

Cannabinoid Receptors Can Activate and Inhibit G Protein-Coupled Inwardly Rectifying Potassium Channels in a *Xenopus* Oocyte Expression System¹

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ABSTRACT

In this study, we focused on the pharmacological characterization of cannabinoid receptor coupling to G protein-gated inwardly rectifying potassium (GIRK) channels. Cannabinoids were tested on *Xenopus laevis* oocytes coexpressing the CB₁ receptor and GIRK1 and GIRK4 channels (CB₁/GIRK1/4) or the CB₂ receptor and GIRK1/4 channels (CB₂/GIRK1/4). WIN 55,212-2 enhanced currents carried by GIRK channels in the CB₁/GIRK1/4 and CB₂/GIRK1/4 system; however, the CB₂ receptor did not couple efficiently to GIRK1/4 channels. In the CB₁/GIRK1/4 system, WIN 55,212-2 was the most efficacious compound tested. CP 55,940 and anandamide acted as partial agonists. The rank order of potency was CP 55,940 > WIN 55,212-2 = anandamide. The CB₁-selective antagonist SR141716A alone acted as an inverse agonist by inhibiting GIRK currents in oocytes expressing CB₁/GIRK1/4, suggesting the

CB₁ receptor is constitutively activated. A conserved aspartate residue, which was previously shown to be critical for G protein coupling in cannabinoid receptors, was mutated (to asparagine, D163N) and analyzed. Oocytes coexpressing CB₁/GIRK1/4 or D163N/GIRK1/4 were compared. The potency of WIN 55,212-2 at the mutant receptor was similar to wild type, but its efficacy was substantially reduced. CP 55,940 did not elicit currents in oocytes expressing D163N/GIRK1/4. In summary, it appears the CB₁ and CB₂ receptors couple differently to GIRK1/4 channels. In the CB₁/GIRK1/4 system, cannabinoids evaluated demonstrated the ability to enhance or inhibit GIRK currents. Furthermore, a conserved aspartate residue in the CB₁ receptor is required for normal communication with GIRK channels in oocytes demonstrating the interaction between receptor and channels is G protein dependent.

The cannabinoid Δ -9-tetrahydrocannabinol (Δ^9 -THC) is the principal active constituent of marijuana (Gaoni and Mechoulam, 1964). It produces a multiplicity of central and peripheral effects, including euphoria, alteration in memory, analgesia, appetite stimulation, and immunomodulation. These effects are thought to be primarily the result of interactions with G protein-coupled receptors (GPCRs) denoted CB₁ and CB₂ (Matsuda et al., 1990; Munro et al., 1993). There are many structurally diverse compounds that act as cannabinoid receptor agonists. The bicyclic analog of Δ^9 -THC, CP 55,940 [(–)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol], and the structurally dissimilar aminoalkylindole, WIN

55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone], are agonists commonly used to test for cannabinimimetic activity. Several endogenous ligands have also been proposed, including anandamide, an arachidonic acid derivative (Devane et al., 1992).

The CB₁ receptor is located primarily in the brain (Herkenham et al., 1991), whereas the CB₂ receptor has been predominantly localized to cells of the immune system (Galiegue et al., 1995). With such diverse pharmacological effects produced from two receptors, it is perhaps not surprising that these receptors have been shown to couple to multiple effector systems. Activation of the CB₁ receptor has been shown to affect cAMP levels, voltage-gated calcium channels, mitogen-activated protein kinase activity, and potassium channels (for a review, see Pertwee, 1997). Unlike the CB₁ receptor, activation of the CB₂ receptor has been shown to alter only

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ABBREVIATIONS: Δ^9 -THC, Δ -9-tetrahydrocannabinol; CB₁ and CB₂, cannabinoid receptor; HP, high potassium; GIRK, G protein-coupled inwardly rectifying potassium; G protein, guanine nucleotide binding protein; GPCR, G protein-coupled receptor; I_{HK}, inward current with an HK solution containing 96 mM K⁺ and 2 mM Na⁺ exchanged for LK; I_{AG}, inward current with application of the cannabinoid agonist WIN 55,212-2 in HK; I_{native}, native current; CP55,940, (–)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride.

cAMP levels and mitogen-activated protein kinase activity. (Bouaboula et al., 1996).

G protein-gated inwardly rectifying potassium (GIRK) channels play an important role in setting the membrane resting potential and influencing cell excitability (Hille, 1992). In the heart, the K(acetylcholine) channel is a GIRK1/GIRK4 (GIRK1/4) heterotetramer (Duprat et al., 1995; Krapivinsky et al., 1995). Binding of acetylcholine to cardiac muscarinic m₂ receptors activates GIRK1/4 channels through G proteins and leads to the classic parasympathetic effect (i.e., decrease in heart rate; Pfaffinger et al., 1985). GIRK channels are also present in many brain regions and have been shown to couple to a variety of inhibitory neurotransmitter receptors, including opioid, muscarinic, and somatostatin receptors (Bausch et al., 1995; Chan et al., 1996; Hans-Jurgen et al., 1997). The physiological relevance of the interaction between receptor and channel protein in the brain is still unclear (for a review, see Dascal, 1997). Because the G_{βγ} subunits of G proteins are direct activators of GIRK, these channels allow the study of G_{βγ} effector outcomes (Logothetis et al., 1987; Reuveny and Jan, 1994).

The CB₁ receptor activates GIRK channels in heterologous expression systems. Mackie et al. (1995) demonstrated in AtT-20 cells transfected with CB₁ cDNA that the cannabinoid agonist WIN 55,212-2 enhanced current flux through an endogenous, pertussis toxin-sensitive, inwardly rectifying potassium channel. Similar results were obtained by Henry and Chavkin (1995) in *Xenopus laevis* oocytes injected with the CB₁ receptor and GIRK1 channel cRNA. On application of the cannabinoid agonist WIN 55,212-2, GIRK1 channel currents were enhanced. In a recent report describing the immunohistochemical distribution of the CB₁ receptor in rat brain, positively stained cap-like structures in the cerebellum were suggested to be basket cell projections. It was noted that these distinct structures appeared similar to projections that had been labeled positive with GIRK channel antibody in rat cerebellum (Tsou et al., 1998).

