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THE PROXIMAL AND DISTAL C-TERMINAL TAIL DOMAINS OF THE CB1 CANNABINOID RECEPTOR MEDIATE G PROTEIN COUPLING

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Abstract—The human CB1 cannabinoid receptor couples to $G_{i/o}$ proteins and inhibits neuronal voltage-gated Ca^{2+} channels. The role of the C-terminal tail of the CB1 cannabinoid receptor in $G_{i/o}$ protein coupling was examined using the superior cervical ganglion neuronal expression system. Deletion of the distal intracellular C-terminal tail (amino acids 418–472) slowed the kinetics and reduced the magnitude of Ca^{2+} channel inhibition. Deletion of the entire intracellular C-terminal tail (amino acids 401–472) abolished Ca^{2+} channel inhibition demonstrating the critical role of the proximal amino acids 401–417 of the C-terminal tail in G protein signaling. Expression of the C-terminal truncated receptors on the cell surface was examined using an N-terminal CB1 antibody. Both the C-terminal truncated receptors were expressed on the cell surface and were no different from wild type CB1 cannabinoid receptors.

This study establishes that the proximal CB1 cannabinoid receptor intracellular C-terminal tail domain (amino acids 401–417) is critical for $G_{i/o}$ protein coupling and that the distal C-terminal tail domain (amino acids 418–472) profoundly modulates both the magnitude and kinetics of signal transduction. Thus, the C-terminal tail of the CB1 cannabinoid receptor has a wider role in G protein coupling than was previously thought.

Key words: G protein-coupled receptors, Ca^{2+} channels, GTP binding protein, neurons, receptors, superior cervical ganglion.

That the brain has a receptor for Δ^9-tetrahydrocannabinol, the active ingredient in *Cannabis*, and that this receptor is coupled to $G_{i/o}$ proteins was discovered by Howlett and colleagues (Howlett, 1985; Howlett et al., 1986; Bidaut-Russell et al., 1990). Subsequently, the brain cannabinoid receptor was cloned and named the CB1 cannabinoid receptor (Matsuda et al., 1990). A second cannabinoid receptor, CB2, was found in cells of the immune system (Munro et al., 1993). The CB1 cannabinoid receptor plays a physiological role in analgesia, learning and memory, appetite stimulation, motor coordination, alleviation of nausea, hypothermia and psychological states such as a sense of well being. Activation of cannabinoid receptors has been shown to alleviate spasticity and stop tremors in a mouse model of multiple sclerosis (Baker et al., 2000). Additionally, improved performance on memory tasks was seen when CB1 receptors were blocked by the inverse agonist SR141716A (Terranova et al., 1996; Lichtman, 2000). Thus, the CB1 cannabinoid receptor is involved in a wide range of physiological functions and interest in the medical use of marijuana has grown significantly in recent years.

On a cellular level, the CB1 receptor signals through $G_{i/o}$ proteins to modulate ion channels and inhibit adenylyl cyclase (Mackie and Hille, 1992; Deadwyler et al., 1995; Mackie et al., 1995; Pan et al., 1996; Twitchell et al., 1997). The structural domains involved in mediating the physiological functions of this receptor have been investigated. The intracellular C-terminal tail of the CB1 cannabinoid receptor functions in a wide range of activities including desensitization, internalization and G protein coupling. Two distinct domains of the distal C-terminal tail of the CB1 receptor mediate desensitization and internalization. Amino acids 418–439 of the rat CB1 cannabinoid receptor contain the phosphorylation sites critical for receptor desensitization mediated by G protein-coupled receptor kinase and β-arrestin (Jin et al., 1999). Amino acids 460–464 located more distally on the C-terminal tail of the rat CB1 cannabinoid receptor are critical for receptor internalization (Hsieh et al., 1999).

Structural domains of the CB1 cannabinoid receptor involved in G protein coupling have been investigated by biochemical means. A peptide fragment from the C-terminal juxtamembrane domain of the mouse CB1 cannabinoid receptor activated GTPγS binding to G proteins and inhibited adenylyl cyclase (Howlett et al., 1998; Mukhopadhyay et al., 1999). This same peptide fragment from the C-terminal juxtamembrane domain competed for $G_{\alpha O}$ and $G_{\alpha i3}$ binding to immunoprecipitated rat brain CB1 receptors which co-immunoprecipitate with these G proteins (Mukhopadhyay et al., 2000). Thus,

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Abbreviations: EDTA, ethylenediaminetetra-acetate; EGTA, ethyleneglycolbis(aminoethyl ether)tetra-acetate; FITC, fluorescein isothiocyanate; GnRH, gonadotropin-releasing hormone; HEPES, *N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid; SCG, superior cervical ganglion neurons; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate.

the CB1 receptor couples tightly with G proteins and the C-terminal juxtamembrane domain can autonomously activate G proteins *in vitro*.

