Differential Roles of CB1 and CB2 Cannabinoid Receptors in Mast Cells[1]

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Cannabinoid modulation of immune responses is a pathological consequence of marijuana abuse and a potential outcome of therapeutic application of the drug. Moreover, endogenous cannabinoids are physiological immune regulators. In the present report, we describe alterations in gene transcription that occur after cannabinoid exposure in a mast cell line, RBL2H3. Cannabinoid exposure causes marked changes in the transcript levels for numerous genes, acting both independently of and in concert with immunoreceptor stimulation via FcεRI. In two mast cell lines, we observed mRNA and protein expression corresponding to both CB1 and CB2 cannabinoid receptor isoforms, contrary to the prevailing view that CB1 is restricted to the CNS. We show that coexpression of the two isoforms is not functionally redundant in mast cells. Analysis of signaling pathways downstream of cannabinoid application reveals that activation of extracellular signal-regulated kinase, AKT, and a selected subset of AKT targets is accomplished by CB2 ligands and nonselective CB1/CB2 agonists in mast cells. CB1 inhibition does not affect AKT or extracellular signal-regulated kinase activation by cannabinoids, indicating that CB2 is the predominant regulatory receptor for these kinases in this cell context. CB1 receptors are, however, functional in these mast cells, since they can contribute to suppression of secretory responses. ***The Journal of Immunology*****, 2003, 170: 4953–4962.**

The active constituents of *Cannabis sativa* have been used for centuries as recreational drugs and medicinal agents. Today, marijuana is the most prevalent drug of abuse in the United States, while therapeutic use of marijuana constituents is gaining mainstream clinical and political acceptance (1–5). An increased molecular understanding of how cannabinoid compounds alter biological processes will facilitate decision making on the medicinal use of cannabinoids and the dispersion of drug control resources. These processes include the documented immunoactivity of some marijuana constituents and the potential for endocannabinoids to be physiological immunomodulators (6–8).

Two cannabinoid receptors, CB1 and CB2, have been cloned (6, 9, 10). Both are members of the seven-transmembrane G protein-coupled receptor superfamily. CB1 and CB2 are coupled to G_i-G_o heterotrimeric G proteins (6, 11, 12). Accordingly, documented signaling events downstream of CB1 and CB2 include effects mediated via suppression of adenylate cyclase and hence inhibition of cAMP-dependent pathways. To date, CB1 and CB2 have been variously shown to regulate a variety of targets via cAMP suppression, including A type and inwardly rectifying potassium channels (6, 13, 14), and focal adhesion kinase (15). CB1 and CB2 also have the potential to impact downstream signaling pathways independently of cAMP via G$\beta\gamma$ subunits (16). G$\beta\gamma$ may transduce cannabinoid signals to phosphatidylinositol (PI)[4] 3-kinase and mitogen-activated protein kinases (6). Cannabinoid regulation of the CREB and NF-κB are among the few transcriptional effects of cannabinoid exposure that have been documented in immune cell contexts (6, 17–21).

The tissue distributions of CB1 and CB2 suggest that they may play widely variant physiological roles (6). CB1 mRNA and protein expression are mainly restricted to cells of the CNS (6). In addition, certain peripheral tissues have documented CB1 content, attributable to expression in either innervating neurons or nonexcitable cell types. In contrast, CB2 is restricted to the periphery. CB2 mRNA and protein have been detected in gut epithelia and, interestingly, in many immune cell subsets (8). Low levels of CB2 are documented in T lymphocytes while B lymphocytes, NK cells, and granulocytes display higher receptor densities (6, 22). In a few tissues, including the murine spleen and the brain-resident macrophage-like microglia, coexpression of mRNA for both CB1 and CB2 has been documented (23). The functional consequences of CB1/CB2 coexpression have not yet been investigated in detail. Ligand selectivity and affinity differences between the two receptors suggest that coexpression may contribute to the complexity of cannabinoid responses in a given cell type.

The presence of CB2 (and CB1) in immune system cells strongly suggests that endocannabinoids are immunomodulators (7, 8, 24, 25). Indeed, cells of the immune system produce a range of endocannabinoids, but the role of these lipids in immunity is not clear. In the context of cannabinoid abuse, immune targets may contribute to associated pathology and increase the health care and societal costs of marijuana usage. In the context of marijuana-based therapeutics, immunomodulation may be a desired clinical

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[4] Abbreviations used in this paper: PI, phosphatidylinositol; ERK, extracellular signal-regulated kinase; ACEA, arachidonyl-2′-chloroethylamide; PEA, palmitoylethandamide; NS, nonstimulated; GSK3β, glycogen synthase kinase 3β; MEK, mitogen-activated protein/extracellular signal-regulated kinase; FKHR, forkhead receptor; MA, *R*-(+)-methanandamide; AC, adenylate cyclase.

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effect or a potentially detrimental side effect. Exogenously applied cannabinoids have a generally immunosuppressive effect (7, 8, 24–29). Documented in vivo effects include impaired responses to viral, bacterial, or protozoan challenge. If translatable to a physiological role for endocannabinoids, these studies suggest that cannabinoid lipids may down-regulate the intensity or duration of an ongoing immune response, or increase the threshold for initiation of immunoactivity.

Mast cells are strategically placed (30–33) in tissues that interface with the external environment (e.g., airways, gastrointestinal tract). A variety of stimuli impact mast cells, including challenges to innate and acquired immunity, CNS-derived agents, and physical stressors. Mast cells release inflammatory mediators that act to increase local vascular permeability, perform limited killing function, and recruit other leukocytes to establish an effective inflammatory site. It is also becoming clear that, in the absence of explicit challenge, mast cells may contribute to the biology of their host tissue through the production of cytokines and growth factors (31, 32). In asthma and allergic disorders, the physiological role of mast cells is subverted and the cells react to innocuous stimuli with devastating results (30–32).

In the context of smoked marijuana, cannabinoids gain access to the systemic circulation within minutes of inhalation. However, airways and the gastrointestinal tract are immediate points of contact for both cannabinoids and tobacco constituents, and the resident mast cells in these areas will be impacted by marijuana smoke (34). Mast cells express CB2 cannabinoid receptors and a variety of responses to cannabinoid application have been described in these cells (35–37). In vitro, suppression of mast cell proinflammatory mediator release by both marijuana constituents and endocannabinoids has been described. The marijuana constituent tetrahydrocannabinol is highly suppressive in in vivo models of mast cell proinflammatory function. These models include passive cutaneous anaphylaxis and substance P- or carageenan-induced hyperalgesia and edema (24, 25, 38–40). In animals where ongoing airway hyperreactivity is being modeled, cannabinoid application has been shown to reduce airway epithelial pathology and decrease the leukocyte infiltrate (24, 25). The fact that mast cells themselves produce endocannabinoids, including anandamide, palmitoylethanolamide (PEA), and 2-arachidonylglycerol, is suggestive of a potential autocrine regulatory loop (37).