The aims of this investigation were to pharmacologically characterize CB₁ receptor effects and to examine possible CB₂ receptor coupling to GIRK channels. Previously, only a single cannabinoid agonist, WIN 55,212-2, had been evaluated for cannabinoid receptor activation of GIRK channels (Henry and Chavkin, 1995; Mackie and Mitchell, 1995). In the present study, multiple cannabinoid agonists were evaluated in a *X. laevis* oocyte system expressing the CB₁ receptor and GIRK1/4 channel proteins (CB₁/GIRK1/4). GIRK1/4 channels were used instead of GIRK1 alone because expression of GIRK channels as heterotetramers led to more consistent expression and increased activity (Duprat et al. 1995; Chan et al., 1996). The CB₁-selective antagonist SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride], which has been reported to have inverse agonist activity (Compton et al., 1996; Bouaboula et al., 1997; Landsman et al., 1997), was also investigated to determine how it would affect the interaction between the CB₁ receptor and GIRK1/4 channels. Finally, a CB₁ receptor aspartate mutation, which has been previously shown to disrupt G protein coupling (Tao and Abood, 1998), was further characterized and used to demonstrate cannabinoid receptor communication with GIRK1/4 channels is dependent on G protein activation.

Experimental Procedures

Materials. *X. laevis* frogs were purchased from *Xenopus* One (Dexter, MI). Reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). CP 55,940 was generously provided by Pfizer (Groton, CT). WIN 55,212-2 was purchased from Research Biochemicals Inc. (Natick, MA). Δ⁹-THC and SR141716A were obtained from NIDA. Dr. Raj Razdan (Organix, Woburn, MA) supplied the anandamide. The human CB₁ cDNA was obtained from Dr. Marc Parmentier (Universite Libre de Bruxelles, Brussels, Belgium). The human CB₂ cDNA was obtained from Dr. Sean Munro (Medical Research Council, Cambridge, UK). The rat GIRK1 cDNA was a gift from Dr. Henry Lester (California Institute of Technology, Pasadena, CA), and the cDNAs for the human homologs of GIRK1 and GIRK4, in the vector pGEM-HE, were graciously supplied by Dr. Diomedes E. Logothetis (Mt. Sinai Medical Center, New York, NY).

Mutagenesis. The Altered Sites (Promega, Madison, WI) in vitro mutagenesis system was used to mutate the CB₁ receptor as previously described by Tao and Abood (1998). To make the D163N mutation, the mutagenic oligonucleotide (5'-CGGTGGCAAACCTCCTGGGGA) containing the desired mutation (GAC to AAC) was used. The mutations were confirmed by sequencing, and the mutated cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA).

cRNA Synthesis. For preliminary studies, cDNAs for rat GIRK1 in pBluescript II KS⁻, human CB₁ in pcDNA3, and human CB₂ in pBluescript II KS⁻ or in pGEM-HE were linearized by *Xho*I, *Xba*I, *Not*I, and *Nhe*I, respectively. For the heteromultimer studies, pGEM-HE containing the cDNAs for the human homologs of GIRK1 and GIRK4 were linearized using *Nhe*I. The cRNAs were transcribed in vitro using a T7 RNA polymerase (mMESSAGE mMACHINE; Ambion, Austin, TX).

Expression in Oocytes and Recordings. Adult oocyte positive *X. laevis* frogs were housed in distilled, dechlorinated water (18–20°C) with 12/12-h light/dark lighting cycle and fed twice weekly. Frogs were anesthetized by partial immersion in a 0.25% solution of MS-222 (Sigma Chemical Co., St. Louis, MO) for 10 to 30 min. A portion of the eggs was removed. The eggs were defolliculated with 1 mg/ml Collagenase type 1A (Sigma Chemical Co.) for 60 to 90 min. Rat GIRK1 or human GIRK1/4 and human CB₁ or human CB₂ cRNAs were coinjected into each oocyte (Drummond Scientific Co., Broomall, PA). Recordings were performed after 7 to 9 days of incubation in 0.5× L-15 media (Sigma Chemical Co., St. Louis, MO) supplemented with L-glutamine and antibiotics. For initial recordings, the eggs were placed in a chamber (total volume, 200 μl) and perfused at 4 ml/min with low potassium (LK) solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES pH 7.5), high potassium (HK) solution (2 mM NaCl, 96 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES pH 7.5), or HK plus drug. Fatty acid-free BSA (3 μM) was added to all drug solutions to minimize adsorption of cannabinoid compounds to the perfusion system. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.5–1.0 MΩ) and were voltage-clamped at reported voltages using an Axon GeneClamp amplifier (Axon Instruments Inc., Foster City, CA). Currents were collected at 100 Hz, filtered at 10 Hz, and analyzed using a Macintosh Centris 650 containing a 16-bit analog-digital interface board and voltage-clamp software running under the IGOR graphics environment (Wavemetrics, Lake Oswego, OR). Exchange of solutions was accomplished using a manual 6-port stream selection valve. In studies used to determine the K_B value for the CB₁-selective antagonist SR141716A, oocytes were pretreated with SR141716A for 5 min before the addition of WIN 55,212-2. When I_{GIRK} amplitudes exceeded 2 μA, small transient changes in the I_{GIRK} time course were often observed. These transient inward changes appeared within the first 20 s on application of drug. Extensive control studies revealed these inflections were not due to drug and/or any component of the vehicle. We concluded these in-

flections were due to an artifact of the solution exchange system that is more apparent at larger I_{GIRK} amplitudes.

Data Analysis. The EC_{50} and E_{max} values with corresponding 95% CLs were calculated using the Prism 2.0 program (GraphPAD, San Diego, CA). The Student's *t* test ($P < .05$) was used for statistical analysis. The apparent equilibrium dissociation constant (K_B) for the interaction of the antagonist and the receptor was calculated from the equation $K_B = [B]/(\text{dose ratio} - 1)$, where $[B]$ is the concentration of antagonist used in the experiment (Griffin et al., 1998).

Results

Characteristics of CB_1 and CB_2 Receptors Coexpressed with GIRK Channels. The addition of WIN 55,212-2 activated an inwardly rectifying potassium current when GIRK1 or GIRK1/4 cRNA were coinjected with human CB_1 cRNA (Fig. 1, A and B). In initial studies, oocytes were voltage-clamped at -80 mV and superfused in an LK solution containing 96 mM Na^+ and 2 mM K^+ . When an HK solution containing 96 mM K^+ and 2 mM Na^+ was exchanged for LK, an inward current (I_{HK}) was produced (Fig. 1B). I_{HK} was a product of basal activation of GIRK1/4 channels (Chan et al., 1996). The application of the cannabinoid agonist WIN 55,212-2 in HK further enhanced the inward current and was defined as I_{Ag} . Coexpression of CB_1 and GIRK1 subunits resulted in production of modest inward currents (Fig. 1A). The average I_{HK} and I_{Ag} (in the presence of 1 μ M WIN 55,212-2) were 772 ± 169 nA ($n = 3$) and 773 ± 78 nA ($n =$

3), respectively. In contrast, coexpression of CB_1 and GIRK1/4 as a heteromultimers led to the production of robust inward currents. The average I_{HK} and I_{Ag} (in the presence of 1 μ M WIN 55,212-2) were 6.4 ± 1.1 μ A ($n = 5$) and 4.7 ± 1.5 μ A ($n = 5$), respectively (Fig. 1B). In six different batches of oocytes injected with CB_1 /GIRK1/4, functional coupling between GIRK1/4 and the CB_1 receptor, as defined by a response to agonist, was more than 96% ($n = 48$). In oocytes injected with CB_1 /GIRK1/4, I_{Ag} was also produced in the presence of the cannabinoid agonists anandamide and CP 55,940 (Fig. 1, C and D).