In the present communication, we used another approach to investigate whether the C-terminal juxtamembrane domain is critical for G protein activation and to determine the role of the distal C-terminal domain in G protein activation. We progressively deleted the C-terminal from the human CB1 receptor (Fig. 1) and tested for its ability to activate G proteins. Both wild type and C-terminal truncated human CB1 cannabinoid receptors were expressed in superior cervical ganglion (SCG) neurons. The advantage of this approach is that the ability of the C-terminal of the CB1 cannabinoid receptor to activate G proteins is tested in the context of the other domains of the CB1 receptor and within the neuronal environment. We tested for the ability of the truncated CB1 receptor to activate G proteins by measuring $G_{i/o}$-mediated inhibition of neuronal Ca^{2+} channels.

EXPERIMENTAL PROCEDURES

Generation of C-terminal truncated CB1 cannabinoid receptor cDNA

The human brain cannabinoid receptor hCB1 cDNA (from Dr. Tom I. Bonner, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD, USA) was subcloned into the mammalian expression vector pCI (Promega, Madison, WI, USA) as previously described (Pan et al., 1998). Two C-terminal truncated hCB1 receptors were constructed: (1) hCB1–417 encoding amino acids 1–417 and (2) hCB1–400 encoding amino acids 1–400. In order to truncate hCB1 receptor cDNA, a DNA segment from a restriction site (*Stu*I) in the middle of the coding sequence of hCB1 to the truncation site was amplified by polymerase chain reaction (PCR) using Taq polymerase (Promega, Madison, WI, USA). The PCR primers were designed to include both the *Stu*I site and the truncation site. A stop codon was added in the downstream primer. Primers: hCB1–417 up: GTG CGT CAT CCT CCA CTC, hCB1–417 down: CGA GAT CTC GTC AGC CTT CAC AAG AGG GAA AC; hCB1–400 up: GTG CGT CAT CCT CCA CTC, hCB1–400 down: TCA CCT CAG AGC ATA GAT GAT. The PCR conditions were 30 cycles of denaturation (95°C, 1 min), annealing (50°C, 1 min), extension (72°C, 1.5 min) in a thermal cycler (MJ Research). The PCR products were then subcloned into pGEM-T vector (Promega). Both pCI-hCB1 and the PCR products in pGEM-T were digested by *Stu*I and *Not*I. The PCR fragment excised from pGEM-T was ligated back into pCI-hCB1 to replace the original excised segment and transformed into JM109 High Efficiency Competent Cells (Promega). Colonies were screened for ligation of the PCR fragment by size restriction analysis using *Stu*I and *Not*I. The correct sequence of the construct was confirmed by sequencing (sequencing facility of the Medical College of Georgia). Preparation of plasmid DNA was accomplished with a plasmid prep kit (Qiagen, Santa Clarita, CA, USA).

Neuron preparation and microinjection

SCG neurons were isolated from adult male Wistar rats (350–375 g; Harlan, Indianapolis, IN, USA) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals in Research and approved by the Committee on Animal Use for Research and Education at the Medical College of Georgia. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Isolated SCG were treated with 0.3 mg/ml trypsin, 0.4 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN, USA) and 0.1 mg/ml DNase in Earle's balanced salt solution for 1 h at 35°C and shaken vigorously for 10 s to dissociate neurons. Dissociated neurons were plated onto poly-L-lysine-coated 35 mm culture dishes in Minimum Essential Medium (Gibco BRL) with 10% fetal calf serum, 1% glutamine and 1% penicillin–streptomycin. Neurons were incubated in a humidified incubator at 37°C in 5% CO_2. After 4–5 h to allow neurons to attach to the culture dishes, hCB1, hCB1–417, hCB1–400 or the empty vector pCI was microinjected directly into the nucleus of single SCG neurons in concentrations of 50, 100 or 200 ng/μl in water. The pEGFP-N1 plasmid (10 ng/μl) containing the coding sequence of the enhanced green fluorescent protein (Clontech, Palo Alto, CA, USA) was used as a co-injection marker. The plasmid solution was centrifuged ($16\,000 \times g$) in non-heparinized hematocrit tubes for 20 min to remove suspended particles. Injection pipettes were pulled from fiber-filled capillary glass (1B120F-4; World Precision Instruments, Sarasota, FL, USA) on a P-97 Flaming–Brown micropipette puller (Sutter Instrument, Novato, CA, USA). SCG neurons were microinjected with an Eppendorf 5246 transjector and 5171 micromanipulator (Madison, WI, USA) using an injection pressure of 75–100 hPa and an injection time of 0.3–0.4 s.