In this study, we report midiarray analysis showing that marked transcriptional changes occur after exposure of mast cells to a potent cannabinoid receptor ligand. We show that cannabinoid exposure may act in concert with, or in opposition to, responses that occur after ligation of the FcϵRI immunoreceptor on mast cells. Analysis of the receptor subtypes that transduce these transcriptional changes reveals functional coexpression of CB1 and CB2 cannabinoid receptors in two mast cell lines. Our data document coexpression of CB1 and CB2 at the mRNA and protein levels. Through use of selective concentrations of CB1 agonists and antagonists, we show that CB2 is the predominant transducer of cannabinoid signals to the AKT and extracellular signal-regulated kinase (ERK) pathways, which are in turn potent regulators of gene transcription. Functionality of CB1 in this cell context is, however, confirmed via the ability of CB1 ligands to suppress mast cell secretory responses. Taken together, these data suggest that pre-exposure or concurrent exposure to marijuana-derived or endocannabinoids may profoundly alter mast cell-mediated tissue responses.

Materials and Methods

Cell lines and culture

RBL2H3M1 mast cells were maintained in media composed of DMEM supplemented with 10% heat-inactivated (55°C for 45 min) FBS with 2 mM glutamine in a 5% CO_2 humidified atmosphere at 37°C. Cath.a catecholaminergic neurons were obtained from the American Type Culture Collection (Manassas, VA) and cultured as above in DMEM supplemented with 8% horse serum, 4% heat-inactivated FBS, and 2 mM glutamine.

Reagents

Cannabinoid compounds AM281, CP55940, arachidonyl-2′-chloroethylamide (ACEA), and WIN552122 were obtained from Tocris Cookson (Ellisville, MO). PEA was obtained from Sigma-Aldrich (St. Louis, MO). LY294002 and PD098059 were obtained from Calbiochem (San Diego, CA). IgE anti-DNP and DNP-BSA were purchased from Sigma-Aldrich and Calbiochem, respectively. CB1 and CB2 Abs were purchased from Affinity Bioreagents (Denver, CO). Phosphospecific Abs to p42/44 ERK (Thr202/Tyr204), AKT (Ser473), forkhead receptor (FKHR; Ser256), and glycogen synthase kinase 3β (GSK3β; Ser9) were obtained from Cell Signaling Technologies (Beverly, MA). Miscellaneous chemicals were obtained from Sigma-Aldrich.

Cell stimulation, vehicle controls, and cell lysis

Cells were harvested by trypsinization and resuspended at 2.5×10^6 cells/ml. Stimulations were performed for the indicated times in 1.0 ml of DMEM/10% FBS in a 37°C water bath. Matched vehicle controls were consistently performed for each stimulation (6), since vehicle effects were observed in the phospho-AKT assays. Diluents were matched as far as possible to decrease the number of vehicle controls necessary. Nonstimulated (NS) refers to cells that were exposed to neither stimulus nor vehicle. Vehicles comprised DMSO or 70% ethanol in dH_2O. Cannabinoids were dissolved freshly for each experiment from a concentrated stock stored for $<$3 mo at −80°C. DNP-BSA was dissolved in PBS/2% DMSO and diluted 1/4000 to achieve final concentration. After stimulation, reactions were stopped by removal to ice and immediate centrifugation at $12{,}000 \times g$ for 1 min in a 4°C centrifuge. Cell pellets were washed once in 1 ml of ice-cold PBS and then lysed for 30 min on ice in 500 μl of a buffer containing 50 mM HEPES (pH 7.4), 75 mM NaCl, 20 mM NaF, 10 mM iodoacetamide, 0.5% (w/v) Triton X-100, 1 mM PMSF, 500 μg/ml aprotinin, 1.0 μg/ml leupeptin, and 2.0 μg/ml chymostatin. Lysates were clarified by microcentrifugation ($10{,}000 \times g$, 5 min). Supernatants were transferred to clean tubes and mixed with 1.4 vol of acetone and placed at −20°C for 1 h. Acetone precipitates were harvested by centrifugation at $10{,}000 \times g$ for 5 min. Protein pellets were resuspended in 70 μl of a reducing SDS sample buffer and heated for 8 min at 95°C. Unless otherwise indicated, samples were resolved by SDS-PAGE in a buffer composed of 192 mM glycine, 25 mM Tris, and 0.05% SDS (pH 8.8).

Western blotting

Resolved proteins were electrotransferred to polyvinylidene difluoride membrane in 192 mM glycine/25 mM Tris (pH 8.8). For Western blotting, membranes were blocked using 5% nonfat milk in PBS for 1 h at room temperature. Primary Abs were dissolved in PBS/0.05% Tween 20/0.05% NaN_3 and incubated with membranes for 16 h at 4°C. Developing Abs comprised anti-rabbit or anti-mouse IgGs conjugated to HRP (Amersham Pharmacia Biotech, Piscataway, NJ). These were diluted to 0.1 μg/ml in PBS/0.05% Tween 20 and incubated with membranes for 45 min at room temperature. A standard washing protocol (four washes of 5 min in 50 ml of PBS/0.1% Tween 20 at room temperature) was used between primary and secondary Abs and following secondary Ab. Signal was visualized using ECL and exposure to Kodak BioMax film (Rochester, NY).

Northern blot analysis

Multiple cell line Northern blots were produced using 1 μg/lane poly(A)$^+$ mRNA isolated from the indicated cell lines via oligo(dT) capture. This RNA was resolved on 1% formaldehyde-agarose gels and transferred to nylon membrane by capillary action. The cDNA probes were generated by restriction digest to generate the following fragments (CB1: 246 bp generated *Bse*RI; CB2: 124 bp generated *Eco*0109I/*Xho*I; designed to maximize cross-species hybridization) and ^{32}P labeled using a random priming reaction. All membranes were hybridized with radiolabeled probe for 2 h at 65°C. After two washes in 2× SSC/0.05% SDS at room temperature for 20 min and two washes in 0.1× SSC/0.1% SDS at 50°C for 20 min, membranes were wrapped in plastic and exposed to Kodak BioMax autoradiograph film for the indicated times at −80°C.

Macroarray analysis of gene expression patterns

Total RNA was purified from adherent RBL2H3 cells left unstimulated or exposed to either IgE (0.5 μg/ml, 16 h) followed by 250 ng/ml DNP-BSA for 3 h or CP55940 (1 μM, 3 h). RNA purification using a Nucleospin

RNAII kit followed the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA). RNA samples were labeled with [α-^{32}P]dATP using an Atlas Pure Total RNA labeling system (Clontech Laboratories). Two sets of matched pairs of Atlas human trial array membranes were hybridized with probes (16 h/68°C/45 rpm). Membranes were washed four times in 2× SSC/1% SDS (55°C/15 min/55 rpm), then once in 0.1× SSC/0.5% SDS (55°C/15 min/55 rpm), and once in 2× SSC (room temperature/5 min/55 rpm). Blots were wrapped and exposed to storage phosphor screens (Packard Biosciences, Meriden, CT) for 3 days. Phosphorimager (Packard Biosciences) data were captured using a Cyclone System (Packard Biosciences). Paired gene spots were analyzed (OptiQuant software; Packard Biosciences) with a spot diameter of 3 mm^2 on the grid template. Blot-to-blot probing differences were normalized based on the average counts of three (pairs) of control spots. The threshold for gene expression to be considered positive was set at two times plus 10% of the normalized background for each blot. Nonhybridizing spots were not analyzed. Each pair of positive spots was averaged and, if above background, expressed as fold changes relative to the corresponding averaged data from the equivalent spots on membranes probed with mRNA derived from unstimulated cells.

