Cannabinoids enhance human B-cell growth at low nanomolar concentrations

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Abstract This study examined the effect of cannabinoid ligands on human tonsillar B-cells activated either through cross-linking of surface immunoglobulins or ligation of the CD40 antigen. The two synthetic cannabinoids, CP55,940 and WIN55212-2, as well as Δ^9-tetrahydrocannabinol (THC), the psychoactive component of marijuana, caused a dose-dependent increase of B-cell proliferation and displayed EC_{50} at low nanomolar concentrations. This cannabinoid-induced enhancing activity was inhibited by pertussis toxin which suggested a G-protein-coupled receptor process. In addition, the absence of antagonistic effect of SR141716A, a specific CB1 receptor antagonist, together with the demonstration that human B-cells displayed large amount of CB2 receptor mRNAs, led us to assume that the growth enhancing activity observed on B-cells at very low concentrations of cannabinoids could be mediated through the CB2 receptor.

Key words: Cannabinoid; Immunomodulation; B-Cell activation; CP55,940; Δ^9-Tetrahydrocannabinol; WIN55212-2

1. Introduction

In addition to psychotropic effects, many reports have described an influence of cannabinoids on immune function (review, see [1]). Most of the in vitro studies showed immunosuppressive effects of cannabinoids (CB): inhibition of mitogen-induced proliferative responses of T-lymphocytes and B-lymphocytes [2,3], of cytotoxic T-cell activity [4], of macrophage microbiocidal activity and TNFα synthesis [5,6], of cytolytic activity and TNFα production of large granular lymphocytes [7]. In some studies, enhancing effects were observed: increase of interleukin-1 bioactivity by mouse resident macrophages or human differentiated macrophagic cell lines due to increased levels of TNFα [8,9].

Kaminsky et al. [10] identified the presence of CB receptors on mouse spleen cells. Bouaboula et al. [11] demonstrated that the first type cannabinoid receptor (CB1) which is present in the brain is also expressed on human leukocytes and Munro et al. [12] cloned a second G-protein coupled cannabinoid receptor, designated CB2, that is only present at the periphery and more particularly on cells of immune origin. More recently, Galiègue et al. [13] demonstrated that CB2 receptors mRNAs are highly expressed in human tonsils and B-cells. Thus, the presence of CB receptors on lymphoid cells could account for the cannabinoid-mediated immune modulation reported above. However, in most cases, the effects were obtained at concentrations of cannabinoids much higher (>1 μM) than those required in binding experiments (nanomolar affinities) (M. Rinaldi-Carmona, submitted), raising the question of receptor-mediated versus non-receptor-mediated mechanisms.

In contrast with the studies which report immunosuppressive effects at physiologically irrelevant concentrations of cannabinoids, we show here that various cannabinoid ligands displayed a growth enhancing activity on human tonsillar B-cells at a nanomolar range of concentrations. This activity was shown to be inhibited by pertussis toxin (PTX), strongly suggesting a specific receptor-mediated mechanism. Further, the presence in these cells of large amounts of CB2 receptor mRNAs and the absence of antagonistic effect of SR141716A, a recently described selective antagonist of the CB1 receptor [14], seemed to indicate a CB2 receptor-mediated process.

2. Materials and methods

2.1. Materials

CP55,940 was generously provided by Pfizer. WIN55212-2 was purchased from RBI (Natick, USA), Δ^9-THC and pertussis toxin were obtained from Sigma (St. Quentin-Fallavier, France). SR141716A and Anandamide were synthesised at Sanofi Recherche. Drugs were dissolved in dimethyl sulfoxide at 10^{-3} M. The final concentration of solvent in assays never exceeded 0.01% and was without effect on experiments. The 3A11 and F111-409 monoclonal antibodies (mAb anti-CD2 and anti-CD3, respectively) used for depletion of T-cells from tonsils were produced at Sanofi Recherche. The polyclonal B-cell activator, rabbit anti-human surface immunoglobulins (anti-Igs) antibody coupled to polyacrylamide beads were purchased from Bio-Rad Laboratories (Richmond, USA). The anti-CD40 mAb, MAB89, was purchased from Immunotech S.A. (Marseille, France). The mouse fibroblast L cells stably transfected with human FcγRII/CDw32 (CDw32 L cells) were obtained from the American Type Culture Collection (Rockville, USA) with the agreement of Dr. J. Banchereau (Schering-Plough, Dardilly, France).

