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Rat Brain Cannabinoid Receptor Modulates N-Type Ca^{2+} Channels in a Neuronal Expression System

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SUMMARY

Modulation of neuronal ion channels by the cloned rat brain CB_1 cannabinoid receptor was investigated with the use of a heterologous neuronal expression system. Transient expression of the rat brain CB_1 cannabinoid receptor was accomplished through microinjection of *in vitro* transcribed cRNA into the cytoplasm of enzymatically dissociated adult rat superior cervical ganglion neurons The cannabimimetic aminoalkylindole WIN 55,212–2 inhibited whole-cell Ca^{2+} currents in neurons injected 16–25 hr previously with rat brain CB_1 cannabinoid receptor cRNA. Inhibition of the Ca^{2+} current was voltage and concentration dependent, with a maximal inhibition of 73% and an IC_{50} value of 47 nM. The synthetic cannabinoid analogue CP 55,940 also inhibited Ca^{2+} currents, with a maximal inhibition of 38% and an IC_{50} value of 7 nM. Ca^{2+} current inhibition was blocked by inclusion of guanosine-5′-*O*-(2-thiodiphosphate) in the intracellular patch pipette solution or by pretreatment with pertussis toxin. Pretreatment with the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA reduced the inhibition by 100 nM WIN 55,212–2 from 44% to 6%, indicating that N-type Ca^{2+} channels are a target of cannabinoid action. On washout of WIN 55,212–2, the Ca^{2+} current amplitude "overrecovered" in 47% of the neurons tested. Anandamide, the endogenous cannabimimetic compound, had an inconsistent effect on the voltage-dependent Ca^{2+} currents in the majority of neurons microinjected with cannabinoid receptor cRNA. Ca^{2+} channels were a specific effector target of the cannabinoid receptor, as two different K^+ currents, the M current and the A current, were not modulated by the cannabimimetic WIN 55,212–2.

Marijuana (*Cannabis sativa*) is a widely used psychoactive substance. The major psychoactive component of marijuana, Δ^9-THC, has a wide range of effects on the central nervous system, including alterations in sensory perception, mood, thinking, and memory (1, 2). Understanding the mechanism of action of cannabinoids has been difficult due to the lipophilic nature of Δ^9-THC. The development of two families of cannabimimetics, the nonclassic cannabinoid CP 55,940 and the aminoalkylindole WIN 55,212–2, with greater potency and better aqueous solubility has greatly facilitated the identification and localization of the cannabinoid receptors (3–7). The cannabinoid receptor, cloned from rat brain, is a member of the family of G protein-coupled receptors (8). Both cannabinoid receptor mRNA and binding of synthetic cannabimimetics have been found in specific regions of the rat brain, including basal ganglia, hippocampus, cerebellum, cerebral cortex, amygdala, and hypothalamus (4, 5, 9).

The identity of the first cannabinoid receptor clone was facilitated by prior research showing that neuroblastoma cell lines have an endogenous cannabinoid receptor (10, 11). Activation of the endogenous cannabinoid receptor in neuroblastoma cells was shown to inhibit N-type voltage-dependent Ca^{2+} channels through a PTX-sensitive G protein (12, 13). It is not known whether the endogenous cannabinoid receptor in neuroblastoma cells is identical to the cloned rat brain receptor; therefore, the current study was designed to directly test the cloned rat brain cannabinoid receptor for its ability to modulate specific neuronal ion channels. This was accomplished by expressing the rat brain cannabinoid receptor in a fully differentiated mammalian neuron. Expression of the cannabinoid receptor was detected 16–25 hr after cytoplasmic microinjection of *in vitro* transcribed cRNA from the rat brain cannabinoid receptor SKR6–14 cDNA (8) into adult rat SCG neurons. We previously reported expression of metabotropic glutamate receptors using this technique (14). SCG neurons are ideal expression hosts for the cannabinoid receptor because they lack endogenous cannabinoid receptors (13) and have well-studied G protein pathways that target ion channels (15). In the current study, we investigated po-

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ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; SCG, superior cervical ganglion; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GDP-β-S, guanosine-5′-*O*-(2-thiodiphosphate); PTX, pertussis toxin; CgTX, conotoxin; I-V, current-voltage; 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; CP 55,940, [1α,2β-(*R*)-5α]-(−)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol.

tential Ca^{2+} and K^+ ion channel targets, G protein coupling, and pharmacology of the rat brain CB_1 cannabinoid receptor heterologously expressed in a neuronal background.

