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# EVIDENCE FOR CANNABINOID RECEPTOR-DEPENDENT AND – INDEPENDENT MECHANISMS OF ACTION IN LEUKOCYTES *

Barbara L. Faubert Kaplan, Cheryl E. Rockwell and Norbert E. Kaminski

Department of Pharmacology and Toxicology and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan, 48824-1317

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Corresponding author: Department of Pharmacology and Toxicology, 315 National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824. Tel: 517-353-3786; Fax: 517-432-3218; e-mail: kamins11@msu.edu.

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Abbreviations: NF-AT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; PMA/Io, phorbol ester plus calcium ionophore; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorphenyl)-4-methyl-H-pyrazole-3 carboxyamidehydrochloride; SR144528, N-[(1S)-endo-1,3,3,-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN-2 (WIN 55212-2), R (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone mesylate; WIN-3 (WIN 55212-3), S (-)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-napthanlenyl) methanone mesylate; $[Ca^{+2}]_i$, intracellular calcium concentration; $[Ca^{+2}]_e$, extracellular calcium concentration

Abstract

Cannabinoids exhibit immunosuppressive actions that include inhibition of interleukin-2 production in response to a variety of T cell activation stimuli. Traditionally, the effects of these compounds have been attributed to cannabinoid receptors, CB1 and CB2, both of which are expressed in mouse splenocytes. Therefore, SR141716A, a CB1 antagonist, and SR144528, a CB2 antagonist, were utilized to investigate the role of cannabinoid receptors in the cannabinoid-induced inhibition of phorbol ester plus calcium ionophore (PMA/Io)-stimulated interleukin-2 production by mouse splenocytes. PMA/Io-stimulated interleukin-2 production was inhibited by cannabinol, cannabidiol and both WIN 55212-2 stereoisomers with a rank order potency of WIN 55212-2 • cannabidiol > WIN 55212-3 • cannabinol. Cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2 was not attenuated by the presence of both SR144528 and SR141716A. Using pertussis toxin to address the role of G protein-coupled receptors in this response, it was determined that pertussis toxin treatment did not attenuate cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2. With the demonstration that cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2 was not mediated via CB1 or CB2, alternative targets of cannabinoids in T cells were examined. Specifically, it was demonstrated that cannabinoids elevated intracellular calcium concentration in resting splenocytes and that the cannabinol-induced elevation in intracellular calcium concentration was attenuated by treatment with both SR144528 and SR141716A. Interestingly, pretreatment of splenocytes with agents that elevate intracellular calcium concentration inhibited PMA/Io-stimulated interleukin-2 production, suggesting that an elevation in intracellular calcium concentration might be

involved in the mechanism of interleukin-2 inhibition. These studies suggest that immune modulation produced by cannabinoids involves multiple mechanisms, which might be both cannabinoid receptor-dependent and –independent.

## Introduction

Cannabinoids are plant-derived molecules that produce a wide variety of biological effects of which the most extensively studied have been on the central nervous system and immune system. Specifically in the immune system, cannabinoids modulate innate and acquired (humoral, cell-mediated) immunity (reviewed in Berdyshev, 2000). With respect to acquired immunity, modulation of interleukin-2 production is one of the more sensitive targets of cannabinoids. It is interesting that cannabinoids either enhance or inhibit interleukin-2 secretion, depending on the experimental conditions, including age of the animals and magnitude of cellular activation (Nakano, *et al.*, 1993; Nakano, *et al.*, 1992; Jan and Kaminski, 2001). Using an optimum stimulatory concentration of PMA/Io, cannabinol treatment inhibited interleukin-2 protein secretion by splenic T cells (Condie et al., 1996). This inhibition correlated with a decrease in expression of phosphorylated ERK MAPK (Faubert and Kaminski, 2000). Furthermore, it was determined that cannabinol inhibited transcription factor binding to the proximal AP-1 and distal NF-AT motifs in the interleukin-2 promoter (Condie et al., 1996; Faubert and Kaminski, 2000; Yea et al., 2000), which might also contribute to the inhibition of interleukin-2. Thus, while the mechanism of cannabinoid-induced inhibition of interleukin-2 in PMA/Io-stimulated splenocyte is understood to occur via inhibition of transcriptional activation of interleukin-2 (Yea et al., 2000), the upstream signals contributing to inhibition of transcription have not been completely elucidated. Likewise, the role of cannabinoid receptors in the inhibition of interleukin-2 by cannabinoid treatment has remained elusive.

The identification of cannabinoid receptors provided a putative mechanism for cannabinoid-mediated effects (Matsuda et al., 1990; Munro et al., 1993). Cannabinoid receptors, both CB1 and CB2, belong to the G protein-coupled family of receptors and can couple negatively to adenylate cyclase (reviewed in Matsuda, 1997). In addition, both receptors have been shown to activate ERK MAPK in cannabinoid receptor-transfected cell lines. The CB1 receptor has also been shown to couple negatively to N- and Q-type calcium channels and positively to inwardly rectifying and A-type potassium channels (reviewed in Matsuda, 1997). CB1 expression is highest in the CNS but has also been detected in several peripheral tissues (including most immune system cells), whereas CB2 expression is highest in cells of the immune system (Bouaboula et al., 1993; Galiegue et al., 1995; Schatz et al., 1997). It is for that reason that many of these studies were conducted with cannabinol, a ligand that exhibits 10-fold greater affinity for the CB2 receptor as compared to CB1 (Munro et al., 1993).

Synthesis of cannabinoid receptor antagonists, SR141716A and SR144528, which antagonize actions at CB1 and CB2, respectively (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998), allowed for the determination of cannabinoid receptor function for many of the effects of these compounds. This is particularly true for the CB1 receptor in that many of the cannabinoid-induced CNS effects were prevented by SR141716A treatment (reviewed in Matsuda, 1997). Furthermore, use of CB1 receptor knockout mice confirmed that the CB1 receptor mediated many of the CNS effects produced by cannabinoids (catalepsy, hypomotility, hypothermia) (Ledent et al., 1999; Steiner et al., 1999; Zimmer et al., 1999). CB1 is present in many immune cells at relatively low levels and there are a few instances in which CB1 was determined to mediate immune system effects of cannabinoids (reviewed in Berdyshev, 2000). On the

other hand, CB2 is believed not to play a role in the CNS effects of cannabinoids (Griffin et al., 1999; Buckley et al., 2000), yet has been shown to be important for immune system effects, particularly for cannabinoid-induced inhibition of antigen processing and presentation in macrophages (McCoy et al., 1999; Buckley et al., 2000). Therefore, the objective of these studies was to investigate the mechanism, including the role of CB1 and CB2, of cannabinoid-induced inhibition of interleukin-2 production in PMA/Io-stimulated mouse splenocytes.

Methods

*Cannabinoid compounds* - SR144528, SR141716A, cannabidiol and cannabinol were provided by the National Institute on Drug Abuse. WIN 55212-2 and WIN 55212-3 were purchased from Sigma (St. Louis, MO).

*Animals* - Pathogen-free female B6C3F1 mice, 6 weeks of age, were purchased from Charles River Breeding. On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage) and quarantined for one week. Mice were given food (Purina Certified Laboratory Chow) and water *ad lib*. Mice were not used for experimentation until their body weight was 17-20 g. Animal holding rooms were maintained at 21-24°C and 40-60% relative humidity with a 12 h light/dark cycle.

*Preparation of lymphocyte culture* - Mice were sacrificed and spleens or thymi were aseptically removed. Single cell suspensions ($5x10^6$ cells/ml) were prepared and cells were cultured in RPMI 1640 supplemented with 100 units penicillin/ml, 100 units of streptomycin/ml, 50 μM 2-mercaptoethanol, and 2% Bovine Calf Serum.

*Interleukin-2 ELISA* – Splenocyte were either treated with antagonist or vehicle for 30 min at 37°C, followed by cannabinoid agonist treatment for 30 min at 37°C. Alternatively, splenocytes were treated with ionomycin, A23187 or thapsigargin for 30 min at 37°C. The cells were then treated with 40 nM PMA/0.5 μM ionomycin for 24 h in media containing 2% Bovine Calf Serum in 48-well culture plates at 0.5 ml/well. Cells were harvested and supernatants were collected and assayed in triplicate for

interleukin-2 using an ELISA. Briefly, 96-well plates were coated for 1 h at 37°C with a purified rat anti-mouse interleukin-2 antibody (Pharmingen, San Diego, CA), followed by blocking with 3% BSA in PBST for 30 min at 37°C. Supernatants from splenocytes were incubated for 1 h at 37°C, followed by addition of biotinylated anti-mouse interleukin-2 antibody (Pharmingen, San Diego, CA) for 1 h at RT. Streptavidin peroxidase was then incubated for 1 h at RT. Finally, TMB substrate and 6N $H_2SO_4$ was added for color development and samples were read with a Bio-Tek EL-808 plate reader at 450 nm. Samples were quantified from a standard curve prepared with interleukin-2 standard (mouse recombinant interleukin-2; Pharmingen, San Diego, CA).