Oocytes coinjected with CB_2 and GIRK1/4 cRNA demonstrated inconsistent coupling of less than 15% ($n = 21$), even when the amount of injected receptor cRNA was raised to 30 ng (injected per egg). In an effort to increase coupling efficiency, the CB_2 receptor was placed into a vector specifically designed to increase expression of foreign cRNA translated proteins in *X. laevis* oocytes (Liman et al., 1992). In oocytes injected with 1 to 5 ng of cRNA, the CB_2 receptor coupled inconsistently, less than 20% ($n = 43$), to GIRK1/4 as was seen originally. When CB_2 cRNA concentrations of 11 to 34 ng were injected into the oocytes, cell integrity was compromised at day 3 (data not shown). We attempted to address the question of whether oocytes were expressing mature CB_2 receptors using binding analysis but were unsuccessful because of high nonspecific binding in the oocyte membrane preparations. In oocytes responding to the cannabinoid ago-

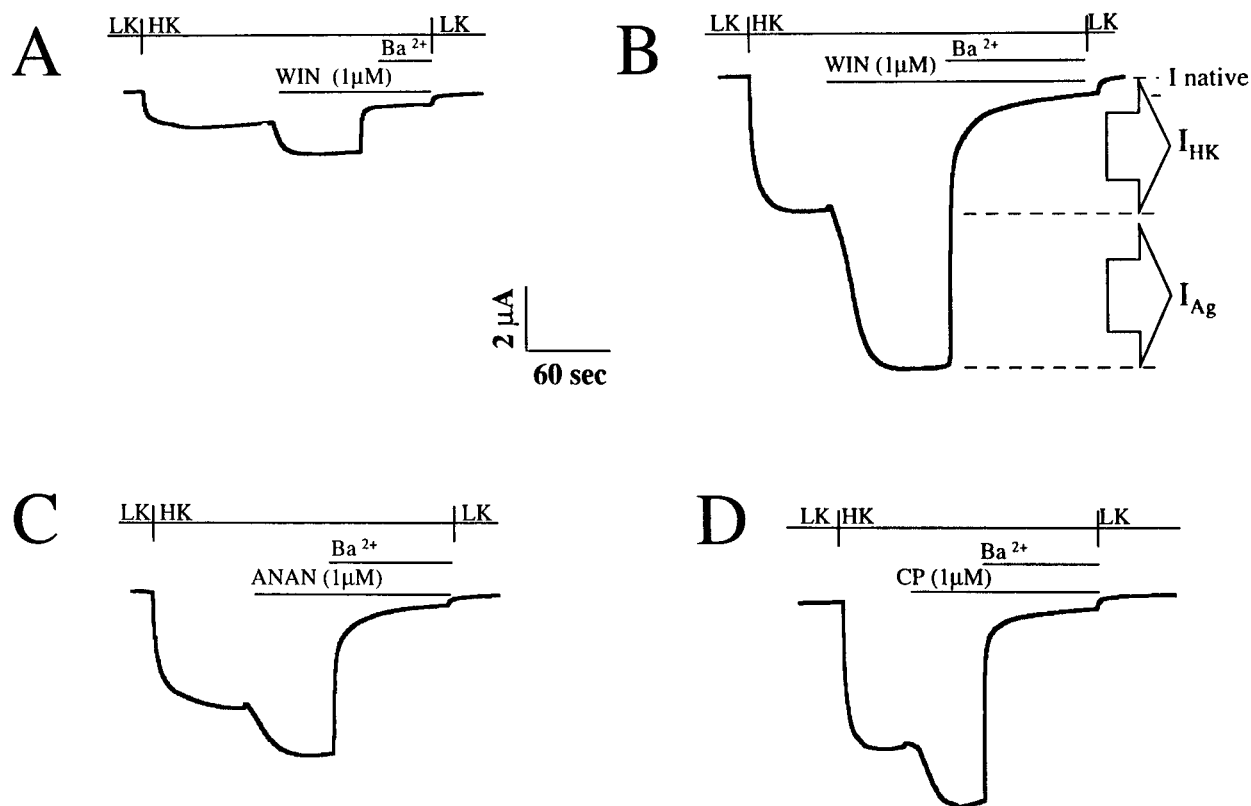


Fig. 1. Cannabinoid agonists activate inwardly rectifying potassium currents in oocytes coexpressing GIRK1 or GIRK1/4 with CB_1 . Currents were induced by exchanging LK with HK while the oocytes were voltage-clamped at -80 mV. Compounds were applied to the perfusion system at the concentrations indicated, and 300 μ M Ba^{2+} was added where illustrated. Representative recordings of oocytes coinjected with 11 ng of GIRK1 and 33 ng of CB_1 cRNA (A) or oocytes coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB_1 cRNA (B, C, and D). A and B, current enhancement observed on application of 1 μ M WIN 55,212-2 (WIN) in the presence of HK. Ba^{2+} (300 μ M) was added where indicated. In the final third of the trace, oocytes were superfused with LK. C and D, addition of 1 μ M anandamide (ANAN) or CP 55,940 (CP) in the presence of HK resulted in current enhancement. Each experiment was repeated in four to six oocytes.

nist WIN 55,212-2, the average I_{HK} and I_{Ag} were $4.8 \pm 1.1 \mu A$ ($n = 5$) and $1.1 \pm 0.6 \mu A$ ($n = 5$), respectively (Fig. 2A).