2.2. Human B-cell purification

B-Cells were isolated as previously described [15]. Briefly, cells from tonsil specimens were incubated with mAbs directed at T-cells (anti-CD2 and anti-CD3) at 2 μg per 10^6 estimated target cells, for 30 min at 4°C, washed and then incubated 30 min at 4°C with sheep anti-mouse IgG-conjugated magnetic beads (Dynabeads, Dynal, Oslo, Norway) at a bead-to-target cell ratio of 5:1. Negative selection of B-cells was performed by magnetic depletion of bead-bound T-cells and resulted in >95% pure B-cells as determined by FACS analysis (Becton Dickinson and Co.) using anti-CD20, -CD4, -CD8 and -CD14 mAbs.

2.3. Detection of CB1 and CB2 receptor mRNAs

The expression of mRNAs for β_2-microglobulin, CB1 and CB2 receptors were examined by reverse transcription coupled to the polymerase chain reaction (RT-PCR) in the exponential phase of amplification as previously described [11]. Briefly, the total human tonsillar B-cell RNA was extracted using guanidinium isothiocyanate, purified by CsCl gradient ultracentrifugation and reverse transcribed to the corresponding cDNA [11]. Human brain cortex cDNA was purchased from Clontech (Palo Alto, USA). DNA amplifications were carried out in PCR buffer supplemented with 1.5 mM $MgCl_2$ containing 1 ng of cDNA with 0.1 mM dNTP, 0.25 μM of each primer, 0.5 U *Taq* polymerase (Perkin

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Elmer-Cetus) in a final volume of 20 μl. The mixture was amplified with the GeneAmp PCR System 9600 thermal cycler (Perkin Elmer-Cetus). The thermal reaction profile consisted of a denaturation step at 95°C for 20 s, annealing at 60°C for 30 s and an extension step at 75°C for 20 s. Reaction was performed for 30 cycles. After PCR, 5 μl loading buffer (Novex, San Diego, USA) were mixed with 10 μl PCR products and separated by electrophoresis on 3% agarose gel in the presence of ethidium bromide with 123 bp DNA markers (Gibco, Eragny, France) as molecular weight controls.

All the primers were 20-residue oligonucleotides with 50% G+C content and lacking 3′ complementary between primer pairs. β_2-Microglobulin primers: *sense* 5′-CCAGCAGAGAATGGAAAGTC-3′; *antisense* 5′-GATGCTGCTTACATGTCTCG-3′. CB1 primers: *sense* 5′-CATCATCATCCACACGTCTG-3′; *antisense* 5′-ATGCTGTTATCCAGAGGCTG-3′. CB2 primers: *sense* 5′-TTTCCCACTATCCCCAATG-3′; *antisense* 5′-AGTTGATGAGGCACAGCATG-3′.

2.4. Anti-Igs assay

Apart from experiment depicted in Fig. 1, all cultures were performed in RPMI 1640 supplemented with 0.5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM HEPES buffer (thereafter named: RPMI-0.5). B-cells were seeded at 1.5×10^5 cells/well of 96-well microplates in a final volume of 0.2 ml. Unless otherwise stated, the anti-Igs polyclonal B-cell activator, used at a final dilution of 1/10000, and the cannabinoid ligands were added at the onset of the culture. Each culture point was performed in 12 replicates. At the times specified in the text, DNA synthesis was determined by pulsing the cells with 1 μCi/well of [^{3}H]thymidine for the last 16 h of the culture period.

