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Endocannabinoid 2-arachidonylglycerol decreases the immunological activation of guinea-pig mast cells: involvement of nitric oxide and eicosanoids.

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Running title: **2-arachidonylglycerol modulates mast cell activation**

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Abbreviations:

AEA, anandamide; 2AG, 2-arachidonylglycerol; CB, cannabinoid receptors; COX, cyclooxygenase; L-NAME, N^G-monomethyl-L-arginine methylester; NOS, nitric oxide synthase; PMSF, phenylmethylsulfonylfluoride.

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Abstract

The antigen-induced release of histamine from sensitised guinea-pig mast cells was dose-dependently reduced by endogenous (2-arachidonylglycerol, 2AG) and exogenous (CP55,940) cannabinoids. The inhibitory action afforded by 2AG and CP55,940 was reversed by SR144528, a selective CB_2 receptor antagonist, and left unchanged by the selective CB_1 antagonist AM251. The inhibitory action of 2AG and CP55,940 was reduced by the unselective nitric oxide synthase (NOS) inhibitor, N-monomethyl-L-arginine methylester (L-NAME) and reinstated by L-arginine, the physiological substrate. The inhibitory action of 2AG and CP55,940 was also reduced by the unselective cyclooxygenase (COX) inhibitor, indomethacin and by the selective COX-2 blocker rofecoxib. Both 2AG and CP55,940 significantly increased the production of nitrite from mast cells, which was abrogated by L-NAME and by 1400W, a selective iNOS inhibitor. Consistently, nitrite production was parallel to a CP55,940-induced increase in the expression of iNOS protein in mast cells. Both 2AG and CP55,940 increased the generation of PGE_2 from mast cells, which was abrogated by indomethacin and by rofecoxib and was parallel to the CP55,940-induced expression of COX-2 protein. Mast cell challenge with antigen was accompanied by a net increase in intracellular calcium levels. Both cannabinoid receptor ligands decreased the intracellular calcium levels which were reversed by SR144528 and L-NAME. In unstimulated mast cells both ligands increased cGMP levels. The increase was abrogated by SR144528, L-NAME, indomethacin and rofecoxib. Our results suggest that 2AG and CP55,940 decreased mast cell activation in a manner that is susceptible to a CB_2 receptor antagonist and to inhibition of nitric oxide and prostanoid pathways.

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The immunosuppressive effect of tetrahydrocannabinol (THC) was formerly reported both in man (Nahas et al., 1974) and in experimental animals (Coffey et al., 1996). More recently, the effects of THC on human immune functions and host defence have been reviewed, providing evidence that THC can suppress the human immune response in a host of leukocyte subsets and alveolar macrophages, further stressing the relationship between marijuana smoking and immunologically-related diseases (Klein et al., 1998).

Of the immunocompetent cells, mast cells are strategically placed in tissues which interface with the external environment and vary in the way in which they release by endocannabinoids. In the rat ear pin, the degranulation of resident mast cells induced by substance P is fully abrogated by the endogenous ligands at cannabinoid receptors (CB) arachidonylethanolamide (anandamide, AEA) and palmitoylethanolamide (Aloe et al., 1993). Our preliminary results showed that 2-arachidonylglycerol (2AG), a CB_2 receptors ligand (Sugiura et al., 2000), significantly reduced the immunological release of histamine from guinea-pig mast cells (Vannacci et al., 2002). Furthermore, endocannabinoids down-regulate the immunological activation of the RBL-2H3 mast cell line, acting through a CB_2 receptor-mediated AKT and ERK-kinase phosphorylation (Samson et al., 2003; Vannacci et al., 2003a). The fact that mast cells themselves produce endocannabinoids, including 2AG, suggests that an autocrine loop exists (Bisogno et al., 1997). However, recent evidence shows that endocannabinoids do not influence the releasability of rat peritoneal mast cells (Lau and Chow, 2003), casting some doubt on the overall effects of cannabinoids.

The prevalent down-regulation of mast cell releasability reported in the literature could be solely accounted for by the activation of CB_2 receptors, or could conceivably imply that there is autocrine generation of inhibitory mediators. Among them, nitric oxide (NO) and prostanoids appear to be linked to the cannabinoid system. The link between endocannabinoids and the NO pathway has been frequently reported. Anandamide (AEA) stimulates NO release

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from human monocytes, which was inhibited by N-monomethyl-L-arginine methylester (L-NAME), a non selective inhibitor of NO synthase (NOS) (Stefano et al., 1996). A stereospecific binding site for AEA is present in *Mytilus edulis* immunocytes, coupled with NO release from these cells (Stefano et al., 1997). Beside the immunocompetent cells, AEA is coupled with the NO pathway in invertebrate microglia (Stefano et al., 1996), in human saphenous vein endothelium (Stefano et al., 1998), in rat brain median eminence (Prevot et al., 1998), and in human arterial endothelial cells (Fimiani et al., 1999b) where it stimulates NO release. It seems likely therefore that the CB_1 receptor ligand AEA could act, at least in part, via the generation of NO. However, in regard to peripheral CB_2 receptors, no studies have addressed the interaction between cannabinoids and the NO pathway on the response of guinea-pig mast cells to allergic stimuli, using endogenous and/or exogenous CB_2 receptor agonists and antagonists.

Modulation of prostaglandin (PG) formation by cannabinoids has been widely established. The metabolically stable analogue of AEA, methanandamide, induces cyclooxygenase (COX) expression in human microglioma (Ramer et al., 2001) and in murine lung cancer cells (Gardner et al., 2003). In addition to the induction of the generating enzyme, AEA has been shown to release arachidonic acid and to generate $PGF_{2\alpha}$ in neuronal cells in culture (Someya et al., 2002). Other interactions between the prostanoid and cannabinoid systems entail the indomethacin-induced shift of arachidonic acid metabolism toward endocannabinoid synthesis, secondary to COX inhibition (Guhring et al., 2002) and the susceptibility of endocannabinoids to oxidative metabolism via COX (Kozak et al., 2000). However, no studies have explored the interaction between CB_2 receptors and the prostanoid pathway using an endogenous agonist and selective agonists and antagonists at CB_2 receptors.

The first aim of the present study was to evaluate the effects of endogenous and exogenous ligands at CB_2 receptors on the immunological activation of guinea-pig mast cells.

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The second aim was to address whether the effect of CB_2 receptor activation could be modulated by manipulation of NO and prostanoid pathways.

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Materials and Methods

Chemicals. Ovalbumin, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), collagenase, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetracetic acid (EGTA), 3'-isobutyl-1-methylxanthine (IBMX), 2AG, phenylmethylsulfonylfluoride (PMSF), nitrate reductase, $NADPH^+$, L-NAME and L-arginine were obtained from Sigma Chemical Co., St. Louis, MO, USA. Ficoll (m.w. 370,000) was purchased from Pharmacia Co., Uppsala, Sweden; bovine serum albumin (BSA) from Pierce, Rockford, IL, USA; O-phthaldialdehyde (OPT), from Carlo Erba Reagenti, Milan, Italy; {1 - [2 - (5-carboxyoxazol - 2-yl)- 6 - aminobenzofuran -5- oxy] -2'- amino -5'- methylphenoxy) - ethane -N,N,N',N'- tetracetic acid pentaacetoxymethyl ester}, (FURA 2-AM) from Calbiochem-Novabiochem Co., Darmstadt, Germany; CP55,940, palmitoyl-ethanolamide and AM251 from Tocris Cookson Ltd., Avonmouth, UK; 1400W and PGE_2 enzymatic immunoassay kit from Cayman Chemical Co., Ann Arbor, MI, USA. SR144528 was kindly provided by Sanofi Recherche (Montpellier, France). The chemicals used to prepare the solutions for the fluorimetric assay were of Suprapur quality, E. Merck AG, Darmstadt, Germany. 2AG, CP55,940, palmitoyl-ethanolamide, AM251, PMSF and 1400W were solubilised in DMSO as stock solutions. Final concentrations of DMSO in experimental samples were always less than 0.1%. All other reagents were of analytical grade.

