Regulation of CB_1R by AT_1-angiotensin and other G_q-coupled receptors

Ph.D. thesis

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The endocannabinoid system

Cannabinoid receptors, endocannabinoids and enzymes involved in their metabolism are parts of the endocannabinoid system. The system is involved in many physiological and pathophysiological regulations. Although its first role has been recognized in the central nervous system, its components are present in other tissues as well. Drugs targeting the cannabinoid system are under development; its components are potential pharmacological targets. Understanding the endocannabinoid system may facilitate the development of more effective and more specific drugs.

Marihuana has been used for thousands of years as a pleasure-inducing drug and as a traditional medicine. Archeological and historical findings indicate that different compounds of the plant were used for medical and ritual purposes since ancient times. The psychoactive effects of marihuana are caused by tetrahydrocannabinol (THC). The effects of THC in humans include euphoria, relaxation, tachycardia and hypothermia. To date, two cannabinoid receptors have been identified by molecular cloning, CB_1 and CB_2 receptors (CB_1R and CB_2R, respectively), although additional GPCRs have also been proposed to function as cannabinoid receptors. CB_1R and CB_2R belong to the family of G-protein-coupled receptors (GPCRs). Cannabinoid receptors recognize certain lipids present in animal tissues termed endocannabinoids, such as arachidonylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). The 2-AG levels in brain homogenates and in many peripheral tissues are near its K_d for the CB_1R. The main route for the production of 2-AG is the production by diacylglycerol-lipase (DAGL) after cleavage of the fatty-acid in the sn-1 position of diacylglycerol (DAG). A number of enzymes are

involved in the hydrolysis of 2-AG, such as monoacylglycerol-lipase, fatty acid amide hydrolase (FAAH) and cyclooxygenase 2.

In neurons, 2-AG is produced following depolarization or activation of certain $G_{q/11}$-coupled receptors. DAGL is localized to the postsynaptic membrane of the synapse, whereas CB_1R is typically found on the presynaptic side. Activation of CB_1R causes inhibition of the release of transmitters, such as GABA, glutamate, acetylcholine, norepinephrine, serotonin and CCK. The localization and function of the CB_1R explain the well known effects of cannabinoids in central nervous system, the depolarization-induced suppression of inhibition (DSI) or depolarization-induced suppression of excitation (DSE). The phenomenon is caused by retrograde cannabinoid signalling in the synapse.

Besides its role in the central nervous system, cannabinoid system has role in a number of peripheral tissues as well. Cannabinoid receptors influence the energy metabolism in several different ways. Cannabinoids regulate food intake in the hypothalamus, they affect the metabolism in the fat cells and in the liver, and the production of insulin in the pancreas. The system is involved in the metabolic syndrome. In addition, cannabinoid effects have been documented in several other tissues and organs as well, including the cardiovascular system, skeletal muscle, eye, gastrointestinal tract, skin, kidneys, lung, immune system and bone.

Angiotensin II and the renin-angiotensin system

The octapeptide angiotensin II is the main effector in the renin-angiotensin system, and acts mainly through the AT_1 angiotensin receptor (AT_1R). The hormone is synthesised by the cleavage of angiotensin I by angiotensin convertase enzyme. Angiotensin I is produced by enzymatic cleavage of angiotensinogen, the reaction is catalysed by renin. According to the classic conception, the renin

production in the kidneys is the regulated step, and this is responsible for the regulation of the whole renin-angiotensin system. Nowadays, it is known, that the system is much more complicated, and its role is not restricted to endocrine regulations. All parts of the system are present in different tissues, where they constitute local renin-angiotensin systems. The concentration of the locally produced angiotensin II may be much higher than its concentration in the plasma. angiotensin II has been also involved in several pathophysiological conditions, like high blood pressure and consequential blood vessel and heart muscle disorders, and in metabolic syndrome.

OBJECTIVES

In the central nervous system, 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. 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. Cannabinoid system is involved in several physiological and pathophysiological regulations that overlap with those in which the renin-angiotensin system is also present. Since AT_1R couples to $G_{q/11}$ proteins, question arise whether AT_1R stimulation leads to endocannabinoid production and cannabinoid receptor activation, analogously to the muscarinic acetylcholine and metabotropic glutamate receptors in neurons. In addition, we tested if stimulation of further $G_{q/11}$-coupled receptors leads to similar response in non-neuronal tissues.

