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SR 141716A Acts as an Inverse Agonist to Increase Neuronal Voltage-Dependent Ca^{2+} Currents by Reversal of Tonic CB1 Cannabinoid Receptor Activity

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ABSTRACT

The CB1 cannabinoid receptor antagonist SR 141716A abolished the inhibition of Ca^{2+} currents by the agonist WIN 55,212–2. However, SR 141716A alone increased Ca^{2+} currents, with an EC_{50} of 32 nM, in neurons that had been microinjected with CB1 cRNA. For an antagonist to elicit an effect, some receptors must be tonically active. Evidence for tonically active CB1 receptors was seen as enhanced tonic inhibition of Ca^{2+} currents. Preincubation with anandamide failed to enhance the effect of SR 141716A, indicating that anandamide did not cause receptor activity. Under Ca^{2+}-free conditions designed to block the Ca^{2+}-dependent formation of anandamide and *sn*-2-arachidonylglycerol, SR 141716A again increased the Ca^{2+} current. The Ca^{2+} current was tonically inhibited in neurons expressing the mutant K192A receptor, which has no affinity for anandamide, demonstrating that this receptor is also tonically active. SR 141716A had no effect on the Ca^{2+} current in these neurons, but SR 141716A could still antagonize the effect of WIN 55,212–2. Thus, the K192 site is critical for the inverse agonist activity of SR 141716A. SR 141716A appeared to become a neutral antagonist at the K192A mutant receptor. Native cannabinoid receptors were studied in male rat major pelvic ganglion neurons, where it was found that WIN 55,212–2 inhibited and SR 141716A increased Ca^{2+} currents. Taken together, our results demonstrate that a population of native and cloned CB1 cannabinoid receptors can exist in a tonically active state that can be reversed by SR 141716A, which acts as an inverse agonist.

Cannabinoids produce a wide range of effects, including analgesia, alterations in cognition and memory, and regulation of endocrine and immune functions. Two subtypes of cannabinoid receptors have been cloned, namely the central nervous system cannabinoid receptor (CB1) and the peripheral cannabinoid receptor (CB2), and both are members of the G protein-coupled receptor family (Howlett, 1995). The discovery of the selective CB1 cannabinoid receptor antagonist SR 141716A [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide hydrochloride] has provided a useful tool for studying the physiological properties of the cannabinoid receptor (Rinaldi-Carmona *et al.*, 1994; Pertwee *et al.*, 1995). The present study was designed to test whether SR 141716A would antagonize the ability of the CB1 cannabinoid receptor to inhibit neuronal Ca^{2+} channels (Pan *et al.*, 1996; Twitchell *et al.*, 1997). Here we report that SR 141716A antagonized the Ca^{2+} current inhibition induced by the cannabinoid agonist WIN 55,212–2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate] in neurons heterologously expressing either rat or human CB1 receptors and, when applied alone, it increased the Ca^{2+} current via a PTX-sensitive pathway. To determine whether the enhancement of the Ca^{2+} current also occurred with native cannabinoid receptors, we studied neurons of the male rat major pelvic ganglion. We found that WIN 55,212–2 inhibited and SR 141716A increased the Ca^{2+} currents in a subpopulation of sympathetic neurons from the rat major pelvic ganglion (Zhu *et al.*, 1995) that natively express cannabinoid receptors.

The experiments reported here with cloned rat CB1, hCB1, and native cannabinoid receptors demonstrate that the cannabinoid receptor can exist in a tonically active state. To determine whether tonic receptor activity is a spontaneous property of the cannabinoid receptor or the result of stimu-

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ABBREVIATIONS: PTX, pertussis toxin; SCG, superior cervical ganglion; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid; Gpp(NH)p, guanylylimidodiphosphate; 2-AG, *sn*-2-arachidonylglycerol; GFP, green fluorescent protein; hCB1, human CB1.

lation by an endogenous agonist, experiments were designed to block the formation of endogenous agonists and to enhance the concentration of an endogenous agonist. The results of these experiments suggest that endogenous agonists are not responsible for tonic receptor activity and that the cannabinoid receptor can spontaneously adopt an active conformational state in the absence of agonists.

Materials and Methods

Single-neuron preparation. Single SCG neurons were dissociated from adult rats using methods previously described (Pan *et al.*, 1996), with modified Earle's balanced salt solution containing 0.9 mg/ml collagenase (type D), 0.3 mg/ml trypsin (from bovine pancreas, lot 13596225–85) (both from Boehringer Mannheim Biochemicals), and 0.1 mg/ml DNase (type I; Sigma). Male rat major pelvic ganglion neurons were dissociated using methods previously described (Zhu *et al.*, 1995), with modified Earle's balanced salt solution containing 0.9 mg/ml collagenase (type D), 0.1 mg/ml trypsin, and 0.1 mg/ml DNase (type I).

Molecular biological procedures. The rat brain cannabinoid receptor cDNA was kindly provided by Dr. Tom I. Bonner (Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD). The SKR6 rat CB1 cDNA (5.7 kilobases) contained ~4100 bases of 3′ untranslated sequence. Another clone, SKR14, contained an incomplete but identical coding sequence with a 3′ untranslated sequence that was ~2900 bases shorter than that of SKR6 (Matsuda *et al.*, 1990). The SKR6 rat CB1 cDNA received from Dr. Bonner was truncated at the alternative polyadenylation site of SKR14 by making a chimera of the SKR6 and SKR14 cDNAs and was inserted into the pSP72 vector. Small-scale preparation of plasmid DNA was accomplished using a mini-prep kit (Qiagen). Plasmid DNA was linearized with *Bam*HI (New England Biolabs). Run-off cRNA transcription was accomplished using the MEGAscript SP6 kit (Ambion), with the addition of $m^7G(5')ppp(5')G$, as previously described (Pan *et al.*, 1996). The cRNA was stored in RNase-free water at −80°. Metabotropic glutamate receptor mGluR2 cRNA was prepared as previously described (Ikeda *et al.*, 1995).

The hCB1 cannabinoid receptor and the K192A mutant human cannabinoid receptor (both in the RcCMV vector) were also provided by Dr. Tom Bonner (Song and Bonner, 1996). The hCB1 and K192A cannabinoid receptors were subcloned into pCI (Promega, Madison, WI) between the *Mlu*I and *Xba*I restriction sites.

Microinjection. Microinjection of CB1 cRNA into SCG neurons was performed with an Eppendorf 5242 microinjector and 5171 micromanipulator system, as previously described (Ikeda *et al.*, 1995; Pan *et al.*, 1996). RNA was mixed with 0.1% fluorescein dextran (10,000 molecular weight; Molecular Probes) to give a final injection concentration of 1.5–2.0 μg/μl, and injections were confirmed by observing the cells for fluorescence (Nikon B2A filter). The concentration of mGluR2 cRNA in the injection pipette was 3.0 μg/μl. Microinjection of hCB1 and K192A receptor cDNA into the nucleus of SCG neurons was accomplished using techniques previously described (Ikeda, 1996). The plasmid containing the receptor cDNA was diluted with water to a final injection concentration of approximately 0.1 μg/μl. To identify neurons that were successfully intranuclearly injected, the cDNA for the S65T mutant of the jellyfish GFP (Heim *et al.*, 1995) subcloned into pCI was coinjected with the receptor cDNA. Alternatively, neurons were coinjected with a commercial plasmid (pEGFP-N1; Clontech, Palo Alto, CA) containing a red-shifted variant of GFP. Successful injections were confirmed by observing the cells for GFP fluorescence.