Materials and Methods

Single-neuron preparation. Single SCG neurons were isolated from adult rats according to methods that have been described previously (14, 16). Briefly, Wistar rats were decapitated, and the SCGs were dissected in cold Hanks' balanced salt solution. The tissue pieces were then incubated in modified Earle's balanced salt solution containing 0.9 mg/ml collagenase type D, 0.3 mg/ml trypsin (both were obtained from Boehringer Mannheim) and 0.1 mg/ml DNase type I (Sigma) at 35° for 1 hr in a shaking water bath. After incubation, the cells were dissociated by vigorous shaking of the flask for 10 sec. The cells were plated onto 35-mm poly-L-lysine-coated polystyrene dishes with minimum essential medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine (all from GIBCO). Neurons were maintained at 37° in a humidified atmosphere containing 5% CO_2 in air.

Molecular biology. The rat brain cannabinoid receptor cDNA (SKR6–14 p2) was kindly provided by Dr. Tom I. Bonner (Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD). Small-scale preparation of plasmid DNA was accomplished with a miniprep kit (QIAGEN). Plasmid DNA was linearized with *Bam*HI (New England Biolabs). Run-off cRNA transcription was accomplished with the MEGAscript SP6 kit (Ambion) with the addition of $m^7G(5')ppp(5')G$. The cRNA was stored in RNase-free water at −80°.

Microinjection. Microinjection of cRNA was performed with an Eppendorf 5242 microinjector and 5171 micromanipulator system as described previously (14). RNA was mixed with 0.1% fluorescein dextran (molecular mass, 10,000 kDa; Molecular Probes) to give a final injection concentration of ~1.5 $\mu g/\mu l$. The RNA solution was centrifuged at 16,000 × *g* for 20 min to remove particulates. Injections were subsequently confirmed by observing the cells for fluorescence (Nikon B2A filter). Neurons were injected 4–6 hr after plating. Expression of the rat brain CB_1 cannabinoid receptor was reproducible as long as the RNA was carefully handled. No expression occurred if the RNA concentration in the injection pipette was <~0.5 $\mu g/\mu l$.

Electrophysiological recording and data analysis. Ca^{2+} currents from SCG neurons were recorded at room temperature (22–26°) 16–25 hr after injection with the whole-cell variant of the patch-clamp technique (17) 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 diameter of pipette tip was ~3–4 μm, and the resistances ranged from 2 to 4 MΩ when filled with the internal solutions 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) with the four-pole Bessel filter of the clamp amplifier.

Voltage-clamp protocols were generated with a Macintosh IIci computer (Apple Computer) equipped with a MacAdios II data acquisition board (GW Instruments) using software written by Dr. Stephen Ikeda (Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA). Current traces were analyzed using the computer program Igor (WaveMetrics). Ca^{2+} currents were elicited by voltage steps from a holding potential of −80 mV and digitized at 200 μsec/point. The M current was activated at a holding potential of −30 mV and deactivated during a 500-msec voltage step to −60 mV. M current amplitudes were measured as the difference between the current amplitudes at the beginning and the end of the voltage step to −60 mV. M currents were digitized at 2 msec/point. Results are presented as mean ± standard error where appropriate. Statistical significance was determined with unpaired Student's *t* test. The differences were considered significant at $p < 0.05$.

Solutions. Earle's balanced salt solution solution was made from 10× concentrated liquid (Sigma) with 10 mM HEPES buffer (Sigma), 20 mM glucose, and 2.6 mM $NaHCO_3$, pH adjusted to 7.4 with 1 M NaOH.

To isolate Ca^{2+} currents for whole-cell recording, cells were bathed in an external solution containing 140 mM tetraethylammonium methane-sulfonate, 10 mM HEPES, 15 mM glucose, 10 mM $CaCl_2$, and 0.0001 mM tetrodotoxin (Calbiochem Corporation), pH 7.4 (adjusted with methanesulfonic acid). The intracellular solution for recording the Ca^{2+} currents 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).

The external solution for recording the M-type K^+ current contained 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 15 mM glucose, 2 mM $CaCl_2$, and 0.0001 mM tetrodotoxin, pH 7.4 (adjusted with NaOH). The internal solution for recording M-type K^+ current contained 150 mM KCl, 10 mM HEPES, 0.1 mM K_4-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 4 mM MgATP, and 0.1 mM Na_2GTP, pH 7.2 (adjusted with KOH).

The external solution for recording the A current contained 130 mM sodium isethionate, 5.4 mM potassium isethionate, 10 mM HEPES, 10 mM $MgCl_2$, and 15 mM glucose, pH 7.4 (adjusted with NaOH). The internal solution for recording A current contained 100 mM potassium isethionate, 15 mM KCl, 10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 4 mM MgATP, and 0.1 mM Na_2GTP, pH 7.4 (adjusted with KOH).

