



Limonene, a natural cyclic terpene, is an agonistic ligand for adenosine A_{2A} receptors

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ARTICLE INFO

Article history:

Received 9 November 2010

Available online 4 December 2010

Keywords:

Limonene

Adenosine A_{2A} receptors

Radioligand binding assay

cAMP

ABSTRACT

Limonene is a major aromatic compound in essential oils extracted from citrus rind. The application of limonene, especially in aromatherapy, has expanded significantly, but its potential effects on cellular metabolism have been elusive. We found that limonene directly binds to the adenosine A_{2A} receptor, which may induce sedative effects. Results from an *in vitro* radioligand binding assay showed that limonene exhibits selective affinity to A_{2A} receptors. In addition, limonene increased cytosolic cAMP concentration and induced activation of protein kinase A and phosphorylation of cAMP-response element-binding protein in Chinese hamster ovary cells transfected with the human adenosine A_{2A} receptor gene. Limonene also increased cytosolic calcium concentration, which can be achieved by the activation of adenosine A_{2A} receptors. These findings suggest that limonene can act as a ligand and an agonist for adenosine A_{2A} receptors.

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1. Introduction

Adenosine is an endogenous nucleoside that modulates numerous physiological processes, including oxygen and metabolic balance in tissues [1], immune response [2], and signaling in the nervous system [3]. Four mammalian subtypes of adenosine receptors (ARs) have been identified and their genes cloned as follows: A₁, A_{2A}, A_{2B}, and A₃. AR subtypes are distributed in a tissue-specific manner [4]. For example, A_{2A} receptors are highly expressed in the brain, immune cells, heart, lung, and blood vessels.

ARs differentially modulate the intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP). Thus, A₁ and A₃ receptor activation inhibits adenylate cyclase to reduce cAMP concentration, whereas A_{2A} and A_{2B} receptor activation elevates cAMP levels [5]. Therefore, in the A_{2A} receptor subtype, ligand binding stimulates protein kinase A (PKA) and induces phosphorylation activation of cAMP response element-binding (CREB), which regulates the transcription of several target genes. In addition, ligand binding to the A_{2A} receptor induces the formation of inositol 1,4,5-triphosphate, which results in opening of endoplasmic reticulum calcium channels [6,7]. These changes are implicated in several clinical conditions, including cardiovascular disease [8], sleep disorders [9], and inflammation [10].

Limonene is a major aromatic compound in essential oils obtained from oranges, grapefruits, and lemons. These essential oils have long been popular in aromatherapeutic practice due to potent calming and sedative effects that alleviate nervous disorders, heart problems, colic, asthma, and depression. In addition, limonene has

anticancer activity [11], but its mechanism has not been elucidated. This study was designed to explore the effects of limonene on cellular metabolism through A_{2A} receptors. We first examined its affinity to the A_{2A} subtype. To further clarify limonene effects on A_{2A} receptor activation, we also investigated the resultant changes in each step of the A_{2A} receptor signaling pathway.

2. Materials and methods

2.1. Membrane preparation

Rat brain tissue membrane fractions were prepared as previously described [12]. Briefly, rat brain tissue was removed and the striata and cortices were dissected out at 4 °C in a 0.32 M sucrose solution containing 50 mM Tris–HCl and 2 mM EGTA (pH 7.6). The tissue was then homogenized in a Potter–Elvehjem homogenizer (Thermo Scientific, Rockford, IL, USA) at 4 °C and the resulting homogenates were centrifuged at 1000g for 10 min at 4 °C. The supernatants were re-centrifuged at 14,000g for 12 min at 4 °C; the pellets were resuspended in a buffer solution containing 50 mM Tris–HCl, 2 mM EGTA, and 1 mM EDTA (pH 7.4) and incubated for 30 min at 37 °C to remove endogenous adenosine. After centrifugation at 14,000 g, the pellets were resuspended in buffer including 50 mM Tris–HCl and 10 mM MgCl₂ (pH 7.4). Aliquots were then frozen at –80 °C until analysis.

2.2. Radioligand binding assays

The [³H]CGS21680 binding assay was performed in triplicate as previously described [13]. Briefly, aliquots of rat cerebral cortex membrane fraction (0.2 mg protein) were incubated in 200 μL of

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50 mM Tris–HCl buffer (pH 7.5) containing 10 mM MgCl_2 with [^3H]CGS21680 (5 nM) in the presence or absence of unlabeled 5'-N-ethylcarboxamidoadenosine (NECA; 10 μM) for determination of nonspecific binding. After 90 min at 25 °C, the binding reaction was stopped by filtration through GF/C fiber glass filters (Whatman, Maidstone, Kent, UK), and then the membrane was washed five times with 5 mL ice-cold buffer. Radioactivity of the punched-out wet filters was counted after adding 5 mL of Ultima Gold scintillation cocktail (Perkin–Elmer, Waltham, MA, USA).

2.3. Cell culture and transient expression of adenosine A_{2A} receptors in CHO cells

Cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a 10% CO_2 incubator at 95% humidity. The plasmid containing the human adenosine A_{2A} receptor (OriGene, Rockville, MD, USA) was transiently transfected into Chinese hamster ovary CHO cells with Hilymax transfection reagent (Dojindo, Seoul, Korea) according to the manufacturer's protocol. Transfections were performed in 100-mm tissue culture dishes and cells were treated with limonene for subsequent cAMP, PKA activity, and calcium assays.

