



Anandamide inhibits FcεRI-dependent degranulation and cytokine synthesis in mast cells through CB₂ and GPR55 receptor activation. Possible involvement of CB₂-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 Ca²⁺-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 CB₂ 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, HU308 and LPI inhibited FcεRI-induced degranulation in a concentration-dependent manner. This effect was mediated by CB₂ and GPR55 receptor activation through a mechanism insensitive to pertussis toxin. Degranulation inhibition was prevented by CB₂ and GPR55 antagonism, but not by CB₁ 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 Ca²⁺ rise in response to IgE/Ag. CB₂ 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 CB₂ and GPR55 receptors acting in close crosstalk, and show that CB₂-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 CB₁ and CB₂ receptors coupled to G_{i/o} proteins causing adenylyl cyclase activity inhibition, increased potassium channels conductance and decreased conductance of calcium

channels. In addition, CB₁ and CB₂ 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 CB₁ and CB₂ receptors [11,12], some others are not [13]. In particular, AEA blocks T-type and L-type Ca²⁺ 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; TRPV₁, transient receptor potential cation channel, subfamily V member 1; NFAT, nuclear factor of activated T-cells

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TRPV₁ 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 Ca²⁺ concentration [Ca²⁺]_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 Ca²⁺ mobilization on these effects. This research shows that AEA inhibits BMMCs degranulation by mechanisms involving both CB₂ 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 (CB₁ receptor antagonist and GPR55 agonist), AM630 (CB₂ receptor antagonist), HU308 (CB₂ agonist), LPI (GPR55 agonist), ML193 (GPR55 antagonist), AMG9810 (TRPV₁ 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 CaCl₂ 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/SvImJ 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 CaCl₂, 1 mM MgCl₂, 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 (Na₂CO₃ 0.1 M/Na₂HCO₃ 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 ([Ca²⁺]_i)

Intracellular calcium concentrations ([Ca²⁺]_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

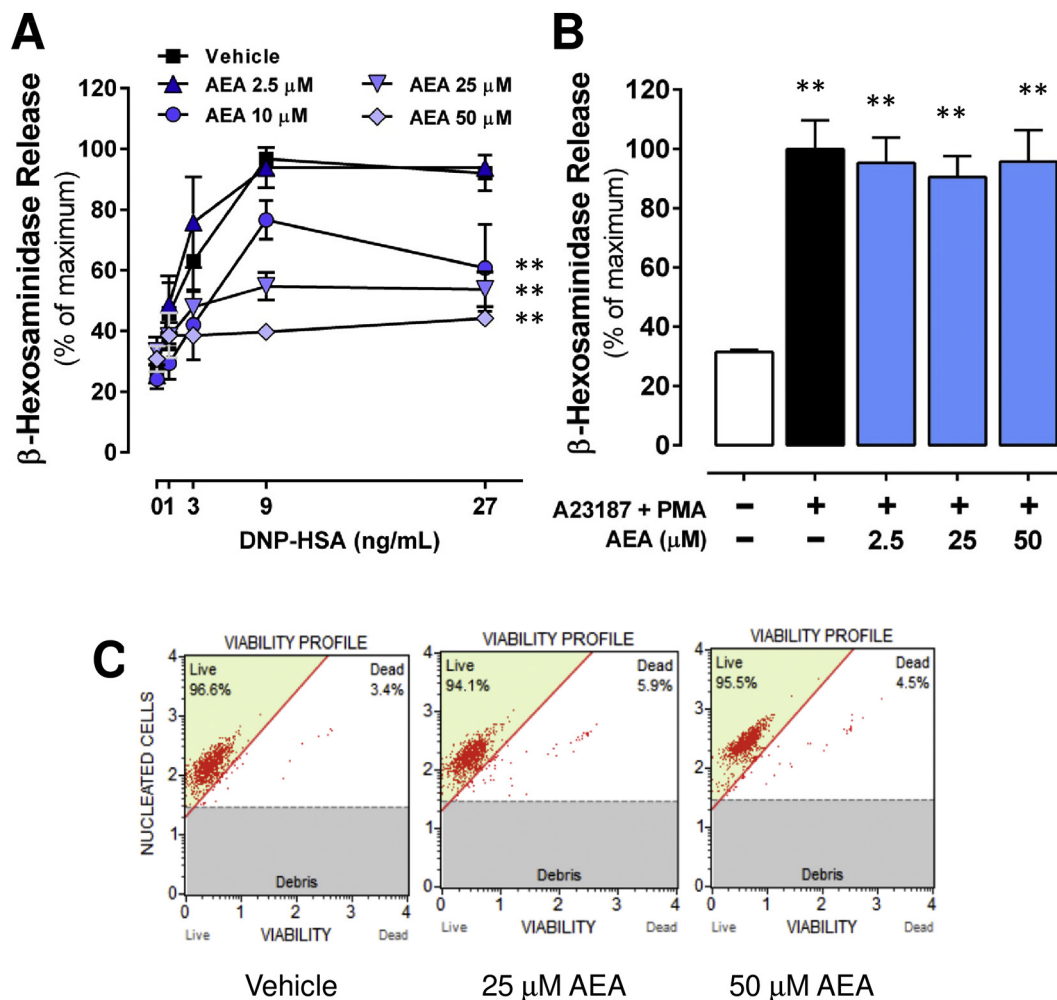


Fig. 1. Anandamide (AEA) inhibits IgE/Ag-induced but not calcium ionophore/PMA-induced β -hexosaminidase release in BMMCs. **A.** Independent groups of IgE-sensitized BMMCs were pre-treated with vehicle or AEA for 15 min and then stimulated with different concentrations of antigen (DNP-HSA) for 30 min at 37 °C. After this time, β -hexosaminidase activity was measured in the supernatant of stimulated cells. Each point represents the mean $\pm$ S.E.M. of 3 to 5 independent experiments. $**p < 0.01$; one-way ANOVA followed by Dunnett's test vs. DNP/HSA (27 ng/mL). **B.** Cells were pre-treated as in **A** but were stimulated with a combination of the calcium ionophore A23187 (200 nM) and the PKC activator PMA (100 nM). β -Hexosaminidase release was measured 30 min after stimulation. Data shown are the mean $\pm$ S.E.M. of 3 to 5 independent experiments. $**p < 0.01$, one-way ANOVA followed by Dunnett's test. **C.** Sample dot blots of cell viability after treatment with the vehicle, 25 μ M and 50 μ M AEA.

spectrofluorometer (Fluoromax 3 Jobin Yvon). Basal fluorescence was recorded during 100 s prior to any pharmacological treatment. $[Ca^{2+}]_i$ was calculated with the parameters and equation described in a previous work [31].