Serotonin release assay

Adherent RBL2H3 (2×10^4 cells/cm^2) were incubated with 1 μCi/ml [^{3}H]5-hydroxytryptamine (NEN, Boston, MA) for 16 h at 37°C. Monolayers were then washed once in Tyrode's buffer (41) at 37°C and cells were incubated with the indicated stimuli or vehicle in 250 μl/cm^2 Tyrode's buffer for 45 min at 37°C. Quenching in ice-cold PBS and/or removal of the plate to ice and immediate transfer of 125 μl of supernatant to scintillation mixture stopped reactions. Scintillation counts were averaged (three replicate points) and expressed as a percentage of the FcϵRI releasable pool of serotonin.

Results

Cannabinoid receptors type 1 and 2 are expressed in various mast cell lines

Mast cells produce a range of endocannabinoids and are sensitive to cannabinoid exposure. We asked which receptors were responsible for transduction of cannabinoid signals in the mast cell context. Fig. 1*A* shows Northern blot analysis of mRNA derived from a variety of immune system-derived cell lines. We noted the presence of CB2 transcripts in both B lymphocyte and mast cell-derived mRNA. Strikingly, we were also able to detect CB1 transcripts in the mast cell line RBL2H3. We validated these data at the protein level using specific Western blotting. In this study, we introduced a control for CB1 expression, the catecholaminergic neuronal line Cath.a (42), and another mast cell line, P815 (43). Fig. 1*B* shows that while CB2 protein is detectable only in the immune-derived mast cell extracts, CB1 is present in both cell types. These data suggest that expression of the CB1 receptor, thought to be mainly CNS-restricted, may be a common feature of mast cells. We have noted a range of reported molecular mass for both CB1 and CB2 cannabinoid receptors. This range is probably attributable to differences in protein isolation and electrophoretic systems as well as differences in the posttranslational modification (glycosylation/phosphorylation) and dimerization status of the receptors between cell types. In our system, the migration data shown in Fig. 1*B* shows approximate molecular mass for CB2 in both mast cell lines of 38 kDa. CB1 migrates at ~60 kDa (mast cell lines) and 55 kDa (Cath.a cells). The theoretical molecular mass for unmodified rat CB1 and CB2 is 52.8 kDa (CB1, NM012784) and 39 kDa (CB2 AF176350), respectively.

Cannabinoids regulate multiple genes in mast cells and may act in concert with or in opposition to immunoreceptor signaling

The mast cells examined here have the unusual characteristic of coexpression of both cannabinoid receptor isoforms. Expression of these receptors suggests that sensitivity to cannabinoids may be an important feature of mast cell physiology. We hypothesized that application of cannabinoid receptor ligands might induce or suppress gene transcript levels in mast cells and that the identity of the affected genes might give insight into the physiological outcome of cannabinoid exposure. The ability to assay the transcript status of multiple genes simultaneously allows a rapid assessment of the impact of an agonist on cell behavior (44, 45). In this system, changes in transcript levels may be attributed to induction or suppression of transcription as well as alterations in transcript stability. We applied this principle to the analysis of cannabinoid effects on mast cells. We selected a potent cannabinoid receptor ligand, CP55940, which has structural features in common with Δ-9-tetrahydrocannabinol, a major psychoactive constituent of marijuana. CP55940 has nanomolar affinity for both CB1 and CB2 cannabinoid receptor isoforms and has been used extensively to probe the in vivo roles of these receptors (6, 17).

We chose to compare the effects of CP55940 exposure with the changes in transcript level caused by ligation of the prevalent immunoreceptor on mast cells, FcϵRI. This high-affinity receptor for the Fc portion of IgE is ligated through cross-linking by multivalent Ag. FcϵRI stimulation drives mast cells to secrete allergic

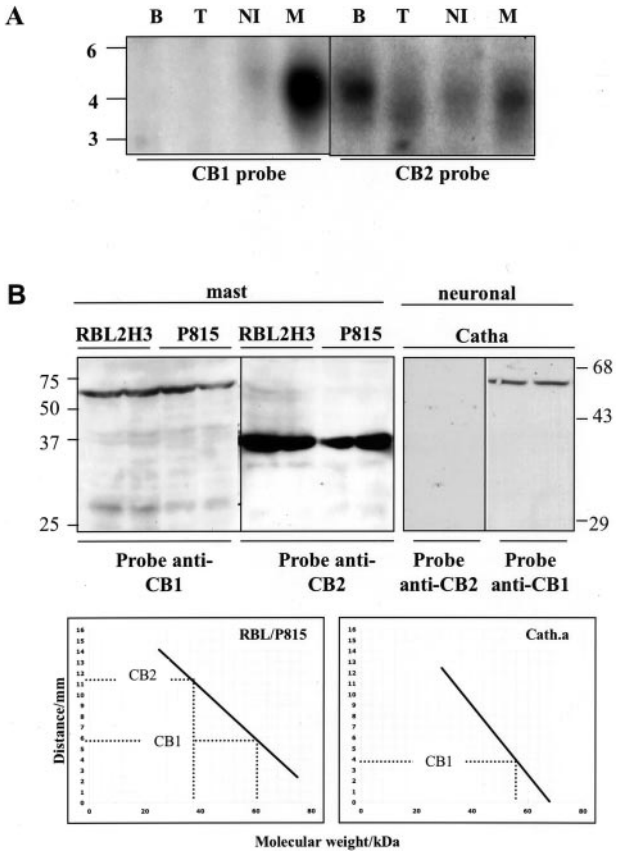

FIGURE 1. *A,* Presence of CB1 and CB2 mRNAs in a rat mast cell line. Northern blot analysis of CB1 and CB2 transcript levels was performed using ^{32}P-labeled probes as described in *Materials and Methods*. Poly(A)$^+$ mRNA (1 μg/lane) was used to prepare multiple cell line Northern blot membranes. Cell types used were: B, Ramos B lymphocytes; T, Jurkat T lymphocytes; NI, nonimmune human embryonic kidney endothelial cells; and M, RBL2H3 mast cell. Size markers are shown in kb. *B,* Western blot analysis of CB1 and CB2 protein levels in mast and neuronal cell backgrounds. Acetone-precipitated protein was prepared from replicate samples of 5×10^6 cells/lane of the indicated cell line. Proteins were resolved by SDS-PAGE and Western blotted using anti-CB1 or anti-CB2 antisera (Affinity Bioreagents). Molecular mass markers are shown in kDa. *Lower panels,* Migration distance plotted against molecular mass to enable estimation of molecular mass for the CB1 and CB2 receptors. The trend line was generated using the estimated midpoint for each molecular mass marker and CB receptor band.

mediators and to transcribe various cytokine and growth factor genes (46). For FcεRI stimulation, adherent RBL2H3 were primed for 16 h with IgE directed against the synthetic Ag DNP. FcεRI stimulation then comprised 3-h exposure to cross-linking Ag DNP-BSA. Cannabinoid stimulation was performed in parallel. In this study, adherent RBL2H3 were exposed for 3 h to CP55940. After harvesting and RNA preparation, hybridization to nylon midiarrays was performed. Two matched pairs of membranes were hybridized with probe derived from (1) control and CP55940-treated cells and (2) control and IgE/DNP-BSA-treated cells.

