THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 25, pp. 16914–16921, June 19, 2009
Printed in the U.S.A.

Paracrine Transactivation of the CB_1 Cannabinoid Receptor by AT_1 Angiotensin and Other $G_{q/11}$ Protein-coupled Receptors*[S]

Received for publication, November 3, 2008, and in revised form, April 3, 2009 Published, JBC Papers in Press, April 7, 2009, DOI 10.1074/jbc.M109.003681

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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 AT_1 receptor (AT_1R) stimulation leads to diacylglycerol lipase-mediated transactivation of co-expressed CB_1Rs in Chinese hamster ovary cells. In the present study we detected a paracrine effect of the endocannabinoid release from Chinese hamster ovary, COS7, and HEK293 cells during the stimulation of AT_1 angiotensin receptors by determining CB_1 cannabinoid receptor activity with bioluminescence resonance energy transfer-based sensors of G protein activation expressed in separate cells. The angiotensin II-induced, paracrine activation of CB_1 receptors was visualized by detecting translocation of green fluorescent protein-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 CB_1 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)[2] 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 phosphatidylinositol (4,5)-bisphosphate, 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 γ-aminobutyric acid 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, CB_1 and CB_2 receptors (CB_1R and CB_2R, 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), 2-arachidonoylglycerol (2-AG), and 2-arachidonoylglyceryl ether (noladin ether) (7, 12–16). In adult and fetal neural tissues, the two major endocannabinoids, anandamide and 2-AG, are produced on demand, usually after depolarization of postsynaptic cells or following stimulation of G_q-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 CB_1R-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 CB_1R (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,

* This work was supported by grants from the Hungarian Scientific Research Fund (OTKA Grants NK-072661, NF-68563, M-045341 and T-034606), the Ányos Jedlik program by the Agency for Research Fund Management and Research Exploitation, Hungary (Grants NKFP1-010/2005), the Hungarian Ministry of Public Health (Grants ETT 447/2006 and 460/2006) and the EC (Grant LSHM-CT-2004-503474).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; 2-AG, 2-arachidononoylglycerol; DAG, diacylglycerol; DAGL, diacylglycerol-lipase; Ang II, angiotensin II; AT_1R, AT_1 receptor; CHO, Chinese hamster ovary; GFP, green fluorescent protein; EGFP, enhanced GFP; RFP, ref Fluorescent protein; EYFP, enhanced yellow fluorescent protein; BRET, bioluminescence resonance energy transfer; AR, adrenergic receptor; V_1, vasopressin receptor; B_2, bradykinin receptor.

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 a 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_1R) leads to DAGL-dependent activation of CB_1Rs 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_1Rs 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_1R or other Ca^{2+}-mobilizing GPCRs. We have further shown that activation of AT_1R 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_1R in neighboring cells.

EXPERIMENTAL PROCEDURES

Materials—Rat α_o-CFP G protein subunit was kindly provided by Dr. N. Gautam (29). Human V_1 vasopressin, α_1 adrenergic, B_2 bradykinin and M_1 muscarinic acetylcholine receptor, β_1 and γ_{11} G protein subunits were obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). The cDNA of the rat vascular CB_1R was provided by Dr. Zsolt Lenkei (CNRS, Paris) (30). β-Arrestin2-EGFP (β-arr2-GFP) was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC) (31).

Plasmid Constructs and Transfection—RFP-tagged CB_1R was constructed by subcloning the CB_1R cDNA into an mRFP containing vector (provided by Dr. R. Tsien, University of California, San Diego, CA). EYFP-β_1 was generated by subcloning human β_1 subunit into the mammalian expression vector pEYFP-C1 (Clontech). α_o-Rluc was constructed by replacing the CFP coding region in α_o-CFP with *Renilla* luciferase. Rat HA-AT_1R receptor and AT_1-EYFP were constructed as described earlier (32). AT_1R-Cerulean was constructed by replacing the cDNA of EYFP with Cerulean coding region (provided by Dr. R. Tsien). CHO cells were transfected with Lipofectamine 2000 according to manufacturer's suggestions using 2 or 16–24 μg of DNA and 2 or 16 μl of Lipofectamine 2000 in 6-well plates or 100-mm tissue culture plates, respectively. CHO cells were maintained in Ham's F-12 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. COS-7 kidney fibroblast cells and HEK293 human embryonic fibroblast cells were grown in complete Dulbecco's modified Eagle's medium containing glucose, glutamine, sodium bicarbonate, and supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin.