1. Does stimulation of AT_1Rs lead to autocrine activation of CB_1Rs in expression system, in non-neural cells?
2. Does DAGL play a role in the transactivation of CB_1Rs after stimulation of AT_1Rs, and is there endocannabinoid production in these cells?
3. Does stimulation of other $G_{q/11}$-coupled receptors lead to endocannabinoid production in non-neuronal tissues?

METHODS

Cell culture and transfection

For experiments, we expressed proteins in different cell cultures. We used simian renal COS-7, human embryonic renal HEK-293, and chinese hamster ovary (CHO) cells. The cells were transfected using Lipofectamine 2000.

Bioluminescence resonance energy transfer (BRET) measurements

We followed the BRET between G-protein subunits and between β-arrestin2-Rluc and YFP-labeled receptors expressed in CHO cells. After the addition of coelenterazine h we measured the intensity of light emitted at 485 and 530 nm. The change in the molecular interaction is revealed by the change in the ratio of light emission at 530 and 485 nm.

Cytoplasmic [Ca^{2+}]-measurements

Cells were transfected with the appropriate proteins, and 24 hours later, the cells were detached, and loaded with Fura-2/AM. The Ca^{2+}-measurements were done in cell suspensions, using excitation at 340 and 380 nm, and emission detected at 505 nm.

Measurement of 2-AG and anandamide levels

2-AG and anandamide levels were determined in Dr. Kunos György's laboratory. Cells were transfected with AT_1R. Following stimulation, lipids were extracted and analysis of endocannabinoid content was done by liquid chromatography/in line mass spectrometry.

Confocal laser microscopy

Cells were grown on coverslips and transfected with β-arrestin2-eGFP and CB_1R. For transactivation experiments, the cells were detached, mixed with cells expressing AT_1R-YFP and placed back on the coverslips. Images were taken using a Zeiss LSM 510 confocal laser microscope, excited by argon and helium/neon lasers. Emission was detected using a 500–530 nm band pass filter for GFP and YFP, and a 560 nm long pass filter for RFP.

Statistical analysis

The differences between groups were analyzed using ANOVA.

Measurement of CB1R activity with BRET

CB_1R activity was measured by energy transfer between heterotrimeric G protein subunits. CB_1R was coexpressed with γ_{11}, α_o-Rluc and EYFP-β_1 subunits in CHO cells. Energy transfer was detected between α_o-Rluc and EYFP-β_1. Stimulation of cells with CB_1R agonist 2-AG caused a decrease of the BRET signal, indicating that subunits were dissociated during activation of the heterotrimeric G_o protein. Following inverse agonist (AM251) addition the BRET signal increased. When the inverse agonist was added first, it increased the BRET signal indicating that non-stimulated CB_1Rs exert basal activity in CHO cells. Stimulation with 2-AG after inverse agonist treatment caused no decrease in BRET signal, as expected.

Angiotensin II induced activation of CB_1Rs

Next, we asked whether CB1R activity can be enhanced by co-expression and stimulation of AT_1R, a G_q-activating GPCR. Endocannabinoid production in CHO cells has not been observed previously, although DAGL is present in almost all tissues. Co-expression of AT_1R with CB_1R, and stimulation with AngII caused readily detectable G_o activation. This activation was prevented by AM251, and was not detected when only one of the two receptors was expressed in cells. Transactivation was also prevented by inhibition of DAGL with tetrahydrolipstatin (THL), indicating the role of DAGL in the process.

Stimulation of AT_1Rs leads to paracrine transactivation of CB_1Rs

We 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 angiotensin II-induced endocannabinoid release by other cells mixed to the cells expressing CB_1R. In this experimental setting, stimulation of AT_1R leads to a decrease of the BRET signal, showing the activation of CB_1Rs. Transactivation also occurred when AT_1R was expressed in HEK-293 or COS-7 cells, and was inhibited by AM251 or THL pretreatment.