Electrophysiological recording and data analysis. Ca^{2+} currents from rat SCG neurons were recorded at room temperature (22–26°), 16–25 hr after injection, using the whole-cell variant of the patch-clamp technique (Hamill *et al.*, 1981), with an Axopatch-1D patch-clamp amplifier (Axon Instruments). Patch electrode pipettes were pulled from borosilicate glass capillaries (Corning 7052; Garner Glass Co.) on a P-87 Flaming-Brown micropipette puller (Sutter Instrument Co.). The patch electrodes were coated with Sylgard 184 (Dow Corning) and fire-polished on a microforge (Narishige). The pipette resistances ranged from 2 to 4 MΩ when the pipettes were filled with the internal solution described below. The cell membrane capacitance and series resistance were electronically compensated to >80%. The whole-cell currents were low-pass filtered at 2–5 kHz (−3 dB) using the four-pole Bessel filter of the clamp amplifier. Ca^{2+} currents from major pelvic ganglion neurons were recorded within 24 hr after plating, using similar techniques.

Voltage-clamp protocols were generated by a Macintosh IIci computer (Apple Computer) equipped with a MacAdios II data acquisition board (GW Instruments), using software written by Dr. Stephen Ikeda. Current traces were analyzed using the computer program Igor (WaveMetrics, Lake Oswego, OR). Ca^{2+} currents were elicited by voltage steps from a holding potential of −80 mV and were digitized at 200 μsec/point. Results are presented as mean ± standard error where appropriate. Statistical significance was determined by unpaired Student's t test or by analysis of variance as needed. The differences were considered significant at $p < 0.05$.

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

To record Ba^{2+} currents, cells were bathed in an external solution that contained 150 mM tetraethylammonium chloride, 5 mM $BaCl_2$, 10 mM HEPES, 0.1 mM EGTA, 30 mM glucose, and 15 mM sucrose, pH 7.4 (adjusted with tetraethylammonium hydroxide). The intracellular solution for recording the Ba^{2+} current consisted of 120 mM *N*-methyl-D-glucamine, 20 mM tetraethylammonium chloride, 10 mM HEPES, 10 mM BAPTA, 4.5 mM $MgCl_2$, 4 mM MgATP, 0.3 mM Na_2GTP, 14 mM phosphocreatine, and 0.0001 mM tetrodotoxin, pH 7.2 (adjusted with HCl and tetraethylammonium hydroxide).

Drug solutions were applied to single neurons that were patched from a macropipette (10–15-μm tip diameter, type N51A glass; Garner Glass Co.) lowered into the bath. Drug application was terminated by removing the macropipette from the bath, which was superfused with external solution at a rate of approximately 1 ml/min. All compounds were diluted into the external solution from concentrated stock solutions, to their final concentrations, just before use. Stock solutions of 10 mM WIN 55,212–2 mesylate (Research Biochemicals International) and SR 141716A (Sanofi Recherche) were prepared in dimethylsulfoxide. Stock solutions of 10 mM anandamide (Biomol Research Laboratories) were prepared in ethanol. Final concentrations of dimethylsulfoxide or ethanol were <0.01%, which had no effect on the Ca^{2+} current. Bovine serum albumin (3 μM, fatty acid-free; Sigma) was added to all solutions to prevent nonspecific binding. All stock solutions were stored at −20°. In experiments with PTX (List Biological Laboratories), neurons were incubated overnight with 500 ng/ml PTX after cRNA injection. In experiments with Gpp(NH)p (Sigma), Gpp(NH)p was added to the internal solution to a final concentration of 500 μM.

Results

Antagonist effect of SR 141716A on the inhibition of the Ca^{2+} current by the cannabinoid receptor agonist WIN 55,212–2 in SCG neurons microinjected with rat CB1 cannabinoid receptor cRNA. Whole-cell Ca^{2+} currents were recorded from SCG neurons that had been microinjected with rat CB1 cannabinoid receptor cRNA. As we

showed previously (Pan *et al.*, 1996), the cannabinoid receptor agonist WIN 55,212–2 inhibited the Ca^{2+} current in SCG neurons injected with CB1 receptor cRNA. Fig. 1A illustrates the time course of the effect of WIN 55,212–2 on the Ca^{2+} current. Ca^{2+} currents were elicited by 70-msec depolarizing voltage steps to +5 mV from a holding potential of −80 mV, every 10 sec, in a SCG neuron that had been previously injected with CB1 cRNA. Application of 0.1 μM WIN 55,212–2 decreased the Ca^{2+} current amplitude (Fig. 1A). The current slowly recovered after washout of the drug, to an amplitude greater than that observed before the application of WIN 55,212–2. Application of 0.1 μM SR 141716A alone slightly increased the Ca^{2+} current amplitude (Fig. 1A). Subsequent application of 0.1 μM WIN 55,212–2 together with 0.1 μM SR 141716A had no effect on the Ca^{2+} current amplitude. To test whether the effect of SR 141716A was reversible, WIN 55,212–2 was applied again after a 5-min washout of SR 141716A. WIN 55,212–2 had no effect on the Ca^{2+} current, indicating that the effect of SR 141716A was not reversible over this time course, which is in agreement with its long duration of action (Rinaldi-Carmona *et al.*, 1994). SR 141716A significantly inhibited the effect of 0.1 μM WIN 55,212–2. WIN 55,212–2 (0.1 μM) decreased the Ca^{2+} current 48.4 ± 4.9% (n = 5) in the absence of SR 141716A but only 3.5 ± 1.4% (n = 5) in the presence of 0.1 μM SR 141716A (Fig. 1B). The reduction in the response to WIN 55,212–2 could be the result of desensitization in response to repeated applications of WIN 55,212–2. However, control experiments with successive applications of WIN 55,212–2 showed little desensitization. Fig. 1C shows an experiment in which three applications of WIN 55,212–2 all inhibited the Ca^{2+} current. Therefore, the effect of SR 141716A is to antagonize the effect of WIN 55,212–2.