Site-directed Mutagenesis—Mutations in the rat CB_1R (D214A and R215A) were performed with the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's suggestions and verified using automated sequencing.

Confocal Laser-scanning Microscopy—CHO cells were grown on glass coverslips and transfected with labeled CB_1R and AT_1R and GFP-labeled β-arrestin2, as described above, 48 h prior to measurement. In transactivation experiments, the cells were detached with Versene 1 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; their emitted fluorescence was detected in multitrack mode with 480–520 nm, 500–530 nm band pass and 560 nm long pass filters, respectively. Because 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 α_o G protein subunit fused with *Renilla* luciferase (α_o-Rluc) and β_1 subunit labeled with enhanced yellow fluorescent protein (EYFP-β_1). Medium was changed to fetal bovine serum-supplemented Ham's F-12 6 h 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 $CaCl_2$, 0.7 $MgSO_4$, 10 glucose, 10 sodium Hepes, pH 7.4 (containing 1 g/liter 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 with the mean of the four control BRET ratio points before the first stimulation (BRET ratio, percent of control).

Measurement of 2-AG and Anandamide Levels—CHO cells transfected with AT_1R were maintained in Ham's F-12 medium in 10-cm tissue culture plates as described above. Aliquots of 4×10^6 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 plus medium were extracted in 2 volumes of ice-cold chloroform:methanol (2:1, v/v) containing 7 ng of $^4H_2$-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 of chloroform, deproteinated with 2 ml of 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).

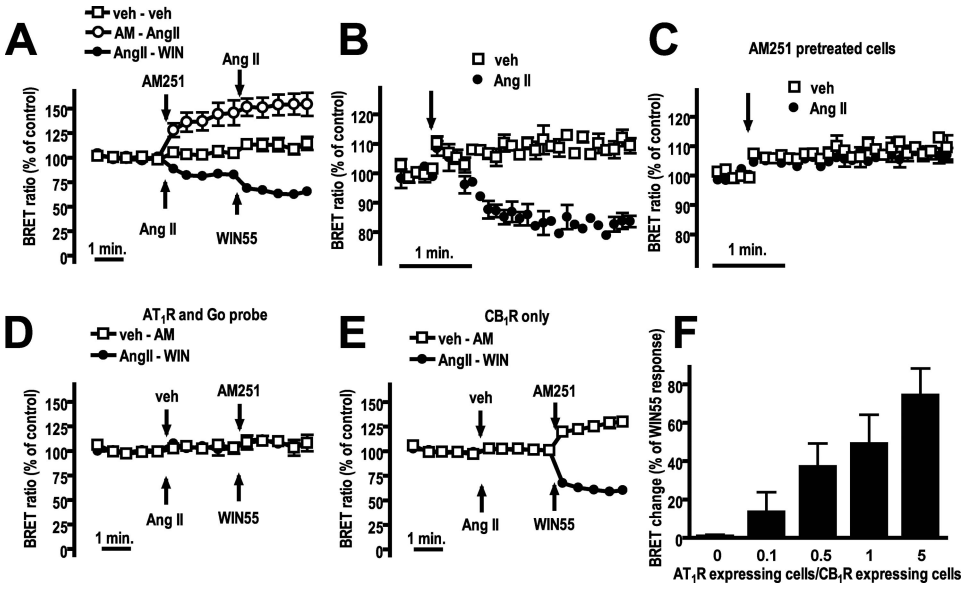

FIGURE 1. **Effects of AT_1R and CB_1R ligands on G_o protein subunit association measured by BRET.** CHO cells expressing AT_1R were mixed with equal quantities of cells expressing CB_1R and G_o protein subunits and CB_1R activity was measured by BRET. *A*, BRET changes are shown in CHO cells treated 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 (*C*) cells ($n = 3$). *D* and *E*, BRET change is shown in vehicle-AM (*open squares*) and Ang II-WIN55-stimulated (*filled circles*) cells when cells expressing AT_1R were mixed with cells expressing G_o probe but no CB_1R (*D*) and when cells expressing only CB_1R and G_o probe were stimulated (*E*). *F*, constant numbers of cells expressing CB_1R were mixed with increasing number of cells expressing AT_1R and change in CB_1R activity was expressed as compared with vehicle treated cells. Relative activity was calculated from BRET ratio values compared with those following WIN55 (1 μM) treatment ($n = 3$). *Arrows* show the time points for the indicated single (*B* and *C*) or sequential (*A*, *D*, and *E*) treatments.

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 $p < 0.05$ was considered significant.

