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# Anandamide inhibits FcεRI-dependent degranulation and cytokine synthesis in mast cells through $CB_2$ and GPR55 receptor activation. Possible involvement of $CB_2$-GPR55 heteromers

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ABSTRACT

Activation of high affinity receptor for IgE (FcεRI) by IgE/antigen complexes in mast cells (MCs) leads to the release of preformed pro-inflammatory mediators stored in granules by a $Ca^{2+}$-dependent process known as anaphylactic degranulation. Degranulation inhibition has been proposed as a strategy to control allergies and chronic inflammation conditions. Cannabinoids are important inhibitors of inflammatory reactions but their effects on IgE/Ag-mediated MCs responses are not well described. In this study, we analyzed the effect of the endocannabinoid anandamide (AEA), the selective $CB_2$ receptor agonist HU308, and the GPR55 receptor agonist lysophosphatidylinositol (LPI) on FcεRI-induced activation in murine bone marrow-derived mast cells (BMMCs). Our results show that AEA, HU380 and LPI inhibited FcεRI-induced degranulation in a concentration-dependent manner. This effect was mediated by $CB_2$ and GPR55 receptor activation through a mechanism insensitive to pertussis toxin. Degranulation inhibition was prevented by $CB_2$ and GPR55 antagonism, but not by $CB_1$ receptor blockage. AEA also inhibited calcium-dependent cytokine mRNA synthesis induced by FcεRI crosslinking, without affecting early phosphorylation events. In addition, AEA, HU308 and LPI inhibited intracellular $Ca^{2+}$ rise in response to IgE/Ag. $CB_2$ and GPR55 receptor antagonism could not prevent the inhibition produced by AEA and HU308, but partially blocked the one caused by LPI. These results indicate that AEA inhibits IgE/Ag-induced degranulation through a mechanism that includes the participation of $CB_2$ and GPR55 receptors acting in close crosstalk, and show that $CB_2$-GPR55 heteromers are important negative regulators of FcεRI-induced responses in MCs.

## 1. Introduction

Endocannabinoids are polyunsaturated fatty acids synthesized on demand upon distinct physiological stimuli. Arachidonoyl ethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) are the best-characterized members of this group [1,2]. Endocannabinoids bind with different affinities to classical $CB_1$ and $CB_2$ receptors coupled to $G_{i/o}$ proteins causing adenylyl cyclase activity inhibition, increased potassium channels conductance and decreased conductance of calcium channels. In addition, $CB_1$ and $CB_2$ receptors regulate phosphorylation and activation of mitogen activated protein kinases (MAPKs) [3]. Endocannabinoids modulate distinct innate and adaptive immune functions [4], including prostaglandin production during inflammation [5], T and B cell proliferation [6–8], neutrophil degranulation and chemotaxis [9], and cytokine production in activated leukocytes [10]. Although many endocannabinoid effects in immune cells are mediated by $CB_1$ and $CB_2$ receptors [11,12], some others are not [13]. In particular, AEA blocks T-type and L-type $Ca^{2+}$ channels [14,15], acts as a partial

*Abbreviations:* AEA, anandamide; BMMCs, bone marrow-derived mast cells; FcεRI, high affinity IgE receptor; 2-AG, 2-arachidonoylglycerol; DNP-HSA, dinitrophenol coupled to human serum albumin (antigen); IgE, immunoglobulin E; TNF, Tumor Necrosis Factor; IL, interleukin; CB, cannabinoid receptor; PKC, protein kinase C; LPI, lysophosphatidylinositol; PI3K, phosphoinositide 3-kinase; $TRPV_1$, transient receptor potential cation channel, subfamily V member 1; NFAT, nuclear factor of activated T-cells

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$TRPV_1$ agonist [16], and activates PPAR [17] and GPR55 receptors [18].

Mast cells (MCs) have an important role in the physiopathology of inflammation [19,20]. They are well known initiators of allergic reactions due to the presence of the high affinity IgE receptor (FcεRI) on their cellular membrane, and secrete a number of pro-inflammatory and immunoregulatory mediators after activation [21,22]. Intensive research, directed to identify compounds able to inhibit IgE/Ag-induced MCs activation, has been conducted worldwide to better cope with the important burden of allergic diseases [23–25]. IgE/Ag-mediated cross-linking of FcεRI activates a complex signaling cascade that involves the Src family kinases Lyn and Fyn [26,27]. In turn, phosphorylation of specific substrates and the formation of multiple protein aggregates lead to the release of preformed mediators stored in granules in a PKC- and calcium-dependent process known as anaphylactic degranulation [28,29]. In addition to granule content exocytosis, the rise in intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ contributes to the activation of specific transcription factors (such as NFAT, AP-1 and NFκB), inducing cytokine mRNA synthesis and cytokine secretion [30–32]. Like other cells in the immune system, MCs respond to endocannabinoids and express cannabinoid receptors [33]. It is assumed that the anti-inflammatory effects of cannabinoids used for the treatment of MCs-associated diseases, such as pain and inflammation [34], arthritis [35], colitis [36], ocular disease [37] and atopic dermatitis [38], are related to MC activity inhibition. Although it is known that endocannabinoids regulate MC maturation [39] and inhibit mediator secretion [40,41], a detailed description of the effects of cannabinoids on MCs, and the identification of the molecular targets of those compounds is far from complete.

The objectives of this work were to study the effects of AEA on IgE/Ag-induced degranulation of bone marrow-derived mast cells (BMMCs) and to determine the role of cannabinoid receptors and $Ca^{2+}$ mobilization on these effects. This research shows that AEA inhibits BMMCs degranulation by mechanisms involving both $CB_2$ and GPR55 receptor activation.