Ninety-six arrayed genes were available for hybridization. After analysis and normalization, significant hybridization was observed for 67 of the 96 genes. The remaining 23 genes are unlikely to be detectably expressed in either resting or stimulated RBL2H3. Of the 67 expressed genes, 31 exhibited no significant changes in hybridization following either FcεRI ligation or CP55940 treatment. In contrast, 26 genes altered status. Eighteen genes were targeted by cannabinoid receptor but not immunoreceptor stimulation. Nine genes were targeted by immunoreceptor but not cannabinoid receptor stimulation. Nine genes were targets of both stimuli. Within the subset of genes that are impacted by both cannabinoid and immunoreceptor stimulation, both concerted and opposing effects of the two stimuli were observed.

Hybridization data are summarized in Fig. 2. The hybridized genes have been organized into loose groupings on the basis of analogous functions. Group A genes involved in metabolic regulation/protein synthesis are largely unaffected by FcεRI stimulation, although several genes in this category are down-regulated following CP55940 treatment. Both FcεRI and/or CP55940 treatment result in transcriptional changes in the genes for multiple cell cycle regulators (group B), including proteins involved in G_2 (murine double minute 2 and cyclin B1), G_1 (cyclin E1), and throughout the cycle (cdc25A). The net effect of these transcriptional changes on proliferation is difficult to predict since an unsynchronized starting cell population was used. Similarly, the mixture of pro- and antiapoptotic genes that are induced by both treatments should be viewed in the light of a heterogeneous starting population and the fact that transcriptional changes do not necessarily represent the induction of the corresponding signaling pathways per se (groups E and H).

We note that more than half of the available genes alter status in response to either or both of the applied treatments. The observed changes in the levels of various transcription factors and nuclear receptors (group C) likely contribute to the gene inductions and repressions that occur during the course of this experiment and at later time points (data not shown). The steroid hormone receptor

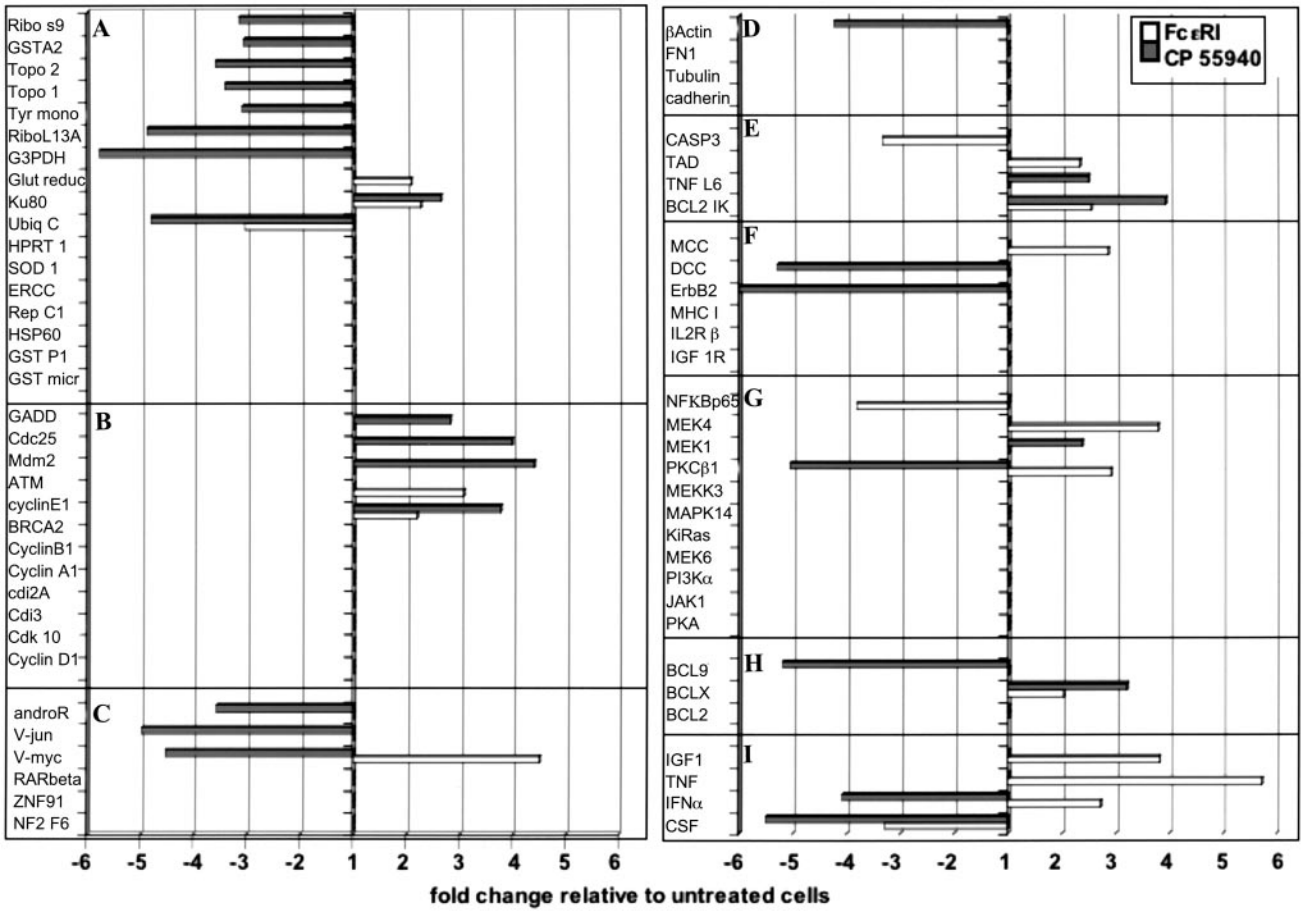

FIGURE 2. Transcript changes following cannabinoid or immunoreceptor ligation in the mast cell line RBL2H3. Midiarray analysis was performed as described in *Materials and Methods*. Abbreviated gene names are listed on the *left*; full names are available at www.clontech.com. Genes are loosely grouped according to function: group A, metabolic regulation and protein synthesis; group B, cell cycle and proliferation; group C, transcription factors; group D, adhesion and cytoskeleton; group E, proapoptotic genes; group F, cell surface receptors; group G, signaling proteins; group H, antiapoptotic genes; and group I, cytokines and growth factors. Data are expressed as fold change (induction or repression) in hybridization levels relative to matched control membranes after background subtraction and normalization. Fold changes less that 2-fold in either direction were defined as nonchanging and are denoted by black bars at the axis. Open bars correspond to FcεRI-stimulated cells (0.5 μg/ml IgE for 1 h at 37°C followed by 250 ng/ml DNP-BSA for 3 h at 37°C); gray bars correspond to CP55940 (1 μM for 3 h at 37°C)-treated cells.

(androgen receptor) examined is repressed by CP55940 treatment but left unaffected by immunoreceptor ligation, as is the jun transcription factor. Strikingly, the myc transcription factor, a potent regulator of multiple gene promoters (47), is up-regulated 4-fold following FcεRI ligation but suppressed to a similar degree following CP55940 treatment.

Relatively little change in the status of genes in the cytoskeletal/adhesion category (group D) is observed, although the β-actin gene is reproducibly repressed following CP55940 treatment. Transcript levels for several signaling molecules (group G) are altered by FcεRI induction but are unaffected by CP55940 (e.g., NF-κB-p65 and mitogen-activated protein/extracellular signal-regulated kinase (MEK) 4). It is striking that in this category, the FcεRI and CP55940 treatments have diametrically opposed effects on the transcript levels of protein kinase Cβ1. The consequences of altered PKCβ1 expression in RBL2H3 have been described previously, comprising dramatic alterations in sensitivity to antigenic stimuli (48). Thus, the alterations in transcript levels seen in this experiment in response to cannabinoid or Ag exposure are likely to have profound effects on cellular responses to subsequent stimuli (48, 49).