RESULTS

Stimulation of AT_1Rs Expressed in CHO, HEK, and COS Cells Leads to Paracrine Transactivation of CB_1Rs—We have previously shown that CB_1R can be transactivated with Ang II in cells co-expressing CB_1R and AT_1R (28). We therefore postulated that, if the transactivation of CB_1R was caused by a released endocannabinoid, it would also occur if AT_1R and CB_1R were expressed in separate, adjacent cells. To examine this possibility, we monitored the activation of CB_1Rs expressed in CHO cells to detect Ang II-induced endocannabinoid release by other cells mixed to the cells expressing CB_1R. CB_1R activity was quantified by detecting bioluminescence resonance energy transfer (BRET) between G_o protein subunits (*Renilla* luciferase-tagged α_o and EYFP-tagged β_1, α_o-Rluc, and EYFP-β_1, respectively) as described previously (28). In this experimental setting, activation of CB_1R leads to a decrease of the BRET signal resulting from the dissociation of activated G_o 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 AT_1R, or were co-transfected with plasmids of CB_1R and tagged G protein subunits. Twenty-four hours later, the cells were mixed and CB_1R 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 CB_1R (Fig. 1*A*, *filled circles*). Administration of the CB_1R 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. 1*A*, *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. 1, *B* 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 ~15–20 s the BRET signal decreased rapidly (Fig. 1*B*), 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. 1*C*).

When AT_1R-transfected cells were mixed with cells expressing the G_o sensor, but no CB_1R, G_o protein activation did not occur, which provides additional evidence that it was mediated through CB_1R (Fig. 1*D*). Similarly, when cells expressing CB_1R, but not AT_1R, were stimulated with Ang II, the BRET signal remained unaffected (Fig. 1*E*). In other experiments, the original 1:1 ratio of AT_1R or CB_1R-expressing cells was increased to 5:1 or decreased to 0.1:1, keeping the number of CB_1R-expressing cells constant at 100,000 cells/well, which resulted in a corresponding increase or decrease, respectively, in G_o protein activation, approaching a plateau between 1:1 and 5:1 cell ratios (Fig. 1*F*). These findings suggest that G_o protein activation in CB_1R-expressing cells was mediated by Ang II-induced endocannabinoid release from CHO cells expressing AT_1R. Transactivation of CB_1R was also inhibited by pretreatment of the cells expressing AT_1R with the DAGL inhibitor tetrahydrolipstatin (1 μM) (Fig. 2*A*, *open triangles*), suggesting that DAGL plays a role in the process. Statistical analysis of the data is shown in Fig. 2*B*.

Transactivation of CHO cells expressing CB_1Rs was also detected when these cells were mixed with equal number of HEK293 or COS7 cells expressing AT_1Rs, and stimulated with Ang II. These data demonstrate that the AT_1R-mediated paracrine transactivation of CB_1Rs is not restricted to CHO cells

and may be a common mechanism in different cell types (Fig. 3). Slightly increased transactivation of CB_1Rs was observed, when these AT_1R-expressing cells were added in a 5-fold excess, suggesting that the response was almost maximal under these conditions (supplemental Fig. S1).

AT_1R Activation Leads to β-Arrestin Translocation to CB_1R in Adjacent Cells—To provide additional evidence that Ang II-induced activation of AT_1R leads to the formation and release of endocannabinoids, β-arrestin translocation to transactivated CB_1Rs 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 CB_1R (CB_1R-RFP) was co-expressed with GFP-tagged β-arrestin2 (β-arr2GFP), CB_1R-RFP localized both to the cell membrane and to intracellular vesicles (Fig. 4*A*), 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 CB_1Rs at the cell surface, and was detected in punctate structures at the plasma membrane (Fig. 4*A*). 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 CB_1R (D214A and R215A) (CB_1R[DRY/AAY]). To study the interaction of the mutant CB_1R with β-arrestin, RFP-tagged CB_1R(DRY/AAY) (CB_1R(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 CB_1R caused its appearance in punctate structures at the plasma membrane (Fig. 4*B*, *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. 4*B*, *middle*). These data demonstrate that co-expression of CB_1R(DRY/AAY)-RFP with β-arr2-GFP is a sensitive tool for visualization of both basal and agonist-induced CB_1R activity.