2.7. Semi-quantitative RT-PCR

Two million IgE-sensitized BMMCs were treated with inhibitors or IgE/Ag for different times and then lysed with TRIzol reagent (Sigma-Aldrich). RNA was isolated following the instructions included with the reagent and suspended in 7 μ L of RNA secure solution (Ambion). Two micrograms of RNA were used for cDNA synthesis utilizing a cDNA synthesis kit from Thermo Scientific. Ten microliters from each cDNA synthesis reaction were used for the amplification of selected cytokines. The following primers were utilized: TNF, 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3' (sense) and 5'-GTATGAGATAGCAATCGGCTGACGGTGTGGG-3' (antisense) [43]; IL-2, 5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG-3' (sense) and 5'-GACAGAAGGCTATCCATCTCTCAGAAAGTCC-3' (antisense) [44]; IL-4, 5'-CCAGCTAGTTGTCATCCTGCTCTTCTTCTCG-3' (sense) and 5'-CAGTGATGTGGACTTGGACTCAATTCATGGTGC-3' (antisense) [44]; IL-6, 5'-ATGAAGTCTCTCTCTGCAAGAGACT-3' (sense) and 5'-CACTAGGTTGCCGAGTAGATCTC-3'

(antisense) [45]; IL-3, 5'-GATACCCACCGTTTAACCAGAACGTG-3' (sense) and 5'-TCCACGGTTAGGAGAGACGGAG-3' (antisense) [46]; IL-10, 5'-ATGCAGGACTTTAAGGGTTACTTGGGTT-3' (sense) and 5'-ATTTCGGAGAGAGGTACAAACGAGGTTT-3' (antisense) [47]; and GAPDH 5'-TGAAGGTCGGTGTGAACGATTGGC-3' (sense) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (antisense) [45]. Alignment temperatures were used as described in the correspondent references, and optimal cycle number for amplification in IgE/Ag-stimulated BMMCs was determined as reported previously [48]. The products were separated on 2% agarose gels with ethidium bromide dye and quantified utilizing a ChemiDoc (BioRad) equipment with the ImageLab 5.0 version program. For endocannabinoid receptor mRNA detection, pellets from two million sensitized cells were solubilized in 0.5 mL of TRI-Reagent (Sigma-Aldrich) at room temperature and total RNA was extracted following instructions suggested by the provider. First strand cDNA synthesis was generated with the Fermentas Life Sciences first-strand synthesis system. Primers for amplification: CB₁, forward, 5'-CGTGGGCGACCTGTTCCTCA-3'; reverse, 5'-CATGCGGGCTTGGTCTGG3' [49], CB₂, forward, 5'-CCGAAAAGAGGATGGCAATGAAT-3'; reverse, 5'-CTGCTGACGCCCTGGAGAAC-3', [49]; and GPR55, forward 5'-GGACTCATTGGTACTCCTAAGCTGT-3'; reverse, 5'-GCAGATCCCAAAGGTCTTCTCT-3' [50]. These primers were obtained from Sigma Aldrich. PCR conditions:

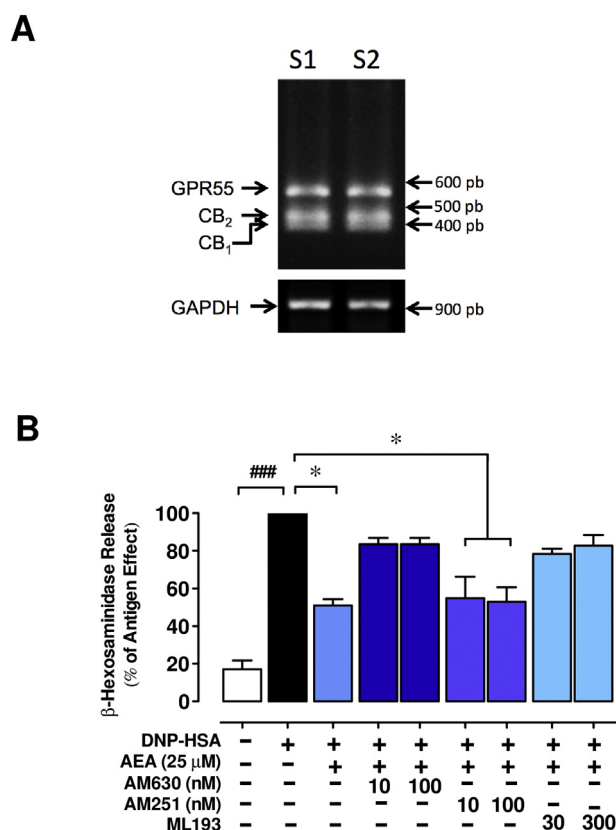


Fig. 2. AEA inhibits FcεRI-dependent degranulation by a mechanism that is independent of CB₁ receptors and G_i proteins, but dependent on the activation of CB₂ and GPR55 receptors. **A.** Semi-quantitative RT-PCRs to detect CB₁, CB₂ and GPR55 receptors in BMMCs. Gel images show results obtained with two samples (S1 and S2) of cells from two independent BMMC cultures. **B.** Two million BMMCs were pre-incubated with different concentrations of AM251, AM630 or ML193 (CB₁, CB₂ and GPR55 receptor antagonists, respectively) for 15 min, then treated with vehicle or 25 μM AEA for 15 min and finally stimulated with DNP-HSA (27 ng/mL) for 30 min. After those treatments, β-hexosaminidase release was measured in the supernatant of stimulated cells. Data shown are the mean ± S.E.M. of 3 to 5 independent experiments. ### *p* < 0.001 vs. non-stimulated cells, Student's *t*-test; **p* < 0.05 vs. antigen-treated cells, one-way ANOVA followed by Dunnett's test.