Detection of CB_1R transactivation by β-arrestin2 binding

To provide additional evidence for the paracrine transactivation of CB_1R by stimulated AT_1Rs, we detected the translocation of β-arrestin2 to the membrane in cells expressing CB_1Rs. Activation of GPCRs is usually followed by translocation of β-arrestins, allowing the visualization of receptor activity. In our experiments we used a mutant CB_1R (CB_1R-DRAA) for more sensitive detection of the translocation. In CB_1R expressing cells, β-arrestin2 translocated to the membrane when AT_1R was stimulated with angiotensin II in adjacent cells.

2-AG formation is enhanced by stimulation of AT_1Rs

To verify whether the angiotensin II-induced decrease in BRET signal was, in fact, due to endocannabinoid release, we measured 2-AG levels in control and angiotensin II-stimulated AT_1R expressing cells. Stimulation of cells with angiotensin II caused sustained elevation of 2-AG levels with similar kinetics to that of CB_1R transactivation measured with BRET. These data show that 2-AG is

produced in CHO cells after AT_1R stimulation, and suggest that the endocannabinoid involved in angiotensin II-induced paracrine CB_1R transactivation is 2-AG.

CONCLUSIONS

We have shown that CB_1R can be activated by AT_1R stimulation in CHO cells.

We confirmed that that AT_1R stimulation is followed by DAGL-dependent endocannabinoid release in CHO, COS-7 and HEK-293 cells, and it can activate CB_1Rs in neighbouring cells.

Transactivation also occurs when other $G_{q/11}$-coupled receptors, M_1R, M_3R, M_5R, V_1R, B_2R or α_1-ARs are stimulated.

We have found using BRET and confocal microscopy techniques, that CB_1R belongs to the class A group of GPCRs. β-arrestin2 coupling was enhanced, when the conserved DRY motif in CB_1R was changed to AAY. We have confirmed AT_1R-mediated paracrine transactivation of CB_1R using β-arrestin2 as a sensor of receptor activation.

We have shown, that AT_2R, unlike AT_1R, does not bind β-arrestin2 after simulation with angiotensin II.

The basal activity of CB_1R was inhibited by THL, suggesting the role of endocannabinoids in the tonic CB_1R activity.

LIST OF PUBLICATIONS

Related to the thesis:

Turu, G.; Simon, A.; Gyombolai, P,; Szidonya, L.; Bagdy, G.; Lenkei, Z.; Hunyady, L.:
The role of diacylgycerol lipase in constitutive and angiotensin AT1 receptorstimulated
cannabinoid CB1 receptor activity. J. Biol. Chem. 282:7753-7, (2007).
IF: 5,581

Turu, G.; Szidonya, L.; Gáborik, Z.; Buday, L.; Spät, A.; Clark, A.J.L.; Hunyady, L.:
Differential ß-arrestin binding of AT1 and AT2 angiotensin receptors. FEBS Letters
580:41-5, (2006).
IF: 3,372

Other publications:

Szaszák, M.; Gáborik, Z.; **Turu, G**.; McPherson, P.S.; Clark, A.J.L.; Catt, K.J.; Hunyady, L.: Role of the proline-rich domain of dynamin-2 and its interactions with SH3 domains during endocytosis of the AT1 angiotensin receptor. J. Biol. Chem.
277:21650-6, (2002).
IF: 6,696.

Hunyady, L., **Turu, G**.: The role of the AT1 angiotensin receptor in cardiac hypertrophy: angiotensin II receptor or stretch sensor? Trends Endocrinol. Metab.
15:405-8, (2004).
IF: 9,058.

Szidonya, L.; Süpeki, K.; Karip, E.; **Turu, G**.; Várnai, P.; Clark, A.J.L.; Hunyady, L.: AT1 receptor blocker-insensitive mutant AT1A angiotensin receptors reveal the presence of G protein-independent signaling in C9 cells. Biochem. Pharmacol. (2007).
IF: 4,006.

Karip, E., **Turu, G.**, Süpeki, K., Szidonya, L.; Hunyady, L.:
Cross-inhibition of angiotensin AT1 receptors support the concept of receptor oligomerization
Neurochemistry International (2007)
IF: 2,975