To test if endocannabinoid release could be detected by β-arr2-GFP translocation to CB_1R, CB_1R(DRY/AAY)-RFP and β-arr2-GFP were co-expressed in CHO cells, and a separate pool of cells were transfected independently with AT_1R-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 CB_1R(DRY/AAY)-RFP and β-arr2-GFP or AT_1R-Cerulean, translocation of β-arr2-GFP to the plasma membrane was observed in cells expressing CB_1R(DRY/AAY)-RFP. This response was observed in 67 ± 7% of cells (n = 3, 86 cells total) with neighboring AT_1R-Cerulean-expressing cells; whereas in parallel experiments, when cells expressing CB_1R(DRY/AAY)-RFP and β-arr2-GFP were stimulated with Ang II in the absence of AT_1R-expressing cells, no translocation of β-arr2-GFP was detected (Fig. 4*C*). These data provide additional evidence that a paracrine endocannabinoid mediator is released as a result of AT_1R activation.

2-AG Formation Is Enhanced by Stimulation of AT_1Rs—To verify whether the Ang II-induced decrease in BRET signal detected by confocal microscopy was, in fact, due to endocannabinoid release, we measured 2-AG and anandamide levels in control and Ang II-stimulated AT_1R-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 CB_1R transactivation measured with BRET (Fig. 1*A*). Anandamide levels were very low and did not show statistically significant changes in response to Ang II stimulation (supplemental Fig. S2.). These data are consistent with our hypothesis that $G_{q/11}$-mediated DAG formation serves as a source of endocannabinoid release and suggest that the endocannabinoid involved in Ang II-induced paracrine CB_1R transactivation is 2-AG.

CB_1R Transactivation by AT_1R Receptor Stimulation Is Not De-

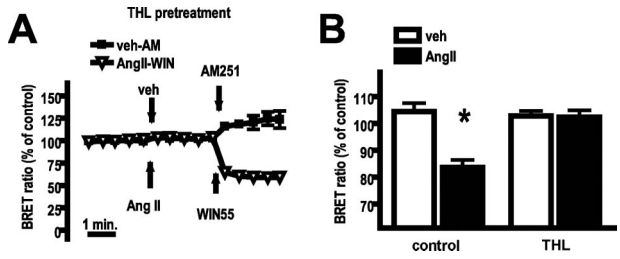

FIGURE 2. **DAG lipase inhibitor (tetrahydrolipstatin) prevents paracrine transactivation of CB_1R by AT_1R.** *A*, cells expressing AT_1R were pretreated with 1 μM tetrahydrolipstatin for 15 min before the start of the experiment, mixed with CB_1R-expressing cells and were immediately moved to plates 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 with average levels of five measurements before stimulation (100%) (n = 3; *, $p < 0.05$).

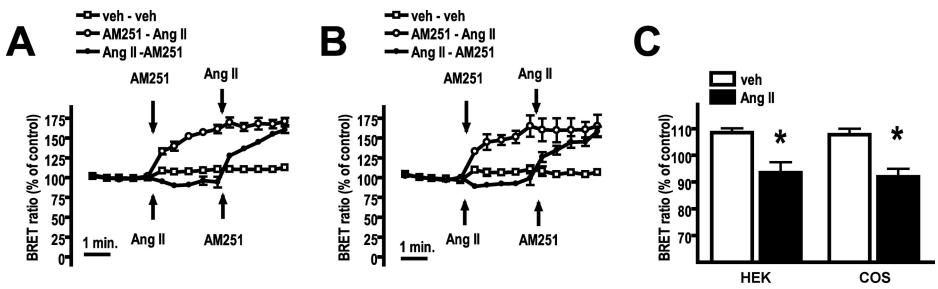

FIGURE 3. **Paracrine transactivation of CB_1R by AT_1R activation in HEK293 and COS7 cells.** HEK293 (*A*) and COS7 (*B*) cells expressing AT_1R were mixed with CHO cells expressing CB_1R and G_o protein subunits and CB_1R activity was measured by BRET. Cells were stimulated with Ang II (100 nM), and BRET signal decreased (*filled circles*) compared with 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* and *B*: mean values of five time points after first stimulation compared with average levels of five measurements before stimulation (100%) (n = 3; *, $p < 0.01$). The *arrows* show the time of the indicated treatments.