Electrophysiological recording of Ca^{2+} currents

Ca^{2+} currents from rat SCG neurons were recorded at room temperature (22–26°C) 16–20 h after injection using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). The pipettes for patch recording were pulled from borosilicate glass capillaries (Corning 7052; Garner Glass, Claremont, CA, USA) on a P-97 Flaming–Brown micropipette puller (Sutter Instrument). The patch electrodes were coated with Sylgard 184 (Dow Corning, Midland, MI, USA) and fire-polished on a microforge (Narishige, Tokyo, Japan). Pipette resistances ranged from 2.8 to 3.5 MΩ when filled with the internal solution described below. The cell membrane capacitance and series resistance were electronically compensated to $>80\%$. Whole-cell currents were low-pass-filtered at 5 kHz using the Bessel filter of the clamp amplifier.

Voltage-clamp protocols were generated with a Power Macintosh 8600/200 computer (Apple Computer, Cupertino, CA, USA) equipped with a PCI-16 Host Interface card connected to an ITC-16 Data Acquisition Interface (Instrutech, Port Washington, NY, USA) using Pulse Control 5.0 XOPs (Richard J. Bookman, Jack D. Herrington, and Kenneth R. Newton, University of Miami, Miami, FL, USA) with Igor software (WaveMetrics, Lake Oswego, OR, USA). Ca^{2+} currents were elicited by voltage steps from a holding potential of −80 mV and digitized at 180 μs per point. A double pulse protocol consisting of two 25 ms steps to +5 mV was used to elicit Ca^{2+} currents. The first step to +5 mV elicits the control Ca^{2+} current. The second step to +5 mV was preceded by a 50 ms step to +80 mV. The current elicited by the second voltage step to +5 mV is facilitated compared to the control current elicited by the first voltage step. Current amplitudes were measured isochronally 10 ms after the first voltage step to +5 mV.

In order to isolate Ca^{2+} currents for whole-cell recording, cells were bathed in an external solution that contained (in mM): 140 tetraethylammonium methanesulfonate, 10 HEPES, 15 glucose, 10 $CaCl_2$, 0.0001 tetrodotoxin, pH 7.4 (adjusted with methanesulfonic acid). The intracellular solution consisted of (in mM): 120 *N*-methyl-D-glucamine, 20 tetraethylammonium chloride, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 4 MgATP, 0.1 Na_2GTP, and 14 phosphocreatine, pH 7.2 (adjusted with methanesulfonic acid).

The SF-77B Perfusion Fast-Step device (Warner Instrument, Hamden, CT, USA) was used to apply the cannabinoid receptor agonist WIN 55,212-2 mesylate (RBI/Sigma). Stock solutions of 10 mM WIN 55,212-2 were prepared in dimethylsulfoxide. On the day of the experiment, the stock solution of WIN 55,212-2

was diluted to 1 μM in external solution and briefly sonicated (20 s) to facilitate dispersion.

Results are presented as means ± S.E.M. where appropriate. Statistical significance was determined by Student's *t*-test. The differences were considered significant at $P < 0.05$.