SR 141716A reversal of enhanced tonic inhibition of the Ca^{2+} current in neurons expressing the CB1 cannabinoid receptor. Ca^{2+} currents were elicited by a double-pulse protocol in a SCG neuron injected with rat CB1 receptor cRNA. The double-pulse protocol consisted of two 25-msec steps to +5 mV. The first step to +5 mV elicited the control Ca^{2+} current. The second step to +5 mV was preceded by a 50-msec step to +80 mV (Fig. 2A, *inset*). The current elicited by the second voltage step was facilitated, compared with the control current elicited by the first voltage step (Fig. 2A). Application of 0.1 μM SR 141716A alone increased the control Ca^{2+} current amplitude while having a minimal effect on the facilitated Ca^{2+} current amplitude. The difference between the amplitudes of the control current and the facilitated current was greatly reduced after SR 141716A application. SR 141716A (0.1 μM) increased the control Ca^{2+} current 32.9 ± 2.9% in neurons injected with rat CB1 receptor cRNA (n = 10) (Fig. 2B). In contrast, SR 141716A changed the Ca^{2+} current by only 0.95 ± 0.9% in uninjected neurons (n = 5) (Fig. 2B). This indicates that the effect of SR 141716A is mediated by the CB1 cannabinoid receptor. To further test the idea that the enhancement of the Ca^{2+} current by SR 141716A is mediated through a G protein-coupled receptor, SCG neurons injected with rat CB1 receptor cRNA were pretreated overnight with 500 ng/ml PTX. PTX pretreatment completely abolished the enhancement of the Ca^{2+} current by SR 141716A (n = 5, 0.76 ± 1.0%) (Fig. 2B). To determine whether the effect of SR 141716A was specific for the CB1 cannabinoid receptor, another PTX-sensitive G protein-coupled receptor, the mGluR2 metabotropic glutamate receptor, was heterologously expressed in SCG neurons by microinjection of mGluR2 cRNA. Expression of mGluR2 receptors was determined by Ca^{2+} current inhibition in response to application of glutamate, as previously reported (Ikeda *et al.*, 1995). SR 141716A had no effect in neurons expressing the mGluR2 receptors (n = 5, 0.27 ± 0.9%) (Fig. 2B). These

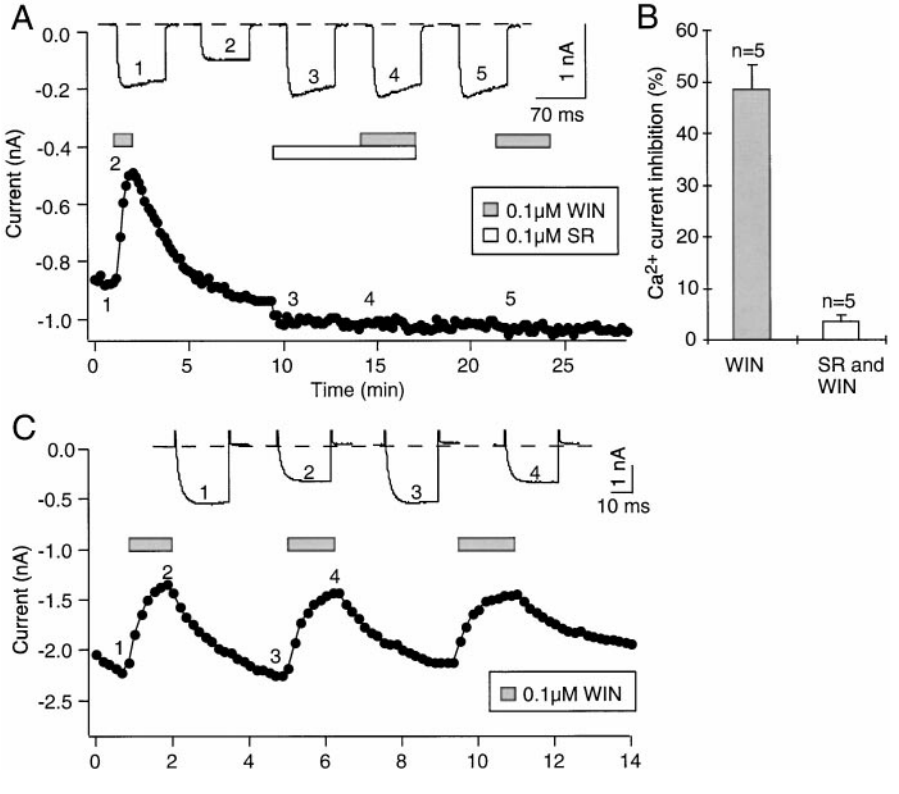

Fig. 1. SR 141716A abolished the WIN 55,212–2-induced inhibition of the Ca^{2+} current in SCG neurons expressing CB1 receptors. A, Ca^{2+} currents were elicited by 70-msec depolarizing pulses to +5 mV, from a holding potential of −80 mV, every 10 sec. Current amplitudes were plotted over the time course of the experiment. The Ca^{2+} current (*point 1*) was inhibited by application of 0.1 μM WIN 55,212–2 (*WIN*) (*point 2*). Application of 0.1 μM SR 141716A alone (*SR*) slightly increased the Ca^{2+} current amplitude (*point 3*). A subsequent application of 0.1 μM WIN 55,212–2 with 0.1 μM SR 141716A had no effect on the Ca^{2+} current (*point 4*). The effect of SR 141716A was not reversible after a 5-min wash, because a subsequent application of WIN 55,212–2 had no effect (*point 5*). *Inset*, Current traces are labeled with *numbers* corresponding to the time points indicated in A. *Dashed line*, zero-current level. B, Ca^{2+} current inhibition by 0.1 μM WIN 55,212–2 (48.4 ± 4.9%) was significantly decreased (p < 0.01) by 0.1 μM SR 141716A (3.5 ± 1.4%). The number of neurons tested is indicated *above the columns*. C, Three applications of WIN 55,212–2 (0.1 μM) inhibited the Ca^{2+} current in neurons expressing CB1 receptors. *Inset*, current traces were elicited by 25-msec depolarizing pulses to +5 mV, from a holding potential of −80 mV, every 10 sec. Current traces are labeled with *numbers* corresponding to the time points indicated in C. *Dashed line*, zero-current level.

results demonstrate that the effect of SR 141716A was specific for the CB1 cannabinoid receptor and was mediated through a PTX-sensitive G protein.

To assess the effect of antagonist concentrations, increasing concentrations of SR 141716A were applied to single neurons injected with CB1 receptor cRNA. The percentage increase of the Ca^{2+} current during the application of SR 141716A is plotted in Fig. 3A. In Fig. 3A, the continuous line represents the best fit of the data to a Hill equation. The EC_{50} of SR 141716A was 32 nM, and the maximal current increase produced by SR 141716A was 41% at 1 μM. The Hill coefficient was 0.6.