2.5. Anti-CD40/CDw32 L cell assay

We employed a modified version of the B-cell in vitro culture system described by Banchereau et al. [16] where mAbs to CD40 antigen are presented in a cross-linked fashion by CDw32 transfected mouse L cells. CDw32 L cells suspended at 2×10^6/ml were treated with 100 μg/ml mitomycin C in RPMI-0.5 for 1 h at 37°C. These cells were then washed four times in the same medium and plated at 2.5×10^4/well of 96-well microtiter tray. They were allowed to adhere for 2 h at 37°C in the presence of 100 ng/ml of the anti-CD40 mAb (MAB 89). B-Cells at 5×10^4/well and cannabinoid ligands at the indicated concentrations were then added in a final volume of 0.2 ml. DNA synthesis was measured in 12 replicates as described above.

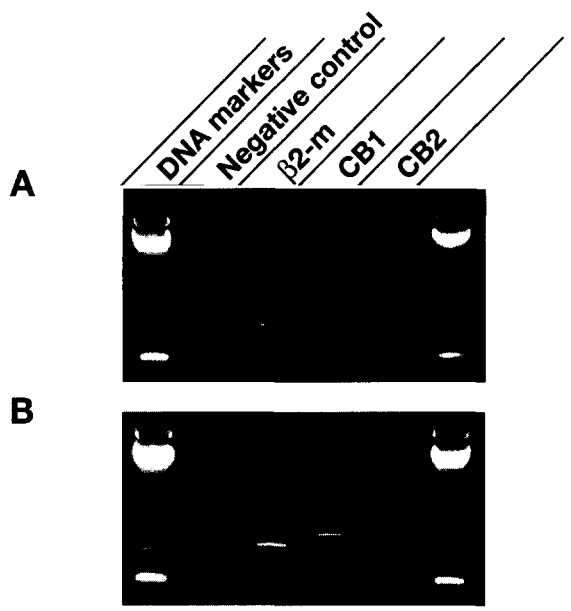

Fig. 1. Cannabinoid-receptor gene expression in human B-cells and human brain cortex. PCR products were obtained after amplification of mRNA-derived cDNA from human tonsillar B-cells (A) and human brain cortex tissues (B) using CB1-and CB2-specific primers. Negative control was performed with no cDNA. Internal control was obtained by PCR of β2-microglobulin gene.

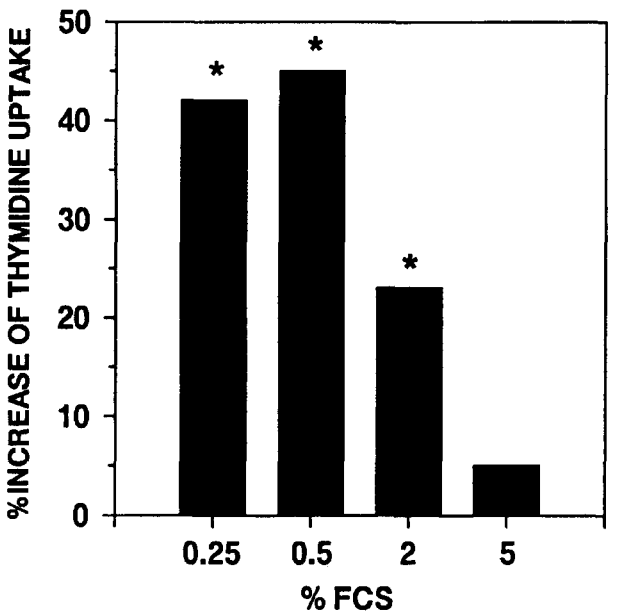

Fig. 2. Influence of serum concentration on cannabinoid-induced B-cell activation. B-cells were costimulated with anti-Igs and 10 nM CP55,940 for 72 h in RPMI medium containing the indicated concentrations of FCS. Results are expressed as % increase of thymidine uptake by comparison with control cells stimulated by anti-Igs only and are based on two independent experiments. $^*P \leq 0.05$.

2.6. Statistical analysis

Data were analysed by the Dunnett's analysis of variance test. A cut-off value of $P \leq 0.05$ was used to indicate statistical significance between cannabinoid-treated cells and control cells. Each experiment was repeated at least twice.