Isolation of serosal mast cells from actively sensitised guinea-pigs. Male albino Dunkin-Hartley guinea-pigs (300-350 g body weight) were used. They were purchased from a commercial dealer (Rodentia, Bergamo, Italy) and quarantined for 7 days at 22-24 °C with a 12-h light, 12-h dark cycle before use. The experimental protocol was designed in compliance with the guidelines of the European Community (86/609/EEC) for animal care and use of

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laboratory animals and was approved by the Animal Care Committee of the University of Florence (Italy), and in agreement with Good Laboratory Practice.

Guinea-pigs were sensitised with 100 mg kg^{-1} i.p. plus 100 mg kg^{-1} s.c. ovalbumin, suspended in saline (40 mg ml^{-1}). The animals were anaesthetised with ethyl ether and killed by decapitation 18-21 days after sensitisation. Mast cells were obtained as previously described (Vannacci et al., 2002). The peritoneal and pleural cavities were washed for 30 min with 15 and 5 ml, respectively, using a solution of the following composition: NaCl 137 mM; glucose 5.6 mM; KCl 2.7 mM; NaH_2PO_4 0.3 mM; $CaCl_2$ 1 mM; 10 mM HEPES and collagenase 1 mg ml^{-1}. Mast cells were then isolated by density gradient centrifugation on Ficoll and washed twice with a medium of the following composition: NaCl 145 mM; KCl 2.4 mM; $CaCl_2$ 0.9 mM; 0.1% glucose adjusted to pH 7.4 with 10% Sörensen phosphate buffer. The procedure yielded a cell population composed of 95% mast cells. The cells were suspended in 2 ml of the same medium as reported before, then exposed for 30 min at 37°C to the drugs under study and stimulated for 30 min with antigen (ovalbumin, 100 μg ml^{-1}). When an antagonist was used, cells were preincubated for 30 min with the antagonist and then for a further 30 min with the agonist. The reaction was stopped by chilling the tubes in an ice-water bath. The cells were then centrifuged at 500 x *g* (15 min, 4°C) and histamine was measured in the supernatants and in the pellets.

Histamine release assay. Histamine was measured fluorimetrically in mast cell suspensions treated as described above and stimulated or not with 100 μg ml^{-1} ovalbumin, using the previously described method (Ndisang et al., 1999). Briefly, the samples were centrifuged at 200 x *g* (10 min, 20°C). In the supernatants 0.1% *O*-phthaldialdehyde (in methanol) was added directly to the samples after alkalinisation with 0.5 ml of NaOH 0.5 N. The reaction was stopped after 4 min by adding 0.2 ml of H_3PO_4 2.5 N. The same procedure was used for the pelleted cells after extraction with HCl 0.1 M. Histamine in the samples was determined

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by fluorimetric measurement using an excitation wavelength of 365 nm and an emission wavelength of 455 nm. Histamine release (supernatant histamine) was expressed as a percentage of the total present in the cells plus supernatants.

Evaluation of NO production. This was performed by determining the nitrite (NO_2^-) amount, the stable end products of NO metabolism, in guinea-pig mast cell supernatants obtained as described. In some experiments, the cells were pretreated for 30 min with L-NAME 10 μM, an NOS inhibitor, before incubation with the tested substances (2AG and CP55,940) or with medium alone. The amount of NO_2^- in cell supernatants was measured spectrophotometrically by the Griess reaction. Briefly, samples were supplemented with 276 mU nitrate reductase and 40 μM $NADPH^+$ and then allowed to react with the Griess reagent (aqueous solution of 1% sulfanylamide and 0.1% naphthylethylendiamine dihydrochloride in 2.5% H_3PO_4) to form a stable chromophore absorbing at 546 nm wavelength. The values were obtained by comparison with reference concentrations of sodium nitrite and expressed as net amounts of nitrite (nmol) per mg of protein (Salvemini et al., 1991). The protein concentrations (mg ml^{-1}) were determined by the Lowry method using BSA as standard (Lowry et al., 1951).

Evaluation of PGE_2 production. Prostaglandin E_2 levels were determined by a competitive enzyme immunoassay kit in guinea-pig mast cell supernatants obtained as described and added with 10 μM indomethacin to inhibit the formation of COX products. In some experiments, the cells were pretreated for 30 min with 100 nM SR144528, a CB_2 blocker, 10 μM indomethacin, an unselective COX blocker, and 100 nM rofecoxib, a selective COX-2 blocker, before 30 min incubation with the tested substances (2AG and CP55,940) or with medium alone. Each sample was assayed in triplicate and the values were normalized with

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respect to protein concentrations. The protein concentrations (mg ml^{-1}) were determined as previously reported. The values were expressed as pg of PGE_2 per mg of protein.

Determination of intracellular calcium concentration ($[Ca^{2+}]_i$). Isolated mast cells were suspended in a buffer of the following composition: HEPES 20 mM; NaCl 127 mM; KCl 50 mM; glucose 0.1 mg ml^{-1}; BSA 1%, adjusted to pH 7.4 and were loaded with 3 mM FURA 2-AM for 1 h in a shaking water bath at 37°C. At the end of the incubation, mast cells were centrifuged at 500 x *g* (15 min, 4°C) and the supernatant was discarded. Cells were then washed twice with the buffer previously reported, aliquoted into the samples and incubated at 37°C in a shaking water bath for 30 min with the drugs under study. When an antagonist was used, cells were preincubated for 30 min with the antagonist and then for a further 30 min with the agonist. Cytosolic-free Ca^{2+} levels were determined spectrofluorimetrically using a Shimadzu DR 15 spectrofluorimeter (Osaka, Japan), which allows the measurement of both peak and plateau values. The fluorescence excitation spectrum was scanned at wavelengths ranging from 300 to 420 nm, with an emission wavelength fixed at 510 nm. The values of cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) were calculated by a computer program using the following equation: $[Ca^{2+}]_i = K_d[(F-F_{min}) / (F_{max}-F)]$, where F_{min} and F_{max} are the fluorescence values at very low and very high Ca^{2+} concentration, respectively. F_{min} was obtained by measuring fluorescence in the presence of 8 mM EGTA and F_{max} was obtained by measuring fluorescence in digitonin-lysed samples. A K_d value of 224 nM was used for the apparent dissociation constant of FURA 2-AM (Ndisang et al., 1999).