*cAMP determinations* – Splenocyte were suspended in 5 ml of 2% BCS and treated with Gey's solution (5 ml/spleen). The splenocytes were swirled on ice for 5 min in order to lyse the red blood cells, followed by 2 washes in 2% Bovine Calf Serum. Splenocyte were resuspended in RPMI 1640 supplemented with 1 mg/ml fatty-acid poor bovine serum albumin (Calbiochem, La Jolla, CA). Cells in 1 ml aliquots of cells were treated in triplicate with cannabinoid agonist for 30 min at 37°C. The cells were then treated with 25 μM forskolin (Sigma, St. Louis, MO) for 15 min at 37°C. The extraction, release, and quantitation of cAMP from cells were performed using cAMP assay kits (Diagnostic Products Inc., Los Angeles, CA). For pertussis toxin treatment, cells were either treated with pertussis toxin (100 ng/ml, Sigma) or not treated for 24 h prior to subsequent agonist treatment.

*Calcium determination* - Splenocyte were treated with Gey's solution in order to lyse the red blood cells. Cells were then washed twice in $Ca^{2+}$-KREB buffer (129 mM NaCl,

5 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 2.8 mM glucose, 0.2% BSA). Intracellular calcium concentration was determined by measuring the fluorescence of fura-2 dye, which is dually excited at 340 nm and 380 nm. Fura-2 AM dye (1 μM, Molecular Research Products, Eugene, OR) was added to the cells and incubated for 30 min at RT in the dark. Cells were harvested, washed three times with $Ca^{2+}$-KREB buffer to remove extracellular fura-2 dye, and readjusted to $5x10^6$ cells/ml in $Ca^{2+}$-KREB buffer. Cells were stored at RT in the dark until used. Cells were placed in a 3 ml quartz cuvette with constant stirring. Calcium determinations were performed at RT with a Spex 1681 0.22 Spectrometer with dual excitation at 340 and 380 nm and emission at 510 nm (all slit widths were 1 mm). Intracellular calcium concentration calculations were based on maximum and minimum calcium values, as assessed with use of 0.1% Triton-X and 250 mM EGTA, respectively. The dissociation constant for the fura-2-calcium complex was $1.45 \times 10^{-7}$. For studies conducted in the absence of extracellular calcium, the KREB buffer was prepared as above without $CaCl_2$ and supplemented with 0.02 mM EGTA.

*Statistical analysis* – The mean ± S.E. was determined by a parametric analysis of variance for each treatment group. When significant differences were detected, treatment groups were compared with the appropriate control with the Dunnett's two-tailed *t* test (Dunnett, 1955).

Results

*Effect of cannabinoid receptor antagonists on cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2 production* – Previous work from this laboratory has established that cannabinoids inhibited PMA/Io-stimulated interleukin-2 production by splenocytes, thymocytes and EL-4 T cells under optimum activating conditions (Condie et al., 1996; Herring and Kaminski, 1999; Yea et al., 2000). Thus, a characterization of the putative involvement of the cannabinoid receptors in cannabinoid-mediated inhibition of interleukin-2 production using splenocytes was conducted. It is notable that splenocytes express mRNA for both cannabinoid receptors (Schatz et al., 1997); thus; the CB1 and CB2 receptor antagonists were used in combination in these studies. As presented in figure 1, interleukin-2 was readily induced by PMA/Io treatment and was inhibited by cannabinol (1-20 μM) in a concentration-dependent manner. These studies were conducted with μM concentrations of cannabinoid compounds as it has been previously demonstrated that these concentrations are required *in vitro* due to both the lipophilicity of the cannabinoids and the serum concentration-dependence for many of the cannabinoid effects (Faubert and Kaminski, 2000). Pretreatment of splenocytes with a combination of antagonists (0.5 μM/0.5 μM, 2.5 μM /2.5 μM and 5 μM/5 μM SR144528/SR141716A) did not significantly attenuate the cannabinol-mediated inhibition of interleukin-2 production at any cannabinol concentration tested. The antagonists alone did not significantly affect the levels of PMA/Io-stimulated interleukin-2 production at concentrations up to 0.5 μM/0.5 μM SR144528/SR141716A. However, it is notable that at higher concentrations, including 2.5 μM /2.5 μM and 5

μM/5 μM of SR144528/SR141716A, the antagonist alone inhibited PMA/Io-induced interleukin-2 production by at least 30% as compared to the vehicle controls. (Fig. 1, inset). In other experiments, SR144528/SR141716A at higher concentrations (2.5 μM /2.5 μM and 5 μM/5 μM) produced an even greater decrease in interleukin-2 production indicating that cells cultured for extended periods of time (24 h) in the presence of these antagonists were functionally affected by both of these agents. No evidence of inverse agonist activity was observed with either antagonist. The estimated $IC_{50}$ values for cannabinol were 15.4 μM in the absence of antagonists and 13.3 μM, 18.1 μM, and 16.8 μM in the presence of 0.5 μM/0.5 μM, 2.5 μM/2.5 μM. and 5 μM/5 μM SR144258/SR141716A, respectively.

*Effect of pertussis toxin on cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2 production* – Due to the inability of the cannabinoid antagonists to attenuate the cannabinol-induced inhibition of interleukin-2 production, pertussis toxin was utilized to examine the potential role of $G\alpha_i/G\alpha_o$ protein involvement in this effect. Pertussis toxin (100 ng/ml) pretreatment for 24 h resulted in an increase (29.0%) in forskolin-stimulated cAMP production in splenocytes, which was statistically different from calcium release-activated calcium channel-stimulated splenocytes in the absence of pertussis toxin (data not shown). However, pertussis toxin pretreatment (10 and 100 ng/ml) did not attenuate the cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2 production (Fig. 2). The estimated $IC_{50}$ values for interleukin-2 inhibition by cannabinol were 16.3 μM in the absence of pertussis toxin and 27.7 μM and 17.9 μM in the presence of 10 ng/ml and 100 ng/ml pertussis toxin, respectively. Pertussis toxin

did not significantly modulate interleukin-2, with the exception that there was a modest but consistent increase in basal interleukin-2 secreted from the cells that were treated with 100 ng/ml pertussis toxin (approximately 10-15 units/ml; Fig. 2, inset).

*Effect of cannabinoid receptor antagonists on WIN-induced inhibition of PMA/Io-stimulated interleukin-2 production* – Another characteristic of a receptor-dependent mechanism is differential effects with stereoisomers. One such cannabinoid stereoisomer pair are the synthetic compounds, WIN 55212-2 and WIN 55212-3. Both WIN 55212-2 ("active" isomer) and WIN 55212-3 ("inactive" isomer) inhibited PMA/Io-induced interleukin-2 production in a concentration-dependent manner; however, WIN 55212-2 demonstrated robust, and consistently greater, inhibition than observed with WIN 55212-3 (Fig. 3A and B). The $IC_{50}$ values for WIN-induced inhibition of interleukin-2 were 3.3 μM for WIN 55212-2 and 14.8 μM for WIN 55212-3. Interestingly, WIN 55212-3 inhibited interleukin-2 as robustly as cannabinol, a ligand that exhibits selectivity for the CB2 receptor. Furthermore, the antagonists (0.5 μM/0.5 μM, 2.5μM/2.5μM, or 5 μM/5 μM SR144528/SR141716A) did not attenuate the inhibition of interleukin-2 by either WIN isomer (Fig. 3A and B). In the presence of SR141716A/SR144528, the $IC_{50}$ values were as follows: 3.9 μM, 5.2 μM, and 4.1 μM for WIN 55212-2 in the presence of 0.5 μM/0.5 μM, 2.5μM/2.5μM, or 5 μM/5 μM SR144528/SR141716A, respectively; 14.9 μM, 15.1 μM, and 12.2 μM for WIN 55212-3 in the presence of 0.5 μM/0.5 μM, 2.5μM/2.5μM, or 5 μM/5 μM SR144528/SR141716A, respectively.