3. Results

3.1. Human B-cells displayed large amount of CB2 receptor mRNAs

Using semi quantitative RT-PCR, we compared in human brain and human B-cells the levels of trancripts for CB1 and CB2 receptors. As shown on Fig. 1, B-cells displayed much more CB2 receptor mRNAs than CB1 receptor mRNAs. This data confirmed the results obtained by Galiègue et al. [13] who showed an about 10-fold higher expression of CB2 mRNAs than CB1 mRNAs in human tonsillar B-cells. In the brain, no CB2 receptor mRNA was detected whereas CB1 receptor transcripts were highly expressed. Thus, in addition to the presence of CB1 receptor trancripts we confirmed here that human B-cells displayed even more CB2 receptor mRNAs. The presence of both types of receptor transcripts provided a molecular basis for the evaluation of the potential effects of cannabinoid ligands on human B-cell activation.

3.2. Effect of cannabinoid ligands on human B-cells activated by cross-linking of Igs

First, we examined the influence of the reference compound CP55,940 at 10^{-8} M on human tonsillar B-cells stimulated by cross-linking of their surface immunoglobulins for 72h in culture medium containing different concentrations of FCS (Fig. 2). The results showed that at low concentrations of serum (0.25% and 0.5%), CP55,940 induced a significant 42% to 45% increase of thymidine uptake. This effect decreased dramati-

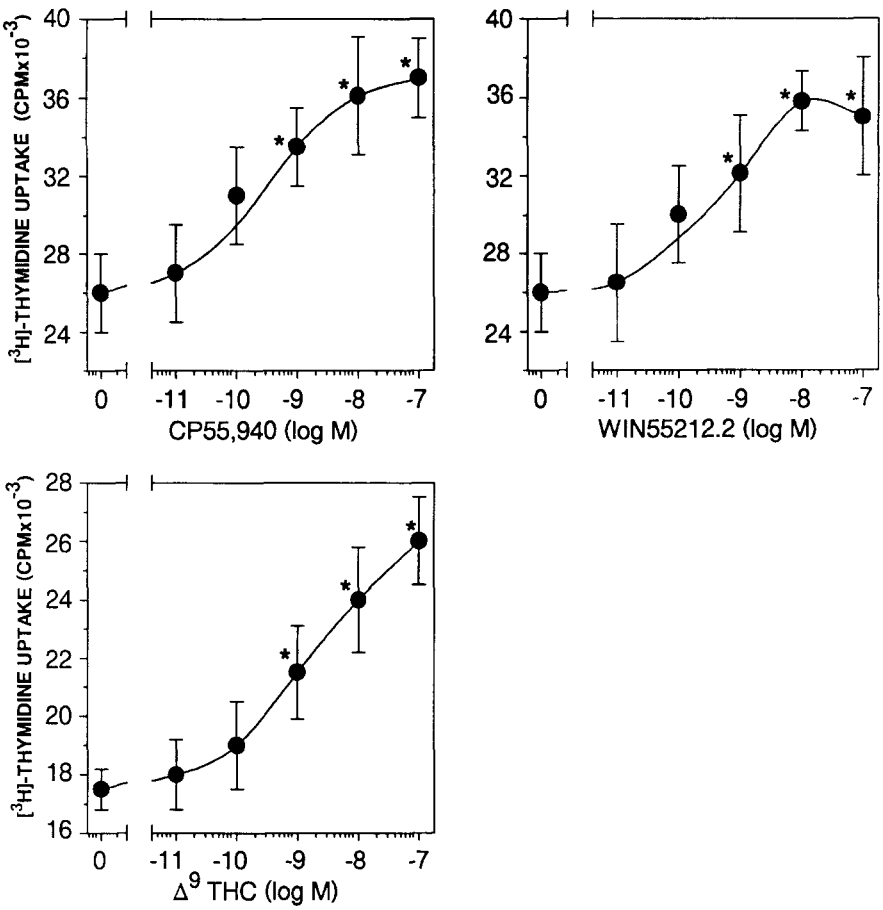

Fig. 3. Effect of different cannabinoid ligands on B-cells activated by cross-linking of Igs. B-cells were co-stimulated with anti-Igs and the indicated concentrations of cannabinoid ligands for 72 h in RPMI 0.5% FCS. The data shown are representative of three separate experiments in which each point (mean ± S.D.) was measured in twelve replicates. $^*P \leq 0.05$.