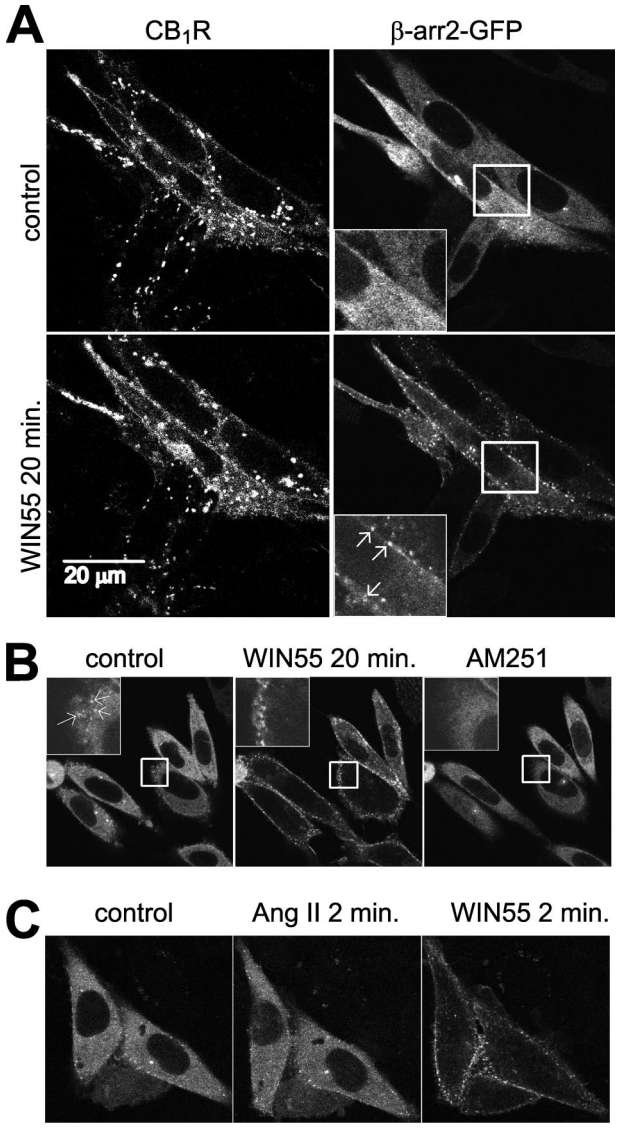

FIGURE 4. **Confocal analysis of β-arr2-GFP coupling to CB_1R in CHO cells.** *A*, CB_1R-RFP and β-arr2-GFP localization in control (*upper panels*) and WIN55 stimulated cells (*lower panels*). *B*, β-arr2-GFP localization in control and stimulated cells expressing CB_1R(DRY/AAY)-RFP. Cells were treated 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 CB_1R(DRY/AAY)-RFP.

pendent on Intracellular Ca^{2+} Signal Generation—2-AG production in neural cells can be stimulated either by the Ca^{2+} signal caused by activation of ionotropic receptors, by stimulation of G_q 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 Ca^{2+} signal with ionomycin (27, 44). Therefore we asked if transactivation of CB_1R by AT_1R is dependent on Ca^{2+}. In control cells, G protein activation occurred after Ang II stimulation and a Ca^{2+} signal was detected (Fig. 7, *A* and *D*). In cells preincubated with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; 60 μM) and preloaded with FURA-2, Ang II-induced Ca^{2+} elevation was not detected even though transactivation of CB_1R still occurred (Fig. 7, *B* and *D*). Moreover, administration of ionomycin (1 μM) was not able to fully mimic the effect of AT_1R stimulation, although a small, reproducible, but in this set of experiments statistically not significant G_o protein activation was detectable (Fig. 7, *C* and *D*). The averaged BRET data are presented in Fig. 7*D*. Because, under these conditions, ionomycin causes larger elevations of cytoplasmic [Ca^{2+}] than those caused by Ang II (Fig. 7, *A* and *C*, *inset*), these results suggest that transactivation was not primarily mediated by intracellular Ca^{2+} elevation.