*Effect of cannabinoid receptor antagonists on cannabidiol-induced inhibition of PMA/Io-stimulated interleukin-2 production* – In light of the above results, cannabidiol, another plant-derived cannabinoid was utilized to assess the role of cannabinoid receptors in cannabinoid-induced interleukin-2 inhibition. Cannabidiol was previously determined to exhibit low-affinity for the CB2 receptor in HL-60 cells (Munro et al., 1993) and did not inhibit forskolin-stimulated cAMP production in CB1-transfected CHO cells (Matsuda et al., 1990). Collectively, these previous studies indicate that cannabidiol exhibits low affinity for both CB1 and CB2. In the present studies we show that in splenocytes, cannabidiol (1-20 μM) robustly inhibited PMA/Io-stimulated interleukin-2 production with an $IC_{50}$ value of 4.1 μM and this inhibition was not attenuated by 0.5 μM/0.5μM or 5 μM/5 μM SR144528/SR141716A (Fig. 4). The $IC_{50}$ values for cannabidiol-induced inhibition in the presence of the antagonists were 2.9 μM and 2.4 μM for 0.5 μM/0.5 μM and 5 μM/5 μM concentrations of the SR144582/SR141716A, respectively. It was notable that the magnitude of inhibition of interleukin-2 by cannabidiol was greater than that of cannabinol, in spite of the fact that cannabinol possesses greater CB1 and CB2 affinity.

*Cannabinol elevated intracellular calcium concentration in resting splenocytes* - With the demonstration that CB1 and CB2 were not involved in cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2, other potential T cell targets were examined. A major target of cannabinoids in T cells is NF-AT (Faubert and Kaminski, 2000; Yea et al., 2000), a critical transcription factor in the regulation of interleukin-2 that is tightly controlled by changes in $[Ca^{+2}]_i$ (reviewed in (Cantrell, 1996). Therefore,

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generation of the calcium signal seemed a likely point of inhibition by cannabinoid compounds. However, these studies revealed that cannabinol treatment elevated $[Ca^{+2}]_i$ for at least 30 min in resting splenocytes (Fig. 5A). While this sustained elevation by cannabinol in $[Ca^{+2}]_i$ was consistent with other agents that increase $[Ca^{+2}]_i$, such as ionomycin and thapsigargin, the shapes of the elevation as well as the overall magnitude of elevation in $[Ca^{+2}]_i$ was different for cannabinol versus ionomycin or thapsigargin (Fig. 5B and C).

*Effect of zero-extracellular calcium concentration on cannabinol-induced elevation in* $[Ca^{+2}]_i$ – In several cell types, thapsigargin is a powerful tool used to determine whether the elevation in $[Ca^{+2}]_i$ is due to an intracellular store release. However, as demonstrated in figure 5C, thapsigargin treatment of resting splenocytes induced a sustained elevation in $[Ca^{+2}]_i$, which was likely due to the activation of cannabinol to subsequently stimulate $[Ca^{+2}]_e$ influx (Zweifach and Lewis, 1993). This sustained elevation prohibited the use of thapsigargin to determine directly whether cannabinol-induced elevation of $[Ca^{+2}]_i$ was due to depletion of intracellular stores. Thus, studies were performed in the presence and absence of extracellular calcium in order to determine whether the cannabinol-induced elevation in extracellular calcium was due to influx of extracellular calcium. The calcium response induced by cannabinol was drastically reduced when the studies were conducted in the absence of $[Ca^{+2}]_e$, indicating the elevation of $[Ca^{+2}]_i$ by cannabinol occurred primarily via influx of extracellular calcium although a modest increase in $[Ca^{+2}]_i$ by cannabinol was still observed (Fig. 6A). Ionomycin-stimulated elevation in $[Ca^{+2}]_i$ was used to confirm the absence of extracellular calcium in the buffer (Fig. 6B). As expected for ionomycin, the first phase was primarily due to

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release of intracellular stores of calcium which was not affected by the zero-extracellular calcium concentration conditions, and the second phase was due to influx of extracellular calcium which was abolished under these conditions (Cantrell, 1996).

*Effect of cannabinoid receptor antagonists on cannabinol-induced elevation in $[Ca^{+2}]_i$* – Induction of $[Ca^{+2}]_i$ elevation with cannabinoid treatment has been demonstrated in a number of other cell types in a cannabinoid receptor-dependent manner (Sugiura et al., 1999; Sugiura et al., 2000). Cannabinol-induced $[Ca^{+2}]_i$ elevation in resting splenocytes was mediated primarily via an influx of extracellular calcium, also suggesting a putative role for membrane-bound receptors. In light of these previous findings, SR144528 and SR141716A were utilized to examine the involvement of CB1 and CB2 in cannabinol-induced elevation of $[Ca^{+2}]_i$ in resting splenocytes and thymocytes. Studies were conducted in thymocytes in order to verify that the elevation in $[Ca^{+2}]_i$ also occurred in a preparation that consisted predominantly of T cells. Cannabinol-induced elevation in $[Ca^{+2}]_i$ was markedly attenuated by both cannabinoid receptor antagonists (either separately or in combination) in resting splenocytes (Table 1). Treatment with either SR141716A and/or SR144528, in the absence of cannabinol, did not significantly elevate the $[Ca^{+2}]_i$ (data not shown). Similar to splenocytes, cannabinol treatment elevated $[Ca^{+2}]_i$ in resting thymocytes. As thymocytes do not express CB1 mRNA (Schatz et al., 1997), only the CB2 antagonist, SR144528, was utilized in order to address the putative role of the CB2 receptor in cannabinol-induced elevation in $[Ca^{+2}]_i$ in thymocytes. As presented in Table 2, SR144528 partially attenuated the cannabinol-induced elevation in $[Ca^{+2}]_i$. Interestingly, in thymocytes only, cannabinol plus SR144528 treatment induced an elevation in $[Ca^{+2}]_i$ (approximately 40 nM) independent

of the cannabinol concentration used, suggesting that SR144528 was acting as a partial agonist.

*Agents that elevate $[Ca^{+2}]_i$ inhibited PMA/Io-stimulated interleukin-2 production* – Results from the above studies suggest that cannabinol elevated $[Ca^{+2}]_i$ in resting splenocytes and thymocytes in a cannabinoid receptor-dependent manner. A number of previous studies have reported that a premature elevation in $[Ca^{+2}]_i$ results in inhibition of T cell activation (Gallichio et al., 1994; Nghiem et al., 1994). Indeed, pretreatment of splenocytes with ionomycin, over a wide concentration range, for 30 min prior to activation resulted in a drastic inhibition of interleukin-2 production. This result was confirmed with two other agents that elevate $[Ca^{+2}]_i$, A23187 and thapsigargin (Fig. 7). The concentrations used for both ionomycin and thapsigargin are in the same range as those used in the $[Ca^{+2}]_i$ determination studies, providing a correlation between the magnitude of $[Ca^{+2}]_i$ elevation and the magnitude of interleukin-2 inhibition.

## Discussion

In the present studies, cannabinoid-mediated inhibition of PMA/Io-stimulated interleukin-2 production in mouse splenocytes was not solely dependent on the presence of CB1 or CB2. The rank order of cannabinoid-induced interleukin-2 inhibition for both potency and efficacy did not correlate with the published $K_i$ values for receptor binding. The rank order potency for cannabinoid agonist-induced inhibition of interleukin-2 in PMA/Io-stimulated splenocytes as assessed by $IC_{50}$ values was: WIN 55212-2 • cannabidiol > WIN 55212-3 • cannabinol. The rank order for efficacy as assessed by the ability of the agonist to produce 100% inhibition of PMA/Io-induced interleukin-2 at the maximum concentration tested was: WIN 55212-2 > cannabidiol > WIN 55212-3 > cannabinol. In splenocytes, the $K_i$ for WIN 55212-2 was lower than that for cannabinol (Schatz et al., 1997). In HL-60 cells, rank order binding activity to the CB2 receptor has been reported to be: $\Delta^9$-THC • cannabinol >> cannabidiol (Munro et al., 1993). In addition, there was a 1000-fold difference in affinity between the WIN stereoisomers in CB2-transfected COS cells (Slipetz et al., 1995). It has also been reported that cannabinol exhibited a 10-fold higher affinity for the CB2 receptor, the predominant cannabinoid receptor expressed in the immune system (Bouaboula et al., 1993; Galiegue et al., 1995; Schatz et al., 1997). Indeed, cannabinol exhibits immunomodulating effects (Condie et al., 1996; Herring et al., 1998; Faubert and Kaminski, 2000; Yea et al., 2000), but, as demonstrated here, so did cannabidiol and WIN 55212-3, both of which exhibit low binding affinity for CB1 and CB2.