Measurement of surface expression

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) with 10% fetal bovine serum in 100 mm culture dishes in a humidified incubator with 5% CO_2 at 37°C. HEK293 cells were transfected using Effectene™ Transfection Reagent (Qiagen, Valencia, CA, USA) following the product protocol with 2 μg of hCB1, hCB1–417 or hCB1–400 cDNA. Twenty-four hours following transfection, the cells were washed with phosphate-buffered saline (PBS; pH 7.0), removed from the plates with 2 ml of 0.02% EDTA, concentrated, washed twice with wash solution (PBS plus 2% goat serum) and incubated with a polyclonal antibody raised against the N-terminus of the CB1 cannabinoid receptor (from Dr. Kenneth Mackie, University of Washington, Seattle, WA, USA). The cells were incubated with the anti-CB1 antibody (diluted 1:500) for 45 min on ice. Control cells were run in parallel by omitting the primary anti-CB1 antibody. Then the cells were washed twice and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (diluted 1:400) (Santa Cruz Biotech, Santa Cruz, CA, USA) for 45 min on ice. Finally, the cells were washed three times and the cell number was adjusted to 10^5–10^6/ml in PBS. For flow cytometry, cell counts and fluorescent intensity events were acquired using a FACScalibur flow cytometer (Becton Dickinson, San Diego, CA, USA). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using CELLQuest (Becton Dickinson). For confocal microscopy, the cells were pipetted onto a glass slide, covered with cover glass and examined on a confocal microscope (Molecular Dynamics). Fluorescence was low in transfected cells in which both antibodies were omitted or when incubated with only the secondary FITC-labeled antibody.

RESULTS

The proximal domain of the C-terminal tail of hCB1 cannabinoid receptor is critical for G protein signaling while the distal C-terminal tail plays a modulatory role

To study the region of the C-terminal tail of the hCB1 cannabinoid receptor critical for G protein coupling and signal transduction, two different truncated hCB1 receptor constructs were generated. Previous work by Howlett and colleagues showed that a synthetic peptide fragment of amino acids 401–417 from the juxtamembrane C-terminal tail of the mouse CB1 receptor, containing a cysteine to serine substitution, could activate GTPγS binding and compete for G protein binding to immunoprecipitated rat CB1 receptors (Howlett et al., 1998; Mukhopadhyay et al., 1999, 2000). We tested two domains, a proximal domain and a distal domain of the C-terminal tail, for interaction with G proteins by deletion mutations of the human CB1 cannabinoid receptor. We used a functional assay, Ca^{2+} current inhibition by $G_{i/o}$ proteins, to determine the functional coupling of the CB1 cannabinoid receptor to $G_{i/o}$ proteins within a neuronal expression system. We predicted that truncation of the entire C-terminal tail would abolish Ca^{2+} current inhibition due to deletion of the proximal, juxtamembrane C-terminal tail domain. We also predicted that deletion of the distal C-terminal tail domain would have no effect on $G_{i/o}$ protein coupling measured as Ca^{2+} current inhibition. Both the wild type and the C-terminal truncated hCB1 receptors were heterologously expressed in SCG neurons by injection of receptor cDNA into the nucleus. The cannabinoid agonist WIN 55,212-2 (1 μM) elicited a robust 43.7 ± 6.5% ($n = 7$) inhibition of the voltage-dependent Ca^{2+} current in SCG neurons expressing wild type hCB1 receptors (Fig. 2). WIN 55,212-2 had no effect in either uninjected SCG neurons (0.2 ± 1.7%, $n = 7$) or in SCG neurons injected with the empty pCI vector (−2.6 ± 0.8, $n = 6$) (Fig. 2). Ca^{2+} current inhibition by the CB1 receptor is mediated by activation of $G_{i/o}$ proteins in SCG neurons (Pan et al., 1996) through the direct interaction of the Gβγ subunit with the N-type Ca^{2+} channels (Herlitze et al., 1996; Ikeda, 1996). Deletion of the C-terminal tail (amino acids 401–472) of the hCB1 receptor (hCB1–400) abolished ($P < 0.01$) the ability of the receptor to inhibit the Ca^{2+} current (Fig. 2). WIN 55,212-2 reduced the Ca^{2+} current only 4.1 ± 2.9% ($n = 7$) in neurons expressing the C-terminal truncated hCB1–400 receptors.

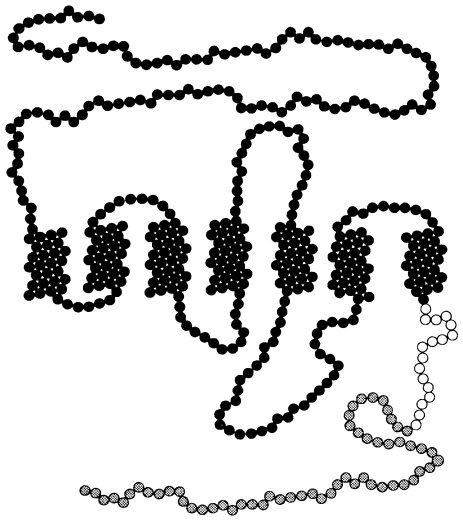

Fig. 1. Schematic of the human CB1 cannabinoid receptor. Two C-terminal deletions were made: hCB1–417 in which amino acids 418–472 were deleted (shaded circles) and hCB1–400 in which amino acids 400–472 were deleted (open circles plus shaded circles).