The currents produced by application of high potassium (I_{HK}) are due to K^+ flux through GIRK1/4 channels as well as rectifying channels endogenous to the oocyte (Kovoor et al., 1995). These currents can be separated using $300 \mu M$ Ba^{2+} (Dascal et al., 1993a), which blocks GIRK-mediated currents. To determine the fraction of native current (I_{native}) that was responsible for I_{HK} , a pool of oocytes injected with only GIRK1/4 cRNA was tested for sensitivity to Ba^{2+} (Fig. 2B). The average I_{HK} was $6.6 \pm 0.6 \mu A$ ($n = 6$). I_{native} (current not sensitive to $300 \mu M$ Ba^{2+}) constituted only a small fraction of total I_{HK} (443 ± 50 nA, $n = 6$). I_{native} was not significantly increased in the presence of $1 \mu M$ WIN 55,212-2 (837 ± 250 nA, $n = 6$). Currents carried by GIRK1/4 were not enhanced on application of WIN 55,212-2 in oocytes not injected with the CB₁ receptor (Fig. 2B). Similar results were obtained in the presence of all cannabinoid agonists tested when the CB₁ receptor was not coinjected (data not shown). Oocytes injected with the CB₁ receptor alone exhibited currents similar to I_{native} in the presence of HK and did not respond significantly to the application of $1 \mu M$ WIN 55,212-2 (Fig. 2C). Similar results were seen in oocytes injected with water (data not shown).

By definition, inwardly rectifying channels will display increasing inward current as the cell membrane is moved from positive to more negative potentials. To study whether cannabinoids can activate inward rectifiers, current-voltage plots were generated. Figure 3 is a representative example of experiments in which oocytes injected with CB₁/GIRK1/4 cRNA were subjected to voltage ramps (-80 to $+50$ mV) during treatment with HK or HK and $1 \mu M$ WIN 55,212-2 (WIN). Inward rectifying current activated under these conditions had a reversal potential near 0 mV, as expected for a potassium current because $[K]_i$ and $[K]_o$ were approximately

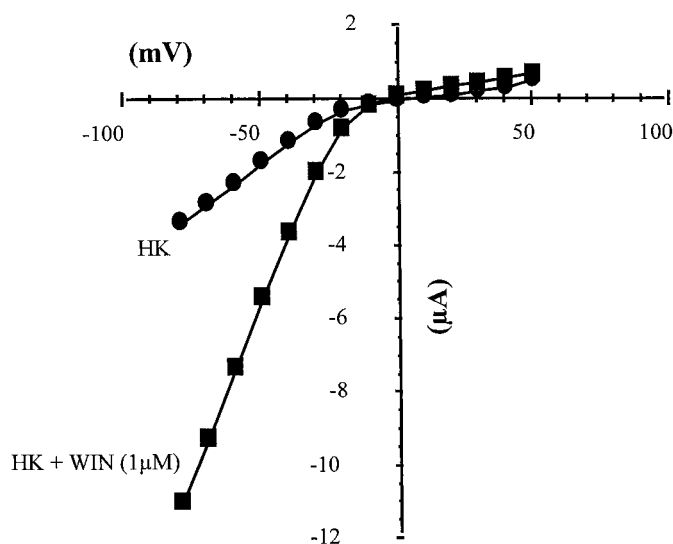


Fig. 3. Current-voltage plots. Oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁ cRNA. Representative current-voltage plot was generated by applying a continuous voltage ramp (-80 to $+50$ mV) to oocytes during treatment with HK (●) or HK and $1 \mu M$ WIN 55,212-2 (■). Leak currents were subtracted in this illustration. This experiment was repeated in more than six oocytes.

equal. WIN 55,212-2 ($1 \mu M$) did not appear to shift the potassium equilibrium potential of the GIRK1/4 current but simply increased its amplitude. Therefore, current enhancement was most likely a result of increased GIRK1/4 channel conductance. Similar results were seen with all cannabinoid agonists tested (data not shown).

The initial recording protocol involved voltage clamping the oocytes at -80 mV. Under these conditions, I_{HK} reached a short-lived steady state within the first 2 min, and then desensitization occurred for the remainder of the recording

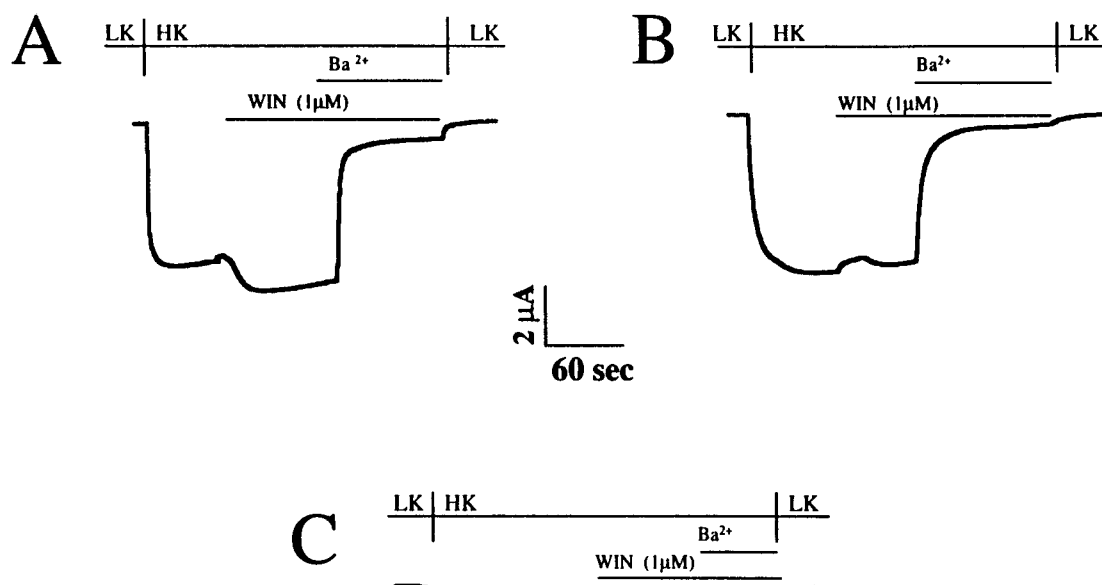


Fig. 2. WIN 55,212-2 enhances GIRK currents in oocytes coexpressing CB₂ and GIRK1/4 but not in oocytes expressing only GIRK1/4 or the CB₁ receptor. A, representative recording of oocytes coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₂ cRNA. Current enhancement seen on the application of WIN 55,212-2 ($1 \mu M$) in the presence of HK. Ba^{2+} ($300 \mu M$) was added where indicated. In the final third of the trace, oocytes were superfused with LK. B, oocytes were injected with only 0.1 to 0.3 ng of GIRK1/4 cRNA. Current enhancement was not observed on application of WIN 55,212-2 ($1 \mu M$) in the presence of HK. C, oocytes were injected with 14 to 25 ng of CB₁ cRNA. Oocytes were subjected to the same recording protocol used in A and B. Each experiment was repeated in four to six oocytes.