cally with the increase of serum concentration and was almost totally abolished at 5% of FCS. This serum inhibition was probably due to a loss of activity of the lipophilic cannabinoid ligand because of non-specific interactions with serum components as it has been already described elsewhere [17]. Therefore, all the experiments depicted thereafter were performed at the optimal concentration of 0.5% FCS.

Different cannabinoid ligands were then compared (Fig. 3). The results showed that CP55,940, WIN55212-2 and Δ^9-THC dose-dependently increased the rate of DNA synthesis induced by anti-Igs. An exposure to 10^{-8} to 10^{-7} M led to an about 40% increase of thymidine uptake. The two potent synthetic cannabinoids, CP55,940 and WIN55212-2, were similarly efficient displaying an EC_{50} of 0.5 and 0.7 nM, respectively, and reached a plateau between 10^{-8} and 10^{-7} M, whereas Δ^9-THC, the psychoactive component of marijuana, was slightly less active with an EC_{50} of 2 nM. No stimulation of B-cell growth was observed when cannabinoids were tested in absence of costimulating agent (data not shown). Anandamide, the putative endogenous cannabinoid ligand 18, proved to be ineffective in this model even at higher concentrations (≥ 1 μM) (data not shown)

When the selective CB1 receptor antagonist SR141716A [14] was used over a wide range of concentrations together with 10^{-9} M CP55,940, no blocking effect was observed (Fig. 4). A similar result was obtained with Δ^9-THC and WIN55212-2 (data not shown)

The kinetics of CP55,940-induced enhancement of B-cell activation were next evaluated by two different approaches. In the first set of experiments (Fig. 5A), CP55,940 at 10 nM and the anti-Igs activator were added together at the onset of the cul-

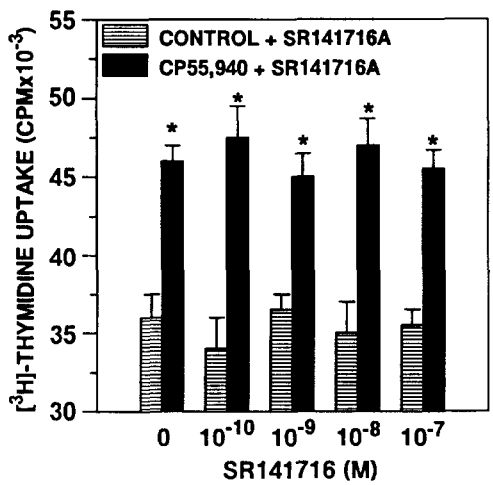

Fig. 4. Absence of antagonistic effect of the CB1-antagonist SR141716A. B-Cells were costimulated with anti-Igs and 1 nM CP55,940 for 72 h in the presence or absence of SR141716A at the indicated concentrations. Results are expresssed as in Fig. 3 and are representative of two separate experiments. $^*P \leq 0.05$.