In experiments designed to study G protein-mediated effects, Na_2GTP was replaced with 2 mM GDP-β-S (Boehringer Mannheim). In experiments with PTX (List Biological Laboratories), neurons were incubated overnight with 500 ng/ml PTX after cRNA injection.

Drug solutions were applied to isolated neurons under study from a macropipette (10–15-μm tip diameter, type N51A glass; Garner Glass Co.). Drug application was terminated by removing the macropipette from the bath, which was superfused with external solution at a rate of 1 ml/min. All compounds were diluted into the external solution from concentrated stock solutions to their final concentrations just before use. ω-CgTX GVIA (Bachem Inc.) and ω-CgTX MVIIC (Peptides International) were made in H_2O as stock concentrations of 1 mM and 100 μM, respectively. Stock solutions of 10 mM (+)-202–791 (a gift from Sandoz, East Hanover, NJ) were made in ethanol. Stock solutions of 10 mM WIN 55,212–2 mesylate (Research Biochemicals Int.) and CP 55,940 (a gift from Pfizer, Groton, CT) were made in dimethylsulfoxide. Final concentrations of dimethylsulfoxide were <0.01%, which had no effect on the Ca^{2+} current. Anandamide (Cayman Chemical Company or Biomol Research Laboratories) was dissolved in ethanol at a stock concentration of 10 mM. To test the quality of anandamide, thin layer chromatography plates were run with a solvent consisting of petroleum ether/ether/methanol (6:40:4) and visualized with nitric acid vapors. The anandamide samples had R_F values of ~0.5. Bovine serum albumin (3 μM, fatty acid free; Sigma) was added to all solutions to prevent nonspecific binding. All stock solutions were stored at −50° or −80°.

Results

Inhibition of Ca^{2+} currents by WIN 55,212–2 in neurons injected with rat brain CB_1 cannabinoid receptor cRNA. Single SCG neurons that had been injected with the rat brain CB_1 cannabinoid receptor cRNA mixed with 0.1% fluorescein dextran 16–25 hr previously were identified with the use of epifluorescent optics. Ca^{2+} currents were recorded from injected neurons with the use of the whole-cell variant of the patch-clamp technique (17). Ca^{2+} current traces were elicited with 70-msec depolarizing pulses from a holding po-

tential of −80 mV to step potentials from −80 mV to +80 mV (Fig. 1A). Activation of the heterologously expressed rat brain CB_1 cannabinoid receptor with WIN 55,212–2 reduced the Ca^{2+} current amplitude and slowed the rising phase of the current in SCG neurons injected with receptor cRNA (Fig. 1B). The maximal inhibition of the Ca^{2+} currents occurred over the voltage range of 0–10 mV or at the peak of the I-V relationship and was reversible on washout (Fig. 1C).

Fig. 2 illustrates the time course and voltage dependence of Ca^{2+} current inhibition. Ca^{2+} currents were elicited with the use of a double-pulse protocol from a holding potential of −80 mV in an SCG neuron that had been previously injected with rat brain CB_1 cannabinoid receptor cRNA. The voltage protocol consisted of two 25-msec steps to +5 mV. The first step to +5 mV elicited the control Ca^{2+} current (Fig. 2, A and B). The second step to +5 mV was preceded by a 50-msec step to +80 mV (Fig. 2, A and B). The current elicited by the second voltage step is facilitated (Fig. 2A, *point 3*, and Fig. 2B, *trace 3*) compared with the control current elicited by the first voltage step (Fig. 2A, *point 1*, and Fig. 2B, *trace 1*). G protein-dependent inhibition of the N-type Ca^{2+} currents can be relieved by a depolarizing prepulse (18–21), and facilitation of the Ca^{2+} current in the absence of an agonist arises from a tonic level of G protein activation in SCG neurons (19). Application of 0.1 μM WIN 55,212–2 (Fig. 2A) resulted in a slow decrease in the Ca^{2+} current amplitude. The control