Radioimmunoassay of cyclic nucleotides. The concentrations of cGMP was determined by means of radioimmunoassay using ^{125}I-labelled cyclic nucleotides as previously described (Ndisang et al., 1999). A suspension of 10^5 guinea-pig mast cells in the presence of 10^{-4} 3'-isobutyl-1-methylxanthine (IBMX) was diluted with Krebs buffer having the following

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composition: NaCl 137 mM; KCl 2.7 mM; $NaHCO_3$ 11.9 mM; Na_2HPO_4 0.3 mM; $MgSO_4$ 0.8 mM; glucose 5.6 mM and $CaCl_2$ 1 mM. 10 μM indomethacin was added to the samples to inhibit the formation of COX products. At the end of the experiment, 500 μl 10% *w/v* of trichloroacetic acid (TCA) were added to each sample containing mast cells and the cyclic nucleotides were extracted from TCA with 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane. Finally, the samples were acetylated with acetic anhydride and the amounts of cGMP in the aqueous phase were measured by radioimmunoassay. The values were expressed as fmol of cGMP mg^{-1} protein. The protein concentrations were determined as previously described.

Western blotting iNOS and COX. Guinea-pig mast cells obtained as described were incubated for 60 min with CP55,940 to evaluate the expression of inducible NOS (iNOS) and COX (COX-1 and -2). The cells were lysed in ice-cold buffer (NaCl 0.9%, TRIZMA-HCl 20 mM pH 7.6, triton X-100 0.1%, phenylmethylsulfonylfluoride (PMSF) 1 mM, leupeptin 0.01%) and centrifuged at 10,000 x *g* (10 min, 4°C). The protein content of the supernatants was determined as previously described. The cell lysate was mixed 1:1 with sample buffer (TRIZMA-HCl 20 mM pH 6.8, glycerol 20%, sodium dodecyl sulfate (SDS) 2%, mercaptoethanol 5%, and bromophenol blue 0.025%) and boiled (Pang and Hoult, 1997). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8% and 5% acrylamide for the separating and stacking gel, respectively. Proteins were transferred to nitrocellulose membrane. Blots were blocked with Blocker BSA 5% solution in phosphate-buffered saline (Pierce) and incubated overnight at 4°C with the primary antibodies. Blots were further incubated with secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature and, finally, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 min and exposed to CL-Xposure Film. The HCT116 cell line served as positive control for iNOS (Jenkins et al, 1994) and the HT29 cell

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line served as a positive control for COX-1 and COX-2 (Liu et al, 2003). Quantitative analysis was performed by means of Scion Image beta 4.02 freeware software (Scion Corporation, Frederick, MA, USA, http://www.scioncorp.com); all values were normalised to actin and to the respective basal levels.

Statistical methods. Statistical analysis was performed using SPSS statistic software (release 11.0; SPSS Inc., Chicago, IL, USA). Groups were compared using Student's t test for unpaired values or the Kruskal Wallis h test followed by the Mann-Whitney u test, when appropriate; p values equal to or less than 0.05 were considered statistically significant.

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Results

The release of histamine from actively sensitised mast cells after *in vitro* challenge with antigen was unaffected by endogenous CB_1 and CB_2 receptor ligands such as AEA (1 nM – 1 μM) and 2AG (1 nM – 1 μM), while it was only partially decreased by palmitoyl-ethanolamide (1 nM – 1 μM) (data not shown), confirming the data of Lau and Chow (2003) on rat peritoneal mast cells. However, when the incubation was carried out in the presence of PMSF (100 μM, 30 min preincubation), AEA and 2AG were effective in decreasing the immunological release of histamine, showing that preserving the hydrolysis of cannabinoids by blocking the fatty acid amide hydrolase with PMSF was crucial to study their effects. In fact, in the presence of PMSF, 2AG decreased the immunological release of histamine, starting at 1 nM and in a concentration-dependent manner (Figure 1, panel a). The effect was dose-dependently reduced by the CB_2 blocker SR144528 (10 nM and 100 nM, 30 min preincubation) and left unchanged by AM251 (100 nM, 30 min preincubation), a selective antagonist at CB_1 receptors, suggesting that the down-regulation of antigen-induced histamine release was mediated by CB_2 receptors (Figure 1, panel a). The participation of CB_2 receptors was strengthened by the observation that compound CP55,940 (10 nM-1 μM), an agonist at CB receptors (Iversen and Chapman, 2002), mimicked the inhibitory action of the endogenous ligand, and this effect was abrogated by SR144528, a selective CB_2 receptor antagonist and not by AM251 (Figure 1, panel b). PMSF, SR144528, nor DMSO modified the basal and the antigen-induced histamine levels (data not shown).

When the incubation with 2AG (1 nM - 100 nM) was carried out in the presence of L-NAME (10 μM, 30 min preincubation), an inhibitor of NO synthase, the decrease in the release of histamine was significantly reduced, and was reinstated in the presence of the physiological substrate L-arginine (10 μM), suggesting that NO participates in the down-regulation of the

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immunological activation of mast cells (Figure 2, panel a). Accordingly, L-NAME also reduced the inhibitory effect of the CB_2 receptor agonist CP55,940 (10 nM – 1 μM) and L-arginine reinstated the inhibitory effect (Figure 2, panel b). Neither L-NAME nor L-arginine were capable of modifying the basal and antigen-induced histamine release. Consistent with these effects is the observation that 2AG (10 nM – 1 μM) determined a concentration-dependent generation of NO from mast cells, as shown by the evaluation of the nitrite production, which was abated by preincubating the cells with L-NAME (10 μM, 30 min preincubation; Figure 3, panel a). The iNOS selective inhibitor 1400W (100 nM, 30 min preincubation) also significantly reduced nitrite generation from mast cells, suggesting an involvement of the inducible isoform of NOS (Figure 3, panel a). Interestingly, also CP55,940 (1 nM – 1 μM) increased the nitrite production from mast cells in a way which was abrogated by L-NAME (10 μM, 30 min preincubation) or 1400W (100 nM, 30 min preincubation) (Figure 3, panel b). Furthermore CP55,940 (1 μM, 60 min incubation) induced the expression of iNOS protein as shown by Western blot analysis (Figure 3, panels c and d). PMSF, L-NAME or 1400W alone were not capable of modifying the nitrite levels (data not shown).

Manipulation of the synthesis of prostanoids also modulated the effect of endogenous and exogenous cannabinoids on antigen-induced histamine release from mast cells. Indeed, either blocking COX-1 and -2 with indomethacin (10μM, 30 min preincubation) or blocking COX-2 with rofecoxib (100 nM, 30 min preincubation), significantly reduced the inhibition of the release of histamine induced by both 2AG and CP (Figure 4, panels a and b). Indomethacin and rofecoxib did not change basal and antigen-induced release of histamine (data not shown). Incubation of mast cells with 2AG or CP55,940 (1 nM – 1 μM) elicited an increased production of PGE_2; this effect was completely inhibited by the CB_2 selective antagonist SR144528 (100 nM, 30 min preincubation), by indomethacin (10 μM, 30 min preincubation) and rofecoxib (100 nM, 30 min preincubation) (Figure 5, panels a and b). A Western blot analysis showed

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that COX-1 protein was constitutively expressed in guinea-pig mast cells, while COX-2 protein was expressed only after the treatment with CP55,940 (1 μM, 60 min incubation) (Figure 5, panel c).

The release of histamine from mast cells is a calcium-dependent process (Foreman et al., 1977). Accordingly, mast cell stimulation with antigen showed a striking increase in the $[Ca^{2+}]_i$ levels (Figure 6). When mast cells were exposed to 2AG (10 nM) before antigen challenge, a significant decrease in $[Ca^{2+}]_i$ was observed which was abrogated preferentially by SR144528 (100nM, 30 min preincubation) and less consistently by L-NAME (10 μM, 30 min preincubation; Figure 6, panel a). The same results were obtained with the exogenous cannabinoid ligand CP55,940 (1 μM) (Figure 6, panel b). Neither SR144528 nor L-NAME alone were capable of modifying $[Ca^{2+}]_i$ levels (data not shown).