95 °C for 10 min; 95 °C for 45 s, 60 °C for 1 min, 72 °C for 45 s and a final step of 72 °C for 7 min. Thirty amplification cycles were performed for the detection of cannabinoid receptors and 25 cycles was used for GAPDH. PCR products were separated on 2% TBE-agarose gels and dyed with ethidium bromide. Pictures of the gels were taken using the MiniBis Pro from BioImaging Systems.

2.8. Immunoprecipitation and western blot

Thirty million IgE-sensitized BMMCs stimulated in specific conditions were suspended in a lysis buffer containing 1% NP-40 (Igepal), 20 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM sodium pyrophosphate, 1 mM orthovanadate and 60 mM β-octylglucoside dissolved in PBS pH 7.4. Lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C and supernatants were kept frozen at –80 °C until use. Then, supernatants were quickly thawed and incubated overnight with 50 μL of protein A-Sepharose beads loaded with 10 μg of the corresponding primary antibodies (Abs) at 4 °C. Beads were then carefully recovered by centrifugation and washed three times with 500 μL of lysis buffer without β-octylglucoside. Beads were resuspended in 50 μL of Laemmli buffer, boiled during 5 min and centrifuged at low velocity for 5 min. Forty microliters of each sample were separated by denaturing

10 to 15% acrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Perkin Elmer, Akron, OH, USA). Membranes were blocked with 6% nonfat milk for 1 h in TBST buffer and washed once with TBST before the addition of primary Abs overnight. After several washes with TBST, blots were incubated with the appropriate dilutions of secondary Abs. Western blots were developed using the Immobilon Western Chemiluminescent HRP substrate from Millipore.

2.9. Statistical analysis

Results in graphs of β-hexosaminidase release are expressed as the mean ± S.E.M. of three to five independent experiments using distinct primary cell cultures. Representative traces taken from three independent experiments from distinct cell cultures are used to illustrate Ca²⁺ mobilization data. Comparisons between two groups were performed using Student's *t*-test and comparisons among several experimental groups were conducted with a one-way or two-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Statistical analyses were conducted using raw values, but some graphs are presented with normalized data for comparison purposes. SigmaPlot 11.0 was utilized for statistical analysis and GraphPad Prism 5 for graphics.

3. Results

3.1. AEA inhibits FcεRI-mediated degranulation of BMMCs in a concentration-dependent manner without altering degranulation machinery or cell viability

First, we assessed the effect of anandamide on BMMCs degranulation. Fig. 1A shows β-hexosaminidase release induced by different concentrations of the antigen (DNP-HSA) in BMMCs pre-treated with vehicle or AEA. As shown, maximal degranulation was obtained after 9 ng/mL of antigen in vehicle-treated cells. AEA pretreatment significantly inhibited antigen-induced degranulation in a concentration-dependent manner. This inhibition was almost complete at 50 μM AEA and occurred for a wide range of DNP concentrations. Fig. 1B depicts the release of β-hexosaminidase produced by the combined administration of the calcium ionophore A23187 and PKC activator PMA in cells treated with distinct amounts of AEA. The endocannabinoid had no effect on FcεRI-independent degranulation even at high concentrations, which suggests that AEA does not inhibit the events that occur after PKC activation and calcium mobilization triggering in BMMCs nor does it alter the general secretory machinery for anaphylactic degranulation. A viability test showed that AEA treatment did not compromise cell survival at any concentration tested (Fig. 1C).

3.2. Neither TRPV₁ nor TRPC3-6 receptors play a role in the anandamide-dependent inhibition of IgE/Ag-induced degranulation

It has been established that TRPV₁ channels are activated and desensitized by AEA at relatively high concentrations [16]. In order to determine if these channels were involved in the inhibitory effects of AEA observed in our experiments, we studied IgE/Ag-triggered degranulation in BMMCs pre-incubated with several concentrations of the TRPV₁ receptor antagonist AMG9810 alone, and in the presence of AEA. AMG9810 by itself did not affect the degranulation produced by DNP-HSA at any concentration (data not shown), and concentrations of 10–1000 nM AMG9810 did not prevent the inhibitory effect of AEA (Supplementary Fig. 1). On the other hand, it is known that activation of TRPC family channels after FcεRI triggering contributes to degranulation in mast cells [51,52]. To test the possible involvement of TRPC3-6 receptors on the inhibitory effects of AEA, we took advantage of the fact that those channels are not activated in BMMCs generated from Fyn kinase-deficient mice [51]. Therefore, we tested the effect of AEA in BMMCs derived from Fyn^{–/–} mice stimulated with IgE/Ag.