## 2. Materials and methods

### 2.1. Drugs

The following compounds were used in this study and bought from Sigma-Aldrich (St. Louis, MO, USA): Anandamide, AM251 ($CB_1$ receptor antagonist and GPR55 agonist), AM630 ($CB_2$ receptor antagonist), HU308 ($CB_2$ agonist), LPI (GPR55 agonist), ML193 (GPR55 antagonist), AMG9810 ($TRPV_1$ receptor antagonist), A23187 (calcium ionophore), Fura-2AM, the thrombin receptor activator peptide 6 (TRAP-6), dinitrophenol coupled to human serum albumin (DNP-HSA, Ag), monoclonal anti-DNP IgE (SPE-7 clone), bovine serum albumin (BSA), phorbol-myristate-acetate (PMA), ethylene glycol-bis (β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), triton X-100, and $CaCl_2$ were obtained from Merck and IL-3 and stem cell factor (SCF) from Peprotech. Anandamide, AM251 and AM630 were initially dissolved in absolute ethanol and then diluted with deionized water until maximal ethanol concentration in cell samples was 0.05%. AMG9810, A23187, Fura-2AM, ML193, and PMA were dissolved in dimethyl sulfoxide (DMSO) and diluted until maximal DMSO concentration in cell samples was 0.05%. Stock of HU308 was prepared in ethanol and diluted in Tyrode's buffer. Stock of LPI was dissolved in a mixture of chloroform: methanol: water 70:27:3 and diluted in Tyrode's buffer to final concentration. DNP-HSA, EGTA, Triton X-100, anti-DNP antibody and TRAP-6 were dissolved in deionized water.

### 2.2. Antibodies

Antibodies (Abs) against p-Tyr (Cat. No. 9411) and p-Src (Tyr 416, Cat No. 2101) were bought from Cell Signaling. Abs against PLCγ1 (Cat No. sc7290), Syk (Cat No. sc1077) and Lyn (Cat No. sc15) were purchased from Santa Cruz Biotechnology. Primary antibodies from Cell Signaling were used at 1:5000 dilution and those from Santa Cruz Biotechnology, at 1:1000. Secondary antibodies were obtained from Millipore and used at 1:15000.

### 2.3. Mice, cell culture and IgE sensitization

BMMCs were differentiated from bone marrow obtained from tibias of C57BL/6J mice (stock No. 000664, Jackson Laboratory, Maine, USA). For data shown on Fig. 5B and C, BMMCs from 129S1/SvlmJ WT (stock No. 002448) and $Fyn^{-/-}$ (stock No. 002271) from Jackson Laboratory were used, as previously described [31]. The protocol for BMMCs generation was approved by the Cinvestav Institutional Committee for the Care and Use of Laboratory Animals (CICUAL, protocol 074-13), following the rules of the Mexican Official Norm for the use and care of laboratory animals (NOM-062-ZOO-1999). Bone marrow was cultured in RPMI 1640 media supplemented with 50 μM β-mercaptoethanol, 25 mM HEPES pH 7.4, 1 mM pyruvate, 20 ng/mL IL-3, 10 ng/mL SCF, antibiotic/antimycotic, non-essential aminoacids and 10% FBS during four to six weeks. BMMCs development was followed by flow cytometry using an anti IgE monoclonal antibody (BD Biosciences) and only cultures showing > 98% FcεRI-positive cells were used for the experiments. For all the experiments BMMCs were sensitized with 300 ng/mL of an IgE anti-dinitrophenol (DNP) monoclonal antibody (Clone SPE-7, SIGMA) overnight at 37 °C. For cytokine mRNA detection, cells were sensitized for 24 h with 100 ng/mL of the same monoclonal IgE. In all cases, unbound antibody was removed by collecting the cells through centrifugation and re-suspension in the corresponding buffer depending on the assay.

### 2.4. Degranulation assay

One million IgE-sensitized cells were centrifuged at 500 × *g* during 5 min and suspended in 1 mL Tyrode's/BSA buffer of the following composition: 20 mM HEPES pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 0.05% bovine serum albumin (BSA). Independent groups of cells were treated with vehicle, 2.5, 10, 25 or 50 μM AEA for 15 min and then stimulated with antigen (1, 3, 9, or 27 ng/mL DNP-HSA) during 30 min at 37 °C. After this treatment, cells were placed on ice for 2 min and centrifuged to 12,000 × *g* for 10 min at 4 °C. Sixty microliters of supernatant or 20 μL of Triton-treated cell pellet were placed in an ELISA plate containing 40 μL of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNAG), and incubated for 1 h at 37 °C before the addition of 120 μL of "stop" solution ($Na_2CO_3$ 0.1 M/$Na_2HCO_3$ 0.1 M). β-Hexosaminidase release was quantified by spectrophotometry in an ELISA plate reader (Tecan Sunrise) at 405 nm, as described [42].

### 2.5. Viability test

To determine the viability of the cells, the Muse™ Count Viability Kit from Millipore was used. Briefly, 1.5 million IgE-sensitized BMMCs were treated with AEA at different concentrations during 15 min and the reagent MCH100102 (included in the kit) was added in a 1:20 dilution. The Viability Intuitive Software included in the analyzer generated the corresponding dot plot providing the percentage of live cells in each sample.

### 2.6. Determination of intracellular calcium concentration ($[Ca^{2+}]_i$)

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured in IgE-sensitized BMMCs. To do this, cells were collected and suspended in Tyrode's/BSA buffer with 5 μM Fura 2-AM for 30 min at 37 °C to load it. Then, eight million of Fura 2-AM-loaded BMMCs were suspended in 2 mL Tyrode's/BSA buffer and placed in the cuvette of a