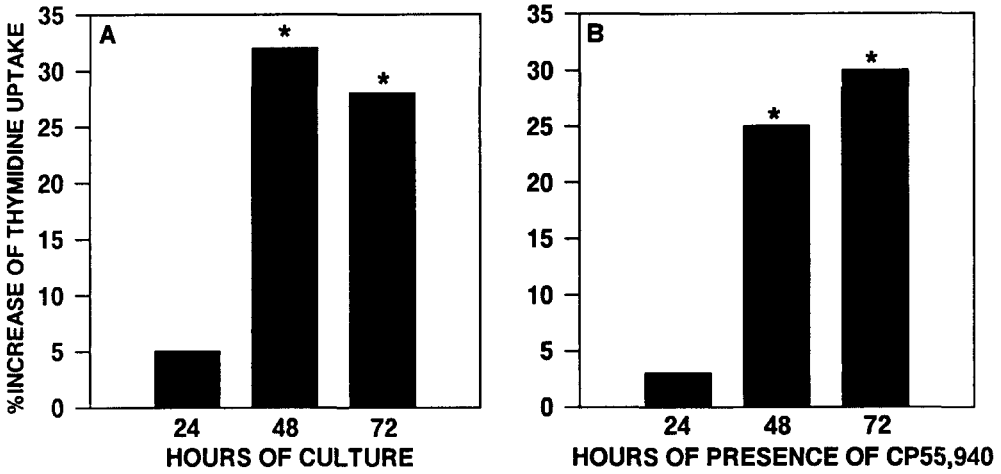

Fig. 5. Kinetics of cannabinoid-induced growth-enhancing activity. B-Cells were stimulated with the anti-Igs activator and 10 nM CP55,940. (A) anti-Igs and CP were added together and [^{3}H]thymidine uptake was measured at each indicated time. (B) cells were stimulated with anti-Igs for 72 h and CP was sequentially added 24, 48, 72 h before [^{3}H]thymidine labeling. Results are expressed as in Fig. 2. Two independent experiments showed similar results. $*P \leq 0.05$.

ture and % increase of DNA synthesis was measured each day from day 1 to day 3. In the second set of experiments (Fig. 5B), the activator was present over the three-day period and the cannabinoid was sequentially added 72, 48 and 24 h before [^{3}H]thymidine labeling. Both types of experiments showed that the cannabinoid-induced B-cell enhancing effect was a late event which required at least a 48 h exposure.

3.3. *Cannabinoid-induced enhancement of B-cell activation after ligation of the CD40 antigen*

The growth promoting activity described above seemed to be a general phenomenon which was also observed when another activation pathway was used. Indeed, when human B-cells were stimulated by engagement of the CD40 antigen by monoclonal antibodies presented by CDW32 L cells, a similar enhancing activity of CP55,940 was observed which reached 60% to 70% above control cells at 10–100 nM after 72 h of culture (Fig. 6A). This type of activation which induced a more sustained B-cell growth 16, allowed to observe that the cannabinoid-induced increase of DNA replication was permanent and within a similar range until 6 days after priming with anti-CD40 antibodies (Fig. 6B).

3.4. *Effect of pertussis toxin on cannabinoid-induced B-cell growth*

Cannabinoid receptors are coupled to an inhibitory subunit of G-protein (G_i) as indicated by numerous data demonstrating substantial cannabinoid suppression of adenylyl cyclase activity [19,20]. In order to confirm that the enhancing effect noticed in our model was a specific receptor-mediated process, we incubated B-cells in the presence of pertussis toxin (PTX) which blocks G_i proteins by preventing the dissociation of their α from β/γ subunits [21]. Fig. 7A shows that the 38% increase of thymidine uptake induced by 10 nM CP55,940 on B-cells costimulated with anti-Igs was completely abolished when cells were cultured with 0.1 μg/ml PTX. In contrast, when a high concen-