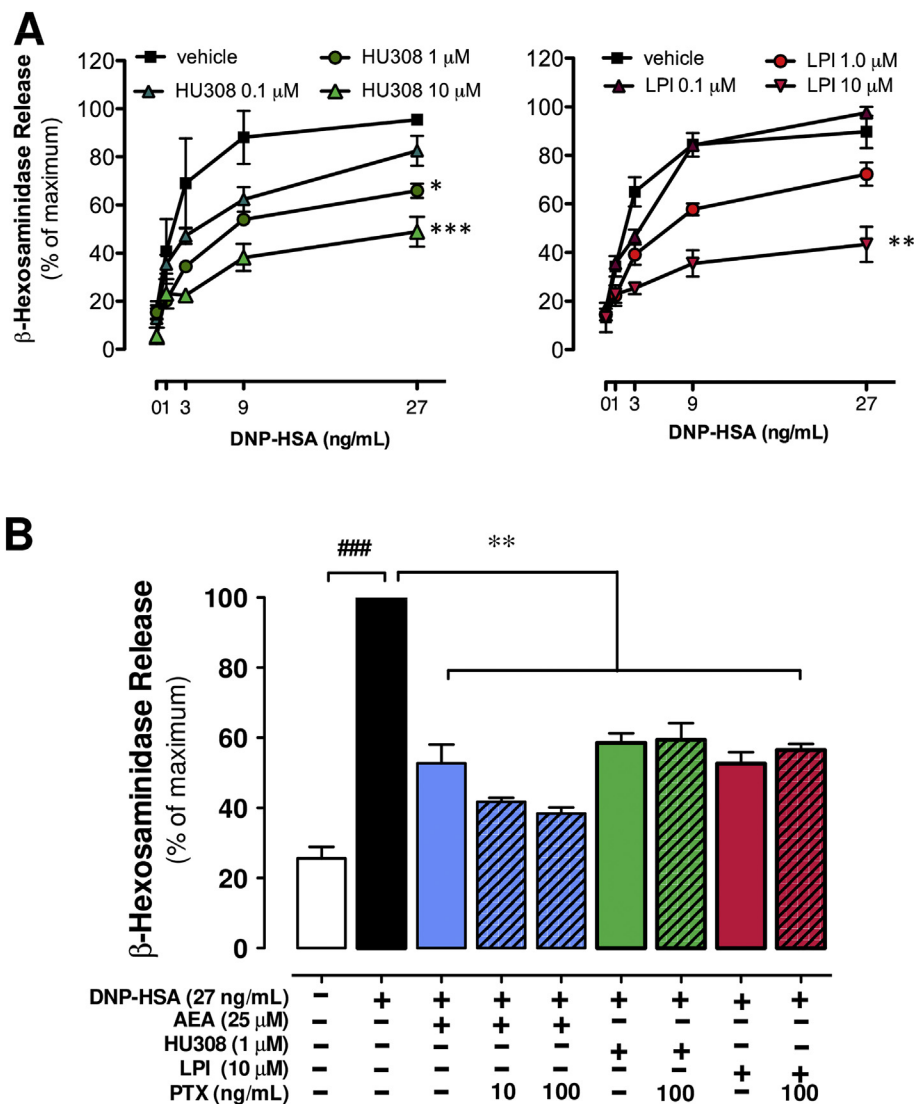


Fig. 3. AEA and LPI concentration-dependently inhibit Fc ϵ RI-dependent degranulation by a mechanism that is insensitive to pertussis toxin. **A.** Independent groups of IgE-sensitized BMMCs were pre-treated with vehicle, HU308 or LPI for 15 min and then stimulated with different concentrations of antigen (DNP-HSA) for 30 min at 37 °C. After this time, β -hexosaminidase activity was measured in the supernatant of stimulated cells. Data shown are the mean $\pm$ S.E.M. of 3 to 5 independent experiments. ** p < 0.01, one-way ANOVA followed by Dunnett's test. **B.** BMMCs were pre-incubated with pertussis toxin (10 or 100 ng/mL) for 2 h and then treated with vehicle, AEA, HU308 or LPI for 15 min before stimulation with DNP-HSA (27 ng/mL) for 30 min. β -Hexosaminidase was measured in the supernatant of stimulated cells. Data shown are the mean $\pm$ S.E.M. of 3 to 5 independent experiments. ### p < 0.001 vs. non-stimulated cells; Student's t -test; ** p < 0.05 vs. antigen-treated cells, one-way ANOVA followed by Dunnett's test.

Because Fc ϵ RI-induced degranulation is lower in Fyn $^{-/-}$ cells [51,52], normalized values of degranulation are presented in Supplementary Fig. 1B to show that AEA inhibited degranulation in the same proportion in WT and Fyn-deficient cells.

3.3. AEA inhibits Fc ϵ RI-induced degranulation with the participation of CB $_2$ and GPR55 receptors

In order to determine if classical cannabinoid receptors were involved in the effects of AEA, we first confirmed, by RT-PCR, that CB $_1$, CB $_2$ and GPR55 receptors were expressed in BMMCs (Fig. 2A). Next, we used distinct antagonists to block the inhibitory actions of AEA. Fig. 2B shows the effects of 25 μ M AEA in cells with different pre-treatments. AEA inhibitory effect was prevented by both the specific CB $_2$ receptor antagonist AM630 and the GPR55 antagonist ML193, but not by AM251 (a CB $_1$ receptor antagonist and GPR55 agonist). None of the antagonists tested altered IgE/Ag-induced degranulation *per se* (data not shown).

3.4. CB $_2$ and GPR55 agonists inhibit Fc ϵ RI-induced degranulation in mast cells via PTX-insensitive G proteins

Because AM630 and ML193 blocked AEA inhibitory actions, we tested the effect of selective agonists for CB $_2$ and GPR55 receptors on Fc ϵ RI-induced degranulation. Fig. 3A shows that the CB $_2$ specific

agonist HU308 and the GPR55 selective agonist LPI inhibited MC degranulation in a concentration-dependent manner, as AEA did. The doses needed for maximal degranulation blockage with HU308 and LPI were lower (10 μ M) than those needed for AEA to produce similar effects (25 μ M). We then evaluated if the inhibition caused by 1 μ M HU308 could be prevented by pre-treatment with 100 nM AM630 or 300 nM ML193 and found that antagonists prevented the effects of those compounds (percentage of β -hexosaminidase release inhibition: HU308 = 58.3 $\pm$ 2.2 vs. AM630 pre-treated = 81.1 $\pm$ 2.9 and ML193 pre-treated = 86.6 $\pm$ 3.4; p < 0.001). A similar situation was observed with LPI, but to a lesser extent (percentage of β -hexosaminidase release inhibition: LPI = 55.4 $\pm$ 2.5 vs. AM630 pre-treated = 74.5 $\pm$ 1.2 and ML193 pre-treated = 82.9 $\pm$ 1.9; p $\leq$ 0.01). These data support the idea that both CB $_2$ and GPR55 receptors are involved in the HU308 and LPI inhibitory effects on degranulation.