The remaining two groupings of genes illustrate the profound effects that cannabinoid and/or Ag exposure may have on the interactions of the mast cell with its tissue context. Various cell surface receptors in group F, including deleted in colorectal cancer, putative surface receptor with external Ig domains) and ErbB2, show decreased transcript levels after CP55940 exposure. Transcripts for mutated in colorectal cancer, a putative receptor with external Ig domains, increase following FcεRI exposure, but are unaffected by CP55940. Finally, we note marked changes in the transcriptional status of several cytokines and growth factors (group I). Changes in IFN-α, colony-stimulating factor 1, TNF, and insulin-like growth factor 1 transcript levels are observed and are established markers of mast cell activation (50–52). Transcript levels for the proinflammatory cytokines insulin-like growth factor 1 and TNF-α are increased following FcεRI stimulation, as has been described in vitro and in vivo for primary mast cells (31, 46, 48, 49). CP55940 exposure does not affect the transcript levels of these factors. Exposure to Ag or CP55940 causes apparent repression of the production of colony-stimulating factor 1. The effects of CP55940 and FcεRI ligation on IFN-α transcript levels are marked and diametrically opposed. Here, FcεRI signaling stimulates the IFN-α transcript levels, whereas CP55940 represses.

Cannabinoid signaling in mast cells leads to induction of both AKT and ERK kinase pathways

Transcriptional effects of cannabinoids are likely to be effected via signaling to target proteins that can impact multiple transcription factor targets. Relatively little is understood of cannabinoid signaling to targets other than adenylate cyclase, although activation (and nonactivation) of AKT and ERK pathways has been documented. We asked whether these kinases were targets of cannabinoid signaling in mast cells. Initially, we selected the synthetic cannabinoid CP55940 as an agonist. As described above, CP55940 has subnanomolar affinity for both CB1 and CB2.

Fig. 3*A* shows that CP55940 causes a dose- and time-dependent increase in AKT phosphorylation relative to a matched vehicle control, and that over a 15-min time course CP55940-induced AKT phosphorylation is as sustained as that achieved via FcεRI ligation. We applied CP55940 to RBL2H3 cells and assayed the phosphorylation status of the p42 ERK1 and p44 ERK2 kinases. Fig. 3*B* shows that CP55940 is an activator of the ERK pathway. We assessed the likely upstream regulators of ERK and AKT kinases in the mast cell context. Predictably, AKT activation via the FcεRI is highly sensitive to application of the PI3-kinase inhibitors LY294002 or wortmannin, but is unaffected by the MEK inhibitor PD098059 (Fig. 3*C, top panel*). FcεRI signaling to the ERK kinases is slightly more complex; here both PI3-kinase inhibition and MEK inhibition suppress ERK kinase activation, although the former is a weaker effect, indicating that multiple pathways converge upon ERK in this system (Fig. 3*C, lower panel*). CP55940 activation of AKT is also extremely sensitive to LY294002 (Fig. 3*D, upper panel*), indicating a dependence on PI3-kinase. LY294002 also reduces CP55940 induction of ERK indicating that, as for the FcεRI, cannabinoid receptor stimulation of ERK is partially achieved via PI3-kinase (Fig. 3*D, lower panel*).

We have proposed that ERK and AKT regulation by cannabinoids in mast cells may lead to potent induction of transcriptional pathways. Each of these kinases can integrate multiple inputs and signal to multiple effectors. Interestingly, we observe that not all of the AKT or ERK targets assayed are affected by cannabinoid application. As an example, Fig. 3*E* shows that cannabinoid application causes phosphorylation of the FKHR but not GSK3β, indicating selectivity in the AKT effectors that are targets for cannabinoid signaling (53, 54). Similarly, we note phosphorylation of the ERK targets ATF2 and c-*myc*, but not the jun transcription factor, following cannabinoid treatment (data not shown).

CB1-selective ligands do not promote AKT or ERK phosphorylation in mast cells

The data presented thus far suggest that our mast cell lines express both CB1 and CB2 cannabinoid receptors. Previous reports of CB1/CB2 coexpression outside the CNS have not established any functional contribution of the two receptors in a given cell context. In addition, we note that cannabinoid application to mast cells results in the activation of key signaling pathways that impact the transcription of multiple genes. In this context, it is important to assess the relative contribution of the two cannabinoid receptor isoforms. We identified a panel of cannabinoid agonists with varying affinities for CB1 and CB2. Fig. 4*A* summarizes the properties of these compounds. Initially, we selected two agonists that are highly selective for CB1 and compared their efficacy to that of CP55940, which is equipotent at the two receptors. The CB1-selective agonists ACEA and *R*-(+)-methanandamide (MA) did not cause an increase in AKT phosphorylation. In contrast, both CP55940 and WIN552122, which are both equipotent at CB1 and CB2, exhibited a potent stimulatory effect (Fig. 4*B*). We validated the efficacy of the same doses of ACEA and MA compounds in a parallel control experiment. Fig. 4*C* shows that both ACEA and MA stimulate ERK phosphorylation in Cath.a neurons, which express only the CB1 receptor. In control experiments (data not shown), doses of ACEA between 0.5 and 100 nM and doses of MA between 1 and 250 nM were without effect on AKT phosphorylation. These data suggest that, while CB1 can couple to ERK phosphorylation in neurons, in the mast cell system cannabinoid activation of ERK kinases occurs via CB2-initiated pathways. Finally, we obtained PEA, which has no significant affinity for CB1 or CB2 (6). The stimulatory effect of PEA on certain in vivo responses has led to the postulation of a third cannabinoid receptor that is ligated by this compound. In our mast cell context, PEA does not cause induction of a signaling pathway that impacts AKT (Fig. 4*D*) or ERK (data not shown).

CB1 does not contribute to AKT or ERK stimulation by cannabinoids in mast cells, but is functional in the suppression of serotonin release

The data presented in Fig. 4 suggest that when a ligand such as CP55940 or WIN552122 is applied to RBL mast cells, its efficacy in promoting AKT and ERK phosphorylation reflects its potency at