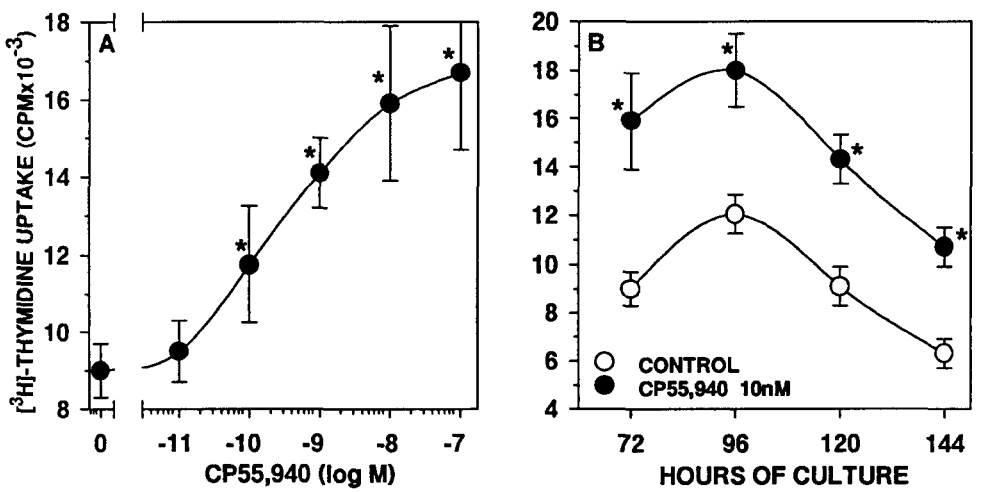

Fig. 6. Cannabinoid-induced enhancement of B-cells activated through ligation of CD40 antigen. B-Cells were stimulated by anti-CD40 mAbs presented by CDW32-transfected L-cells. (A) Dose–effect of CP55,940 was studied over a 72 h period of culture. (B) Kinetics of cannabinoid-induced enhancement of B-cell activation were evaluated at 10 nM CP55,940. The results are the mean ± S.D. of twelve determinations from one representative experiment out of three. $*P \leq 0.05$.

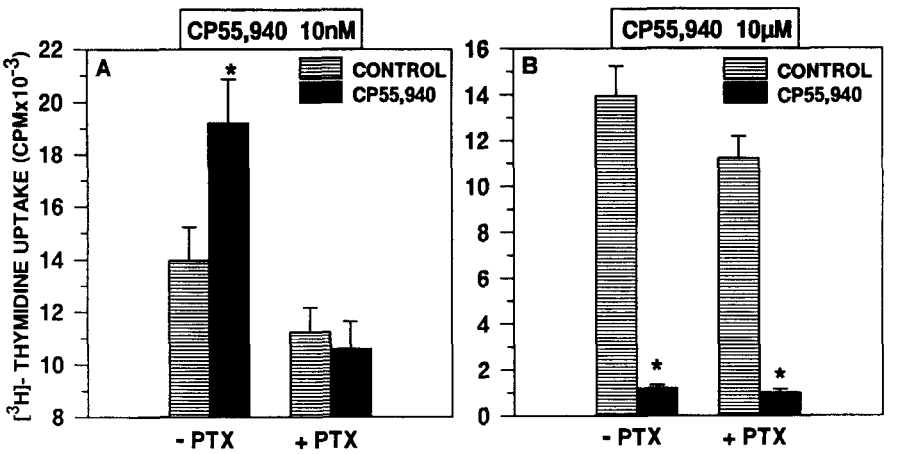

Fig. 7. Effect of pertussis toxin on cannabinoid-induced B-cell activation. B-Cells, stimulated by anti-Igs, were treated with CP55,940 at 10 nM (A) or 10 μM (B) for 72 h in the presence or absence of 100 ng/ml PTX. Data are from one representative experiment out of three. *$P \leq 0.05$.

tration of CP55,940 (10 μM) was used in the same conditions, a major inhibition of DNA synthesis was observed which was not blocked by PTX (Fig. 7B).

4. Discussion

To date, most studies that have examined the effects of cannabinoids on immune functions have demonstrated some degree of immunosuppression after exposure to high concentrations of ligands [22–24]. In this study, we have concentrated on exposure of human B-cells to physiologically relevant concentrations of cannabinoids and in conditions of low level of serum, since non-specific associations of cannabinoids with serum lipoproteins and albumin have been demonstrated [17]. The dramatic loss of activity due to these interactions already observed in other studies, was experienced in the model presented here where high concentrations of serum inhibited the cannabinoid-induced effect on B-cells.

In optimal conditions, our study shows that nanomolar concentrations of the two synthetic cannabinoids, CP55,940 and WIN55212-2, as well as Δ^9-THC, the psychoactive component of marijuana, were able to significantly increase DNA synthesis of human B-cells co-stimulated with either anti-Igs or anti-CD40 antibodies. This range of concentrations which correlated with the nanomolar affinities observed in binding studies on CHO cells tranfected with CB1 or CB2 receptors (M. Rinaldi-Carmona, submitted) was the first indication for a receptor-mediated process. The demonstration of a plateau between 10 and 100 nM which indicated a saturation process, represented an additional evidence. Cannabinoid receptors are coupled to G-proteins and numerous laboratories have demonstrated that cannabinoids inhibit adenylyl cyclase most likely by interaction with G_i [20,21]. Using pertussis toxin to block G_i protein, we demonstrated that the stimulating effect of low concentrations of cannabinoid on B-cells was abolished. The pertussis toxin sensitivity of this phenomenon provided a third strong indication for a receptor-mediated mechanism.