Our previous results have shown that when mast cells from actively sensitised guinea-pigs were challenged with the antigen, there was a significant increase in cAMP levels while cGMP levels were unaffected (Ndisang et al., 1999). In unstimulated mast cells, the exposure to 2AG or to CP55,940 significantly raised cGMP concentrations. The increased levels of cGMP were abated by blocking CB_2 receptors with SR144528 (100 nM, 30 min preincubation), or inhibiting NOS and COX pathways with L-NAME (10 μM, 30 min preincubation), indomethacin (10 μM, 30 min preincubation) and rofecoxib (100 nM, 30 min preincubation; Figure 7). SR144528, L-NAME, indomethacin and rofecoxib alone did not modify the levels of cGMP (data not shown).

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Discussion

The present results demonstrate the putative role of CB_2 receptors, of NO and prostanoids in the modulation of the allergic activation of guinea-pig mast cells *in vitro*. In fact, 2AG and CP55,940 down-regulate the response of mast cells to antigen, as shown by the diminution of the antigen-induced release of histamine. The suppression of histamine release is rescued by SR144528, a selective CB_2 antagonist, by L-NAME, a NOS inhibitor, and by the COX-1 and COX-2 blockers indomethacin and rofecoxib, and is left unchanged by AM251, a selective antagonist at CB_1 receptors. The effect of L-NAME is reversed by an excess of L-arginine, showing that the production of NO is intermediate between the activation of CB_2 receptors and the inhibition of histamine release. Moreover, 2AG and CP55,940 generate NO and PGE_2 from guinea-pig mast cells and promote the expression of iNOS and COX-2 proteins.

These data suggest the presence of functional CB_2 receptors on mast cells. However, the function of CB_2 receptors on mast cells is uncertain. Following the demonstration that palmitoyl-ethanolamide abrogates the peptidergic degranulation of rat mast cells *in vivo* (Aloe et al., 1993), the same Authors have demonstrated that rat peritoneal mast cells express both the gene and the protein of a CB_2 receptor (Facci et al., 1995). These data were criticized on the basis that the secretion of histamine induced by THC in rat mast cells was not antagonized by SR144528 (Bueb et al., 2001). In line with this observation, Lau and Chow (2003) have recently shown that cannabinoid receptor agonists do not modify the immunological histamine release from rat mast cells, although their experiments were carried out in the absence of the inhibition of fatty acid amide hydrolase.

On the other hand, our experiments are in keeping with previous reports on the anti-inflammatory effects of N-acethylethanolamines (Ganley et al., 1958) and the involvement of endocannabinoids in controlling mast cell activation (Aloe et al., 1993). They also suggest that

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the down-regulation of the immunological activation of guinea-pig mast cells afforded by both endogenous (2AG) and exogenous (CP55,940) cannabinoids entails selective CB_2 receptor activation.

However the concentration-dependent inhibitory effect of both compounds could also be attributable to CB_1 receptors, since 2AG and CP55,940 are known to behave as unselective agonists at both receptor subtypes (Barth and Rinaldi-Carmona, 1999). This is not the case, since compound SR144528, a selective CB_2 receptor antagonist, concentration-dependently abated their effects, while compound AM251, a CB_1 receptor antagonist was ineffective. These findings strengthen the prevailing involvement of CB_2 receptors.

The present experiments also show that the stimulation by 2AG and CP55,940 of the G-protein coupled CB_2 receptors could parallel a variety of intracellular events, relevant in understanding the inhibitory effect. In fact, the intracellular signals set in motion by 2AG and CP55,940 entail the production of NO and PGE_2 involving the induction of iNOS and COX-2, the increase in the intracellular levels of cGMP, and the blunting of the antigen-induced increase in intracellular calcium. The link between endocannabinoids and the NO pathway is well established by experiments showing that AEA stimulates the generation of NO in a host of experimental conditions, both *in vivo* and *in vitro* (Stefano et al., 1996; 1997; 1998; Prevot et al., 1998; Fimiani et al., 1999a). The present experiments add further evidence to the relationship between the endocannabinoids and the NO system by showing that 2AG and CP55,940 generate NO from guinea-pig mast cells. Gilchrist et al. (2002) have recently described in rat mast cells the presence of a functioning eNOS system and the ability to produce NO through iNOS upon exposure to various immunological stimuli. In our experiments the use of the iNOS-specific inhibitor 1400W and the Western blot analysis present evidence for a cannabinoid-induced expression of iNOS. Nitrite production seems to be more evident upon treatment with CP55,940 than with 2AG; nevertheless further studies are

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needed to ascertain wether this effect is due to the lability of the endogenous cannabinoid or to different pharmacodynamic properties.

Nitric oxide generated via iNOS induction (Salvemini et al., 1991; Vannacci et al., 2003b) or released by NO donors (Masini et al., 1994) has been shown to inhibit the immunological and non-immunological response of guinea-pig and rat mast cells, even if the mechanism by which NO modulates mast cell activation is still a matter of debate (Brooks et al., 1999). Our previous studies demonstrated an involvement of the guanylate cyclase/cGMP system, and the decrease of the intracellular calcium available for the exocytosis (Ndisang et al., 1999). Also in the present experiments 2AG and CP55,940 increased the intracellular levels of cGMP and blunted the rise of the intracellular calcium induced by antigen challenge. The effects were abrogated by SR144528, by L-NAME and by COX-1 and COX-2 inhibitors, suggesting that the generation of NO and PGE_2 could in turn inhibit the immunological activation of mast cells by raising the intracellular cGMP and decreasing the amount of calcium necessary for the exocytosis (Ndisang et al., 1999).

Less consistent are the links between the prostanoid and cannabinoid systems. In any case, the present experiments clearly show that 2AG and CP55,9440 increase the generation of PGE_2 from mast cells, an effect linked to COX-1 and the induction of COX-2 expression.

Whether the increased generation of PGE_2 has an inhibitory effect on mast cell activation is a debated issue. In rat mast cells PGE_1 inhibits the immunological release of histamine (Kaliner, 1979). In the same cells, using a different secretagogue (compound 48/80), PGE_1 inhibits the release of histamine, although at rather high concentrations (Loeffler et al., 1971). Ennis et al. (1983) have shown that low concentrations of PGD_2 and PGE_1 are without significant effects on the immunological release of histamine from rat peritoneal mast cells, in contrast with the reported inhibition of the anaphylactic histamine release with low doses of PGD_2**.** In addition, it has been shown that high concentrations of PGD_2 inhibit histamine release only in combination with phosphodiesterase inhibition (Wescott and Kaliner, 1981).

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In conclusion, circumstantial evidence suggests that the mechanism by which CB_2 ligands modulate mast cell activation is by generating NO and PGE_2, acting in an autocrine manner in the inhibition of allergic histamine release. As alternative explanation, the activation of CB_2 receptors and the generation of NO and PGE_2 could be separate events. However, also in this case, 2AG may be considered an endogenous inhibitor of mast cell activation, and the CB_2 receptor agonists as potential therapeutic drugs useful for controlling inflammation and modulating tissue immune response.