In contrast to the data presented here, Buckley, *et al.* determined that $\Delta^9$-THC–induced inhibition of interleukin-2 in $\alpha$-CD3 plus peritoneal macrophage-stimulated T

cells was lost in CB2 receptor knockout mice (Buckley et al., 2000); however, the experimental design was very different from the studies described here. In the present studies, cannabinoid-induced inhibition of interleukin-2 from splenic T cells was measured directly. In the study by Buckley, *et al.*, activated peritoneal macrophages derived from either wild type or CB2 receptor knockout mice were treated with vehicle or $\Delta^9$-THC for 4 h. Subsequently, the macrophages were co-cultured with a helper T cell hybridoma, which produced interleukin-2 in response to the macrophages plus α-CD3. In these studies, the macrophages were utilized as co-stimulators for optimal interleukin-2 production as they presumably express the B7 molecule required to bind the co-stimulatory T cell molecule, CD28 (reviewed in (Harris and Ronchese, 1999). However, the expression level of B7 on the macrophages was not measured in either the wild type or CB2 receptor knockout mice, nor in response to $\Delta^9$-THC treatment. Furthermore, the helper T cell hybridoma that produced interleukin-2 in response to α-CD3 plus peritoneal macrophages was not assayed for cannabinoid receptor expression. These critical issues greatly influence the interpretation of the results. Nevertheless, the authors concluded that the loss of cannabinoid-induced inhibition of interleukin-2 was due to the loss of CB2 in the stimulator macrophages, and not through direct effects on the helper T cell hybridoma (Buckley et al., 2000). Concordant with the aforementioned studies, McCoy, *et al.* demonstrated that the CB2 antagonist was involved in $\Delta^9$-THC-induced inhibition of antigen processing and presentation (McCoy et al., 1999). Again, although interleukin-2 production from T cells was used as an endpoint, the effect by $\Delta^9$-THC was exerted on the macrophages used to stimulate the T cells (not the T cells themselves) (McCoy et al., 1999). Thus, under certain

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experimental conditions, the CB2 receptor appears to be important for antigen processing and/or presentation in macrophages (McCoy et al., 1999; Buckley et al., 2000). However, it is important to emphasize that neither of the two experimental designs critically evaluated the role of CB1 and CB2 in the direct effects that cannabinoid treatment exerts on T cells to inhibit interleukin-2.

The observation that cannabinoids elevated $[Ca^{+2}]_i$ in a cannabinoid receptor-dependent manner is consistent with several other studies (Sugiura et al., 1999; Sugiura et al., 2000). Interestingly, in splenocytes, SR141716A provided more effective antagonism than did SR144528, but both cannabinoid receptor antagonists partially attenuated the cannabinol-induced elevation in $[Ca^{+2}]_i$. In contrast, in thymocytes, CB2 mediated the cannabinol-induced elevation in $[Ca^{+2}]_i$ as SR144528 almost completely attenuated the response to cannabinol. Although the exact mechanism responsible for the elevation in $[Ca^{+2}]_i$ by cannabinoids has not been elucidated, it is tempting to speculate that cannabinoid receptors coupled to $G\alpha_s$ in lymphocytes mediate the cannabinoid-induced elevation in $[Ca^{+2}]_i$. In fact, the ability of cannabinoid receptors to couple to $G\alpha_s$ has been suggested, particularly for CB1 (Glass and Felder, 1997; Maneuf and Brotchie, 1997). Another interesting observation was the gradual rise in $[Ca^{+2}]_i$ induced by cannabinol. Although it is notable that the calcium determinations in this study represent the mean from many cells at any given time point, rather than for a single cells, the gradual rise in $[Ca^{+2}]_i$ could be attributed to at least one of two possibilities. One possible explanation is that cannabinol induced only a partial depletion of intracellular stores. Alternatively, a rapid re-uptake of intracellular calcium occurred concomitantly with the calcium influx. Additional studies will be required to resolve the specific mechanism.

While the cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2 was not attenuated by either the CB1 or CB2 antagonists, the cannabinoid receptor antagonists attenuated (both alone and in combination) the cannabinol-induced elevation in $[Ca^{+2}]_i$ in resting splenocytes. This differential role of the cannabinoid receptors in these two cannabinoid-mediated effects suggests either that the elevation in $[Ca^{+2}]_i$ did not contribute to the cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2 or it is one of several contributing factors. The present investigation suggests the latter since cannabinoids as well as agents that elevated $[Ca^{+2}]_i$ (i.e., ionomycin, A23187 and thapsigargin) inhibited PMA/Io-stimulated interleukin-2 production in splenocytes. In concordance with these observations, T cells become unresponsive (anergic) if they receive an inappropriate or incomplete activation signal. Anergy is a state of unresponsiveness in T cells as characterized by decreased production of interleukin-2, and AP-1 and NF-κB DNA binding activity (reviewed in Maier and Greene, 1998). In fact, T cells become anergic, as characterized by > 90% block in the ability to produce interleukin-2, if prematurely stimulated with the calcium ionophore A23187 (Gallichio et al., 1994). In addition, PMA/Io-induced interleukin-2 reporter gene activity was inhibited in response to an 8 h, 2 μM ionomycin pre-treatment (Nghiem et al., 1994). This suggests that a premature elevation in $[Ca^{+2}]_i$ induces anergy depending on the time of activation of the calcium signal relative to cellular activation and/or the magnitude of the overall elevation in $[Ca^{+2}]_i$. Presently, it is unclear whether the time of generation or magnitude of the calcium signal (or both) contributes to an unresponsive state. Nevertheless, this might explain how the $[Ca^{+2}]_i$ elevation by cannabinoids contributes to the inhibition of interleukin-2 production in PMA/Io-stimulated splenocytes.

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The differential role of the cannabinoid receptors in cannabinoid-induced $[Ca^{+2}]_i$ and cannabinoid-induced interleukin-2 inhibition in PMA/Io-stimulated splenocytes might be due to the requirement for cellular activation in the studies in which interleukin-2 production was measured. The discrepancy might be associated with the observation that cellular activation modulates cannabinoid receptor expression levels in T cells and B cells (Carayon et al., 1998; Noe et al., 2000). The ability of cellular stimulation to increase or decrease cannabinoid receptor expression depends on cell type, cell maturity, and activation stimulus. In T cells, CB1 receptor mRNA expression was decreased in response to PMA/Io or α-CD3 treatment, suggesting that T cell activation inhibits expression of cannabinoid receptors (Noe et al., 2000). This explanation is only plausible under the assumption that the cannabinoid effects are not solely mediated via the cannabinoid receptors. Thus, assuming a minimal role of cannabinoid receptors in cannabinoid-induced inhibition of PMA/Io-stimulated interleukin-2, the CB1 and/or CB2-dependent component would not be detected in activated cells. A second possible explanation for the discrepancy is that these two responses occurred in distinct cell populations in the splenocyte cell preparation. However, this latter scenario seemed unlikely based on the demonstration that SR144528 attenuated the cannabinol-induced $[Ca^{+2}]_i$ elevation in thymocytes, a predominantly T cell preparation.

Another interesting observation was that despite the fact that both WIN isomers inhibited interleukin-2 production, there were consistent differences in efficacy especially at concentrations above 10 μM suggesting that the two isomers differentially affected a specific cellular target, as opposed to a non-specific mechanism of cell perturbation. In addition, the inability of pertussis toxin to attenuate cannabinol-

mediated inhibition of PMA/Io-stimulated interleukin-2 suggests that the cannabinoid receptors might couple to G proteins, other than $G\alpha_i/G\alpha_o$. Again, it has been suggested that CB1 might couple to $G\alpha_s$ (Glass and Felder, 1997; Maneuf and Brotchie, 1997).

Taken together, these data support the notion that cannabinoid-induced inhibition of interleukin-2 production is not solely dependent on the presence of CB1 and CB2 in mouse splenocytes. Furthermore, the present results suggest a strong likelihood of multiple parallel mechanisms for the cannabinoid-induced inhibition of interleukin-2 production based on the observation that cannabinol-induced elevation in $[Ca^{+2}]_i$ was attenuated by the cannabinoid receptor antagonists while the cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2 production was not affected by the antagonists. Collectively, these results point toward the possibility that cannabinoids modulate interleukin-2 expression via a combination of cannabinoid receptor-mediated mechanisms, and CB1/CB2-independent mechanisms.

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Footnotes

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Reprint requests: Norbert E. Kaminski, Ph.D., Department of Pharmacology and Toxicology, Michigan State University, 315 National Food Safety and Toxicology Center, East Lansing, MI 48824-1317

Figure Legends

*Figure 1. Effect of cannabinoid receptor antagonists on cannabinol-induced inhibition of PMA/Io-stimulated IL-2 production.* Splenocyte (5 x $10^6$ cells/ml) were treated with both SR141716A and SR144528 or 0.1% DMSO vehicle for 30 min followed by cannabinol treatment for 30 min. Cells were stimulated with 40 nM PMA/0.5 µM ionomycin for 24 hours. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was • 80% for all treatment groups as assessed by trypan blue exclusion. * $p < 0.05$ as compared to the vehicle (VH) group, ** $p < 0.05$ as compared to the SR 0.5µM/0.5µM + VH group, ‡ $p < 0.05$ as compared to the SR 2.5µM/2.5µM + VH group, # $p < 0.05$ as compared to the 5µM/5µM + VH group. Results are representative of four separate experiments with three replicates per treatment group.