(Fig. 4A). This slow agonist-independent desensitization has been reported to occur at the channel level (Kovoor et al., 1995). To carry out concentration-response experiments in a single oocyte, I_{HK} was allowed to reach a stable steady state before application of the drug. Using this method, we observed compromised cell membrane integrity; therefore, the protocol was modified. Figure 4B is a representative example of typical concentration-response experiments using the modified recording approach. Oocytes injected with CB₁/GIRK1/4 were voltage-clamped at 0 mV. In the presence of LK, HK, or HK plus increasing concentrations of WIN 55,212-2, 800-ms pulses of -80 mV were periodically applied to an oocyte to monitor the current (Fig. 4B). This protocol allowed for I_{HK} to reach a stable steady state (arrow) within approximately 17 min ($n = 33$). At this point, drug was added. Incomplete washout of drug is typically seen with cannabinoid compounds, presumably due to their lipophilicity; therefore, cumulative concentration-response experiments were performed to circumvent this problem. The application of increasing concentrations (4–5 concentrations per experiment) of WIN 55,212-2 led to a concentration-dependent enhancement of I_{Ag} (Fig. 4C). A more detailed view of the I_{Ag} is shown in Fig. 4D. Figure 4B shows an example of the pulse recordings used to generate Fig. 4B. By following this recording protocol, stress to oocytes was minimal, allowing for increased data acquisition time and more reproducible concentration-response experiments.

Cannabinoid Receptor Agonists. The responses to representative cannabinoid agonists CP 55,940, WIN 55,212-2, anandamide, and Δ^9 -THC were compared (Fig. 5). These agonists produced a concentration-dependent enhancement of ionic current carried by GIRK1/4 channels in oocytes coinjected with CB₁/GIRK1/4 cRNA. WIN 55,212-2 was chosen as the standard reference agonist and was run as a control in each set of experiments. A WIN 55,212-2 concentration-response curve was run each week, in each set of experiments, to standardize all agonist responses. The maximal response to WIN 55,212-2 was considered 100%. All responses were expressed as the percent of current enhancement: ($I_{Ag}/\text{maximal } I_{Ag} \text{ produced with WIN 55,212-2} \times 100$). Nonlinear regression analysis of WIN 55,212-2 gave EC_{50} and E_{max} values of 127 nM (64–251 nM) and 100% (81–118%), respectively. The potency of anandamide was not significantly different from WIN 55,212-2, but anandamide-stimulated currents were approximately half those seen with WIN 55,212-2. The EC_{50} and E_{max} values generated for anandamide were 89 nM (64–122 nM) and 46% (43–49%), respectively. CP 55,940 was the most potent compound tested, but it was the least efficacious ($EC_{50} = 0.8$ nM (0.2–3.2 nM) and $E_{max} = 28\%$ (19–37%), respectively). I_{Ag} was not consistently produced on application of Δ^9 -THC (data not shown); therefore, EC_{50} and E_{max} values could not be calculated.

Activity of SR141716A. To determine whether the CB₁-selective antagonist SR141716A (Rinaldi-Carmona et al.,

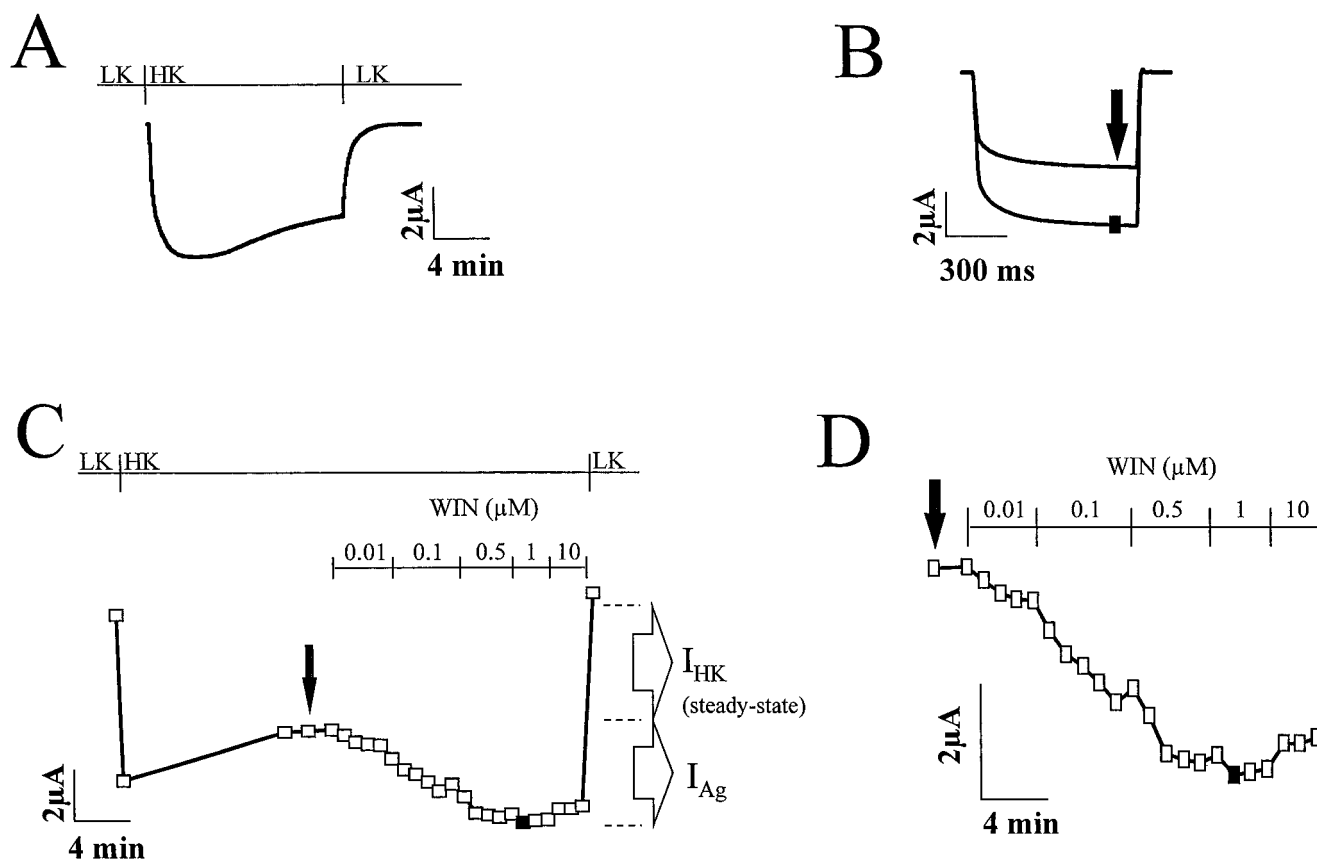


Fig. 4. Alternative recording approach for concentration-response analysis. Oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁ cRNA. A, representative example of oocytes subjected to a constant voltage-clamp of -80 mV. Inward currents were induced by exchanging LK with HK. B, two pulse recordings used in generating Fig. 2C. The inward currents at 750 ms, marked with an arrow (I_{HK}) or filled box (I_{Ag}), correspond to single points on C. C, representative concentration-response experiment generated by pulse recording. Oocytes were voltage-clamped at 0 mV, and pulses of -80 mV were periodically applied in the presence of LK, HK, or HK and increasing concentrations of WIN 55,212-2 as indicated. D, portion of the recording denoted I_{Ag} in Fig. 4C was enlarged. These experiments were repeated in more than six oocytes.