2.4. Cyclic AMP measurement

Cells were washed with phosphate-buffered saline and treated with various concentrations of limonene. After incubation with limonene for 30 min, intracellular cAMP was quantified with a commercially available cAMP radioimmunoassay system (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacturer's recommendations.

2.5. PKA activity assay

PKA activity was determined using a commercially available kit (Enzo Life Sciences) according to the manufacturer's instructions.

2.6. Measurement of limonene-induced cytosolic calcium release

Cells were seeded at a density of 30,000 cells/well into black 96-well plates with clear bottoms to allow cell visualization and fluorescence measurements from the bottom of each well. The dye-loading buffer consisted of a final concentration of 4 μM 4-(6-acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthonyl)-4'-methyl-2,2'-(ethylenedioxy)dianiline- N,N,N',N' -tetraacetic acid tetrakis (acetoxymethyl) ester (fluo-3-acetomethyl ester; Sigma, St. Louis, MO, USA) in serum-free cell culture maintenance medium containing 20 mM HEPES and 2.5 mM probenecid. Assay conditions were as described previously [14]. Briefly, each 96-well plate containing dye-loaded cells was placed into a multilabel plate reader (VICTOR3; Perkin–Elmer) and laser intensity was adjusted to an optimum level to obtain basal values of approximately 10,000 fluorescence units. After determining the baseline fluorescence of the fluo-3- Ca^{2+} complex, limonene and NECA were added into the 96-well plate. Fluorescence readings were obtained every 10 s for 100 s. The maximum fluorescent signal was recorded and normalized to a positive control of 10 μM NECA. Each experiment was performed in quadruplicate on every plate. Each 96-well plate contained four wells dedicated to a positive control (10 μM NECA) and four wells to a negative control (assay buffer alone).

2.7. Immunoblotting analysis

Cells were lysed in RIPA buffer (10 mM Tris–HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, pH 7.5)

containing protease inhibitor cocktail at 4 °C. Samples were separated using 7.5% SDS–PAGE and blotted onto a nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4 °C. After washing several times with 0.5% TBS-E wash buffer, the membrane was incubated with the secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence Western blotting detection reagents (Amersham–Pharmacia Korea, Seoul, Korea) and quantified with ChemoDoc™ XRS system (Bio-Rad, USA) using the Quantity One software.

3. Results and discussion

We demonstrated for the first time that a cyclic terpene, limonene, was a direct ligand for A_{2A} receptors and activated receptor-mediated signaling pathways, as confirmed by cytosolic cAMP and calcium concentrations, and induced phosphorylation of CREB transcription factor. Taken together, our data demonstrate that limonene is an A_{2A} receptor agonist.

First, we performed a radioligand binding assay to quantitate the binding affinity of limonene to A_{2A} receptors with a rat brain cerebral cortex membrane fraction. Specifically, we employed competitive binding of limonene with a known selective A_{2A} receptor agonist, [^3H]CGS21680. We found that [^3H]CGS21680 displayed a saturable binding profile in the absence of limonene, and the binding of [^3H]CGS21680 to A_{2A} receptors decreased with increasing concentrations of limonene in a concentration-dependent manner (Fig. 1). At the highest limonene concentration, [^3H]CGS21680 binding decreased by 91.2% as compared to the control group. This indicated that limonene competitively bound to the [^3H]CGS21680-binding site of the A_{2A} receptor. Therefore, we found that limonene has a high affinity toward A_{2A} receptors and can act as a ligand for them.

In the next step, we investigated whether limonene binding activated the A_{2A} receptor-mediated signaling pathway. A series of experiments was performed with CHO cells transfected with the human A_{2A} receptor gene. A_{2A} receptors are coupled to a G_s G protein [15] and activate adenylyl cyclase [16]. The activated G_s subunit can couple to downstream effectors to regulate the amount of second messengers, including cAMP and PKA. cAMP-induced gene transcription is generally accepted to be mediated through the activation of PKA, whose two regulatory subunits bind to cAMP and induce a conformational change, which produces subunit dissociation resulting in enzymatic activation [17] and subsequent phosphorylation of the transcription factor CREB.

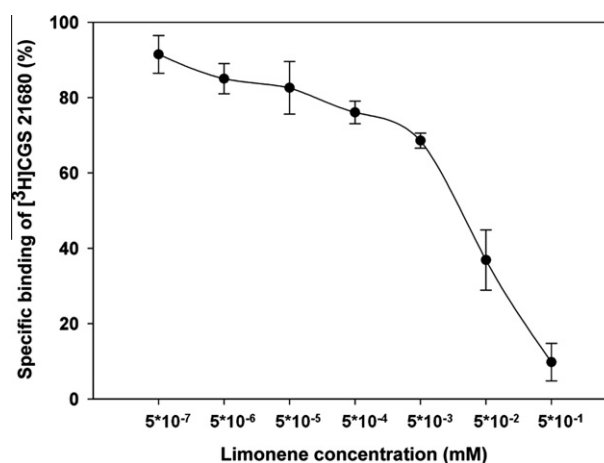


Fig. 1. Competitive binding assay of limonene with [^3H]CGS21680 in a rat brain membrane fraction. Binding of [^3H]CGS21680, an A_{2A} receptor ligand, was reduced by limonene in a concentration-dependent manner. Values are means \pm SEM.

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