*Figure 2. Effect of pertussis toxin on cannabinol-induced inhibition of PMA/Io-stimulated interleukin-2 production.* Splenocyte (5x$10^6$ cells/ml) were treated with 10 or 100 ng/ml pertussis toxin for 24 h. The cells were harvested, washed, and resuspended to a cell density of 5x$10^6$ cells in 2% Bovine Calf Serum. Cells were treated with either cannabinol or 0.1% ethanol vehicle for 30 min followed by 40 nM PMA/0.5 µM ionomycin for 24 h. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Average stimulation with PMA/Io was 948 ± 184 units IL-2/ml. Cellular viability was • 80% for all treatment groups as assessed by trypan blue exclusion. * $p < 0.05$ as compared to the vehicle (VH) group; ** $p < 0.05$ as compared to the pertussis toxin 10 ng/ml + VH group; ‡ $p < 0.05$ as compared to the

pertussis toxin 100 ng/ml + VH group. Results represent five separate experiments with three replicates per treatment group. One of the representative graphs is shown in the inset.

*Figure 3. Effect of the cannabinoid receptor antagonists on inhibition of PMA/Io-stimulated interleukin-2 production mediated by the WIN stereoisomers.* Splenocyte (5 x $10^6$ cells/ml were treated with both SR141716A and SR144528 or 0.1% DMSO vehicle for 30 min followed by either A.) WIN55,212-2 or B.) WIN55,212-3 treatment for 30 min. Cells were stimulated with 40 nM PMA/0.5 μM ionomycin for 24 hours. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. Cellular viability was • 80% for all treatment groups as assessed by trypan blue exclusion. * $p < 0.05$ as compared to the vehicle (VH) group, ** $p < 0.05$ as compared to the SR 0.5μM/0.5μM + VH group, ‡ $p < 0.05$ as compared to the SR 2.5μM/2.5μM + VH group, # $p < 0.05$ as compared to the 5μM/5μM + VH group. Results are representative of two separate experiments with three replicates per treatment group.

*Figure 4. Effect of cannabinoid receptor antagonists on CBD-induced inhibition of PMA/Io-stimulated interleukin-2 production.* Splenocyte were treated with both SR141716A and SR144528 (0.5 μM/0.5 μM or 5 μM/5 μM) for 30 min followed by CBD treatment for 30 min. Cells were stimulated with PMA/Io (40 nM/0.5 μM) for 24 h. Cells were stimulated with 40 nM PMA/0.5 μM ionomycin for 24 h. Average

stimulation with PMA/Io was 1639 ± 342 units IL-2/ml. Cellular viability was • 80% for all treatment groups as assessed by trypan blue exclusion. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the VH group. ** $p < 0.05$ as compared to the SR (0.5 μM/0.5 μM) + VH group; ‡ $p < 0.05$ as compared to the SR (5 μM/5 μM) + VH group. Results represent three separate experiments with three replicates per treatment group. One of the representative graphs is shown in the inset.

*Figure 5. Cannabinol, ionomycin, and thapsigargin elevate intracellular calcium concentration in resting splenocytes.* Splenocyte were loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in $Ca^{2+}$-KREB buffer to remove excess fura-2-AM dye from the buffer. Three ml of cells were added to a quartz cuvette and calcium concentration was determined. Cannabinol (A.), ionomycin (B.) or thapsigargin (C.) were added by glass syringe to the cuvette at either 50 or 300 s and the increase in intracellular calcium concentration was measured for 1800 s total. Maximum and minimum values were obtained with 0.1% Triton-X at 1600 s and 0.5 mM EGTA at 1700 s, respectively. Intracellular calcium concentration was calculated from the change in the ratio of bound to free calcium. Results represent at least two separate experiments.

*Figure 6. Effect of zero-extracellular calcium concentration on cannabinol- and ionomycin-induced elevation in intracellular calcium concentration.* Splenocyte were

loaded with fura-2-AM dye for 30 min at RT in the dark. Cells were harvested and washed three times in $Ca^{2+}$-KREB buffer to remove excess fura-2-AM dye from the buffer. Cells were resuspended in either buffer that contained or did not contain extracellular calcium concentration. Assays were conducted as described in figure 6 with cannabinol (A.) or ionomycin (B.). Results represent three separate experiments.

*Figure 7. Inhibition of PMA/Io-stimulated interleukin-2 production by ionomycin, A23187 and thapsigargin.* Splenocyte were treated with ionomycin, A23187, or thapsigargin followed by activation with PMA/Io (40 nM/0.5 μM) for 24 h. For the ionomycin -treated samples, the concentrations of ionomycin are in addition to that given in combination with PMA (i.e., 0.5 μM plus the indicated concentrations). Cellular viability was • 80% for all treatment groups as assessed by trypan blue exclusion. The supernatants were harvested and interleukin-2 production was measured by ELISA analysis. * $p < 0.05$ as compared to the vehicle group. VH is 0.1% DMSO. Results represent three separate experiments with three replicates per treatment group.

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**TABLE 1**

**Summary of effects of cannabinoid receptor antagonists on CBN-induced elevation intracellular calcium in resting SPLC***

| | | CBN 15 μM + SR144528 (μM) | | |
|---|---|---|---|---|
| VH | CBN 15 μM | 1 | 5 | 10 |
| 17 | 222 | 207 | 124 | 124 |

| | | CBN 15 μM + SR141716A (μM) | | |
|---|---|---|---|---|
| VH | CBN 15 μM | 1 | 5 | 10 |
| 17 | 190 | 184 | 112 | 60 |

| | | CBN 15 μM + SR144528 (μM)/SR141716A (μM) | | |
|---|---|---|---|---|
| VH | CBN 15 μM | 0.5/0.5 | 2.5/2.5 | 5/5 |
| 20 | 206 | 197 | 147 | 117 |

* values are $\Delta [Ca^{2+}]_i$ as measured from base to peak.

Results are representative of two separate experiments.

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**TABLE 2**

**Summary of effects of cannabinoid receptor antagonists on CBN-induced elevation in intracellular calcium in resting THMC***

| | | CBN (μM) | | |
|---|---|---|---|---|
| | VH | 5 | 10 | 15 |
| CBN (μM) | 8 | 27 | 81 | 108 |
| CBN (μM) + SR144528 10 μM | 23 | 49 | 45 | 39 |

* values are $\Delta [Ca^{2+}]_i$ as measured from base to peak.

Results are representative of two separate experiments.