Next, we tested if classical pertussis toxin (PTX)-sensitive G $_i$ proteins mediated the inhibitory effects on degranulation produced by AEA, HU308 and LPI. BMMCs were pre-treated with different amounts of PTX and then stimulated with IgE/Ag, measuring the activity of secreted β -hexosaminidase 30 min after stimulation. As shown in Fig. 3B, PTX pre-treatment did not prevent the inhibitory effect of AEA, HU308 or ML193. A control test for the activity of the PTX used in these experiments was performed. As expected, PTX inhibited β -hexosaminidase release triggered by the G $_i$ -coupled PAR-1 receptor agonist

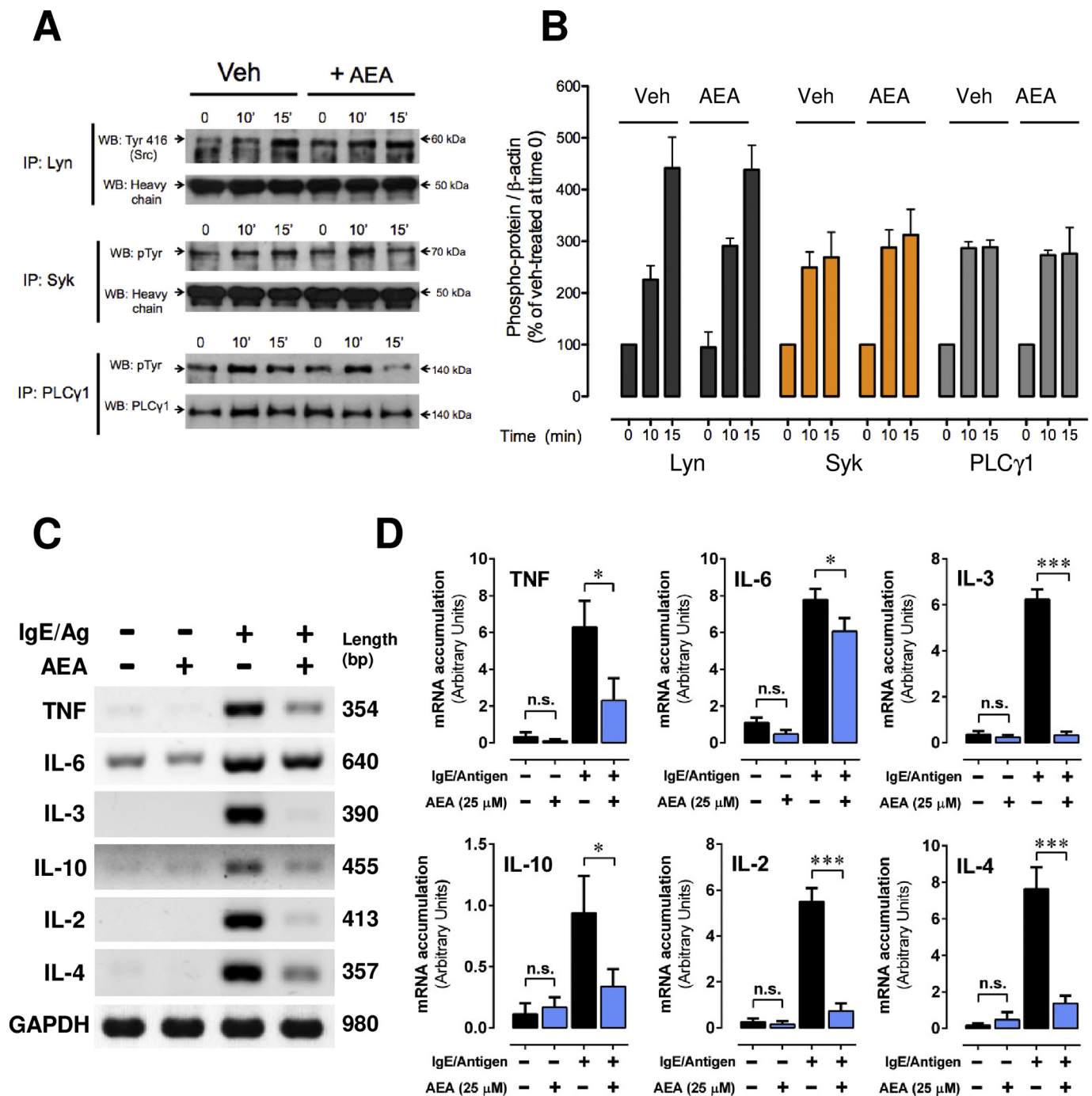


Fig. 4. AEA does not inhibit initial phosphorylation events, but blocks Ca^{2+} -dependent cytokine mRNA synthesis stimulated by $\text{Fc}\epsilon\text{RI}$ receptor in BMMCs. **A.** Western blot of Lyn, Syk and $\text{PLC}\gamma 1$ obtained by immunoprecipitation after stimulation of $\text{Fc}\epsilon\text{RI}$ receptors in cells pre-treated with vehicle or AEA (25 μM) for 0, 10 or 15 min. **B.** Densitometric quantification of the bands shown in Panel A. **C.** Semi-quantitative RT-PCR to detect the production of TNF, IL-6, IL-3, IL-10, IL-2, and IL-4 mRNA in cells pre-treated with vehicle (Veh) or AEA (25 μM) for 15 min and then stimulated with DNP-HSA antigen (27 ng/mL) for 1 h. Western blot and agarose gel images are representative of three independent experiments performed with distinct cell cultures. **D.** Quantification of cytokine mRNA production shown in Panel C. Bars represent the mean $\pm$ S.E.M. * $p < 0.05$, *** $p < 0.001$ vs. antigen-stimulated cells, Student's *t*-test.

(TRAP-6) in a concentration-dependent fashion (Supplementary Fig. 2).

3.5. Anandamide does not block early phosphorylation events triggered by $\text{Fc}\epsilon\text{RI}$ receptor, but inhibits IgE/Ag-induced cytokine synthesis

$\text{Fc}\epsilon\text{RI}$ receptor activation promotes rapid kinase activation and calcium mobilization needed for degranulation in mast cells [26]. After those initial events, $\text{Fc}\epsilon\text{RI}$ triggering leads to *de novo* cytokine mRNA synthesis. We explored if AEA could prevent early phosphorylation

events triggered by $\text{Fc}\epsilon\text{RI}$ crosslinking. As observed in Fig. 4A, pre-treatment with AEA did not alter tyrosine phosphorylation of Lyn and Syk kinases or $\text{PLC}\gamma 1$.