Anandamide, a candidate endogenous ligand isolated from porcine brain [18], was evaluated over a wide range of concentrations and found to be ineffective in this model. In the rat, it has been reported that anandamide displayed an affinity for the peripheral receptor 30-fold less than that reported for the brain receptor [12]. The lower affinity for the CB2 receptor could account, at least in part, for the absence of strictly peripheral effect on human B-cells. In addition, some enzymatic breakdown leading to inactivation of anandamide could occur [20]. Because no steps were taken to curtail catabolism in the model presented here, degradation of anandamide could not be totally ruled out and might represent another possibility for the lack of enhancing effect on B-cell growth. These different hypotheses need to be explored before drawing definitive conclusion.

The stimulating effects observed here with cannabinoid ligands were in contrast with the inhibiting effects on lymphocyte proliferation reported in other studies [3,24]. However, it is noteworthy that inhibition was obtained at micromolar concentrations (10–100 μM), raising the question of high-concentration non-specific membrane effects probably due to lipophilic interactions [19]. When cannabinoid was used at the high concentration of 10 μM, we also found an inhibition of B-cell activation which confirmed the experiments reported above by other laboratories. However, in this case, the pertussis toxin was ineffective in blocking this effect, thus reinforcing the likelihood of a non-receptor-mediated process at high concentrations of cannabinoids. Nevertheless, it cannot be completely excluded that other receptor-coupled second messenger systems could be involved. Watz et al. [25] also found a differential effect of cannabinoids on interferon- secretion by human macrophages: stimulatory effect at physiologically achievable concentrations of Δ^9-THC (10–100 ng/ml), suppressive effect at concentrations of THC above physiologically obtainable levels (5–20 μg/ml). As in our model, this could account for receptor-mediated effect versus non-specific effect on the membranes of the lymphoid cells.

Lynn et al. [19] in an extensive study on localization of cannabinoid receptors in peripheral tissues in the rat found that CB receptors were mainly confined to B lymphocyte-enriched areas and Munro et al. [12] identified in the same species the gene for the CB2 receptor which is expressed in splenic cells but not in the brain. Recently, Bouaboula et al. [11] demonstrated that,

among the different human leukocyte subsets tested, B-lymphocytes are the cells expressing the higher level of central type cannabinoid receptor transcripts. In this study, we confirmed the results of Galiègue et al. [13] who demonstrated that in addition to CB1 receptor mRNAs, human B-cells express very high level of CB2 receptor mRNAs. With regard to CB1 versus CB2 receptor-mediated process, it is noteworthy that CP55,940 and WIN55212-2 displayed a same potency on human B-cell activation. This result was consistent with the similar affinity of both ligands for the CB2 receptor whereas a 10-fold lower affinity of WIN55212-2 compared to that of CP55,940 was reported for the CB1 receptor (M. Rinaldi-Carmona, submitted). These data together with the absence of blocking effect of the cannabinoid-induced B-cell activation by the specific CB1 antagonist SR141716A 13 led us to assume that the enhancing activity observed on B-cells could be mediated through the peripheral-restricted CB2 receptor. Definitive information will depend on the availability of a CB2 specific antagonist or blocking antibodies.

Whatever the subtype of CB receptor involved, we demonstrated that immunomodulation by cannabinoids is not limited to supression after exposure to very high concentrations of ligands but that B-lymphocytes which expressed both type of receptors were sensitive to the stimulating effect of nanomolar concentrations of cannabinoids. This could have an as yet unappreciated influence on human B-cell humoral activity and immune response in general as well.

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