Surprisingly, deletion of the distal C-terminal domain (amino acids 418–472) of the hCB1 receptor (hCB1–417) had a profound effect on the ability of the hCB1 receptor to signal through G proteins. Inhibition of the Ca^{2+} current by WIN 55,212-2 was significantly reduced ($P < 0.05$) by truncation of the distal C-terminal tail. Application of WIN 55,212-2 inhibited the Ca^{2+} current 22.6 ± 3.0% ($n = 5$) in SCG neurons expressing hCB1–417 C-terminal truncated receptors. Thus, restoration of the proximal, juxtamembrane domain (amino acids 401–417) proved to be critical for $G_{i/o}$ protein coupling. These results confirm *in vitro* biochemical studies that identified this region as a key domain in G protein coupling (Howlett et al., 1998; Mukhopadhyay et al., 1999, 2000). However, our results also demonstrate that the

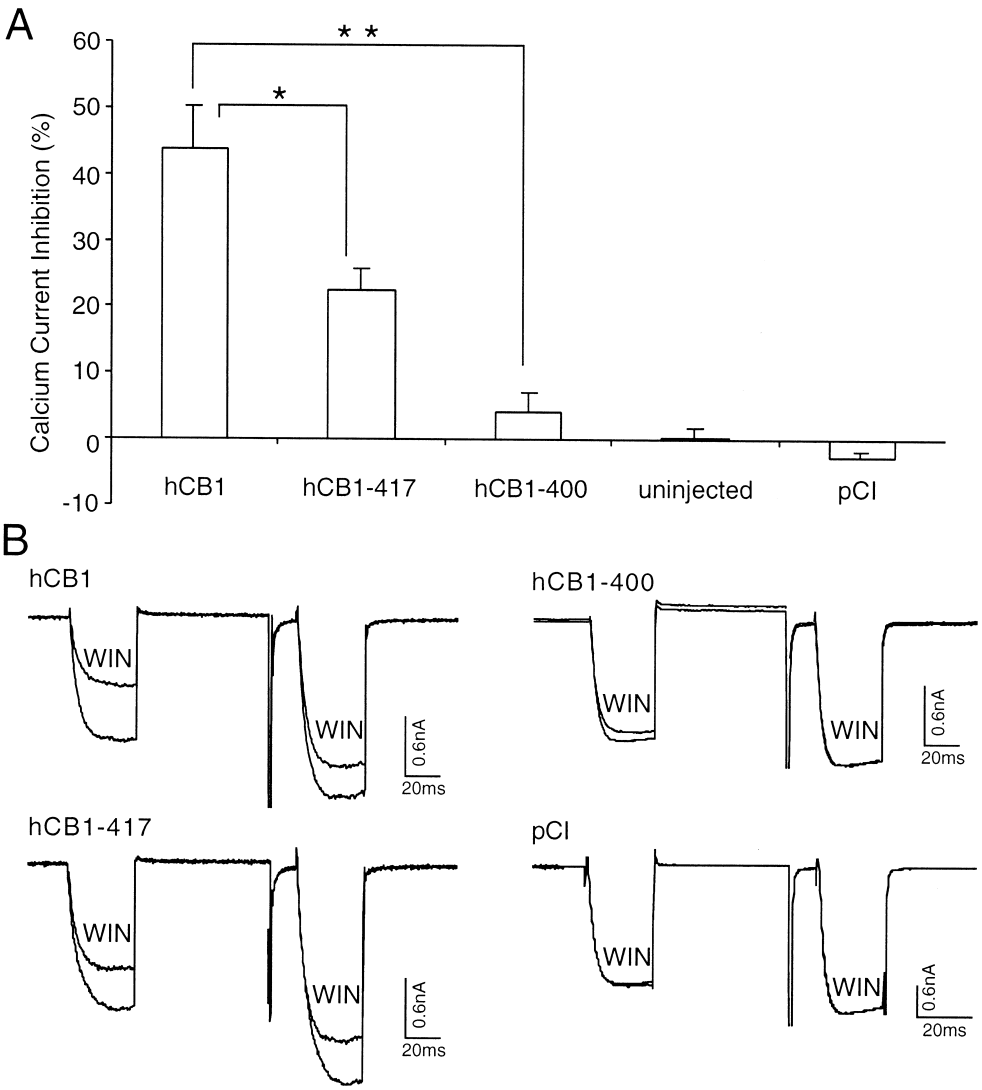

Fig. 2. The effect of WIN 55,212-2 on the Ca^{2+} current in SCG neurons expressing wild type and C-terminal truncated CB1 cannabinoid receptors. (A) Inhibition of Ca^{2+} currents in SCG neurons by 1 μM WIN 55,212-2 (WIN) was significantly reduced (*$P < 0.05$) by deletion of amino acids 418–472 from the distal C-terminal tail of the hCB1 receptor (hCB1–417) compared to the wild type hCB1 receptor. Deletion of the entire C-terminal tail of the hCB1 cannabinoid receptor (hCB1–400) abolished the ability of the receptor to inhibit the Ca^{2+} current (**$P < 0.01$). Neither uninjected SCG neurons or neurons injected with the empty vector pCI responded to WIN 55,212-2. (B) Superimposed current traces elicited by a double pulse voltage paradigm from SCG neurons injected with 100 ng/μl hCB1, hCB1–400 and hCB1–417 cDNA or the empty vector pCI in the absence and presence of WIN 55,212-2.