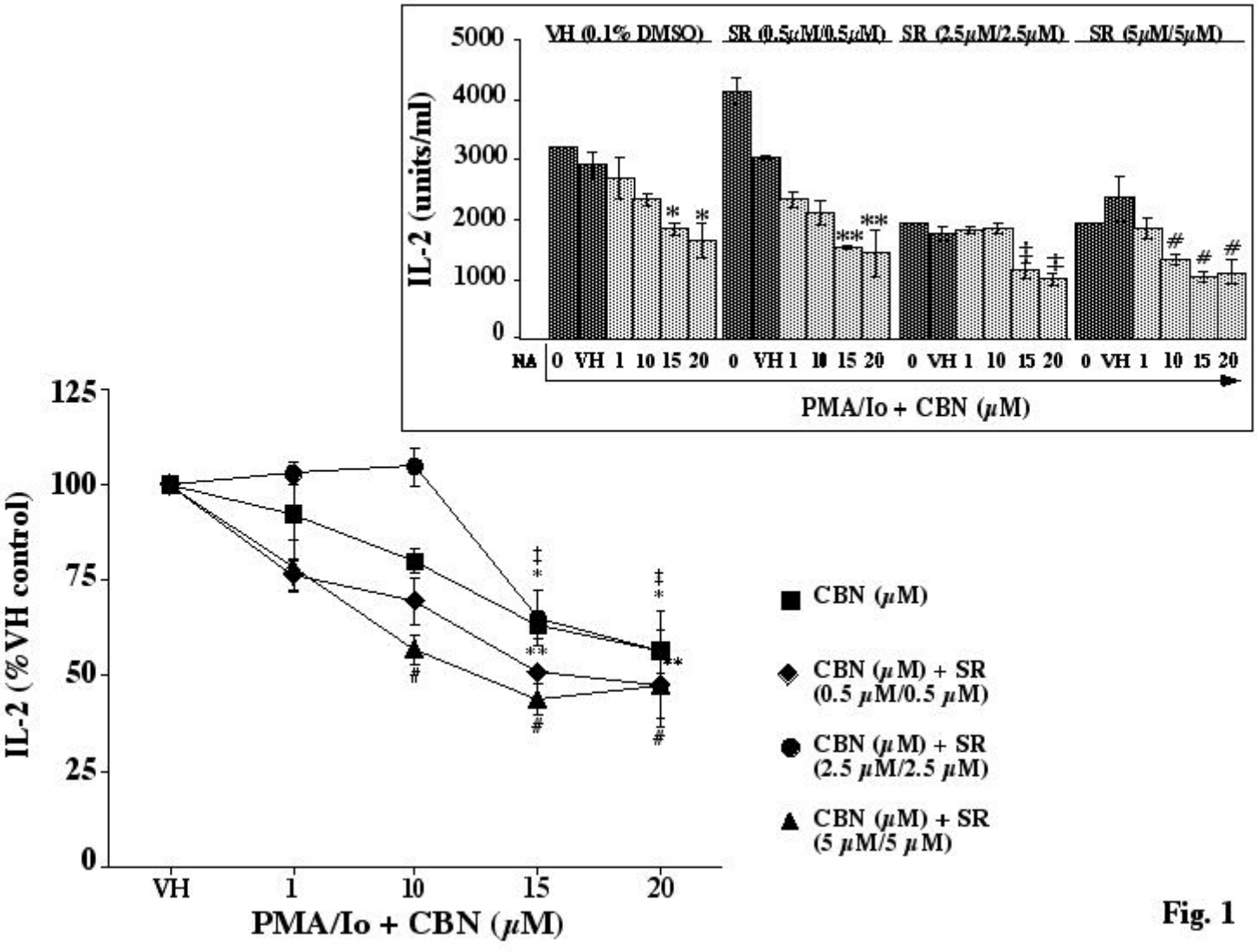
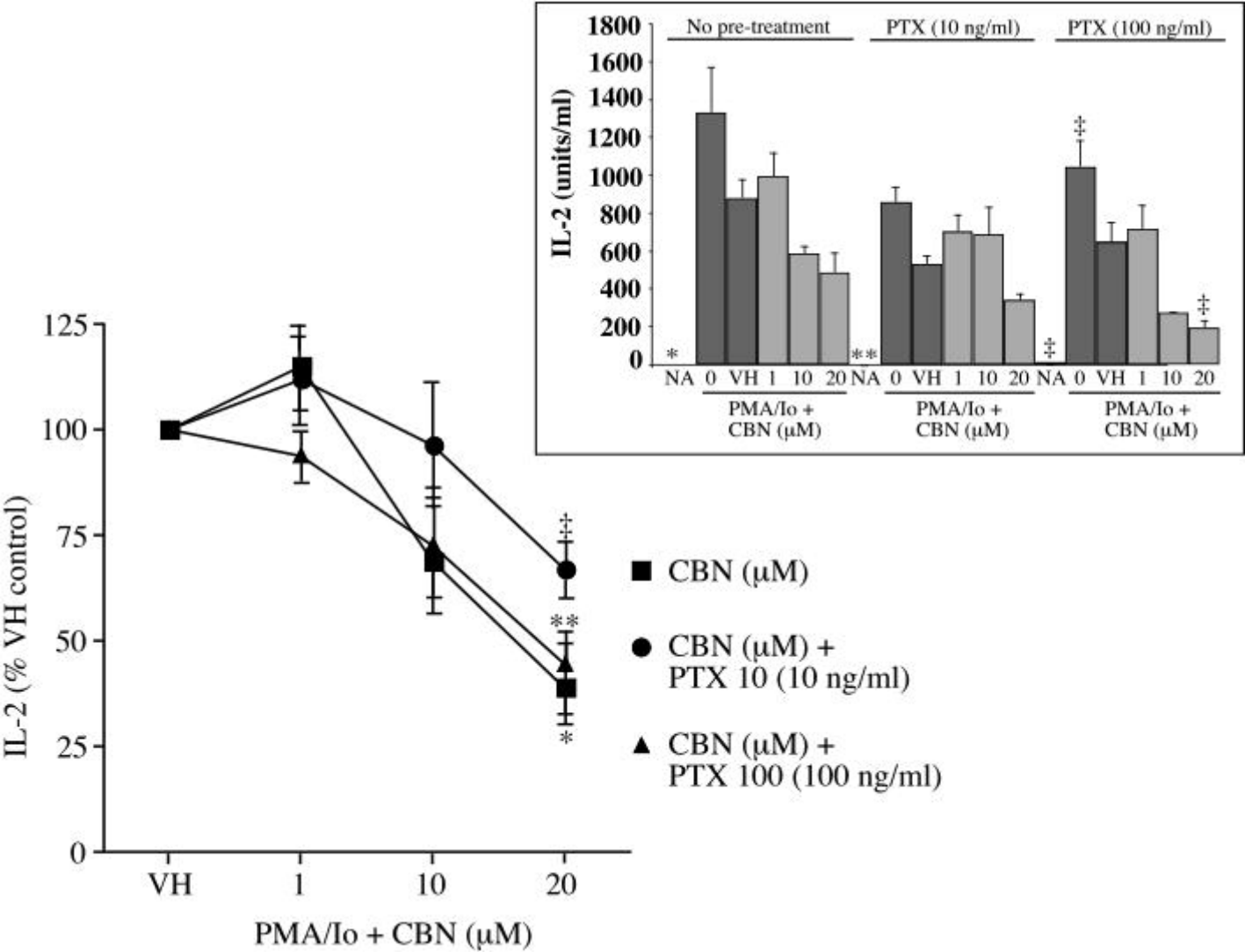

VH (0.1% DMSO)
SR (0.5μM/0.5μM)
SR (2.5μM/2.5μM)
SR (5μM/5μM)
IL-2 (units/ml)
5000
4000
3000
2000
1000
0
NA
0 VH 1 10 15 20 0 VH 1 10 15 20 0 VH 1 10 15 20 0 VH 1 10 15 20
PMA/Io + CBN (μM)
IL-2 (% VH control)
125
100
75
50
25
0
VH
1
10
15
20
PMA/Io + CBN (μM)
CBN (μM)
CBN (μM) + SR (0.5 μM/0.5 μM)
CBN (μM) + SR (2.5 μM/2.5 μM)
CBN (μM) + SR (5 μM/5 μM)

Fig. 1


IL-2 (units/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
No pre-treatment
PTX (10 ng/ml)
PTX (100 ng/ml)
NA 0 VH 1 10 20 NA 0 VH 1 10 20 NA 0 VH 1 10 20
PMA/Io + CBN (μM)
PMA/Io + CBN (μM)
PMA/Io + CBN (μM)
IL-2 (% VH control)
125
100
75
50
25
0
VH
1
10
20
PMA/Io + CBN (μM)
CBN (μM)
CBN (μM) + PTX 10 (10 ng/ml)
CBN (μM) + PTX 100 (100 ng/ml)