G protein-dependent inhibition of N–type Ca^{2+} channels has been shown to be relieved or facilitated by depolarizing voltages (Bean, 1989; Ikeda, 1991; Ehrlich and Elmslie, 1995). Because the depolarizing voltage step relieves most of the inhibition of the N–type Ca^{2+} current in SCG neurons (Ikeda, 1991), the ratio of the facilitated current (elicited after the prepulse to +80 mV) to the control current (elicited without a prepulse) increases with the magnitude of tonic Ca^{2+} current inhibition (Ehrlich and Elmslie, 1995). The facilitated/control Ca^{2+} current ratio in uninjected SCG neurons was 1.17 ± 0.01 (n = 11), but the ratio was significantly increased to 1.51 ± 0.05

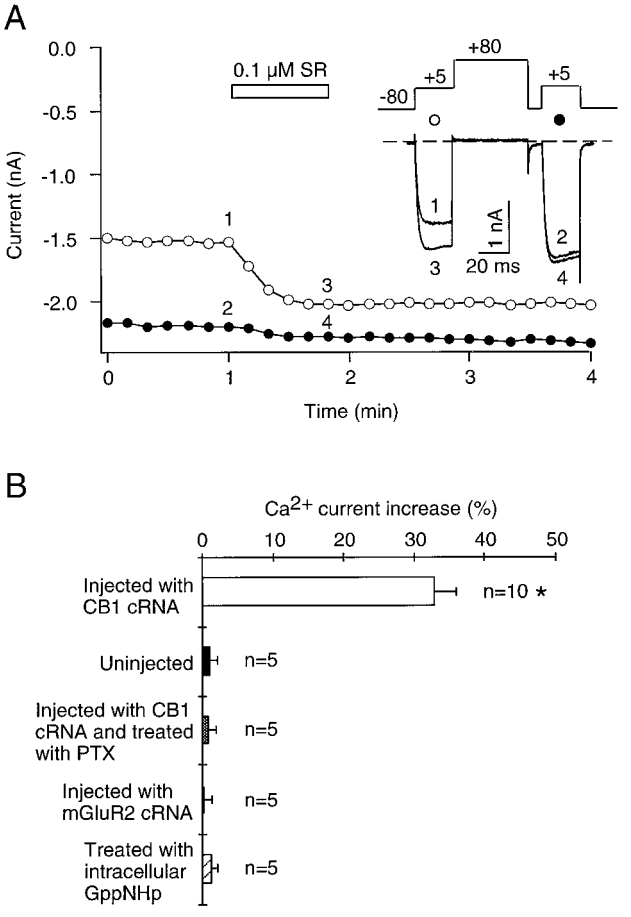

Fig. 2. SR 141716A increased the Ca^{2+} current in SCG neurons expressing CB1 cannabinoid receptors. A, A double-pulse protocol (*inset*) was used to elicit control (○) and facilitated (●) Ca^{2+} currents in a SCG neuron expressing CB1 receptors. The double-pulse protocol was repeated every 10 sec, and the current amplitudes were plotted over the time course of the experiment. Tonic inhibition of the control Ca^{2+} current (*point 1*) was relieved by a 50-msec depolarizing prepulse to +80 mV (*point 2*). Application of 0.1 μM SR 141716A (*SR*) resulted in an increase of the control Ca^{2+} current (*point 3*) to a level close to the facilitated current (*point 4*). *Inset*, *upper*, the double-pulse protocol used to elicit control (○) and facilitated (●) Ca^{2+} currents. *Lower*, superimposed current traces elicited by the double-pulse protocol, in the absence (*traces 1* and *2*) and presence (*traces 3* and *4*) of 0.1 μM SR 141716A, at the time points indicated in A. B, SR 141716A (0.1 μM) significantly increased (*, $p < 0.01$, analysis of variance) the Ca^{2+} current amplitude in SCG neurons injected with cannabinoid receptor cRNA (*Injected with CB1 cRNA*), compared with uninjected neurons (*Uninjected*), neurons pretreated with PTX (*Injected with CB1 cRNA and treated with PTX*), neurons expressing mGluR2 metabotropic glutamate receptors (*Injected with mGluR2 cRNA*), or uninjected neurons recorded using an intracellular solution containing 500 μM Gpp(NH)p (*Treated with intracellular GppNHp*). There was no significant difference between the uninjected, PTX-pretreated, mGluR2-injected, and intracellular Gpp(NH)p-treated groups. The numbers of neurons tested are indicated.

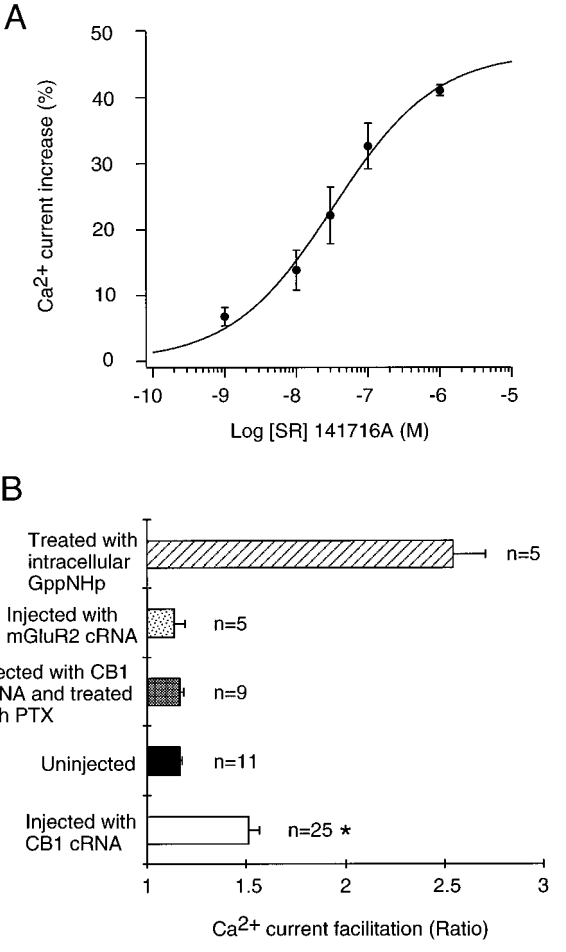

Fig. 3. A, SR 141716A increased the Ca^{2+} currents in SCG neurons injected with CB1 receptor cRNA in a concentration-dependent manner. SR 141716A increased Ca^{2+} currents with an EC_{50} of 32 nM and a maximal current increase of 41% at 1 μM (n = 2–5). B, Heterologous expression of the rat brain CB1 cannabinoid receptor resulted in enhanced tonic inhibition of the Ca^{2+} current in SCG neurons. Ca^{2+} current facilitation was measured as the ratio of the facilitated current to the control current. (The ratio equals 1 when there is no facilitation.) The ratio in cells injected with cannabinoid receptor cRNA (*Injected with CB1 cRNA*) was significantly greater (*, $p < 0.01$, analysis of variance) than in uninjected neurons (*Uninjected*), neurons injected with mGluR2 metabotropic glutamate receptor cRNA (*Injected with mGluR2 cRNA*), or neurons injected with CB1 receptor cRNA and pretreated overnight with PTX (*Injected with CB1 cRNA and treated with PTX*). The maximal facilitation ratio in SCG neurons was obtained with intracellular application of Gpp(NH)p (*Treated with intracellular GppNHp*), which was significantly different from all other treatments ($p < 0.01$, analysis of variance). There was no significant difference between the groups of uninjected neurons, mGluR2-injected neurons, and PTX-pretreated neurons. The numbers of neurons tested are indicated.