Also, we analyzed changes in the expression of different relevant cytokines in cells treated with AEA to obtain information about the elements of the $\text{Fc}\epsilon\text{RI}$ signal transduction system that could be affected. As previously reported in this cell preparation [48], basal expression of TNF, IL-6, IL-3, IL-10, IL-2 and IL-4 was very low or not detectable in the absence of stimulation (Fig. 4B). AEA at 25 μM had no effect by

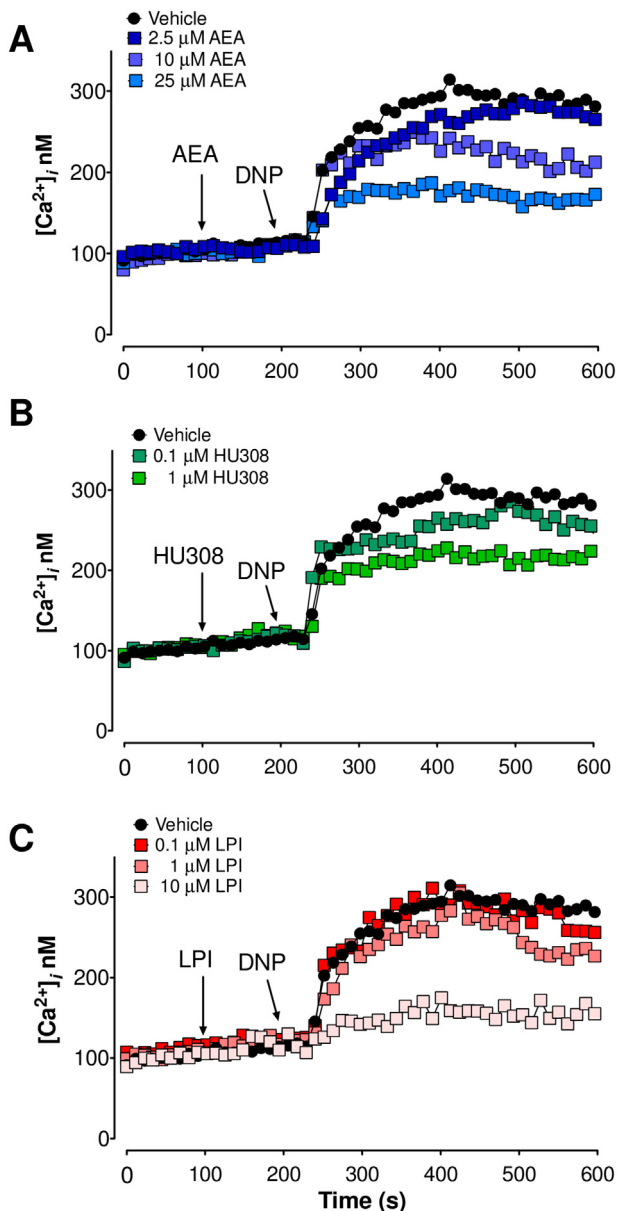


Fig. 5. AEA, HU308 and LPI inhibit maximal intracellular calcium increase induced by FcεRI receptor triggering. BMMCs were loaded with Fura-2AM as described in the **Materials and methods** section. After basal fluorescence registration, vehicle, AEA (Panel A), HU308 (Panel B) or LPI (Panel C) was added to the cuvette. After 100 s, cells were stimulated with 27 ng/mL antigen (DNP-HSA). Fluorescence was registered to reach a final time of 500 s. Data shown are the mean $\pm$ S.E.M. of two to three independent experiments performed with distinct cell cultures.

itself, but significantly prevented cytokine mRNA synthesis stimulated by IgE/Ag at optimal time. Quantification of these data is shown in Fig. 4C. Inhibition was particularly evident in calcium-dependent cytokine expression [30], *i.e.*, IL-2, IL-3, IL-4 and TNF. These results suggest that AEA inhibits calcium-dependent cytokine gene expression triggered by FcεRI stimulation.

3.6. AEA, HU308 and LPI inhibit antigen-induced calcium mobilization in BMMCs with the participation of GPR55 cannabinoid receptors

To analyze the effects of AEA on calcium mobilization, we measured changes in intracellular calcium concentration ($[Ca^{2+}]_i$) elicited by IgE/Ag in BMMCs pre-treated with different concentrations of AEA,

HU308 and LPI. None of the three agonists had an effect on basal calcium levels, but they did reduce the increase in $[Ca^{2+}]_i$ induced by DNP-HSA in a concentration-dependent manner (Fig. 5). We then tested the effects of the CB₂ antagonist AM630 and the GPR55 antagonist ML193 on calcium mobilization inhibition produced by AEA, HU308 and LPI. Neither CB₂ receptor blockage nor GPR55 antagonism prevents AEA or HU308 effects. However, AM630 and ML193 partially prevent LPI effects (Fig. 6).

4. Discussion

Main results obtained in this work are presented in Table 1 and can be summarized as follows: 1) AEA inhibits FcεRI receptor-induced degranulation with the participation of CB₂ and GPR55 receptors by a PTX-insensitive mechanism; 2) AEA does not block early FcεRI-induced phosphorylation events, but prevents calcium-dependent cytokine production; 3) BMMCs express CB₁, CB₂ and GPR55 receptor mRNAs. When CB₂ and GPR55 receptors are stimulated separately, they mimic the inhibitory actions of AEA on degranulation; 4) specific antagonism of CB₂ and GPR55 shows that both receptors play an important role in the inhibitory effects of AEA, HU308 and LPI on degranulation. As to FcεRI-induced $[Ca^{2+}]_i$ rise: 5) AEA, HU308 and LPI inhibit this parameter; 6) neither CB₂ receptor blockage nor GPR55 antagonism prevent AEA inhibitory effects; 7) ML193, but not AM630, blocks the inhibitory actions of HU308; and 8) both AM630 and ML193 partially prevent LPI-induced inhibition of $[Ca^{2+}]_i$ rise.

Cannabinoids have been proven to be important regulators of the immune system [4]. Together with the control of immune cell proliferation and differentiation, they exert important effects on chronic and acute inflammatory reactions [12]. Although the effects of those compounds have been widely documented and their use is considered a promising therapeutic strategy for inflammation-related diseases [53], the molecular mechanisms by which they exert their actions are not fully understood.