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References

Aloe L, Leon A and Levi Montalcini R (1993) A proposed autacoid mechanism controlling mastocyte behaviour. *Agents Actions* **39**:C145-C147.

Barth F and Rinaldi Carmona M (1999) The development of cannabinoid antagonists. *Curr Med Chem* **6**:745-755.

Bisogno T, Maurelli S, Melck D, De Petrocellis L and Di Marzo V (1997) Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J Biol Chem* **272**:3315-3323.

Brooks AC, Whelan CJ and Purcell WM (1999) Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. *Br J Pharmacol* **128**:585-590.

Bueb JL, Lambert DM and Tschirhart EJ (2001) Receptor-independent effects of natural cannabinoids in rat peritoneal mast cells in vitro. *Biochim Biophys Acta* **23**;1538(2-3):252-9.

Coffey RG, Yamamoto Y, Snella E and Pross S (1996) Tetrahydrocannabinol inhibition of macrophage nitric oxide production. *Biochem Pharmacol* **52**:743-751.

Ennis M, Robinson C and Dollery CT (1983) Action of 3-isobutyl-1-methylxanthine and prostaglandins D_2 and E_1 on histamine release from rat and guinea pig mast cells. *Int Arch Allergy Appl Immunol* **72**:289-293.

Facci L, Dal Toso R, Romanello S, Buriani A, Skaper SD and Leon A (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc Natl Acad Sci U. S. A* **92**:3376-3380.

Fimiani C, Liberty T, Aquirre AJ, Amin I, Ali N and Stefano GB (1999a) Opiate, cannabinoid, and eicosanoid signaling converges on common intracellular pathways nitric oxide coupling. *Prostaglandins Other Lipid Mediat* **57**:23-34.

Fimiani C, Mattocks D, Cavani F, Salzet M, Deutsch DG, Pryor S, Bilfinger TV and Stefano GB (1999b) Morphine and anandamide stimulate intracellular calcium transients in human arterial endothelial cells: coupling to nitric oxide release. *Cell Signal* **11**:189-193.

Foreman JC, Hallett MB and Mongar JL (1977) The relationship between histamine secretion and ^{45}calcium uptake by mast cells. *J Physiol* **271**:193-214.

Ganley O, Graessle O and Robinson H (1958) Antiinflammatory activity of compound obtained from egg yolk, peanut oil and soybean lecithin. *Lab Clin Med* **51**:709-714.

Gardner B, Zhu LX, Sharma S, Tashkin DP and Dubinett SM (2003) Methanandamide increases COX-2 expression and tumor growth in murine lung cancer. *FASEB J.* **17**:2157-2159.

Gilchrist M, Savoie M, Nohara O, Wills FL, Wallace JL and Befus AD (2002) Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J Leukoc Biol* **71**:618-624.

Guhring H, Hamza M, Sergejeva M, Ates M, Kotalla CE, Ledent C and Brune K (2002) A role for endocannabinoids in indomethacin-induced spinal antinociception. *Eur J Pharmacol* **454**:153-163.

Iversen L and Chapman V (2002) Cannabinoids: a real prospect for pain relief? *Curr Opin Pharmacol* **2**:50-55.

Jenkins DC, Charles SA, Baylis SA, Lelchuk R, Radomski MW and Moncada S (1994) Human colon cancer cell lines show a diverse pattern of nitric oxide synthase gene expression and nitric oxide generation. *Br J Cancer* **70**:847-849.

Kaliner M (1979) Human lung tissue and anaphylaxis: cyclic nucleotide response and prostaglandin synthesis. *Monogr Allergy* **14**:119-121.

Klein TW, Friedman H and Specter S (1998) Marijuana, immunity and infection. *J Neuroimmunol* **83**:102-115.

Kozak KR, Rowlinson SW and Marnett LJ (2000) Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J Biol Chem* **275**:33744-33749.

Lau AH and Chow SS (2003) Effects of cannabinoid receptor agonists on immunologically induced histamine release from rat peritoneal mast cells. *Eur J Pharmacol* **464**:229-235.

Liu Q, Chan STF and Mahendran R (2003) Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. *Carcinogenesis* **24**:637-642.

Loeffler LJ, Lovenberg W and Sjoerdsma A (1971) Effects of dibutyryl-3',5'-cyclic adenosine monophosphage, phosphodiesterase inhibitors and prostaglandin E_1 on compound 48-80-induced histamine release from rat peritoneal mast cells in vitro. *Biochem Pharmacol* **20**:2287-2297.

Lowry O, Rosebrough N, Farr A and Randall P (1951) Protein measurement with the foin-phenol reagent. *J Biol Chem* **193**:265-275.

Masini E, Di Bello MG, Pistelli A, Raspanti S, Gambassi F, Mugnai L, Lupini M and Mannaioni PF (1994) Generation of nitric oxide from nitrovasodilators modulates the release of histamine from mast cells. *J Physiol Pharmacol* **45**:41-53.

Nahas GG, Suciu-Foca N, Armand JP and Morishima A (1974) Inhibition of cellular mediated immunity in marihuana smokers. *Science* **183**:419-420.

Ndisang JF, Gai P, Berni L, Mirabella C, Baronti R, Mannaioni PF and Masini E (1999) Modulation of the immunological response of guinea pig mast cells by carbon monoxide. *Immunopharmacology* **43**:65-73.

Pang L and Hoult JR (1997) Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E_2 and other cyclic AMP stimulants in J774 macrophages. *Biochem Pharmacol* **53**:493-500.

Prevot V, Rialas CM, Croix D, Salzet M, Dupouy JP, Poulain P, Beauvillain JC and Stefano GB (1998) Morphine and anandamide coupling to nitric oxide stimulates GnRH and CRF release from rat median eminence: neurovascular regulation. *Brain Res* **790**:236-244.

Ramer R, Brune K, Pahl A and Hinz B (2001) R(+)-methanandamide induces cyclooxygenase-2 expression in human neuroglioma cells via a non-cannabinoid receptor-mediated mechanism. *Biochem Biophys Res Commun* **286**:1144-1152.

Salvemini D, Masini E, Pistelli A, Mannaioni P F and Vane J (1991) Nitric oxide: a regulatory mediator of mast cell reactivity. *J Cardiovasc Pharmacol* **17**:S258-S264.

Samson MT, Small-Howard A, Shimoda LM, Koblan-Huberson M, Stokes AJ and Turner H (2003) Differential Roles of CB1 and CB2 Cannabinoid Receptors in Mast Cells. *J Immunol* **170**:4953-4962.

Someya A, Horie S and Murayama T (2002) Arachidonic acid release and prostaglandin F(2alpha) formation induced by anandamide and capsaicin in PC12 cells. *Eur J Pharmacol* **450**:131-139.

Stefano GB, Liu Y and Goligorsky MS (1996) Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia, and human monocytes. *J Biol Chem* **271**:19238-19242.

Stefano GB, Salzet B and Salzet M (1997) Identification and characterization of the leech CNS cannabinoid receptor: coupling to nitric oxide release. *Brain Res* **753**:219-224.

Stefano GB, Salzet M, Magazine HI and Bilfinger TV (1998) Antagonism of LPS and IFN-γ induction of iNOS in human saphenous vein endothelium by morphine and anandamide by nitric oxide inhibition of adenylate cyclase. *J Cardiovasc Pharmacol* **31**:813-820.

Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, Suhara Y, Takayama H and Waku K (2000) Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB_2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J Biol Chem* **275**:605-612.