($n = 25$) in neurons microinjected with CB1 cRNA (Fig. 3B). The facilitation ratio in SCG neurons heterologously expressing the mGluR2 receptor was 1.14 ± 0.05 ($n = 5$) (Fig. 3B), similar to that in uninjected SCG neurons. Thus, in SCG neurons expressing the CB1 cannabinoid receptor, there was enhanced tonic inhibition of the Ca^{2+} current. This increase in the facilitation ratio in neurons injected with CB1 cRNA was abolished by overnight pretreatment of the neurons with PTX ($n = 9$, 1.16 ± 0.02) (Fig. 3B). These results suggest that the enhanced facilitation ratio is specific for expression of CB1 cannabinoid receptors and is mediated by a population of active receptors coupled to PTX-sensitive G proteins. To test whether the effect of SR 141716A resulted from enhanced facilitation, uninjected neurons were recorded with a patch pipette containing 500 μM Gpp(NH)p, a nonhydrolyzable analogue of GTP. Although Gpp(NH)p enhanced the facilitation ratio in uninjected neurons ($n = 5$, 2.55 ± 0.15) (Fig. 3B), SR 141716A had no effect on the Ca^{2+} current ($n = 5$, 1.2 ± 0.8%) (Fig. 2B).

Modulation of Ca^{2+} current by cannabinoids in male rat pelvic ganglion neurons. To determine whether the enhancement of the voltage-dependent Ca^{2+} current by SR 141716A occurred with native cannabinoid receptors, we examined the effect of SR 141716A in male rat major pelvic ganglion neurons. The rat major pelvic ganglia consist of both sympathetic and parasympathetic postganglionic neurons (Dail, 1992). Electrical stimulation of sympathetic nerve terminals has been shown to evoke a contractile response in the vas deferens (Stjärne and Åstrand, 1985). This contractile response has been shown to be inhibited by cannabinoids by inhibition of norepinephrine and ATP release from sympathetic nerve terminals (Stjärne and Åstrand, 1985; Pacheco *et al.*, 1991; Pertwee *et al.*, 1992; Pertwee and Griffin, 1995; Ishac *et al.*, 1996). These results suggest that sympathetic neurons of male major pelvic ganglia might express native cannabinoid receptors coupled to N-type Ca^{2+} channels. Therefore, we sought to study the sympathetic neurons from the male major pelvic ganglia. To identify the sympathetic neurons of the male major pelvic ganglia, we took advantage of a study by Zhu *et al.* (1995), who found that all neurons from the major pelvic ganglia that express a low-threshold, T-type Ca^{2+} current are tyrosine hydroxylase-immunopositive sympathetic neurons. Thus, sympathetic neurons of the male rat major pelvic ganglia could be easily identified by the presence of the low-threshold, T-type Ca^{2+} current. Current-voltage curves were elicited either by voltage steps from −100 to +80 mV from a holding potential of −80 mV or by 160-msec voltage ramps from −100 to +80 mV. Neurons with T-type Ca^{2+} currents were identified by the presence of low-threshold currents (Fig. 4A, *inset*). Neurons identified as having low-threshold, T-type Ca^{2+} currents were then stimulated by the double-pulse protocol to elicit high-threshold Ca^{2+} currents. Application of 1 μM WIN 55,212–2 reversibly inhibited the high-threshold Ca^{2+} current (Fig. 4A). In 6 of 23 pelvic ganglion neurons with low-threshold, T-type Ca^{2+} currents, 1 μM WIN 55,212–2 inhibited the high-threshold Ca^{2+} current 26.1 ± 1.8% ($n = 6$) (Fig. 4C). Application of 1 μM SR 141716A enhanced the control Ca^{2+} current amplitude but had little effect on the facilitated Ca^{2+} current, as illustrated in another pelvic ganglion neuron recorded with the double-pulse protocol (Fig. 4B). The difference between the control and facilitated current amplitudes was reduced after SR 141716A application. In 5 of 20 pelvic ganglion neurons with low-threshold, T-type Ca^{2+} currents, SR 141716A (1 μM) increased the high-threshold Ca^{2+} current 27.4 ± 6.9% ($n = 5$) (Fig. 4C). These results indicate that sympathetic neurons of the rat major pelvic ganglia have native cannabinoid receptors that can modulate voltage-dependent Ca^{2+} channels in a manner similar to that of the cloned rat brain CB1 cannabinoid receptor heterologously expressed in SCG neurons.

Experiments with the mutant K192A hCB1 receptor. Tonic inhibition of the Ca^{2+} current by the CB1 cannabinoid receptor expressed in rat SCG neurons could be the result of activation of the CB1 receptor by endogenous ligands such as anandamide (Devane *et al.*, 1992). Mutation of lysine to alanine at position 192 (K192A) in the third transmembrane domain of the hCB1 receptor was reported to change the affinity of the CB1 receptor for anandamide and CP 55940, such that they were unable to compete for [^{3}H]WIN55,212–2 binding. The affinity of WIN 55,212–2 for the mutant K192A