In the present study, AEA inhibited IgE/Ag-dependent degranulation in a concentration-dependent fashion without altering the viability of the cells or the general secretory machinery of MCs. AEA is an important endocannabinoid produced in response to nerve and immune cell stimulation [54]. It is a partial agonist of CB₁ ($K_i = 61\text{--}543$ nM) and CB₂ receptors ($K_i = 279\text{--}1940$ nM) [55], as well as a GPR55 receptor agonist at low nanomolar concentrations [56]. CB₁ and CB₂ cannabinoid receptors are GPCRs coupled to PTX-sensitive G_{i/o} proteins [56,57], while the GPR55 receptor couples to the PTX insensitive G_{12/13} proteins. The inhibition on degranulation caused by AEA observed in the present study was the same when PTX was added to the cells; therefore, neither CB₁ nor CB₂ receptors mediate this effect. Our data are in line with those reported previously, where inhibitory actions of endocannabinoids were observed using distinct models of MCs activation. For example, palmitoylethanolamide (PEA) blocks the PMA-induced secretion of nerve growth factor [58] and 2-AG inhibits antigen-dependent histamine release in sensitized guinea pig MCs [59]. However, the sensitivity to PTX was not addressed in those studies.

The insensitivity to PTX found in our study could be interpreted as the result of direct GPR55 activation by AEA. However, our findings suggest that CB₂ receptors are also involved. This is based on the fact that both HU308 (a highly selective CB₂ receptor agonist) [60] and LPI (GPR55 endogenous agonist) prevented MC degranulation. In support to this idea, GPR55 is expressed in our BMMCs and is functional because selective blockage of this receptor with ML193 inhibits AEA actions. To the best of our knowledge, this is the first time that an inhibitory action of GPR55 on the FcεRI signaling system is reported. Our results are in line with those reported by Cantarella and coworkers, who observed that GPR55 activation prevents the release of nerve growth factor stimulated by PMA in the human cell line HMC-1 [58]. Our data also add to the notion that LPI is an important lipid mediator capable of modulating the intensity of inflammatory reactions through GPR55

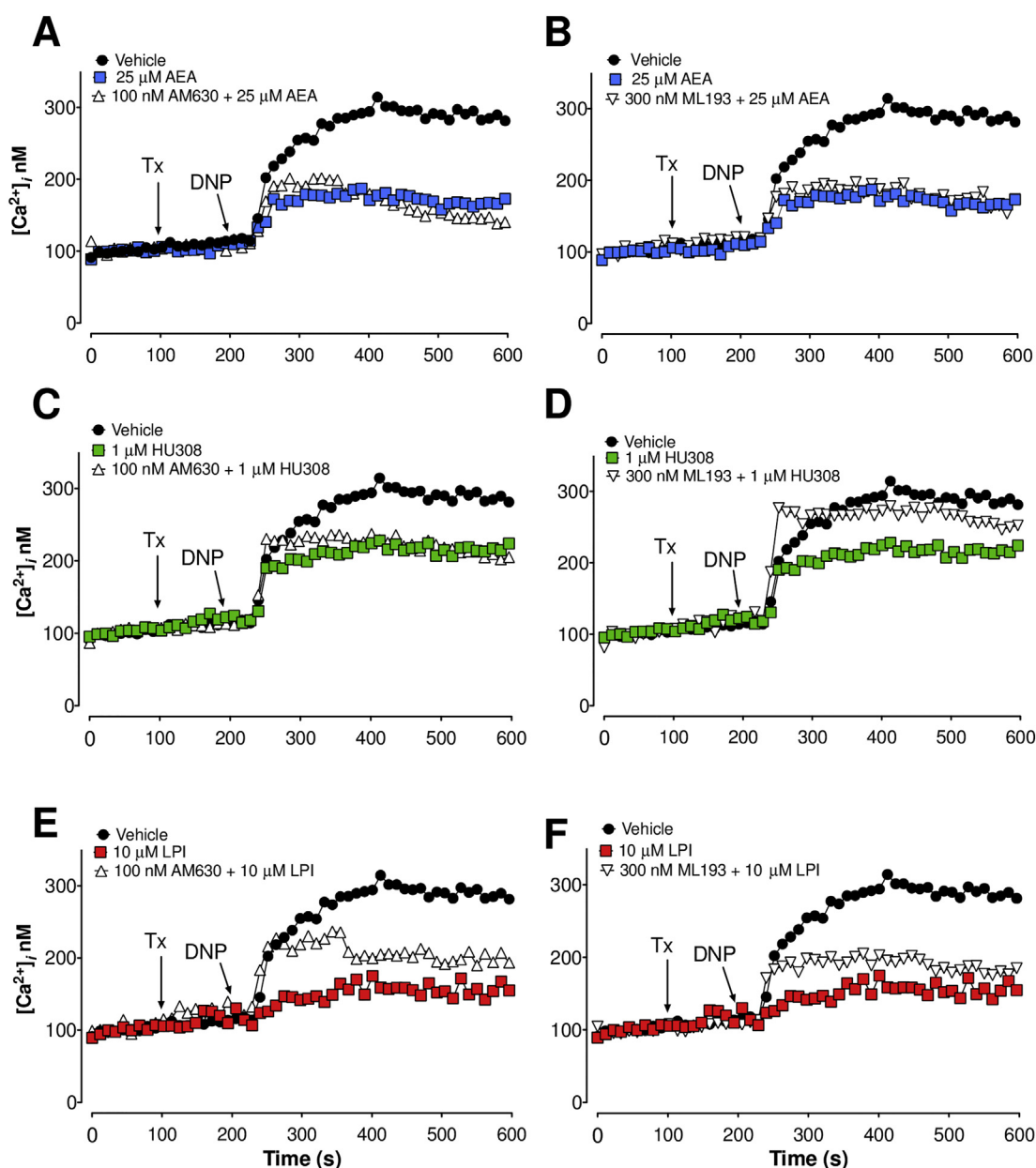


Fig. 6. Blockage of CB₂ has no effect on AEA and HU308 inhibitory actions on Fc ϵ RI-receptor mediated calcium rise, but partially reverses LPI-mediated effects. GPR55 receptor antagonist prevents HU308 and LPI inhibitory actions, but not those produced by AEA. BMMCs were loaded with Fura-2AM and AM630 or ML193 as described in the [Materials and methods](#) section. After basal fluorescence registration, vehicle, AEA (A, B), HU308 (C, D) or LPI (E, F) was added to the cuvette (Tx). After 100 s, cells were stimulated with 27 ng/mL antigen (DNP-HSA). Fluorescence was registered to reach a final time of 500 s. Data shown are the mean $\pm$ S.E.M. of two to three independent experiments performed with distinct cell cultures.

activation [61]. In this regard, it has been shown that LPI levels increase in different inflammatory conditions [62,63]. Also, GPR55 stimulation has been proposed as a therapeutic strategy for pathologies related to intestinal contractility, colonic motility and vasoconstriction [61] where MCs contribute to tissue damage. We hope that our finding of the inhibitory role of GPR55 on MCs would open new avenues in the search for molecules able to limit diseases related to MCs.