$G_{q/11}$-coupled GPCRs Cause Paracrine Transactivation of CB_1R—It has been demonstrated previously that activation of G_q-coupled muscarinic or metabotropic glutamate receptors induces endocannabinoid release in neurons (17–19). To test if endocannabinoid release common occurs after stimulation of G_q-coupled receptors, we expressed M_1, M_3, and M_5 cholinergic (M_1R, M_3R, and M_5R, respectively), V_1 vasopressin (V_1R), α_{1A} adrenergic ($\alpha_{1A}AR$), and B_2 bradykinin (B_2R) receptors in CHO cells, and tested if paracrine transactivation of CB_1R occurs. We also tested the effect of stimulation of $G_{i/o}$-coupled M_2 and M_4 muscarinic cholinergic receptors (M_2R and M_4R, respectively). $G_{q/11}$- or $G_{i/o}$-activating receptors were expressed in one set of cells, and CB_1R and G_o 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, CB_1R was transactivated when $G_{q/11}$ protein-activating M_1R, M_3R, M_5R, $\alpha_{1A}AR$, 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. 2*B*). Transactivation did not occur in the absence of either CB_1R or the $G_{q/11}$-activating receptor (supplemental Fig. S3). These findings demonstrate that paracrine CB_1R 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 the case of $G_{q/11}$-coupled receptors, such as the AT_1R, early events include cleavage of phosphatidylinositol 1,4,5-bisphosphate by phospholipase Cβ and formation of second messengers such as inositol 1,4,5-trisphosphate and DAG (2, 3). Inositol 1,4,5-trisphosphate initiates Ca^{2+} release from intracellular stores, whereas DAG activates protein kinase C (52). Because DAG is a common signaling molecule in different cell types and DAGL is found in almost every tissue (27), we hypothesized that 2-AG

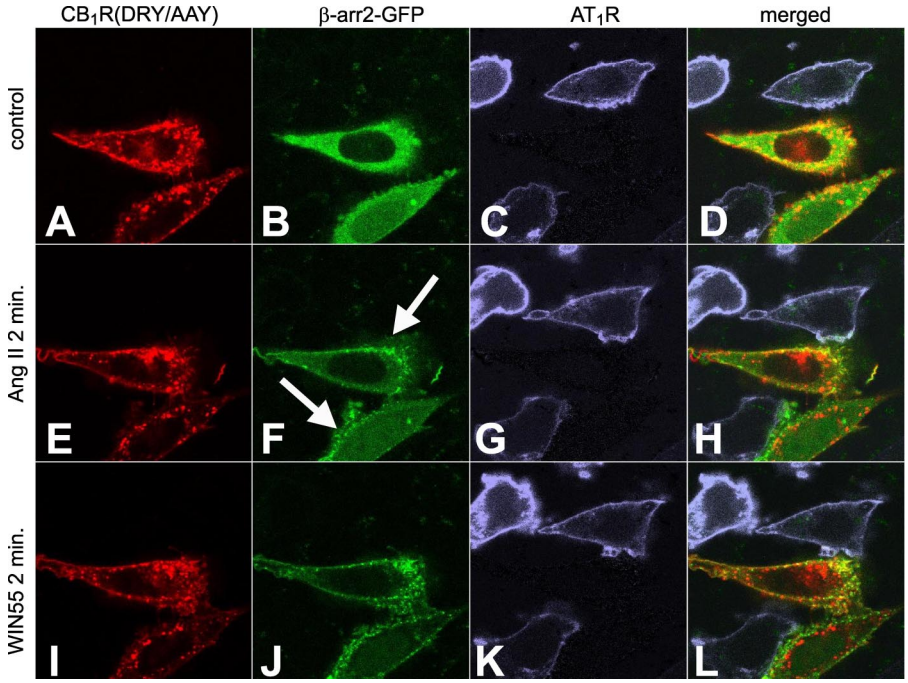

FIGURE 5. **Visualization of AT_1R-mediated paracrine transactivation of CB_1R using β-arr2-GFP.** AT_1R-Cerulean-expressing cells (see *C*, *G*, and *K*) were mixed with those transfected with CB_1R(DRY/AAY)-RFP and β-arr2-GFP (see *A*, *E*, *I*, *B*, *F*, and *J*), and were placed on coverslips. Cells were treated with 100 nM Ang II (*E*, *F*, *G*, and *H*), followed by stimulation with WIN55,212-2 (*I*, *J*, *K*, and *L*). The *arrows* in *panel F* point at β-arr2-GFP translocated to the membrane of cells expressing CB_1Rs. Merged images are presented in *panels D*, *H*, and *L*.