Vannacci A, Giannini L, Masini E and Mannaioni PF (2003a) Cannabinoid-induced activation of ERK and AKT in mast cells may be mediated by intracellular NO production. *J Immunol* **171**:2767.

Vannacci A, Passani MB, Pierpaoli S, Giannini L, Mannaioni PF and Masini E (2003b) Nitric oxide modulates the inhibitory effect of cannabinoids on the immunological activation of guinea pig mast cells. *Inflamm Res* **52**:S07-S08.

Vannacci A, Zagli G, Marzocca C, Pierpaoli S, Passani MB, Mannaioni PF and Masini E (2002) Down-regulation by cannabinoids of the immunological activation of human basophils and guinea pig mast cells. *Inflamm Res* **51**:S09-S10.

Wescott S and Kaliner M (1981) The effects of histamine and prostaglandin D2 on rat mast-cell cyclic AMP and mediator release. *J Allergy Clin Immunol* **68**:383-391.

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Footnotes

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Legends for figures

Figure 1. 2-arachidonylglycerol (2AG, panel a) and CP55,940 (CP, panel b) in the presence of 100 μM phenylmethylsulfonylfluoride reduced in a concentration-dependent way the release of histamine from actively sensitised guinea-pig mast cells after ovalbumin (OA, 100 μg ml^{-1}) challenge. The effect was prevented by SR144528 (SR, 10nM and 100nM, 30 min preincubation) and left unchanged by AM251 (AM, 100 nM, 30 min preincubation). Data are expressed as mean ± s.e.mean of 6 experiments performed in duplicate. $^{\#\#}p<0.01$ vs OA; $^{**}p<0.01$ vs 2AG or CP.

Figure 2. 2-arachidonylglycerol (2AG, panel a) and CP55,940 (CP, panel b) in the presence of 100 μM phenylmethylsulfonylfluoride reduced in a concentration-dependent way the release of histamine from actively sensitised guinea-pig mast cells after ovalbumin (OA, 100 μg ml^{-1}) challenge in a concentration-dependent way. The effect was prevented by L-NAME (10 μM, 30 min preincubation) and reinstated in the presence of L-arginine (L-arg, 10 μM). Data are expressed as mean ± s.e.mean of 6 experiments performed in duplicate. $^{\#\#}p<0.01$ vs OA; $^{**}p<0.01$ vs 2AG or CP.

Figure 3. 2-arachidonylglycerol (2AG, panel a) and CP55,940 (CP, panel b) in the presence of 100 μM phenylmethylsulfonylfluoride increased the generation of nitrite by guinea-pig mast cells in a concentration-dependent way. The effect was prevented by L-NAME (10 μM, 30 min preincubation) or 1400W (100 nM, 30 min preincubation). Data are expressed as mean ± s.e.mean of 6 experiments performed in triplicate. $^{\#\#}p<0.01$ vs basal and 2AG + L-NAME; $^{*}p<0.05$ vs 2AG.

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CP55,940 (CP, 1 μM, 60 min incubation) increased the expression of inducible NOS (iNOS) protein evaluated by Western blot analysis (HCT116 cell line served as positive control for iNOS; panel c), as shown by densitometric analysis (panel d). Data are normalized to actin and to basal levels and expressed as the mean ± s.e.mean of 3 experiments; **$p<0.01$ vs basal.

Figure 4. 2-arachidonylglycerol (2AG, 10nM, panel a) and CP55,940 (CP, 1μM, panel b) in the presence of 100 μM phenylmethylsulfonylfluoride reduced the amount of histamine released from actively sensitised guinea-pig mast cells after ovalbumin (OA, 100 μg ml^{-1}) challenge. The effect was prevented pretreating the cells for 30 min with indomethacin (INDO, 10 μM) and rofecoxib (ROFE, 100 nM). Data are expressed as mean ± s.e.mean of 6 experiments performed in duplicate. **$p<0.01$ vs 2AG alone or CP alone.

Figure 5. 2-arachidonylglycerol (2AG, panel a) and CP55,940 (CP, panel b) in the presence of 100 μM phenylmethylsulfonylfluoride increased the generation of PGE_2 from guinea-pig mast cells. The effect was prevented pretreating the cells for 30 min with SR144528 (SR, 100 nM), indomethacin (INDO, 10 μM) and rofecoxib (ROFE, 100 nM). Data are expressed as mean ± s.e.mean of 6 experiments performed in duplicate. $^{\#\#}p<0.01$ vs basal; **$p<0.01$ vs 2AG alone or CP alone.

CP55,940 (CP, 1 μM, 60 min preincubation) increased the expression of inducible COX (COX-2) protein while COX-1 expression was unchanged, as shown by Western blot analysis (HT29 cell line served as positive control for COX-1 and COX-2; panel c) and densitometric analysis (panel d). Data are normalized to actin and to basal levels and expressed as the mean ± s.e.mean of 3 experiments; $^{\S}p<0.05$ vs basal.

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Figure 6. 2-arachidonylglycerol (2AG, panel a) and CP55,940 (CP, panel b) in the presence of 100μM phenylmethylsulfonylfluoride blunted the increase of $[Ca^{2+}]_i$ levels in guinea-pig mast cells challenged with ovalbumin (OA, 100 μg ml^{-1}). The effect was abolished preincubating the cells for 30 min with SR144528 (SR, 100nM) and L-NAME (10 μM). $^{\#\#}p<0.01$ vs OA; **$p<0.01$ vs 2AG alone or CP alone; § $p<0.05$ vs SR.

Figure 7. 2-arachidonylglycerol (2AG, white bars) and CP55,940 (CP, grey bars) in the presence of 100 μM phenylmethylsulfonylfluoride increased the levels of cGMP in guinea-pig mast cells. The effect was prevented preincubating the cells for 30 min with SR144528 (SR, 100nM), L-NAME (10 μM), indomethacin (INDO, 10 μM) and rofecoxib (ROFE, 100 nM). Data are expressed as mean ± s.e.mean of 6 experiments performed in duplicate. $^{\#\#}p<0.01$ vs basal; **$p<0.01$ vs 2AG alone or CP alone.

Figure 1

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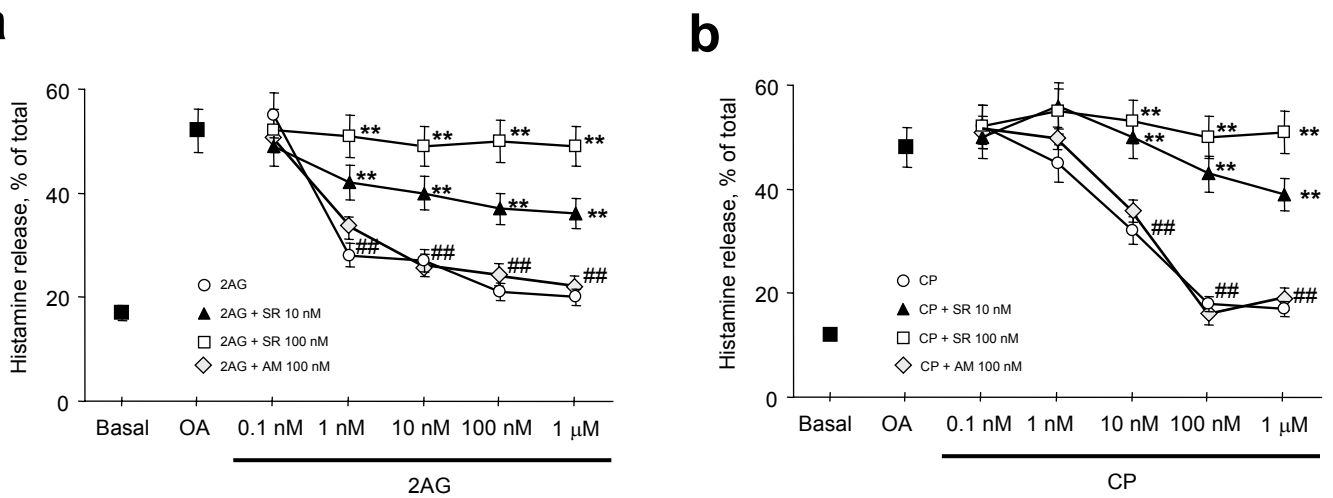