It has been shown that GPR55 receptors coupled to G_{12/13} proteins down-regulate reactive oxygen species (ROS) production and degranulation in human neutrophils [9]. MCs degranulation stimulated by IgE/Ag also requires the generation of ROS [64]; therefore, the possibility to explore the inhibitory actions of AEA on ROS production in MCs is an attractive idea. On the other hand, there is evidence that activation of another G_{12/13}-coupled receptor (LPA5) by lysophosphatidic acid inhibits B cell antigen receptor (BCR) by diminishing

calcium mobilization. Our data support the hypothesis that GPR55 and other receptors coupled via PTX insensitive proteins (particularly G_{12/13}) can modulate selected effector functions in immune cells.

Interestingly, we observed that not only GPR55 but also CB₂ receptor antagonist blocked the inhibitory actions of AEA, HU308 and LPI on Fc ϵ RI-induced degranulation. These results strongly suggest that a CB₂-GPR55 heteromer mediates this inhibition. Our findings are in line with the observation that a CB₂ receptor antagonist prevents the inhibitory effects of 2-AG on histamine release in MCs [59] and also blocks the effects of the CB₂ receptor agonist S-777469 on skin MCs [65]. However, the participation of GPR55 was not addressed in those studies.

As to the mechanism behind the inhibitory actions of AEA, we found that it does not block early Fc ϵ RI-induced phosphorylation events, but prevents calcium-dependent cytokine production. Fc ϵ RI signal

Table 1

Summary of the effects of AEA, HU308 and LPI, and their antagonists on degranulation and intracellular calcium increase produced by IgE/Ag stimulation on BMMCs.

	Pre-Tx	Degranulation	Ca ²⁺ rise
IgE/Ag AEA (CB ₁ ,CB ₂ and GPR55 receptor agonist)	Saline	↑↑↑↑↑	↑↑↑↑↑
	Saline	↓↓↓	↓↓↓
	AM251 (CB ₁ antagonist, GPR55 agonist)	↓↓↓	N/A
	AM630 (CB ₂ antagonist)	Reverses inhibition	↓↓↓
	ML193 (GPR55 antagonist)	Reverses inhibition	↓↓↓
HU308 (CB ₂ receptor agonist)	Saline	↓↓↓	↓↓↓
	AM630	Reverses inhibition	↓↓↓
	ML193	Reverses inhibition	Reverses inhibition
LPI (GPR55 receptor agonist)	Saline	↓↓↓	↓↓↓
	AM630	Reverses inhibition	Partially reverses
	ML193	Reverses inhibition	Partially reverses

transduction system is composed by multiple branches connecting the IgE/Ag-dependent crosslinking of the receptor with degranulation, lipid mediator release and cytokine production [26,66,67]. One of the most important initial events is the tyrosine phosphorylation of protein kinase Lyn that continues with the activation of protein kinase Syk. Then, tyrosine phosphorylation of distinct adapters allows the recruitment of effector enzymes, such as phospholipase C (PLC) γ , which controls protein kinase C activation and calcium release from intracellular stores. Our data show that AEA does not affect those early events on Fc ϵ RI signaling, but importantly blocks cytokine mRNA production. It is well known that a number of transcription factors, implicated in the accumulation of IL-2, IL-4, TNF and other cytokines, are regulated by calcium mobilization in MCs. Present results indicate that the point(s) of the inhibitory actions of anandamide is (are) located downstream the early kinase activation and before the activation of transcription factors. These results are in line with data generated in T cells, where Δ -9-tetrahydrocannabinol suppressed the DNA-binding activity of NFAT and NF κ B transcription factors on the *CD40L* gene promoter and inhibited T-cell receptor (TCR)-induced calcium rise without significant impairment of ZAP70, PLC γ 1/2, Akt and GSK3 β phosphorylation [68]. Our data add to the description of inhibitory actions of endocannabinoids on the activation of transcription factors involved in cytokine synthesis in immune cells. They also suggest that common negative regulatory pathways operate in innate and adaptive immune cells.

The formation of active dimers between distinct GPCRs has been identified as a biochemical mechanism that modifies the intensity and quality of signals generated by a given ligand [69]. It is known that CB₂ and GPR55 receptors are co-expressed in several cell types, such as neutrophils and cancer cells [70,71]. These receptors activate different pathways *via* ligand- and concentration-specific crosstalk [72]. The formation of CB₂ and GPR55 receptor heterodimers is capable of down-regulating MC activation *via* non-classical mechanisms in response to cannabinoids, opening the possibility of using CB₂ receptor agonists to trigger GPR55-mediated inhibitory effects to limit MC-dependent inflammatory reactions, where the levels of LPI could not reach the threshold to trigger GPR55 activation.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2018.09.006>.

Authorship

SLC and CGE performed the study conception and experimental design, wrote the paper and directed the activities of this work. ESM and JICA performed most of the degranulation assays, [Ca²⁺]_i measurements regarding to the effects of anandamide, HU308 and LPI on BMMCs, some RT-PCRs and contributed to the writing of the article; RDCV performed initial degranulation and [Ca²⁺]_i measurements, together with analysis of Fyn KO cells and the effect of AM9810. AIS generated all BMMCs needed for the study, performed the RT-PCR for cytokine mRNA analysis and immunoprecipitation experiments, and contributed to the discussion of the results.

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Conflict of interest disclosure

Authors declare no conflict of interests.

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