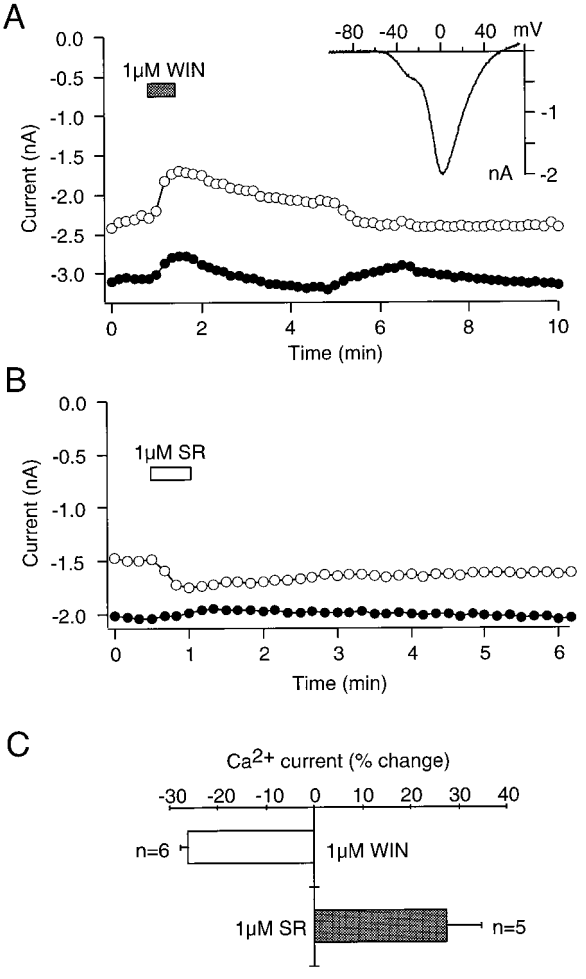

Fig. 4. WIN 55,212–2 and SR 141716A modulated the Ca^{2+} current in male rat major pelvic ganglion neurons. A, The double-pulse protocol was used to elicit control (○) and facilitated (●) Ca^{2+} currents. Application of 1 μM WIN 55,212–2 (*WIN*) decreased the Ca^{2+} current, which slowly recovered after washout. *Inset*, current-voltage relationship obtained using a continuous voltage ramp protocol (from −100 mV to +80 mV over 160 msec, from a holding potential of −80 mV). B, Application of 1 μM SR 141716A (*SR*) increased the control Ca^{2+} current (○) but not the facilitated Ca^{2+} (●) current. C, A summary of the percentage changes in Ca^{2+} currents produced by 1 μM WIN 55,212–2 (−26.1 ± 1.8%, $n = 6$) and by 1 μM SR 141716A (27.4 ± 6.9%, $n = 5$) in male rat major pelvic ganglion neurons is shown.

cannabinoid receptor was only slightly changed; the K_d of the mutant receptor was twice that of the wild-type hCB1 receptor (Song and Bonner, 1996). We tested the K192A mutant cannabinoid receptor to determine whether anandamide could be responsible for the tonic activity of the CB1 cannabinoid receptor.

WIN 55,212–2 inhibited the Ca^{2+} current in SCG neurons injected with hCB1 receptor cRNA but not in neurons injected with K192A mutant hCB1 receptor cRNA. This suggests either that the mutant K192A hCB1 receptor was not successfully expressed after cytoplasmic cRNA microinjection or that the mutant K192A hCB1 receptor could not be activated by WIN 55,212–2 as well as could the wild-type receptor. Functional expression of both hCB1 and K192A hCB1 receptors was accomplished by microinjection of receptor cDNA directly into the nuclei of SCG neurons. WIN 55,212–2 inhibited and SR 141716A increased the Ca^{2+} current in a single neuron microinjected with hCB1 receptor cDNA (Fig. 5A). Among neurons microinjected with hCB1 receptor cDNA, 1 μM WIN 55,212–2 inhibited the Ca^{2+} current 55 ± 3.6% (n = 3) and 1 μM SR 141716A increased the Ca^{2+} current 49.8 ± 17.6% (n = 3) (Fig. 5B). In neurons microinjected with the mutant K192A receptor cDNA, 1 μM WIN 55,212–2 inhibited the Ca^{2+} current by 43.3 ± 5.7% (n = 4) (Fig. 5B), which was not significantly different from the wild-type hCB1 receptor value. However, 1 μM SR 141716A did not increase the Ca^{2+} current (3.1 ± 1.9%, n = 7) in neurons microinjected with the mutant K192A receptor cDNA (Fig. 5B). SR 141716A was, however, capable of antagonizing the effect of WIN 55,212–2 on the K192A mutant receptor. SR 141716A (1 μM) significantly reduced, from 43.5 ± 5.7% (n = 4) to 13.4 ± 2.8% (n = 3), but did not abolish the inhibition of the Ca^{2+} current by WIN 55,212–2 in neurons microinjected with the mutant K192A receptor (data not shown). These results suggest that endogenous anandamide might be responsible for the tonic activity of the wild-type hCB1 receptor.

If endogenous anandamide is responsible for activating the wild-type hCB1 receptor, then the Ca^{2+} current facilitation ratio in neurons expressing the wild-type hCB1 receptor should be significantly greater than that in neurons expressing the mutant K192A receptor, which is insensitive to anandamide. Contrary to this prediction, the Ca^{2+} current facilitation ratios were not significantly different between the hCB1 and K192A mutant cannabinoid receptors. Facilitation ratios were 1.41 ± 0.09 (n = 4) for neurons expressing wild-type hCB1 receptors and 1.42 ± 0.06 (n = 8) for neurons expressing mutant K192A cannabinoid receptors. The Ca^{2+} current facilitation ratios for wild-type hCB1 and mutant K192A receptors were both significantly different ($p < 0.001$) from the Ca^{2+} current facilitation ratio in uninjected neurons (1.17 ± 0.01, n = 11). The finding that the mutant K192A cannabinoid receptor has the same facilitation ratio as the wild-type hCB1 receptor suggests that anandamide is not responsible for tonic receptor activation and that both mutant and wild-type receptors can exist in a spontaneously active, G protein-coupled state. The fact that SR 141716A was not able to enhance the Ca^{2+} current in neurons expressing the mutant K192A cannabinoid receptor suggests that the mutant receptor is able to adopt an active conformational state but is less able to transit to the inactive conformational state.

Evidence that the cannabinoid receptor is not activated by an endogenous ligand present in neuronal cultures. In our previous studies we found that anandamide had no effect on the Ca^{2+} current in 23 of 33 neurons expressing the rat CB1 cannabinoid receptor (Pan *et al.*, 1996). Given the lack of effect of anandamide, it seemed unlikely that anandamide could be acting as an endogenous agonist under our experimental conditions. Experiments were designed to test whether anandamide or another endogenous cannabinoid agonist, 2-AG (Mechoulam *et al.*, 1995), could be