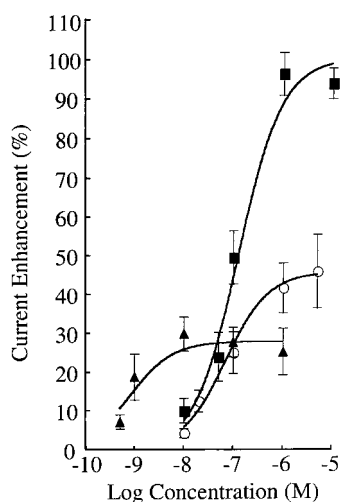


Fig. 5. Concentration-response analysis with cannabinoid agonists. Oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁ cRNA. Current enhancement was produced on application of WIN 55,212-2 (■), CP 55,940 (▲), and anandamide (○). Each data point is the mean $\pm$ S.E. of three to seven determinations from two batches of oocytes.

1994) acts as an inverse agonist on GIRK1/4 channels, a concentration-response curve was generated. Substantial inhibition of I_{HK} was produced by SR141716A in oocytes injected with CB₁/GIRK1/4 cRNA but not in oocytes injected with only GIRK1/4 cRNA (Fig. 6). SR141716A inhibited I_{HK} in a concentration-dependent manner (Fig. 7A). The calculated EC_{50} and E_{max} values were 200 nM (100–300 nM) and –54% (48–61%), respectively. Figure 7B illustrates the magnitude of current inhibition produced by 1 μ M SR141716A compared with 1 μ M WIN 55,212-2. SR141716A or WIN 55,212-2 was applied to oocytes injected with CB₁/GIRK1/4 cRNA when I_{HK} reached steady state. The application of WIN 55,212-2 enhanced I_{HK} by $104 \pm 11\%$ ($n = 7$), whereas SR141716A inhibited I_{HK} by $-46 \pm 7\%$ ($n = 9$).

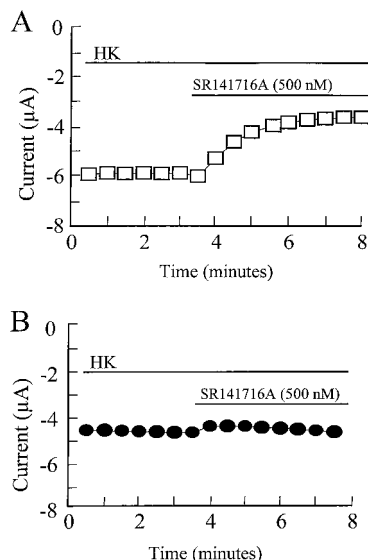


Fig. 6. SR141716A inhibition of GIRK1/4 currents is dependent on expression of the CB₁ receptor. A, oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 cRNA and 14 to 25 ng of CB₁ cRNA (□) or (B) coinjected with 0.1 to 0.3 ng of GIRK1/4 (●). Application of 500 nM SR141716A in HK to oocytes injected with CB₁/GIRK1/4 cRNA resulted in current inhibition. This inhibition was not seen when oocytes were injected with only GIRK1/4 cRNA. These experiments were repeated in six oocytes.

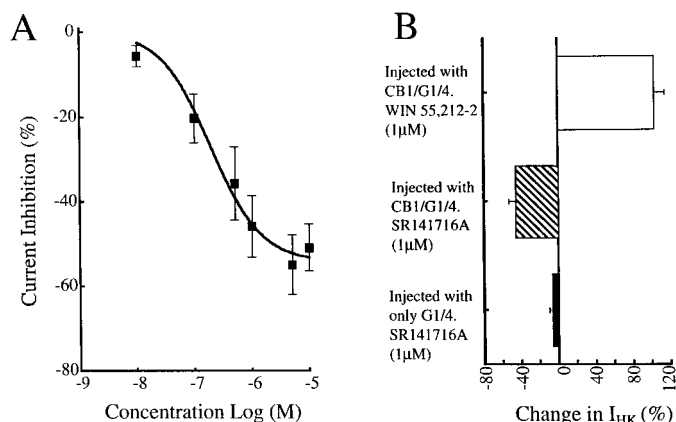


Fig. 7. The current inhibition produced by SR141716A is concentration dependent and opposite of the effect produced by the agonist WIN 55,212-2. Oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁ cRNA. A, current inhibition produced on application of SR141716A (■). B, SR141716A inhibits, whereas WIN 55,212-2 enhances, GIRK currents. Each data point or column is the mean $\pm$ S.E. of at least six determinations from two batches of oocytes.

In the next set of experiments, a concentration of SR141716A that did not produce significant inverse agonism (10 nM) was used to determine whether SR141716A could antagonize the effects of WIN 55,212-2 and anandamide (Fig. 8A). Concentrations that were the approximate EC_{80} values for the agonists were used in the presence of 10 nM SR141716A. WIN 55,212-2- and anandamide-stimulated currents were substantially reduced from 64 ± 4 to $25 \pm 5\%$ and 42 ± 5 to $27 \pm 4\%$, respectively. In both the presence and absence of 10 nM SR141716A, two concentrations of WIN 55,212-2 located in the linear portion of the respective concentration-response curves were compared to calculate a dose ratio of 11.98 (7.82–18.57; Fig. 8B). The apparent equilibrium dissociation constant (K_B) of SR141716A calculated in the presence of WIN 55,212-2 was 0.91 nM (0.57–1.46 nM).

Evaluation of Cannabinoid Receptor Mutant D163N.