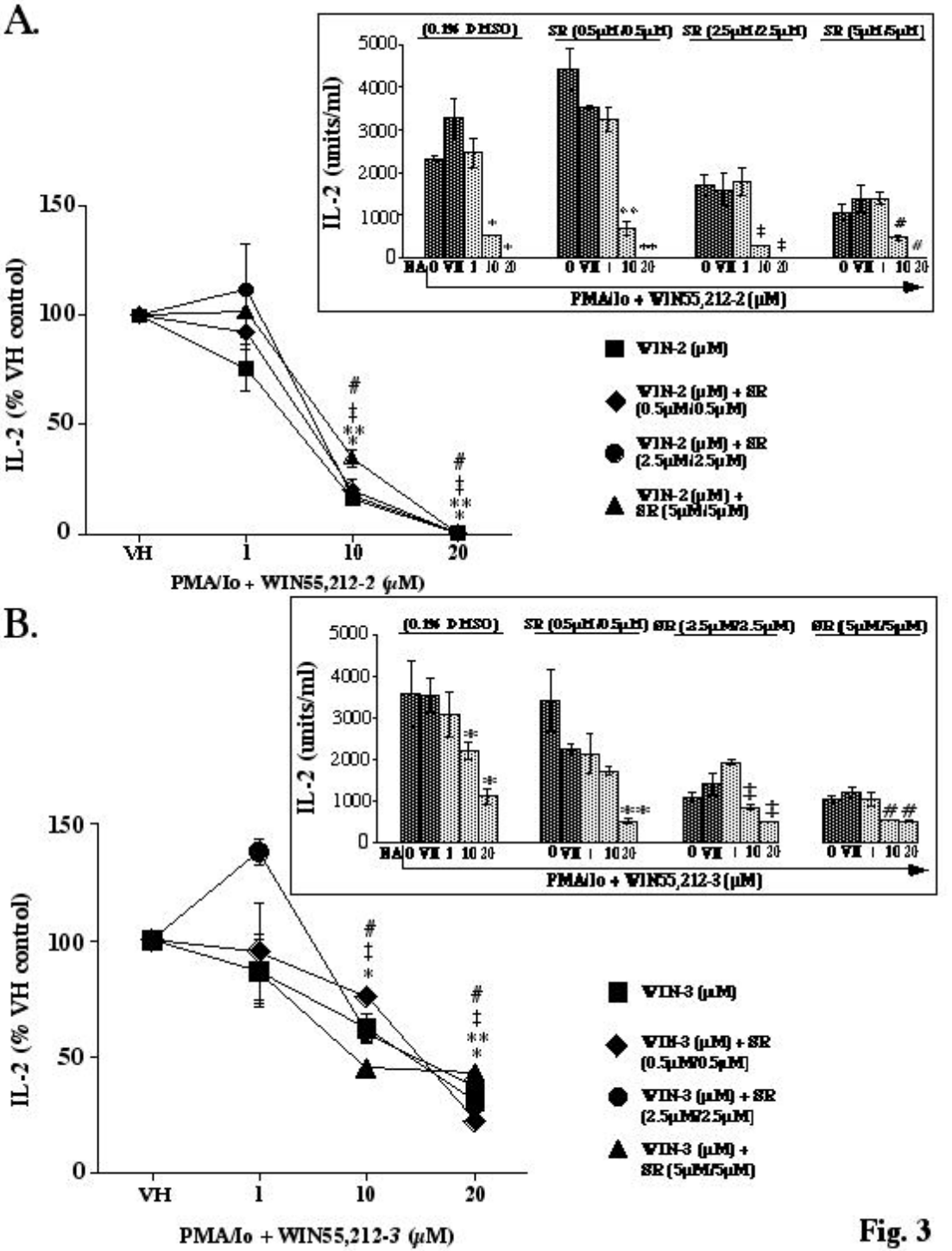

A.
(0.1% DMSO)
SR (0.5μM/0.5μM)
SR (2.5μM/2.5μM)
SR (5μM/5μM)
IL-2 (units/ml)
5000
4000
3000
2000
1000
0
HA 0 VH 1 10 20
PMA/Io + WIN55,212-2 (μM)
150
100
50
0
IL-2 (% VH control)
VH
1
10
20
PMA/Io + WIN55,212-2 (μM)
WIN-2 (μM)
WIN-2 (μM) + SR (0.5μM/0.5μM)
WIN-2 (μM) + SR (2.5μM/2.5μM)
WIN-2 (μM) + SR (5μM/5μM)
B.
(0.1% DMSO)
SR (0.5μM/0.5μM)
SR (2.5μM/2.5μM)
SR (5μM/5μM)
IL-2 (units/ml)
5000
4000
3000
2000
1000
0
HA 0 VH 1 10 20
PMA/Io + WIN55,212-3 (μM)
150
100
50
0
IL-2 (% VH control)
VH
1
10
20
PMA/Io + WIN55,212-3 (μM)
WIN-3 (μM)
WIN-3 (μM) + SR (0.5μM/0.5μM)
WIN-3 (μM) + SR (2.5μM/2.5μM)
WIN-3 (μM) + SR (5μM/5μM)

Fig. 3

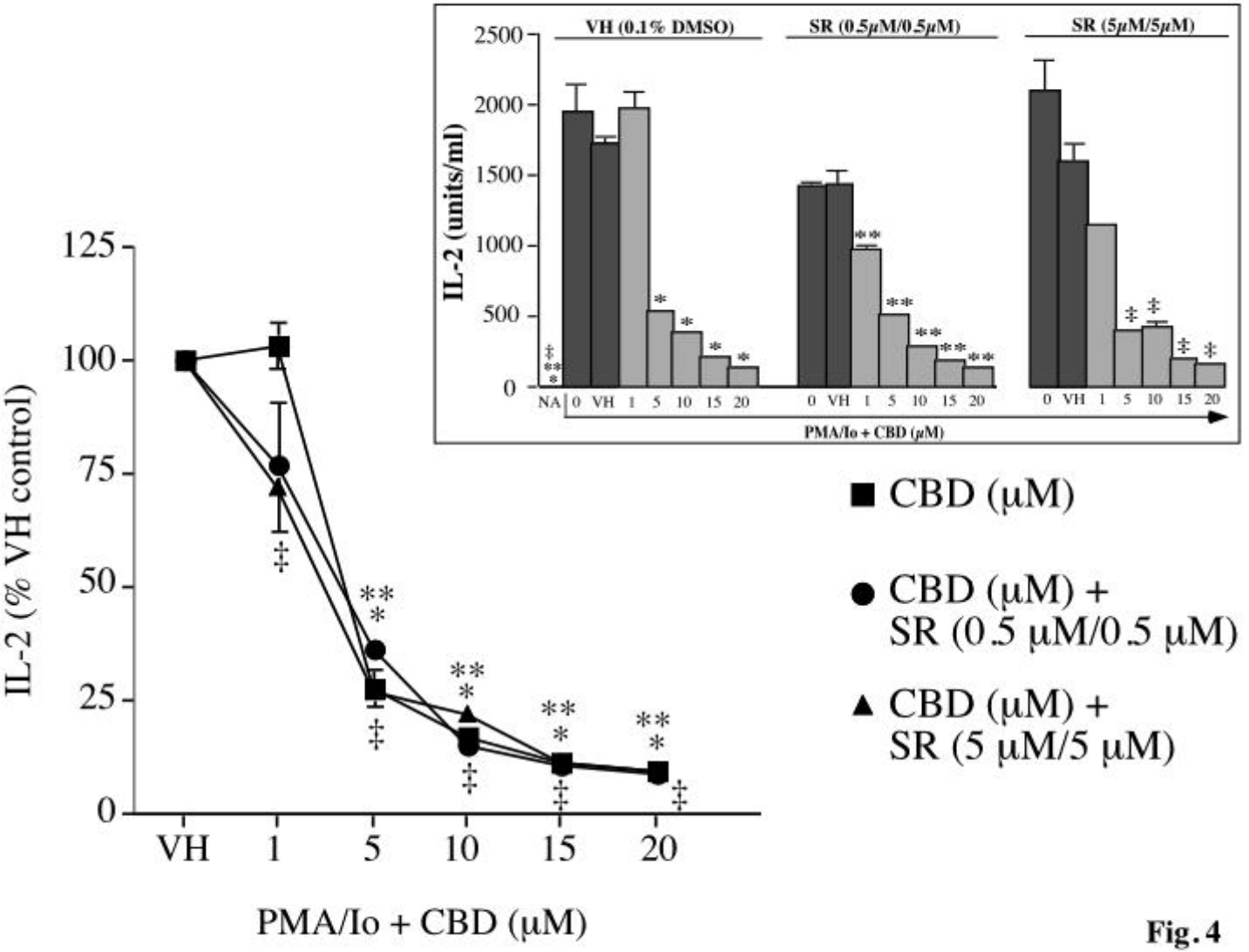
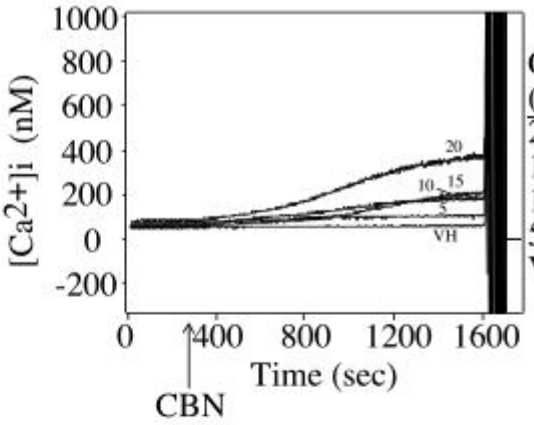

VH (0.1% DMSO)
SR (0.5μM/0.5μM)
SR (5μM/5μM)
IL-2 (units/ml)
2500
2000
1500
1000
500
0
NA
0 VH 1 5 10 15 20
PMA/Io + CBD (μM)
IL-2 (% VH control)
125
100
75
50
25
0
VH 1 5 10 15 20
PMA/Io + CBD (μM)
CBD (μM)
CBD (μM) + SR (0.5 μM/0.5 μM)
CBD (μM) + SR (5 μM/5 μM)

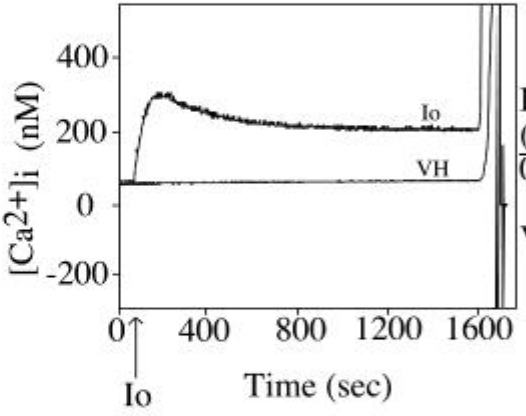
**Fig. 4**

A.