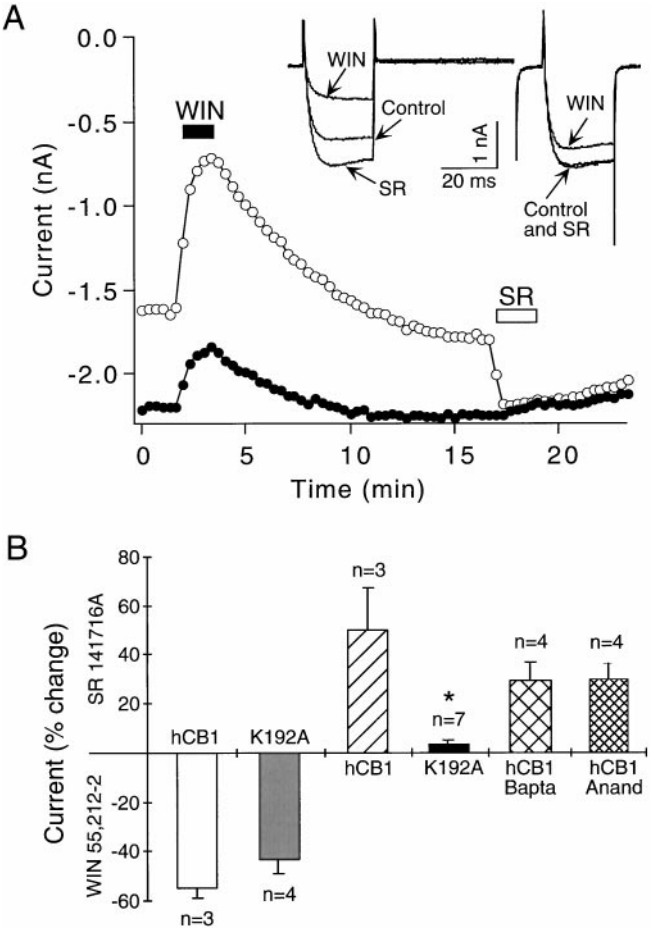

Fig. 5. A, WIN 55,212–2 (*WIN*) decreased and SR 141716A (*SR*) increased the Ca^{2+} currents in a SCG neuron expressing hCB1 receptors after cDNA microinjection. The double-pulse protocol was used to elicit control (○) and facilitated (●) Ca^{2+} currents, and current amplitudes were plotted over the time course of the experiment. Application of 1 μM WIN 55,212–2 decreased the Ca^{2+} current, which slowly recovered to a greater amplitude. A subsequent application of 1 μM SR 141716A rapidly increased the Ca^{2+} current. *Inset*, superimposed current traces recorded at the beginning of the experiment (*Control*), in the presence of 1 μM WIN 55,212–2, and in the presence of 1 μM SR 141716A. B, A summary of the changes in Ca^{2+} current amplitudes in the presence of 1 μM WIN 55,212–2 (*negative axis*) or 1 μM SR 141716A (*positive axis*) in neurons microinjected with hCB1 or mutant K192A receptor cDNA is shown. In neurons microinjected with hCB1 receptor cDNA (*hCB1*), WIN 55,212–2 inhibited the Ca^{2+} current and 1 μM SR 141716A increased the Ca^{2+} current. In neurons microinjected with mutant K192A receptor cDNA (*K192A*), 1 μM WIN 55,212–2 inhibited the Ca^{2+} current but 1 μM SR 141716A did not increase the Ca^{2+} current. In neurons microinjected with hCB1 cDNA and recorded in the absence of Ca^{2+}, using Ba^{2+} as the charge carrier and BAPTA to chelate intracellular Ca^{2+} (*hCB1 Bapta*), SR 141716A increased the current. SR 141716A also increased the current in neurons microinjected with hCB1 cDNA and preincubated with 300 nM anandamide (*hCB1 Anand*).

acting as an endogenous ligand of the heterologously expressed CB1 cannabinoid receptors in the neuronal cultures.

2-AG is present in rat brain and acts as a full agonist in hippocampal neurons (Stella *et al.*, 1997). Production of 2-AG is Ca^{2+}-dependent and is mediated by phospholipase C and diacylglycerol lipase (Stella *et al.*, 1997). Anandamide (arachidonoylethanolamide) has been found to be synthesized by two routes; one involves Ca^{2+}-dependent, hydrolytic cleavage from a phospholipid precursor (Di Marzo *et al.*, 1994) and one is Ca^{2+}-independent and involves a condensation reaction (Devane and Axelrod, 1994; Kruszka and Gross, 1994). Experiments were performed to block the Ca^{2+}-dependent synthesis of anandamide and 2-AG. Electrophysiological recordings were performed in solutions without Ca^{2+}, using Ba^{2+} as the charge carrier. The external solution contained 5 mM Ba^{2+} and 0.1 mM EGTA, and the intracellular solution contained 10 mM BAPTA to chelate intracellular Ca^{2+} (see Materials and Methods). SR 141716A increased the Ba^{2+} current 29.2 ± 7.5% (n = 4), which was not significantly different from the effect of SR 141716A when Ca^{2+} was used as the charge carrier (Fig. 5B). The results of these experiments show that the cannabinoid receptor antagonist SR 141716A can still enhance current through Ca^{2+} channels in the absence of Ca^{2+}.

Because anandamide can be formed enzymatically by condensation of arachidonic acid with ethanolamine (Devane and Axelrod, 1994; Kruszka and Gross, 1994), an additional experiment was performed. All of our experiments were performed with neuronal cultures that were superfused with extracellular solution for approximately 20 min after the cultures were removed from the incubator. Endogenous anandamide would have to remain in the cultures throughout this superfusion with extracellular solution to function as an agonist. If the cannabinoid receptor antagonist SR 141716A was acting as a competitive antagonist, then the effect of SR 141716A should be greater in the presence of additional anandamide. Neurons were preincubated with anandamide (300 nM) for 20 min, and then SR 141716A (1 μM) was tested after a 20-min washout of anandamide, to mimic the recording conditions without supplemental anandamide. SR 141716A increased the Ca^{2+} current 29.8 ± 6.5% (n = 4), which was not significantly different from the effect of SR 141716A on the Ca^{2+} current without anandamide preincubation (Fig. 5B). The results of the experiments without Ca^{2+} and with added anandamide suggest that neither anandamide nor 2-AG is responsible for the activity of the CB1 cannabinoid receptor.

Discussion

The pharmacological effects of the CB1 cannabinoid receptor antagonist SR 141716A were studied in preparations of adult neurons that expressed both native and cloned CB1 receptors. SR 141716A antagonized the inhibitory effect of the agonist WIN 55,212–2 on the voltage-dependent Ca^{2+} current in SCG neurons heterologously expressing the rat CB1 receptor. However, SR 141716A, when given alone, increased the Ca^{2+} current both in SCG neurons with heterologously expressed CB1 receptors and in pelvic ganglion neurons with native cannabinoid receptors. For an antagonist to have an effect, some receptors must be in an active state. Evidence that CB1 receptors were in a tonically active state was seen as enhanced tonic inhibition of voltage-dependent Ca^{2+} currents in neurons expressing CB1 receptors. The active state of the receptor could arise through two different mechanisms, 1) activation by an endogenous agonist or 2) adoption of a spontaneously active state. In the former case the effect of SR 141716A would be that of a classical antagonist, whereas in the latter case SR 141716A would be an inverse agonist. Inverse agonists have been recognized recently by their ability to block the signal transduction effects mediated by constitutively active receptors.

To account for the phenomenon of inverse agonism, a two-state receptor model was proposed (Costa *et al.*, 1992; Chidiac *et al.*, 1994; Samama *et al.*, 1994). In the two-state receptor model, receptors exist in an equilibrium between inactive (R) and active (R*) states. Agonists stabilize the R* state, inverse agonists stabilize the R state, and antagonists have equal preferences for both states. Thus, for an antagonist to be an inverse agonist some receptors must be in the active R* state.