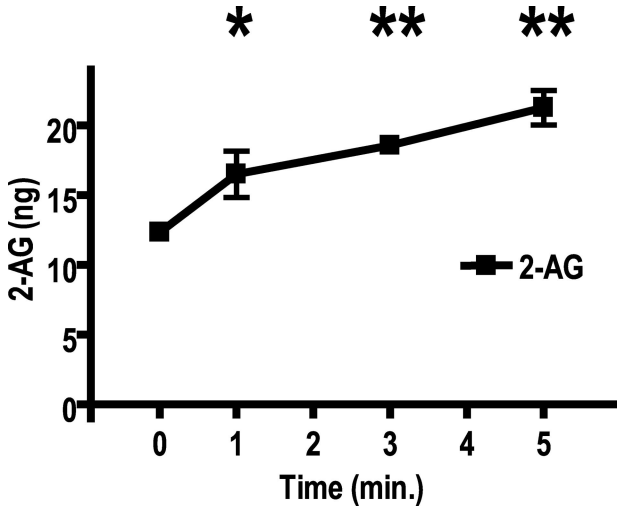

FIGURE 6. **Effect of Ang II on 2-AG levels in CHO cells expressing AT_1Rs.** CHO cells (4 million cells/10-cm tissue culture plates with 3.5 ml of medium) expressing AT_1R were treated with 100 nM AngII for indicated times, and lipids were extracted from cells and the medium. Tissue levels of 2-AG was quantified by liquid chromatography/in-line mass spectrometry and statistical analysis of the data with analysis of variance was performed as described under "Experimental Procedures" ($n = 3$; *, $p < 0.05$; **, $p < 0.01$).

formation can be a common early signaling event in most cells. 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 CB_1R 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 CB_1Rs. Our data also indicate that activation of $G_{q/11}$-coupled receptors can cause paracrine activation of adjacent cells, because in our experiments the stimulated $G_{q/11}$-coupled receptors and the CB_1R 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 CB_1R (28). These data support our hypothesis that the Ang II-induced DAGL-dependent transactivation of CB_1Rs 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 (53). However, we could not detect transactivation when the supernatant from the stimulated cell population was transferred to cells expressing CB_1Rs.[3] It is possible that endocannabinoids, being lipophilic molecules, remain membrane-associated and require direct contact with adjacent CB_1R-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_1ARs, V_1Rs, and B_2Rs. Our data suggest that 2-AG release is a common consequence of the activation of various $G_{q/11}$-coupled receptors. Because 2-AG can be released by cells (54), 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 (inositol 1,4,5-trisphosphate 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_1R can also be triggered indirectly via $G_{q/11}$-coupled receptors, which means that the primary

[3] G. Turu and L. Hunyady, unpublished observation.