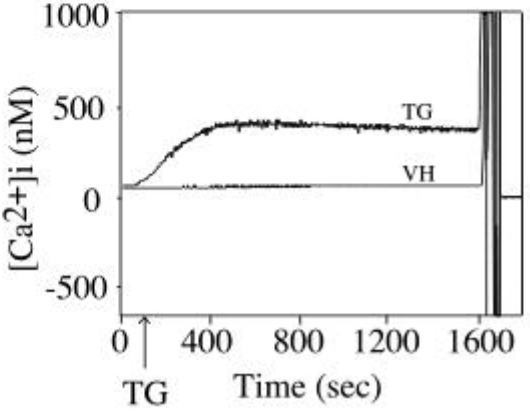

| CBN (μM) | Δ[Ca2+]i (nM) |
|---|---|
| 20 | 274 |
| 15 | 153 |
| 10 | 115 |
| 5 | 34 |
| VH | 8 |

B.




| Io (μM) | Δ[Ca2+]i (nM) |
|---|---|
| 0.5 | 259 (peak) |
| | 159 (plateau) |
| VH | 11 |

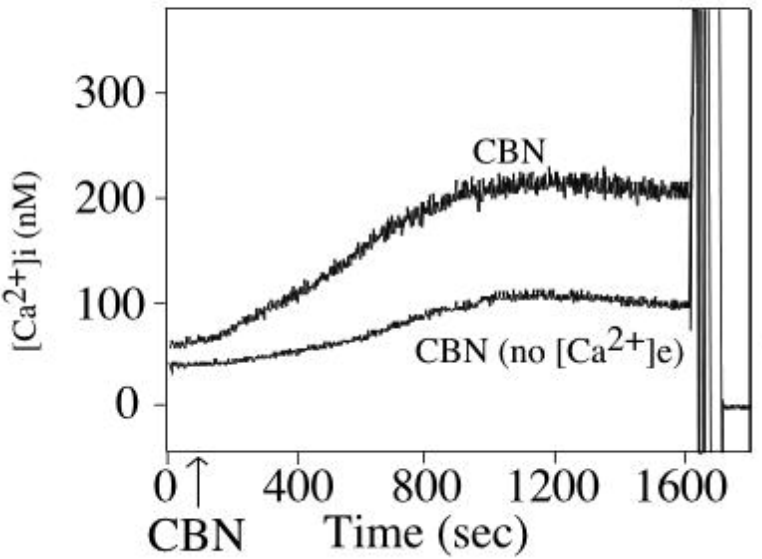
C.




| TG (nM) | Δ[Ca2+]i (nM) |
|---|---|
| 50 | 333 |
| VH | 19 |

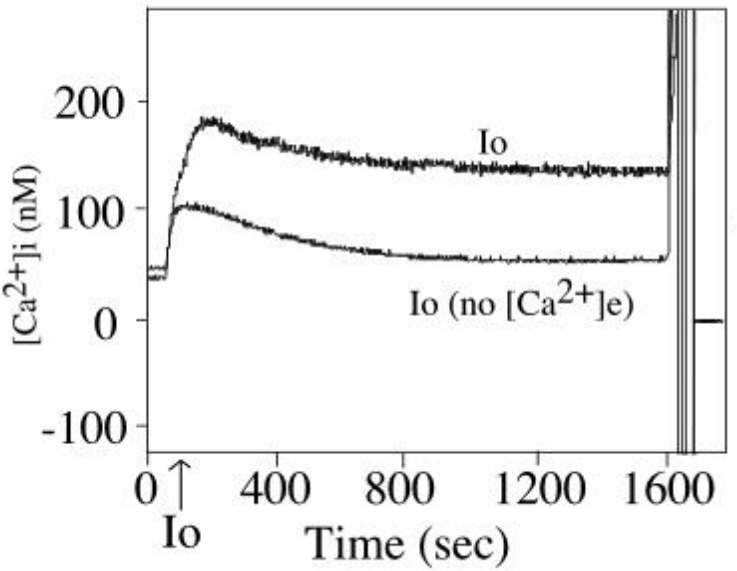
**Fig. 5**

A.




| Δ [Ca2+] (nM) | |
|---|---|
| CBN 15 μM | 160 |
| CBN 15 μM (no [Ca2+]e) | 72 |

B.




| Δ [Ca2+] (nM, peak) | |
|---|---|
| Io 0.5 μM | 135 |
| Io 0.5 μM (no [Ca2+]e) | 63 |

**Fig. 6**

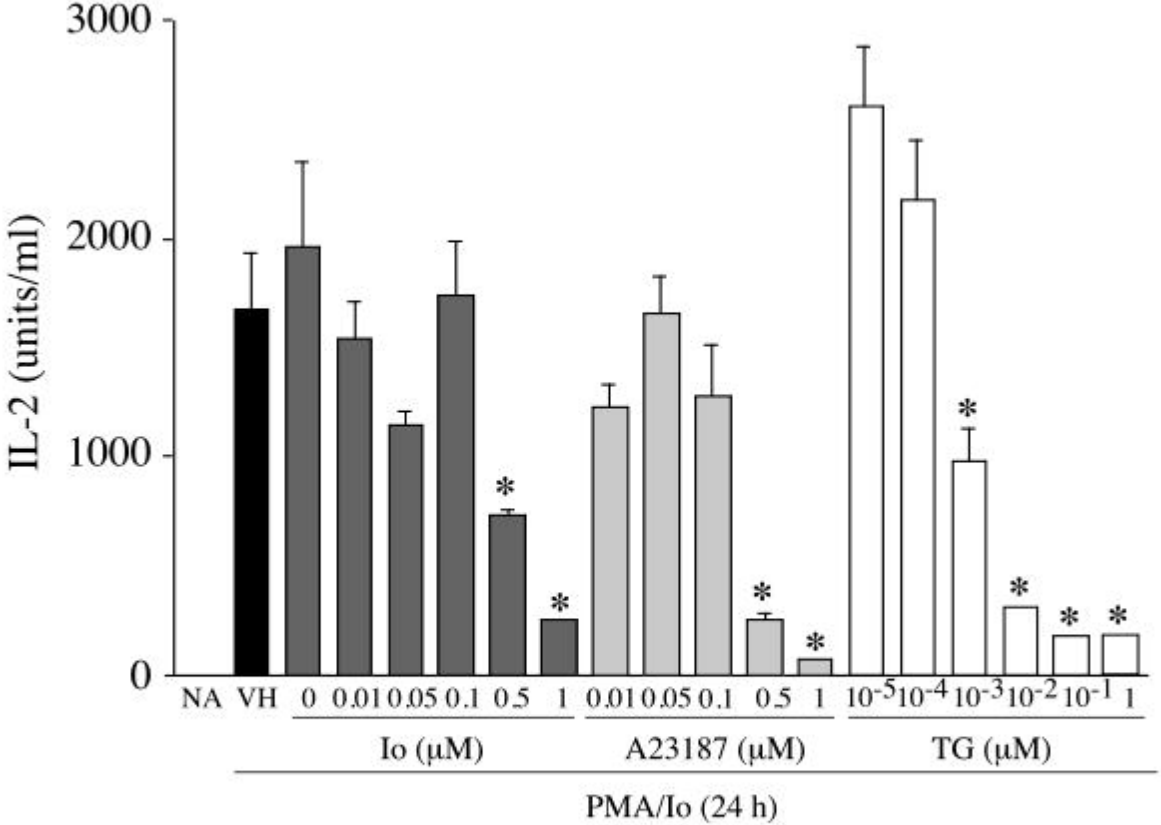

3000
2000
1000
0
IL-2 (units/ml)
NA
VH
0
0.01
0.05
0.1
0.5
1
0.01
0.05
0.1
0.5
1
10-5
10-4
10-3
10-2
10-1
1
Io (μM)
A23187 (μM)
TG (μM)
PMA/Io (24 h)

**Fig. 7**