R(DRY/AAY)-RFP. 

Figure 5: Visualization of AT₁R-mediated paracrine transactivation of CB₁R using β-arr2-GFP. AT₁R-Cerulean expressing cells (see C, G and K panels) were mixed with those transfected with CB₁R(DRY/AAY)-RFP and β-arr2-GFP (see A, E, I, B, F and J panels), and were placed on coverslips. Cells were treated with 100 nM Ang II (E, F, G and H panels), followed by stimulation with WIN55,212-2 (I, J, K and L panels). The arrows in panel F point at β-arr2-GFP translocated to the membrane of cells expressing CB₁Rs. 

Figure 6: Effect of Ang II on 2-AG levels in CHO cells expressing AT₁Rs. CHO cells (4 million cells/10 cm tissue culture plates with 3.5 ml medium) expressing AT₁R were treated with 100 nM AngII for indicated times and lipids were extracted from cells and the medium. Tissue levels of 2-AG was quantified by liquid chromatography/in-line mass spectrometry and statistical analysis of the data with ANOVA was performed as described in Materials and Methods (n=3; *, p<0.05; **, p<0.01). 

Figure 7: Transactivation of CB₁R by coexpressed AT₁R is independent of cytoplasmic Ca²⁺ signal generation. CB₁R activity was measured by BRET between Go protein subunits in cells coexpressing CB₁R and AT₁R. Inserts: To study the cytoplasmic Ca²⁺ signal generation, the cells were loaded with FURA 2 for 45 minutes, and Ca²⁺ was measured in suspended cells by detecting the ratio of fluorescent emissions ratio at 510 nm during excitation at two wavelengths (380/340 nm). A. Cells were treated with vehicle (veh, empty squares) or Ang II (100 nM, filled circles) at the time indicated by the first arrow, and BRET signal was measured in cells expressing CB₁R. At the second arrow the Ang II-stimulated cells were also treated with WIN55 (1 μM). B. Cells expressing the same constructs were preloaded with 60 μM BAPTA-AM for 45 min on room temperature and Go activation was measured. C. The effect of ionomycin (1 μM) on Go protein activation in cells expressing the Go protein sensors. D. Quantification of data in panels A, B and C: mean values of five time points after stimulation with vehicle (white bars), Ang II or ionomycin (black bars) (n=3; *, p<0.01). 

Figure 8: Paracrine transactivation of CB₁R by different GPCRs. M₁-, M₂-, M₃-, M₄-, and M₅, AchR, α₁AR, B₂, V₁ receptor expressing cells were mixed with cells expressing CB₁R and Go protein subunits as described in Materials and Methods and CB₁R activity was measured by BRET after simulation with the corresponding agonists (carbachol 10 μM, phenylephrine 100 μM, bradykinin 100 nM, AVP 100 nM). Mean values of five time points after stimulation with vehicle or agonists (black bars) are presented (n=3, * p<0.01, ). P values: 0.002 (M₁R), 0.604 (M₂R), below 0.001 (M₃R), 0.489 (M₄R), below 0.001 (M₅R), below 0.001 (α₁-AR), below 0.001 (B₂R), below 0.001 (V₁R). 

Supplementary Figure 1: Paracrine transactivation of CB₁R by AT₁R expressed in HEK293 and COS7 cells at different cell ratios. HEK293 (A and B) and COS7 (C and D) cells expressing AT₁R were mixed with CHO cells expressing CB₁R and Go protein subunits and CB₁R activity was measured by BRET. AT₁R and CB₁R expressing cells were mixed in 1:1 (A and B) or
5:1 (C and D) ratios. Averages of three time points before and after Ang II stimulation are presented compared to vehicle stimulated samples (n=3-4; *, p<0.01).

Supplementary Figure 2: Effect of Ang II on AEA levels in CHO cells expressing AT1Rs. CHO cells (4 million cells/10 cm tissue culture plates with 3.5 ml medium) expressing AT1R were treated with 100 nM AngII for indicated times and lipids were extracted from cells and the medium. Tissue levels of AEA were quantified by liquid chromatography/in-line mass spectrometry spectrometry. Ang II did not affect significantly the AEA levels of the cells. Statistical analysis of the data with ANOVA was performed as described in Materials and Methods (n=3).

Supplementary Figure 3: Both CB1R and Gq-activating receptors are required for paracrine transactivation. Gq protein activity was measured by BRET. Data quantification was done by calculating mean values of five time points after first stimulation compared to average levels of 5 measurements before stimulation (100 %) A. CHO cells expressing CB1R and Gq protein subunits were stimulated by different ligands (carbachol 10 μM, phenylephrine 100 μM, bradykinin 100 nM, AVP 100 nM) B. and C. Cells expressing Gq-activating receptors were mixed with cells expressing Gq probe but no CB1R and BRET change was measured after stimulation with appropriate ligands.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

| Control          | β-arr2-GFP | AT_1R | Merged          |
|-----------------|-----------|-------|----------------|
| CB₁R(DRY/AAY)   |           |       |                |
| Ang II 2 min.   |           |       |                |
| WIN55 2 min.    |           |       |                |

A B C D  
E F G H  
I J K L
Figure 6.
Figure 8.