distal C-terminal domain of amino acids 418–472 profoundly modulates the ability of the receptor to couple to $G_{i/o}$ proteins.

Disruption of signaling by deletion of the distal C-terminal tail (amino acids 418–472) of the hCB1 receptor is independent of differences in receptor expression

To test whether the ability to activate $G_{i/o}$ proteins by the distal C-terminal truncated cannabinoid hCB1–417 receptor was due to differences in receptor expression we compared neurons microinjected with three different receptor cDNA concentrations (50, 100 and 200 ng/μl). Ca^{2+} current inhibition in the presence of WIN 55,212-2 was significantly reduced in neurons microinjected with all three hCB1–417 receptor cDNA concentrations when compared to neurons injected with these concentrations of wild type hCB1 receptor cDNA. WIN 55,212-2 inhibited the Ca^{2+} current $13.5 \pm 3.6\%$ ($n = 5$) in neurons injected with 50 ng/μl hCB1–417 cDNA compared to $32.2 \pm 4.7\%$ ($n = 4$) in neurons injected with 50 ng/μl wild type hCB1 cDNA. In neurons injected with 100 ng/μl cDNA, the wild type hCB1 receptor inhibited the Ca^{2+} current $43.7 \pm 6.5\%$ ($n = 7$) while the truncated hCB1–417 receptor inhibited the current only $22.6 \pm 3.0\%$ ($n = 5$). With the highest concentration of cDNA injected (200 ng/μl), the wild type hCB1 receptor inhibited the Ca^{2+} current $43.4 \pm 3.7\%$ ($n = 5$) while the truncated hCB1–417 receptor inhibited the current only $18.8 \pm 2.4\%$ ($n = 5$). Thus, at all cDNA concentrations injected the effect of WIN 55,212-2 was significantly reduced ($P < 0.05$) by the distal C-terminal truncated