Mutation of a conserved aspartate to an asparagine in the second transmembrane domain of the CB₁ receptor disrupts G protein coupling (Tao and Abood, 1998). To compare the

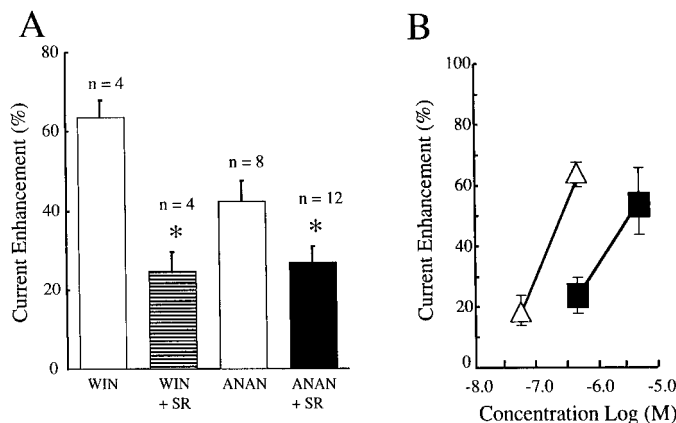


Fig. 8. Antagonism of WIN 55,212-2 and anandamide-stimulated currents by SR141716A. Oocytes were coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁ cRNA. A, current enhancement observed on application of 500 nM WIN 55,212-2 (WIN) or anandamide (ANAN) was blocked by 10 nM concentration of the CB₁-selective antagonist SR141716A (SR; * $p < .05$). B, WIN 55,212-2-stimulated currents in the absence (△) and presence (■) of 10 nM SR141716A. Each data point is the mean $\pm$ S.E. of at least four determinations from two batches of oocytes.

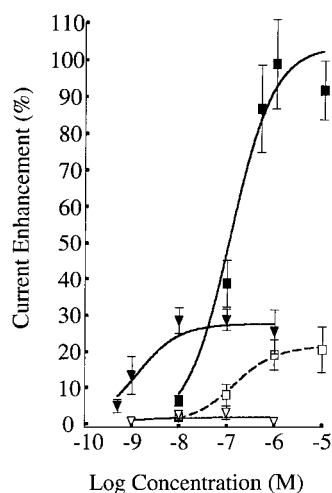


Fig. 9. Concentration-response analysis in oocytes coinjected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of D163N (■) and CP 55,940 (▼) in oocytes injected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of CB₁. The analysis was repeated with WIN 55,212-2 (□) and CP 55,940 (▽) in oocytes injected with 0.1 to 0.3 ng of GIRK1/4 and 14 to 25 ng of D163N. Each data point in the dose-response curve is the mean $\pm$ S.E. of at least four determinations from two batches of oocytes.

wild-type CB₁ receptor with the mutant D163N receptor, concentration-response curves were generated with WIN 55,212-2 and CP 55,940 in oocytes expressing CB₁/GIRK1/4 or D163N/GIRK1/4 (Fig. 9). Wild-type controls were compared with mutants in the same pool of oocytes. The potencies of WIN 55,212-2 and CP 55,940 in the wild-type (CB₁/GIRK1/4) system were EC₅₀ values of 103 nM (80–127 nM) and 1.3 nM (0.4–4.4 nM), respectively. Agonist-stimulated currents in the presence of WIN 55,212-2 and CP 55,940 in the wild-type system were $E_{\max}$ values of 103% (80–127%) and 27% (21–34%), respectively. The potency of WIN 55,212-2 was similar in the mutant (D163N/GIRK1/4) system, but agonist-stimulated currents in the presence of WIN 55,212-2 were substantially reduced. The calculated EC₅₀ and $E_{\max}$ values were 155 nM (76–316 nM) and 21% (18–24%), respectively. $E_{\max}$ and EC₅₀ values could not be calculated for CP 55,940 in the mutant system because significant current enhancement was not seen.

Discussion

The results of this study demonstrate that a variety of cannabinoid agonists can enhance the activity of GIRK1/4 channels in oocytes coexpressing CB₁/GIRK1/4. The effects produced by agonists were dependent on expression of the cannabinoid receptor. The current enhancement produced on application of WIN 55,212-2 was rapidly blocked by barium, which is known to block GIRK channel responses (Kovoor et al., 1995). In oocytes not injected with CB₁/GIRK1/4, only native channel activity was observed in the presence of WIN 55,212-2. These results together suggest that the majority of current enhancement observed on application of cannabinoid agonists is due to the activation of GIRK1/4 channels. The current-voltage relationships generated in the presence and absence of cannabinoid agonists revealed that current enhancement is a result of increased GIRK1/4 channel activity.

There are several differences between the CB₁ and CB₂ receptors, including structure, tissue localization, ligand specificity, and, undoubtedly, function (Pertwee, 1997). The

present investigation revealed the CB₂ receptor did couple to GIRK1/4 channels but not consistently. In addition, the agonist-stimulated currents, produced by WIN 55,212-2 in oocytes expressing CB₂/GIRK1/4, were approximately four times less than those recorded in oocytes expressing CB₁/GIRK1/4. A detailed dose-response analysis was not carried out in oocytes expressing CB₂/GIRK1/4 because of the inconsistent coupling seen between receptor and channel. Perhaps the pertussis toxin-sensitive G proteins the CB₂ receptor normally uses (Bouaboula et al., 1996) are only expressed at low levels in oocytes, and therefore activation of this pool of G proteins is inconsistent. However, it is more likely the receptor coupled promiscuously to G proteins it does not normally associate with in a native environment. The CB₂ receptor has been shown not to couple to GIRK or calcium channels in AtT-20 cells (Mackie and Mitchell, 1995). Additionally, there is little evidence to suggest colocalization of the CB₂ receptor and GIRK1/4 channels. CB₂ receptors are restricted primarily to cells of the immune system, a population of cells not associated with expression of GIRK channels (Dascal et al., 1993b; Munro et al., 1993). In contrast, the CB₁ receptor is located in many of the same regions of the central nervous system as GIRK channels (Herkenham et al., 1991; Ponce et al., 1996). CB₁ receptors and GIRK channels could be colocalized on the action of γ -aminobutyric acid-positive neurons in the cerebellum (Tsou et al., 1998). In the brain, some of the effects of inhibitory neurotransmitter receptors are probably the result of the activation of GIRK channels (Dascal, 1997).

Whether CB₁ receptors interact with GIRK channels in native tissue has yet to be determined. In this study, the EC₅₀ values calculated for the cannabinoid agonists are in reasonable agreement with potency values reported in other functional assays, such as cannabinoid inhibition of forskolin-stimulated cAMP accumulation and cannabinoid stimulation of guanosine-5'-O-(3-thio)triphosphate binding (Felder et al., 1995; Griffin et al., 1998). WIN 55,212-2 was the most efficacious agonist tested in oocytes expressing CB₁/GIRK1/4. In studies evaluating inhibition of calcium channels and neurotransmitter release, WIN 55,212-2 was reported to be the most efficacious compound tested (Pan et al., 1996; Shen et al., 1996). In this investigation, anandamide produced approximately 46% of the current enhancement seen with WIN 55,212-2, whereas CP 55,940 produced only 28% of the effect seen with WIN 55,212-2. The efficacy values reported for cannabinoid receptor inhibition of presynaptic glutamate release in rat hippocampal cultures (Shen et al., 1996) were similar to the efficacy values calculated in this study. It was suggested that ion channel modulation was the mechanism contributing to inhibition of glutamate release. The relationship between potencies in stimulation of GIRK channels and other pharmacological measures at least supports the premise that CB₁ receptors may be coupled to GIRK channels.