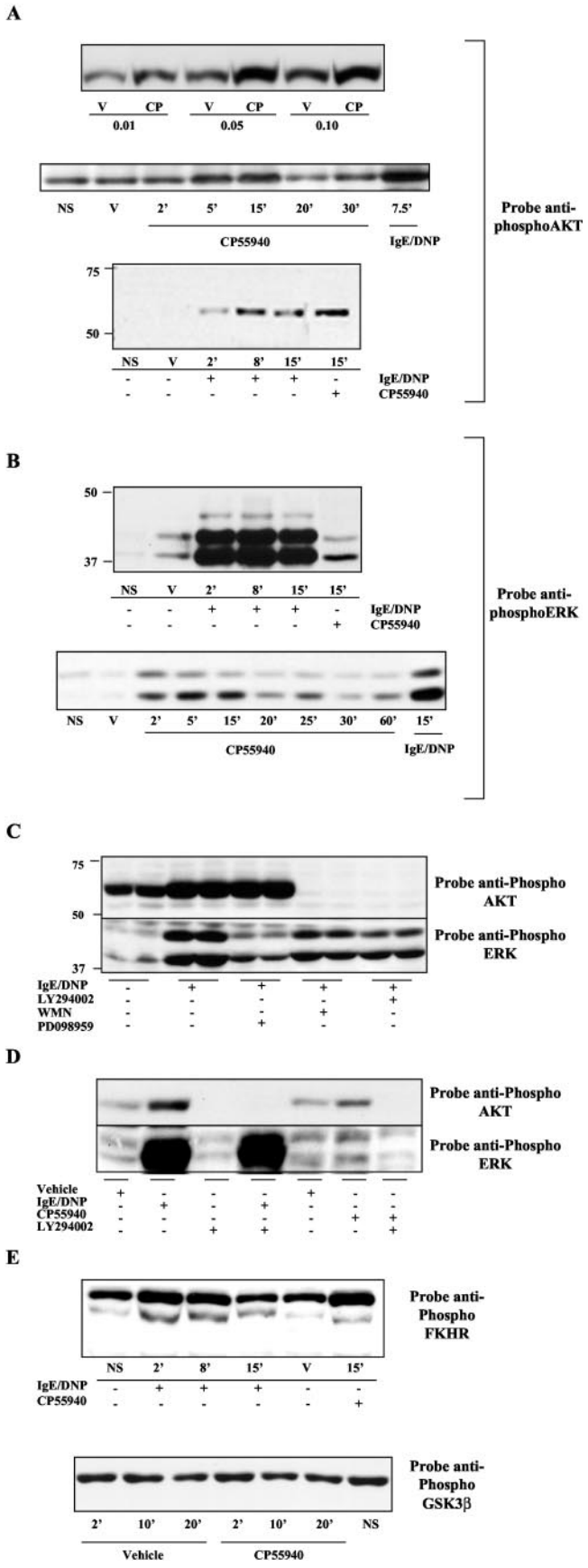

FIGURE 3. *A,* AKT phosphorylation following CP55940 treatment of mast cells. *Top panel,* RBL2H3 (5×10^6 cells/lane) were exposed to vehicle or the indicated doses of CP55940 (in μM) for 15 min at 37°C. *Center panel,* RBL2H3 (5×10^6 cells/lane) were left untreated (NS) or exposed to vehicle (V) or 1 μM CP55940 for the indicated times (in min) at 37°C. *Lower panel,* RBL2H3 (5×10^6 cells/lane) were left NS, exposed to vehicle (V), or exposed to either IgE followed by 250 ng/ml DNP-BSA or 1 μM CP55940 for the indicated times. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 10% SDS-PAGE and Western blotted using anti-phospho-AKT Ab. Note importance of matched vehicle controls in experiments using cannabinoid receptor ligands. *B,* ERK kinases are phosphorylated after both FcεRI and cannabinoid receptor ligation in mast cells. *Top panel,* RBL2H3 (5×10^6 cells/lane) were left NS, exposed to vehicle (V), or exposed to either IgE followed by 250 ng/ml DNP-BSA or 1 μM CP55940 for the indicated times (in minutes). *Lower panel,* RBL2H3 (5×10^6 cells/lane) were left NS, exposed to vehicle (V), or exposed to either IgE followed by 250 ng/ml DNP-BSA or 1 μM CP55940 for the indicated times. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 15% SDS-PAGE and Western blotted using anti-phospho-ERK Ab. *C* and *D,* Upstream regulation of AKT and ERK kinases following FcεRI or cannabinoid exposure in mast cells. RBL2H3 (5×10^6 cells/lane) were left NS, exposed to vehicle (V), or exposed to either IgE followed by 250 ng/ml DNP-BSA or 1 μM CP55940 for the indicated times. Inhibitors were all applied 5 min before exposure to either CP55940 or DNP-BSA. Inhibitor concentrations were: 15 μM LY294002; 100 nM wortmannin (WMN); and 10 μM PD098059. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 15% SDS-PAGE and Western blotted using anti-phospho-AKT or anti-phospho-ERK Ab. *E,* AKT substrates were selectively phosphorylated after FcεRI or cannabinoid receptor ligation in mast cells. RBL2H3 (5×10^6 cells/lane) were left NS, exposed to vehicle (V), or exposed to either IgE followed by 250 ng/ml DNP-BSA or 1 μM CP55940 for the indicated times. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 10% SDS-PAGE and Western blotted using anti-phospho-FKHR Ab (*top panel*) or anti-GSK3β Ab (*lower panel*) as indicated.

CB2, not CB1, cannabinoid receptors. To address this point more directly, we used an antagonist/inverse agonist that selectively targets CB1. AM281 has nanomolar affinity for CB1 but micromolar affinity for CB2. AM281 application, like its structural analog SR141716A, prevents or reverses CB1-mediated signaling. We proposed that treatment of RBL cells with AM281 would leave CB2-mediated signaling events intact but disrupt those pathways that depend on CB1. Fig. 5*A* shows that in RBL2H3 cells, CP55940 and FcεRI stimulation both result in ERK phosphorylation. Pretreatment of cells with AM281 does not affect the ability of either stimulus to induce ERK phosphorylation. Similarly, Fig. 5*B* shows that AKT activation by either immunoreceptor or CP55940 is not affected by pretreatment with AM281. In Fig. 5*C*, a control experiment is presented that demonstrates the efficacy of AM281. In the CB1-expressing Cath.a cells, the induction of ERK phosphorylation by CP55940 is severely attenuated following pretreatment with AM281. Taken together, the data in Figs. 4 and 5 suggest that it is CB2, not CB1, that mediates CP55940 signaling to AKT or ERK kinases, and their downstream effectors, in mast cells.

We asked whether CB1 ligation produced any functional effects in the RBL2H3 context. We have previously noted that cannabinoid application suppresses the secretion of serotonin from this cell line, although the degree of this effect is highly variable (H. Turner and M. Koblan-Huberson, unpublished observations). Ligation of FcεRI causes secretion of serotonin from RBL2H3 (shown in Fig. 6). Application of either CP55940 (CB1/CB2 ligand) or methanandamide (at a CB1-selective concentration) does not stimulate serotonin release, but these cannabinoids repress IgE receptor responses. The CB1 antagonist AM281 rescues the repression of secretion caused by both cannabinoids, indicating involvement of CB1 receptors in this cannabinoid effect. AM281 alone does not affect secretion and does not itself inhibit FcεRI stimulation of serotonin release.