Figure 2

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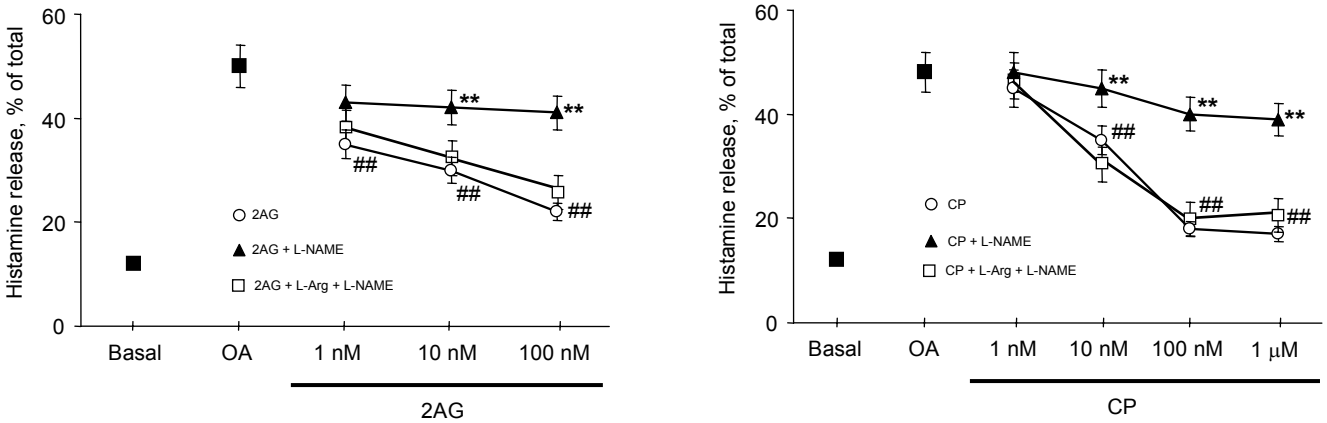

Figure 3

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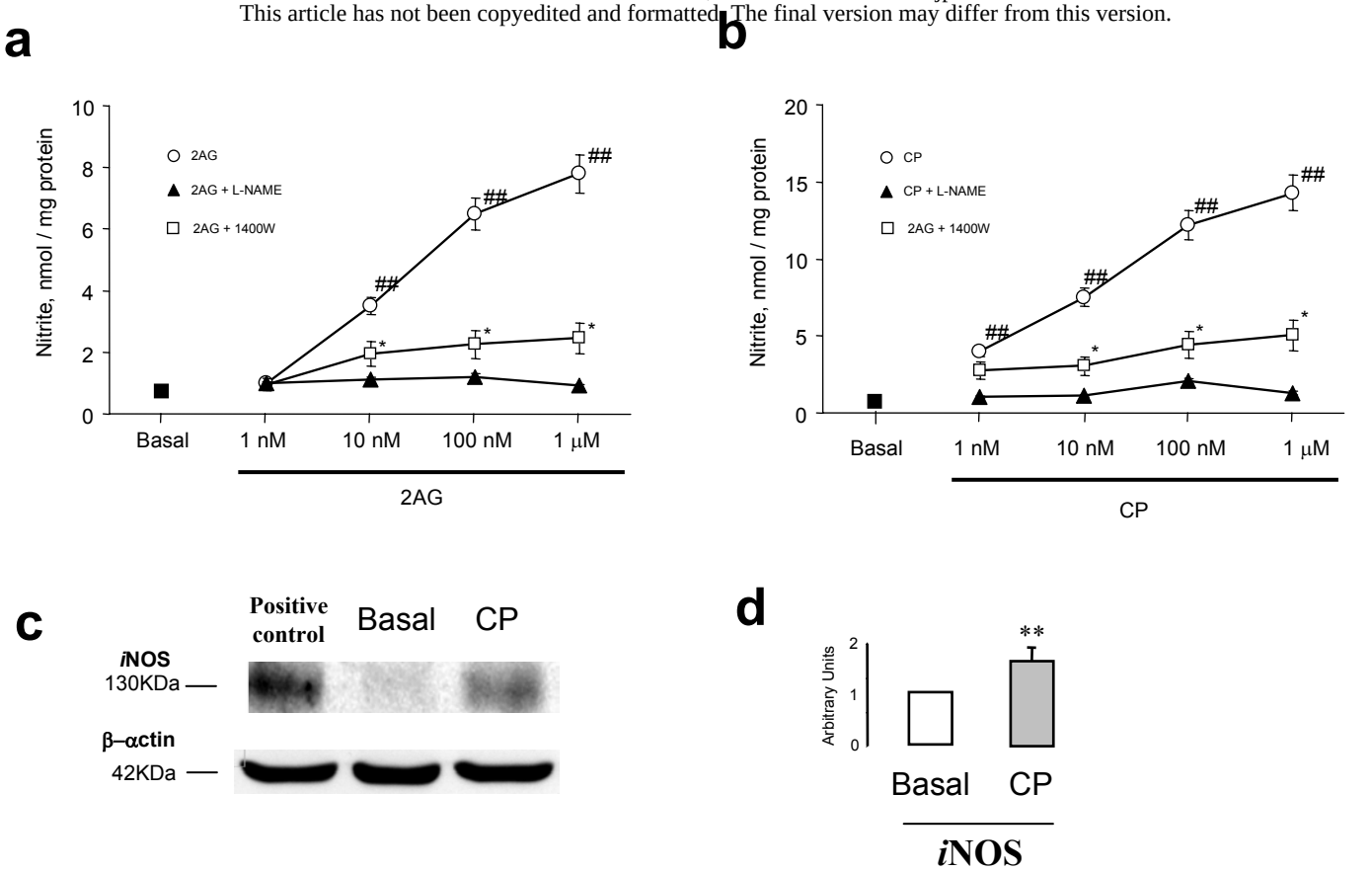