The CB1 receptor antagonist SR 141716A has been reported to act as an inverse agonist. Bouaboula *et al.* (1997) reported that SR 141716A reversed a constitutively active hCB1 receptor, as measured by adenylyl cyclase and mitogen-activated protein kinase activity. Both SR 141716A and AM630 were reported to be inverse agonists, because they reduced basal guanosine-5′-*O*-(3-thio)triphosphate binding in cells with hCB1 receptors (Landsman *et al.*, 1997; Landsman *et al.*, 1998). Earlier evidence for constitutively active cannabinoid receptors came from studies on electrically evoked contractions of the mouse urinary bladder, where SR 141716A alone was reported to significantly increase contractions (Pertwee and Fernando, 1996). SR 141716A alone has also been reported to potentiate acetylcholine release from hippocampal slices (Gifford and Ashby, 1996) and to decrease neuronal firing in the substantia nigra (Tersigni and Rosenberg, 1996). However, none of those studies, except that by Bouaboula *et al.* (1997), addressed the issue of whether the CB1 receptor was being activated by an endogenous ligand. Bouaboula *et al.* (1997) reported that the EC_{50} value of SR 141716A was similar to the binding affinity of SR 141716A and concluded that SR 141716A could not be competing with an endogenous agonist. A more recent study by MacLennan *et al.* (1998) argued that endogenous agonists are not responsible for CB1 cannabinoid receptor activity, because cannabinol, unlike SR 141716A, had no effect on basal guanosine-5′-*O*-(3-thio)triphosphate binding in cells expressing hCB1 receptors. Our study demonstrates that SR 141716A is not competing with two endogenous cannabinoid agonists (i.e., anandamide and 2-AG) but acts to reverse a tonically active CB1 receptor.

The active state of a G protein-coupled receptor can be assessed in SCG neurons by the Ca^{2+} current facilitation ratio. Facilitation is thought to arise from a voltage-dependent reversal of G protein-mediated Ca^{2+} current inhibition (Bean, 1989; Ikeda, 1991; Ehrlich and Elmslie, 1995). Thus, if the cannabinoid receptor is in an active state, the following two predictions can be made: 1) the facilitation ratio would be larger in neurons expressing the cannabinoid receptor than in neurons without the receptor and 2) an inverse agonist would enhance the Ca^{2+} current to a level equal to the maximal amplitude that can be obtained using voltage to reverse Ca^{2+} channel inhibition. Consistent with the first

prediction, we found that the facilitation ratio was larger in SCG neurons expressing CB1 receptors than in uninjected neurons or in neurons expressing another G protein-coupled receptor (the mGluR2 metabotropic glutamate receptor). SR 141716A consistently enhanced the Ca^{2+} current to equal the maximal facilitated amplitude, consistent with the second prediction. Taken together, these results are consistent with the idea that cannabinoid receptors can adopt an active conformational state.

To test the possibility that the active state of the cannabinoid receptor is induced by an endogenous agonist, the mutant K192A hCB1 receptor was studied. The K192A receptor has no affinity for anandamide but has affinity similar to that of the wild-type receptor for WIN 55,212–2 (Song and Bonner, 1996). If anandamide was activating the wild-type receptor, then the mutant receptor would be expected to be inactive. SR 141716A would be predicted to have no effect and the Ca^{2+} current facilitation ratio would not be enhanced. In SCG neurons expressing wild-type hCB1 receptors, WIN 55,212–2 decreased and SR 141716A increased the Ca^{2+} current. However, in neurons expressing K192A receptors, WIN 55,212–2 inhibited the Ca^{2+} current but SR 141716A had no effect. This result is consistent with the idea that endogenous anandamide could be responsible for activation of the wild-type CB1 receptor. However, in neurons expressing the K192A receptor, the Ca^{2+} current facilitation ratio was equal to the facilitation ratio in neurons expressing wild-type hCB1 receptors, indicating that the mutant receptor can adopt an active R* conformational state. Because the K192A mutant cannabinoid receptor is insensitive to anandamide, the mutant receptor must be spontaneously active. If the mutant receptor is in an active R* conformational state, then SR 141716A should increase the Ca^{2+} current by stabilizing the inactive R conformational state. SR 141716A, however, had no effect on the Ca^{2+} current in neurons expressing the K192A mutant receptor. One possible explanation is that the K192A mutation alters the ability of the receptor to transit from the active R* conformational state to the inactive R state. SR 141716A could still bind to the K192A receptor, because it antagonized the effect of WIN 55,212–2. Thus, the K192 site appears critical for SR 141716A action as an inverse agonist. When this site is mutated, as in the K192A mutant receptor, SR 141716A can no longer act as an inverse agonist. Instead, SR 141716A appears to behave as a neutral antagonist. Molecular modeling studies indicate that the lysine at position 192, referred to as K3.28, is one of several amino acids that interacts with SR 141716A. As an inverse agonist, SR 141716A would prefer the inactive R state of the receptor, and interaction with K3.28 might result in the preference of SR 141716A for the R state (Reggio P, personal communication).

Chin *et al.* (1998) reported that another mutation, K192E, altered the ability of the cannabinoid receptor to adopt an active conformational state. They reported that the binding affinities for WIN 55,212–2 were similar with wild-type and mutant receptors, but the EC_{50} for inhibition of cAMP was 10-fold greater with the mutant receptor. These results suggest that the positively charged lysine in the third transmembrane domain plays a role in receptor activation. Movement of the third transmembrane domain of the prototypical G protein-coupled receptor rhodopsin has also been shown to influence receptor activation (Sakmar, 1998). Our results with the K192A receptor also suggest that this lysine may be critical for receptor transitions between R* and R states.

Experiments designed to test whether anandamide and 2-AG are responsible for tonic cannabinoid receptor activity yielded negative results. If endogenous agonists were responsible for tonic receptor activity, then blocking their synthesis should block the effect of SR 141716A. The effect of SR 141716A was not significantly different in the absence of Ca^{2+} to block the Ca^{2+}-dependent synthesis of anandamide (Di Marzo *et al.*, 1994) and 2-AG (Stella *et al.*, 1997). However, anandamide has also been reported to be synthesized through a Ca^{2+}-independent pathway (Devane and Alelrod, 1994; Kruszka and Gross, 1994). Under conditions with an increased concentration of anandamide, SR 141716A would be predicted to have a greater effect. We found that the effect of SR 141716A was no different in neurons supplemented with exogenous anandamide. These experiments suggest that it is unlikely that these two endogenous ligands are responsible for tonic CB1 receptor activity. However, the possibility remains that not all cannabinoid agonists have been discovered.

The results of our experiments in neurons using Ca^{2+} channels as effector targets of the cannabinoid receptor demonstrate that significant populations of both native and cloned CB1 cannabinoid receptors can exist in a constitutively active conformational state. SR 141716A acts as an inverse agonist to enhance the voltage-dependent Ca^{2+} current by relief of Ca^{2+} current inhibition by constitutively active CB1 receptors, an effect opposite that of the cannabinoid agonist WIN 55,212–2. Inhibition of constitutively active CB1 receptors by SR 141716A has also been reported to inhibit mitogen-activated protein kinase and enhance forskolin-stimulated adenylyl cyclase activity (Bouaboula *et al.*, 1997). Additionally, SR 141716 has been reported to improve short-term olfactory memory in rodents (Terranova *et al.*, 1996). Although animal experiments cannot always predict effects in humans, these memory experiments, together with the human memory impairment produced by marijuana, suggest that the inverse agonist effect of SR 141716A might have important therapeutic benefits in the treatment of human memory impairments.

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