A

LIGAND	$K_{i/d}$ at CB1(nM)	Affinity ratio CB1/CB2	Selected concentration (nM)
ACEA	1.4	>1400	20
MA	19	~45	25
CP55940	0.6	0.36	10
Win552122	2	1.6	100
AM281	12	~400	100

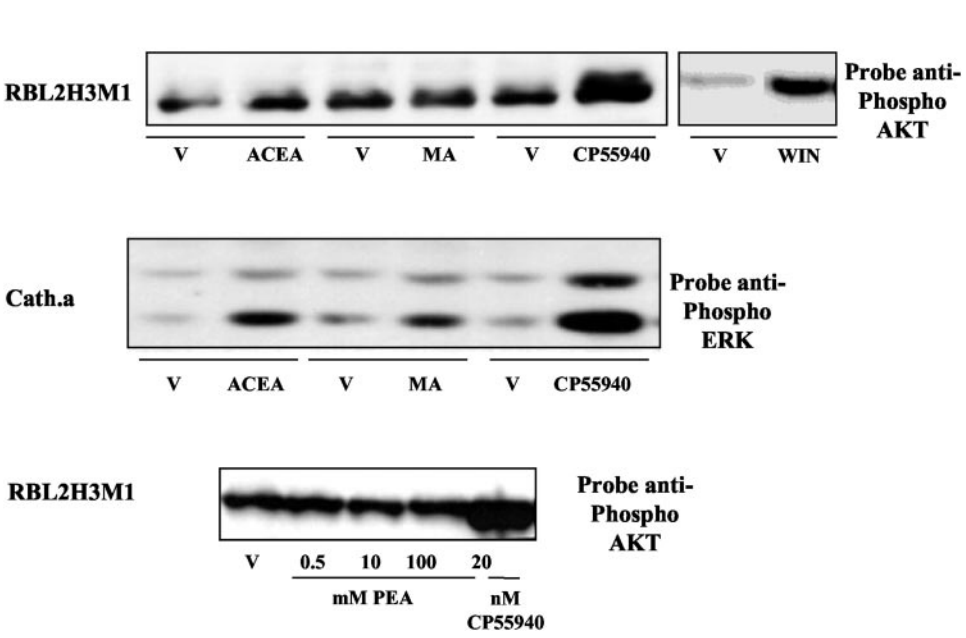

FIGURE 4. *A,* Cannabinoid receptor ligand affinities. This table compiles published affinity measurements for ligation of CB1 and CB2 by various cannabinoids and cannabimimetic compounds. Values were taken from primary reports and Ref. 6. *Right column,* Selected concentrations of ligands used in subsequent experiments. *B,* CB1/CB2 coagonists but not CB1-selective ligands activate AKT phosphorylation in RBL2H3. RBL2H3 (5×10^6 cells/lane) were exposed to vehicle (V) or exposed to ACEA, MA, CP55940, or WIN552122 at 20, 25, 10, and 100 nM, respectively, for 15 min at 37°C. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 10% SDS-PAGE and Western blotted using anti-phospho-AKT Ab. *C,* CB1/CB2 coagonists and CB1-selective ligands activate ERK phosphorylation in RBL2H3. RBL2H3 (5×10^6 cells/lane) were exposed to vehicle (V) or exposed to ACEA, MA, CP55940, or WIN552122 at 20, 25,10, and 100 nM, respectively, for 15 min at 37°C. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 15% SDS-PAGE and Western blotted using anti-phospho-ERK Ab. *D,* PEA does not impact AKT phosphorylation in RBL2H3. RBL2H3 (5×10^6 cells/lane) were exposed to vehicle (V) or exposed to PEA at the indicated doses for 15 min at 37°C. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 10% SDS-PAGE and Western blotted using anti-phospho-AKT Ab.

Discussion

Cannabinoid effects on immune system cells are components of the pathology associated with drug abuse and may be desired or unsolicited features of medicinal marijuana use. Endogenous cannabinoids may also be important physiological immunoregulators. In the present report, we have examined the consequences of cannabinoid exposure in the mast cell line RBL2H3. Interestingly, this cell line expresses two isoforms of the cannabinoid receptor, CB1 and CB2. Cannabinoid exposure leads to induction of multiple transcriptional events, some of which are common to cannabinoid and immunoreceptor responses. We observe a range of independent, concerted and opposing effects on transcription of individual genes in response to the ligation of Ag or cannabinoid receptors in mast cells. Cannabinoids induce several signaling pathways with transcriptional targets, and we have been able to attribute the induction of AKT and ERK kinase phosphorylation to CB2-mediated pathways. Coexpression of CB1 and CB2 clearly does not imply functional redundancy, since CB1 apparently has the unique ability to cause suppression of FcεRI-induced mast cell secretory responses.

Overlapping tissue distributions for the CB1 and CB2 cannabinoid receptor isoforms have been described in both primary and immortalized immune system cells (6). CB1 transcripts and/or protein have been observed previously in murine spleen (55), microglia, and the following cell lines: Raji and Daudi (B lymphocyte), THP-1 (monocyte), CTLL2 and Jurkat (T lymphocyte) (22, 23, 35, 56). In the present report, we document coexpression of CB1 and CB2 in two mast cell lines, although we have not yet confirmed that CB1/CB2 coexpression is present in tissue-derived primary mast cells or basophils. The presence of CB1 in multiple immune system contexts suggests that CB1-mediated responses may be important aspects of immunity and that CB2 is not sufficient to mediate all cannabinoid effects on cells of the immune system. Accordingly, it is logical to suggest that CB1 and CB2 are able to mediate distinct responses or to respond to unique physiological stimuli and that their coexpression is not redundant. Indeed, our

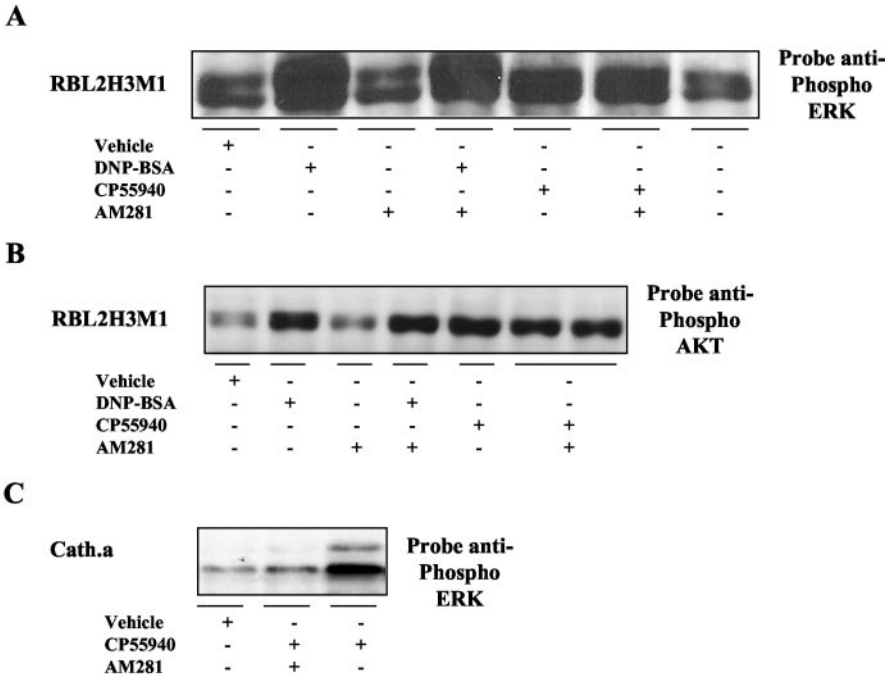

FIGURE 5. *A*, The CB1 antagonist AM281 does not affect CP55940-mediated ERK phosphorylation in RBL2H3. RBL2H3 (5×10^6 cells/lane) were exposed to vehicle (V) or exposed to AM281 (100 nM) for 5 min before a 15-min treatment with either vehicle, DNP-BSA (250 ng/ml), or CP55940 (20 nM) as indicated. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 15% SDS-PAGE and Western blotted using anti-phospho-ERK Ab. *B*, The CB1 antagonist AM281 does not affect CP55940-mediated AKT phosphorylation in RBL2H3. RBL2H3 (5×10^6 cells/lane) were exposed to vehicle (V) or exposed to AM281 (100 nM) for 5 min before a 15-min treatment with either vehicle, DNP-BSA (250 ng/ml) or CP55940 (20 nM) as indicated. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 10% SDS-PAGE and Western blotted using anti-phospho-AKT Ab. *C*, The CB1 antagonist AM281 suppresses ERK kinase activation mediated by CB1 in Cath.a neuronal cells. Cath.a (2×10^6 cells/lane) were exposed to vehicle (V) or exposed to AM281 (100 nM) for 5 min before a 15-min treatment with either vehicle or CP55940 (20 nM) as indicated. Lysates were acetone precipitated to recover total protein, and proteins were resolved by 15% SDS-PAGE and Western blotted using anti-phospho-ERK Ab.

data clearly show that CB1 and CB2 are not functionally equivalent in the mast cell context.