Δ^9 -THC was tested in oocytes expressing CB₁/GIRK1/4, but the production of concentration-dependent current enhancement was inconsistent, whereas WIN 55,212-2, CP 55,940, and anandamide were consistently effective as agonists. In other in vitro studies, Δ^9 -THC has acted as a weak partial agonist or has not produced an effect (Pertwee, 1997; Griffin et al., 1998). Receptor/G protein stoichiometry probably contributes to differences in the effects of the same

compound between cell systems (for a review, see Kenakin, 1996).

The CB₁-selective antagonist SR141716A (Rinaldi-Carmona et al., 1994) blocked the effects of WIN 55,212-2 and anandamide in oocytes injected with CB₁/GIRK1/4 cRNA. The apparent equilibrium dissociation constant of SR141716A calculated in the presence of WIN 55,212-2 was similar to that reported for antagonism of agonist-stimulated guanosine-5'-O-(3-thio)triphosphate binding in cerebellar membranes and agonist-induced inhibition of smooth muscle contraction (Pertwee and Griffin, 1995; Griffin et al., 1998). Although blockade of anandamide effects by SR141716A has previously been shown (Rinaldi-Carmona et al., 1994; Felder et al., 1995), a recent study demonstrated that SR141716A was not able to block a number of behavioral effects produced by anandamide in an established cannabinoid *in vivo* model, the mouse tetrad bioassay (Compton et al., 1993; Adams et al., 1998). Adams et al. (1998) suggested that metabolism may play a role in the effects of anandamide, albeit even anandamide metabolites acting at the CB₁ receptor should be sensitive to SR141716A blockade. In the present study, a single oocyte is constantly perfused (4 ml/min) with anandamide; therefore, effects produced by anandamide metabolites are unlikely. Thus, in oocytes expressing CB₁/GIRK1/4, anandamide enhances GIRK currents through a CB₁ receptor-mediated pathway that is sensitive to blockade by SR141716A.

It was quite evident that SR141716A was an effective antagonist when evaluated at concentrations (10 nM) that were devoid of any direct effects. However, the application of SR141716A alone at concentrations greater than 10 nM inhibited GIRK currents. This latter effect was dependent on expression of the CB₁ receptor and demonstrates that a single GPCR subtype can both stimulate and inhibit the activity of GIRK1/4 channels. There is increasing evidence that SR141716A can behave as an inverse agonist *in vivo* and *in vitro* (Compton et al., 1996; Bouaboula et al., 1997; Landsman et al., 1997). If the CB₁ receptor can affect GIRK channels in native tissue, then perhaps some of the inverse agonist activity produced by SR141716A *in vivo* is related to this interaction.

A recent report by Pan et al. (1998) proposed that the inverse agonist qualities of SR141716A are a result of inhibition of constitutively active CB₁ receptors. In *X. laevis* oocytes, even in the absence of expressed GPCRs, GIRK1/4 channels have a substantial basal activity that is G protein dependent (Chan et al., 1996). If constitutively activated CB₁ receptors were shifted into an inactive state by SR141716A, the signaling moieties responsible for maintaining basal activity of GIRK channels (i.e., free G_{βγ} subunits) could be sequestered by the inactivated CB₁ receptors. The result would be a decrease in the basal activity of the GIRK channels. Alternatively, if an oocyte could synthesize a sufficient amount of an endogenous agonist, such as anandamide, to activate CB₁ receptors, then a neutral antagonist could appear to be acting as an inverse agonist. However, it is highly unlikely that a single oocyte, which is rapidly perfused, could continually produce a significant concentration of an endogenous agonist.

The use of pertussis toxin in oocytes to suggest an effect is G_i/G_o protein dependent can lead to conflicting results because oocytes can contain pertussin toxin-insensitive forms

of G_i/G_o (Dascal et al., 1993a). We previously reported that mutation of the CB₁ receptor to D163N resulted in a receptor that retained binding affinity for cannabinoid ligands but lost the ability to fully couple to G proteins (Tao and Abood, 1998). Specifically, the D163N receptor could only partially inhibit cAMP accumulation even when high doses of cannabinoid agonists were used. An analogous mutation in the α₂-adrenoreceptor resulted in a receptor that was selectively uncoupled from one of the two (cAMP and GIRK) second messenger systems studied (Surprenant et al., 1992).

The potency value for WIN 55,212-2 was not significantly different in oocytes expressing D163N/GIRK1/4 versus the wild-type system. However, current enhancement produced by WIN 55,212-2 was substantially reduced in the mutant system. A potency value for CP 55,940 could not be obtained because significant current enhancement was not produced by this ligand in the D163N/GIRK1/4 system. The results with WIN 55,212-2 suggest that the ligand was able to bind to the mutant receptor but that uncoupling of receptor and G proteins resulted in an substantial attenuation of current enhancement. Although CP 55,940 may have still bound to the mutant receptor, its lower efficacy compared with WIN 55,212-2 probably prevented it from producing a significant response. These interpretations would be in agreement with our previously published results. The results of the mutation experiments suggest the interaction between the CB₁ receptor and GIRK1/4 channel is G protein dependent. In the CB₁ receptor, the aspartate residue is part of a critical structural requirement that allows efficient coupling to not only cAMP but also GIRK channels.

In summary, the results demonstrate the functional coupling of CB₁ and CB₂ receptors to GIRK1/4 channels, albeit the CB₂ receptor couples less efficiently. Although previous studies have used only a single cannabinoid agonist, this investigation provides detailed pharmacological analysis in a system that demonstrated G protein-dependent coupling between cannabinoid receptors and GIRK channels. In oocytes expressing CB₁/GIRK1/4, SR141716A can act as an antagonist at low concentrations. At higher concentrations, it acts as an inverse agonist, demonstrating that GIRK channels can be both inhibited and activated by a single GPCR subtype. Finally, it appears the aspartate residue in the second transmembrane of the CB₁ receptor is critical for coupling to multiple effector systems. Future research into cannabinoid receptor second messenger systems and structure activity should provide us with insight into the role of the cannabinoid system in humans.

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