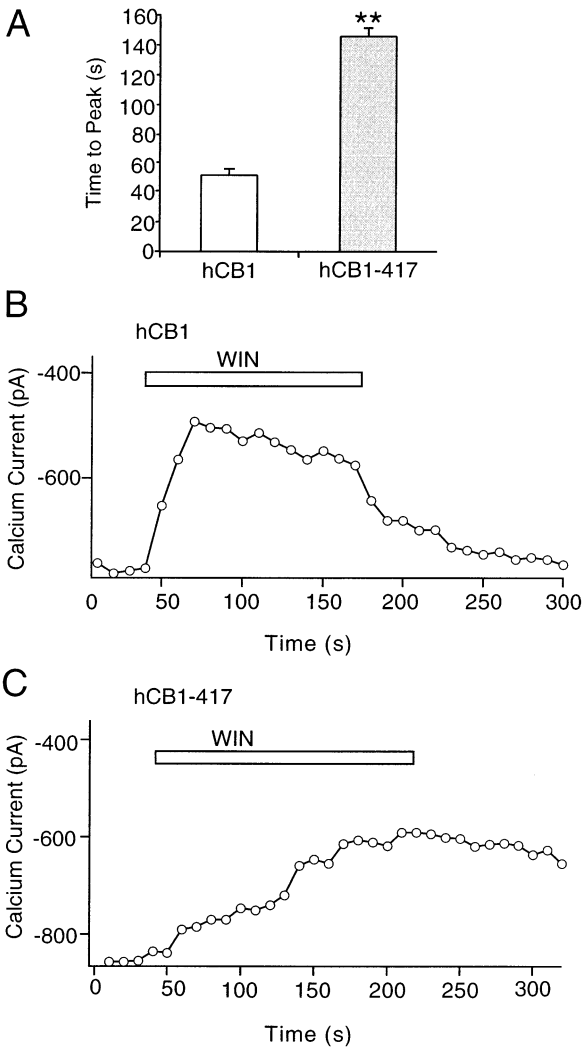

Fig. 3. course of Ca^{2+} current inhibition by the wild type and C-terminal truncated CB1 cannabinoid receptors. (A) Bar graph of the time to peak inhibition of the Ca^{2+} current in the presence of 1 μM WIN 55,212-2 in neurons injected with wild type hCB1 (open bar) or C-terminal truncated hCB1–417 cDNA (shaded bar). Truncation of the distal C-terminal tail of the hCB1 cannabinoid receptor resulted in a much slower inhibition of the Ca^{2+} current ($P < 0.01$). (B) Time course of Ca^{2+} current inhibition in the presence of WIN 55,212-2 (open bar) in neurons injected with hCB1 cDNA (100 ng/μg). (C) Time course of Ca^{2+} current inhibition in the presence of WIN 55,212-2 (open bar) in neurons injected with hCB1–417 cDNA (100 ng/μg).

hCB1–417 receptor. These results indicate that it is unlikely that the difference in the effect of WIN 55,212-2 between the wild type hCB1 receptor and the C-terminal truncated hCB1–417 receptor is due to differences in receptor expression.

Deletion of the distal C-terminal tail (amino acids 418–472) of the hCB1 receptor slowed the kinetics of signal transduction

The time course of Ca^{2+} current inhibition in the presence of WIN 55,212-2 was significantly ($P < 0.01$) slower in neurons expressing the distal C-terminal truncated hCB1–417 cannabinoid receptor compared to the wild type hCB1 receptor (Fig. 4). The time to peak Ca^{2+} current inhibition upon application of WIN 55,212-2 (1 μM) was 146.0 ± 8.7 s ($n = 5$) for hCB1–417 versus 51.5 ± 5.5 s ($n = 7$) for the wild type hCB1 receptor (Fig. 3).

Both C-terminal truncated hCB1 receptors, hCB1–417 and hCB1–400, successfully traffic to the cell surface

The C-termini of some G protein-coupled receptors play a role in trafficking of receptors to the membrane. For example, the mammalian gonadotropin-releasing hormone (GnRH) receptor does not have a C-terminal tail and is trafficked to the cell surface. In contrast, the catfish GnRH receptor has a C-terminal tail and its deletion decreased surface expression of the receptor (Blomenrohr et al., 1999). Deletion of the C-terminal of the mGluR7 metabotropic glutamate receptor prevents trafficking to the cell membrane (McCarthy et al., 2001). To address the possibility that the C-terminal truncated hCB1 receptors are not expressed on the cell surface and that this difference may account for the differences in the effect of WIN 55,212-2 on the Ca^{2+} current we measured cell surface expression using confocal microscopy and flow cytometry (FACS) techniques.

A polyclonal antibody raised against the extracellular amino-terminus 77 amino acid residues of the rat CB1 receptor (Twitchell et al., 1997) (provided by Dr. Kenneth Mackie, University of Washington) was used to detect surface expression. HEK293 cells transiently transfected with hCB1, hCB1–417 or hCB1–400 cDNA (2 μg/100 mm dish) were collected, incubated with the CB1 receptor antibody, washed and incubated with an FITC-labeled secondary antibody. The live cells were then detached from the dish and examined for surface expression by flow cytometry (FACS) and confocal microscopy. Wild type hCB1, hCB1–417 and hCB1–400 receptors were similarly distributed in patches on the membrane surface of HEK293 cells as shown by confocal microscopy (Fig. 4). Flow cytometry was used to quantify the number of cells expressing the CB1 receptors on their cell surface. Control cells in which the primary antibody was omitted showed low fluorescence intensity and were clearly separated from cells expressing hCB1 and the C-terminal truncated hCB1–417 and hCB1–400 receptors (data not shown). Thus, both flow cytometry and confocal imaging demonstrate that the C-terminal truncated hCB1 receptors are trafficked to the cell surface and have a pattern of expression similar to the wild type hCB1 cannabinoid receptor.