Agonists with equipotency at CB1 and CB2 cannabinoid receptors alter transcriptional status of multiple genes and suppress serotonin release in the RBL2H3 mast cell line studied here. CB1-mediated responses may be isolated through the use of appropriate agonists at selective concentrations or via the application of CB1/CB2 agonists in the presence of a CB1-selective antagonist. In these experiments, we note that CB2 is the predominant mediator of cannabinoid signaling to ERK and AKT kinases (and hence, we presume, to their downstream transcriptional targets) in the RBL2H3. In contrast, our data document that CB1 ligation suppresses FcεRI-induced serotonin release in RBL2H3, but that CB2 does not couple to the secretory apparatus. These strongly imply some qualitatively or quantitatively specific signaling events downstream of the two receptors in the RBL2H3 cell context.

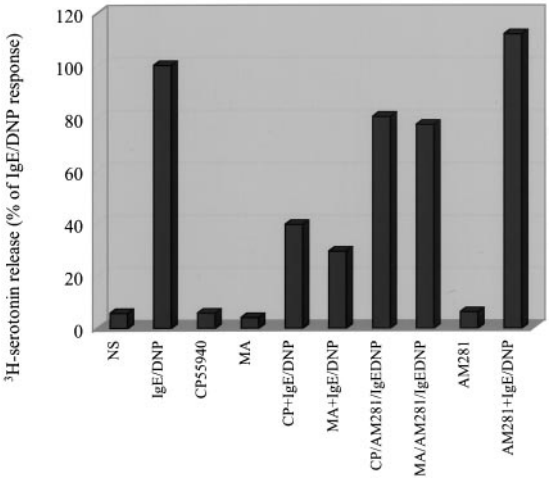

FIGURE 6. CB1 receptor functionality in RBL2H3 is evidenced by CB1 ligand-mediated suppression of secretory responses. RBL2H3 were loaded with 1 μCi/ml [^{3}H]serotonin for 16 h at 37°C in the absence or presence of 1 μg/ml IgE anti-DNP. Pretreatments of 5-min duration were performed at 37°C as indicated followed by a 45-min exposure to the indicated cannabinoids (100 nM CP55940, 25 nM MA, 100 nM AM281) or 250 ng/ml DNP-BSA at 37°C. Results are representative of three experiments.

We have considered the possible signaling pathways that may connect CB2 to the AKT and ERK kinases and enable CB1 agonists to suppress serotonin release. It is established that CB1 and CB2 couple to $G_{i/o}$ heterotrimeric G proteins, and that G_i activation causes suppression of adenylate cyclase (AC) and hence a decrease in intracellular cAMP. In addition, free $\beta\gamma$ dimers may regulate PI3-kinase and ERK activation pathways (6, 16). We propose to investigate whether a $\beta\gamma$-mediated signaling mechanism is important in CB2-mediated induction of ERK and AKT phosphorylation (both of which are PI3-kinase dependent in this system).

The mechanism through which CB1 mediates suppression of FcεRI-induced serotonin release also remains to be elucidated. CB1 activity through G_i-G_o coupling will tend to decrease cAMP levels. It is established that global elevations in cAMP (via AC activation or cytosolic perfusion) tend to suppress basal secretory responses (57, 58). Hence, CB1/$G_{i/o}$ signals would be expected to be neutral or to enhance serotonin release. Similarly, the documented ability of CB1 signals to activate inward rectifier-type

potassium channels (12, 13) would be predicted to have a net enhancement effect upon secretion via an increase in membrane potential difference, and hence an increase in the driving force for calcium entry that follows FcεRI ligation and is required for secretion (59). Nevertheless, we (and others) report that cannabinoid application does exert a suppressive effect on mediator release from mast cells (35, 60). These data have also been extended to the in vivo inflammatory consequences of mast cell activation (29); therefore, a mechanism must exist to explain the apparent paradox.

It has been suggested that, in the absence of $G_{i/o}$ coupling, CB1 can modulate cellular responses via $G_{\alpha s}$ (61). The net effect of this coupling is for CB1-targeted cannabinoids to stimulate AC and elevate cAMP. As described above, such an elevation would be expected to suppress FcεRI-induced mediator release via several mechanisms. We do not yet know whether CB1 is coupled to $G_{i/o}$ or $G_{\alpha s}$ in the RBL2H3. Rhee et al. (62) report that a key determinant of the outcome of CB1 ligation, in terms of stimulatory or inhibitory effects on cAMP levels, is the representation of AC isoforms in a given cell context. In cells expressing AC isoforms 1, 3, 5, or 8, cannabinoid ligation results in a net suppression of cAMP levels. Net elevations in cAMP are observed in cells expressing AC isoforms 2, 4, and 7. Preliminary expression array analysis in our laboratory shows that RBL2H3 express transcripts for AC isoforms 4 and 8 (A. L. Small-Howard, unpublished data). Further studies, including cAMP measurements and reporter assays, are clearly required to generate a clear mechanistic model for cannabinoid effects on mast cell secretion and inflammation.

The data in this report show a small scale midiarray analysis intended to establish whether transcription is regulated through cannabinoid receptors in this cell context. Interestingly, we observe a range of responses to cannabinoids in which changes in transcript levels are apparent. In parallel, we examined the same gene set in cells stimulated via FcεRI. When compared, the two data sets raise a number of interesting issues. Although a number of genes are clearly independently regulated by FcεRI or cannabinoid application, several are targets for both stimuli. Moreover, completely opposite regulation of a number of key genes (*IFN-α*, c-*myc*, and *PKCβ1*) is observed in cells exposed to FcεRI ligand or CP55940. Future expression analysis at the protein level and concurrent functional analysis will validate these data and enable us to establish their consequences for mast cell-mediated immune responses.

Mast cells are likely to be exposed to endogenous cannabinoids from autocrine or paracrine sources and to marijuana constituents during drug inhalation. Both transcriptional events and the secretion of allergic mediators can be modified by cannabinoids in the model mast cell line used here. Preliminary data from a more extensive array analysis suggest that the immunoreceptor and cannabinoid signaling systems may cross-talk, since we observe that FcεRI regulates transcript levels for components of the cannabinoid signaling pathway (A. L. Small-Howard, unpublished data). We propose that exposure to cannabinoids before or during an Ag-driven mast cell response may profoundly alter the outcome of the activation process.

Acknowledgments

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