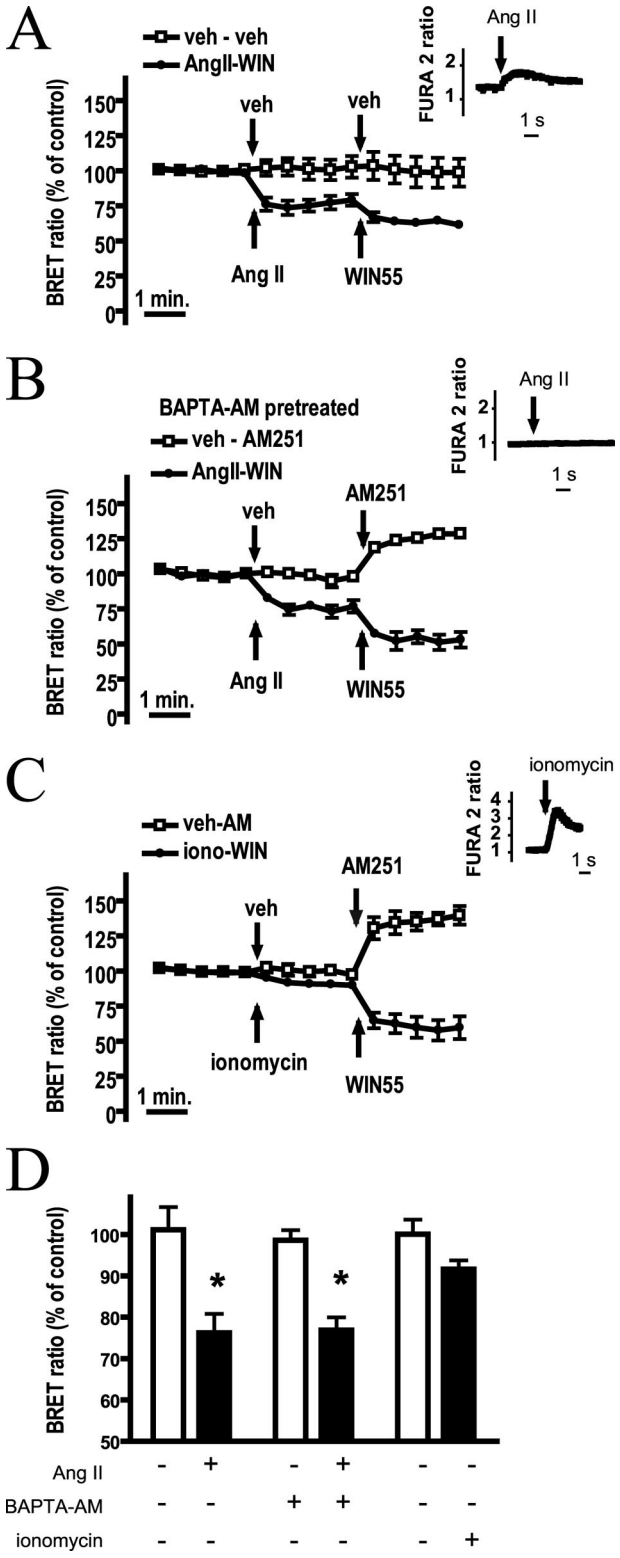

FIGURE 7. **Transactivation of CB_1R by coexpressed AT_1R is independent of cytoplasmic Ca^{2+} signal generation.** CB_1R activity was measured by BRET between Go protein subunits in cells coexpressing CB_1R and AT_1R. *Insets*: to study the cytoplasmic Ca^{2+} signal generation, the cells were loaded with FURA 2 for 45 min, and Ca^{2+} 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 CB_1R. 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 G_o activation was measured. *C*, the effect of ionomycin (1 μM) on G_o protein activation in cells expressing the G_o protein sensors. *D*, quantification of data in *A–C*: mean values of five time points after stimulation with vehicle (*white bars*), Ang II or ionomycin (*black bars*) ($n = 3$; *, $p < 0.01$).

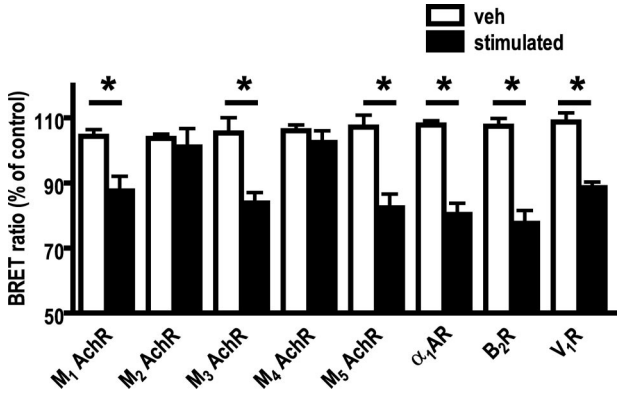

FIGURE 8. **Paracrine transactivation of CB_1R by different GPCRs.** M_1-, M_2-, M_3-, M_4-, and M_5, AchR, α_1AR, B_2, V_1 receptor-expressing cells were mixed with cells expressing CB_1R and G_o protein subunits as described under "Experimental Procedures," and CB_1R activity was measured by BRET after simulation with the corresponding agonists (carbachol (10 μM), phenylephrine (100 μM), bradykinin (100 nM), and 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 (M_1R), 0.604 (M_2R), below 0.001 (M_3R), 0.489 (M_4R), below 0.001 (M_5R), below 0.001 (α_1-AR), below 0.001 (B_2R), below 0.001 (V_1R).

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 Gq/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_1R 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 addition, 2-AG released as a paracrine mediator may activate CB_2R, because it is a full agonist at both CB_1R and CB_2R (19) and may also activate additional receptors (12). We have also tested whether G_q protein activation leads to endocannabinoid release in different types of cell lines. CHO cells are ovarian, whereas HEK293 cells are from the kidney and COS cells are fibroblasts. We detected CB_1R activation after stimulation of G_q protein-activating AT_1R expressed in all three cell lines tested and also following stimulation of other G_q protein-activating receptors transfected into these cells. This strongly suggests that endocannabinoid release is not dependent of cell type or specific $G_{q/11}$-coupled receptors and may be a general property of all tissues upon G_q protein activation. Endocannabinoid release triggered by activation of $G_{q/11}$-coupled receptors could be inhibited by a DAGL inhibitor, suggesting that the underlying mechanism was an increase in the DAGL-catalyzed hydrolysis of DAG to yield 2-AG. However, we cannot exclude the alternative possibility that the increased cellular levels of 2-AG result, at least in part, from inhibition of its degradation via monoacylglycerol lipase. Thus, the magnitude of the basal

"endocannabinoid tone" and endocannabinoid release may depend on relative activity of these enzymes.

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 inositol 1,4,5-trisphosphate- 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. Because 2-AG 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.

Acknowledgment—The excellent technical assistance of Judit Bakacsiné Rácz is greatly appreciated.

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