Figure 4

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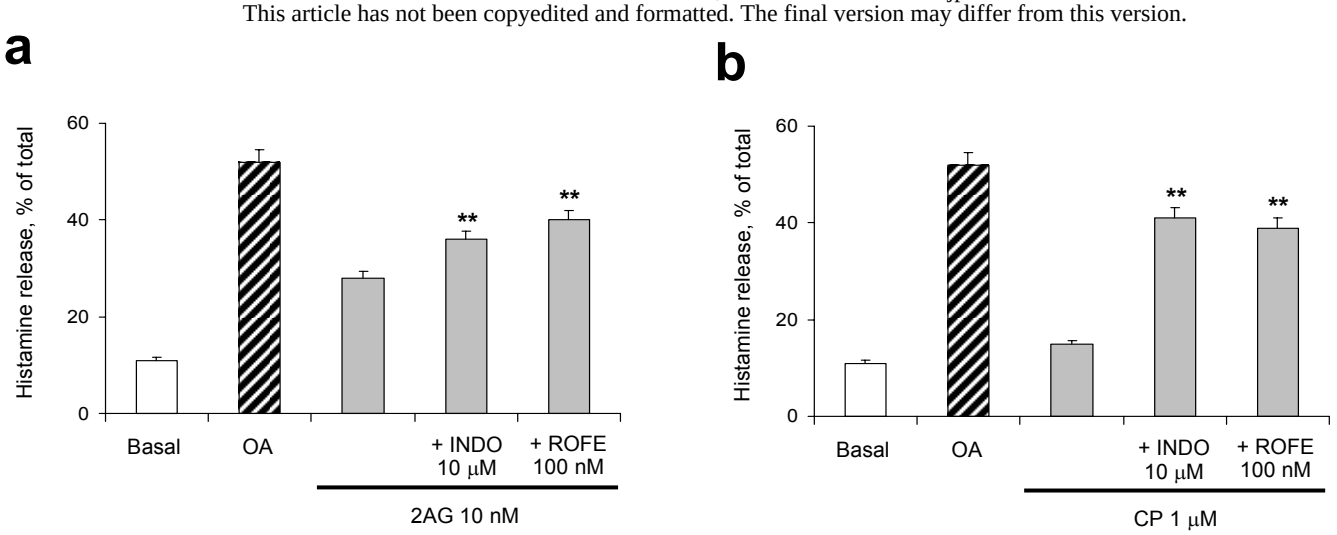

Figure 5

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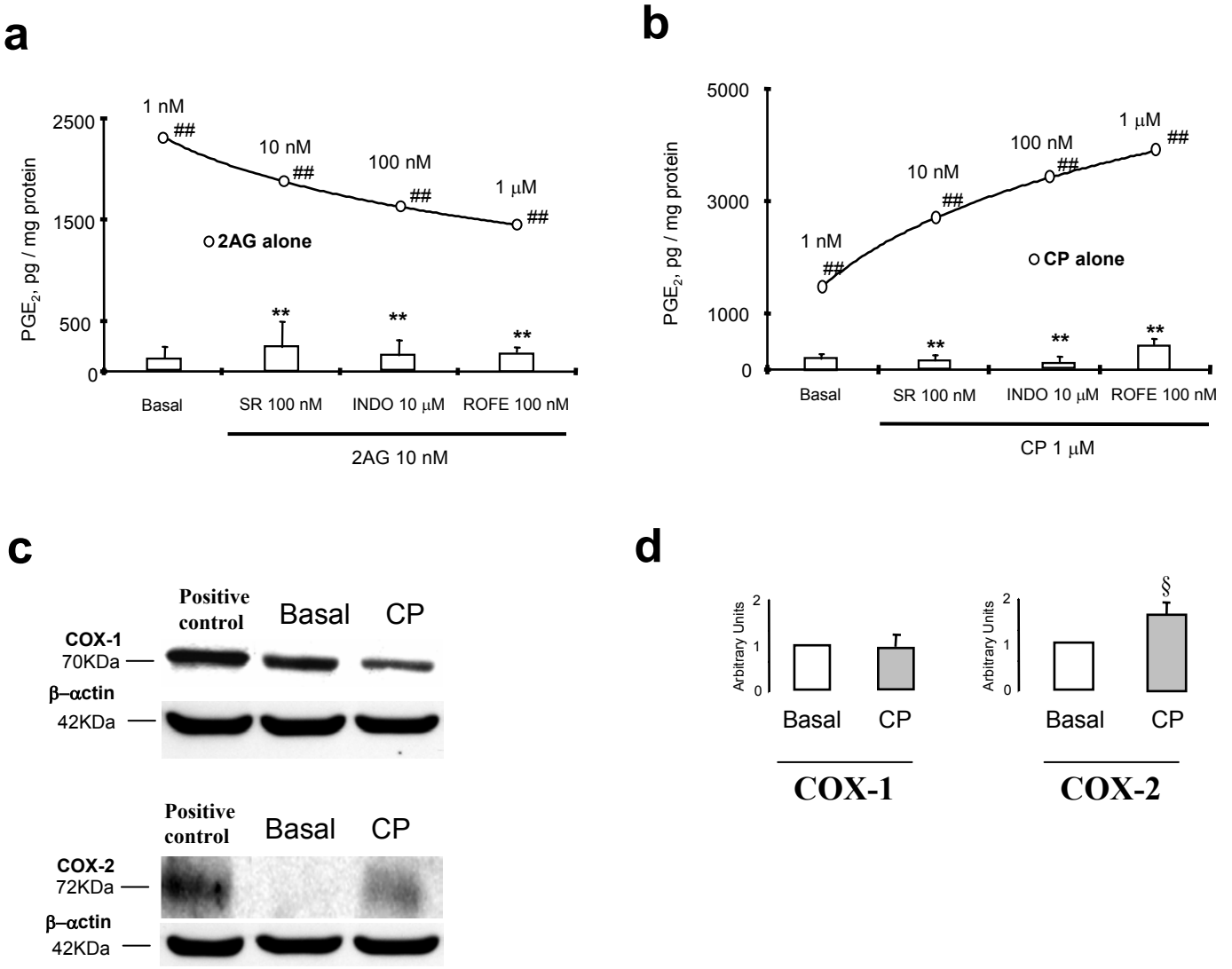

Figure 6

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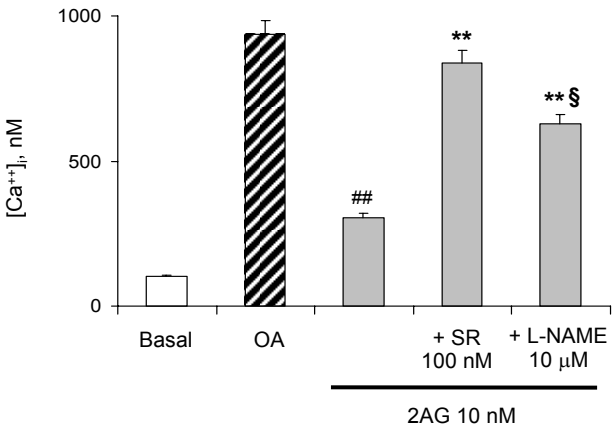
a



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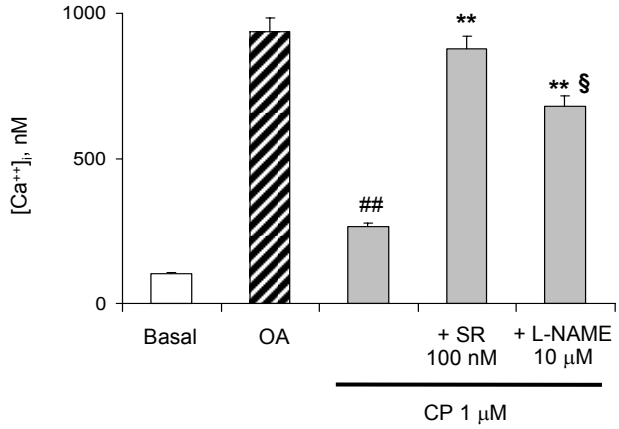

Figure 7

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