DISCUSSION

The principal findings in this study are (1) that the proximal domain of the C-terminal tail of the CB1 cannabinoid receptor is critical for G protein coupling and (2) that the distal C-terminal tail profoundly alters G protein coupling. Expression of the wild type CB1 cannabinoid receptor activates $G_{i/o}$ proteins that, in turn, inhibit voltage-dependent Ca^{2+} currents in SCG neurons.

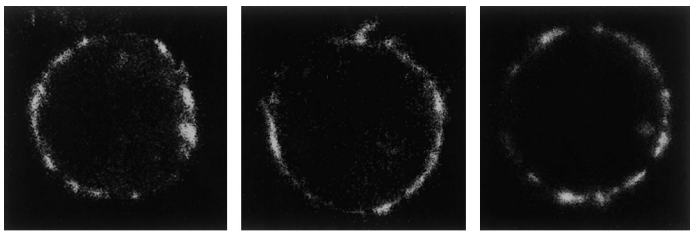

Fig. 4. Surface expression of wild type and C-terminal truncated CB1 cannabinoid receptors. Confocal photomicrographs of resuspended, living HEK293 cells transfected with wild type hCB1, hCB1–417 or hCB1–400 C-terminal truncated receptors and labeled with an N-terminal anti-CB1 antibody.

Deletion of the entire C-terminal tail of the CB1 cannabinoid receptor (hCB1–400) abolished the ability of the cannabinoid agonist WIN 55,212-2 to inhibit the Ca^{2+} current. This destruction of receptor signaling was not from failure of the truncated hCB1–400 receptor to traffic to the cell surface because an N-terminal antibody detected surface labeling similar to wild type receptors when expressed in HEK293 cells. It remains possible, however, that trafficking in neurons may be different than trafficking in HEK293 cells. Removal of amino acids 418–472 from the distal C-terminal tail (hCB1–417) slowed the kinetics and reduced the magnitude of Ca^{2+} current inhibition by the CB1 cannabinoid receptor. Again, cell surface labeling of the hCB1–417 cannabinoid receptor indicated that it was trafficked to the cell surface. There was no difference between cell surface expression of the wild type hCB1 receptor and either of the truncated hCB1 receptors (hCB1–400 and hCB1–417). Thus, amino acids 401–417 located in the proximal region of the C-terminal tail are critical for G protein coupling to the CB1 cannabinoid receptor. This result provides physiological confirmation of biochemical studies that demonstrated that a peptide from this region activated G proteins and competed for $G_{i/o}$ protein binding to the CB1 cannabinoid receptor (Howlett et al., 1998; Mukhopadhyay et al., 1999, 2000).

In addition, the present study provides evidence that the distal C-terminal tail (amino acids 418–472) of the hCB1 cannabinoid receptor plays a modulatory role in receptor G protein coupling. It took a much longer time for the cannabinoid agonist to produce its peak effect and the peak effect was greatly reduced. Similar results can be seen in the paper by Jin et al. (1999) in which a C-terminal truncated Δ418 mutant rat CB1 receptor activated inwardly rectifying currents of smaller amplitude compared to wild type CB1 receptors. The slow response of WIN 55,212-2 in the C-terminal truncated CB1 receptors may reflect an influence of the distal C-terminal domain on G protein coupling. There are several possible mechanisms whereby the distal C-terminal domain could influence G protein coupling. First, the distal C-terminal tail could influence G protein binding by direct interaction with the G protein. Second, the C-terminal tail could interact with other proteins or other parts of the CB1 receptor that could influence G protein coupling. Third, the C-terminal tail might constrain the CB1 receptor to an inactive state. Deletion of the C-terminal might cause the receptor to be in an active G protein-coupled state that would mask further activation by WIN 55,212-2. Fourth, although not yet proven, there is some evidence to suggest that CB1 cannabinoid receptors may exist as dimers (Mackie et al., 2000). If dimer formation occurs it may be through C-terminal tail interactions. A receptor dimer may be the optimal G protein binding conformation of the receptor. Deletion of the C-terminal tail could prevent receptor dimerization and G protein binding.

In summary, the C-terminal tail of the human CB1 cannabinoid receptor contains two functional domains: (1) a proximal C-terminal domain that is critical for coupling to $G_{i/o}$ proteins and (2) a distal C-terminal domain that profoundly modulates the ability of the human CB1 cannabinoid receptor to couple to $G_{i/o}$ proteins.

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