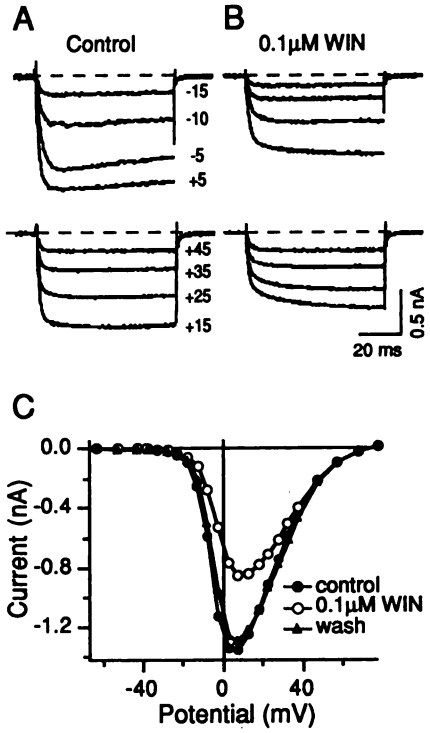

Fig. 1. WIN 55,212–2 inhibits Ca^{2+} currents in an SCG neuron injected with rat brain CB_1 cannabinoid receptor cRNA. A, Control Ca^{2+} current traces (*Control*) elicited by 70-msec depolarizing pulses from a holding potential of −80 mV to the indicated potentials. *Dashed line*, zero current level. B, Ca^{2+} current traces recorded in the presence of the cannabimimetic agonist WIN 55,212–2 (0.1 μM) (*WIN*) for the corresponding step potentials indicated in A. C, Superimposed I-V curves of control Ca^{2+} currents (●), Ca^{2+} currents recorded in the presence of 0.1 μM WIN 55,212–2 (○), and Ca^{2+} currents recorded after a 3-min washout of WIN 55,212–2 (▲). Current traces shown in A and B and the I-V curves were from the same neuron.

Ca^{2+} current was inhibited to a greater extent than the facilitated current (Fig. 2A, *points 2 and 4*; Fig. 2B, *traces 2 and 4*). The time to peak inhibition decreased with increasing concentrations of WIN 55,212–2. The time to peak for 0.03, 0.1, and 1 μM WIN 55,212–2 was 100 ± 6 sec (three experiments), 62 ± 2 sec (10 experiments), and 32 ± 2 sec (seven experiments), respectively. Control, noninjected SCG neurons lack endogenous cannabinoid receptors, as indicated by the absence of Ca^{2+} current modulation in the presence of WIN 55,212–2 (Fig. 2C). In summary, WIN 55,212–2 (0.1 μM) inhibited the control Ca^{2+} current in neurons injected with rat brain CB_1 cannabinoid receptor cRNA 44.3 ± 6.4% (eight experiments) (Fig. 2D) compared with 1.5 ± 0.9% (seven experiments) in noninjected neurons (Fig. 2D). This inhibition was characteristic of a G protein-mediated block because it could be relieved by a depolarizing prepulse. WIN 55,212–2 (0.1 μM) inhibited the control Ca^{2+} current by 45.0 ± 2.8% (10 experiments) but inhibited the facilitated Ca^{2+} current by only 15.6 ± 1.3% (10 experiments). During washout of WIN 55,212–2, both the control and facilitated Ca^{2+} currents slowly increased to amplitudes even greater than before WIN 55,212–2 exposure (Fig. 2A). In 17 of 36 neurons tested, washout of WIN 55,212–2 resulted in an "overrecovery" of the Ca^{2+} currents. Overrecovery of both the control and facilitated Ca^{2+} currents suggests that this phenomenon is not simply a reversal of a tonic G protein-dependent Ca^{2+} current inhibition.

Functional expression of the cannabinoid receptor was detected in every neuron injected with rat brain CB_1 cannabinoid receptor cRNA. Because expression was both robust and reliable, receptor pharmacology and second messenger systems could be investigated.

Concentration-dependent Ca^{2+} current inhibition by WIN 55,212–2 and CP 55,940. Ca^{2+} currents were recorded from neurons injected with rat brain CB_1 cannabinoid receptor cRNA. One or two concentrations of WIN 55,212–2 were tested on each neuron. When two concentrations of WIN 55,212–2 were tested on a single neuron, the lower concentration was tested first and the higher concentration was tested after the current had returned to the initial control amplitude. Because of poor reversibility, only one concentration of CP 55,940 was tested on each neuron. Normalized Ca^{2+} current inhibition is plotted in Fig. 3 as a function of agonist concentration. The continuous lines in Fig. 3 represent the best fit of the data to a single binding site model as determined from nonlinear regression analysis. The IC_{50} and the maximal block for WIN 55,212–2 were 47 nM and 73%, respectively. The IC_{50} and the maximal block for CP 55,940 were 7 nM and 38%, respectively. Application of CP 55,940 attenuated the effect of a subsequent application of WIN 55,212–2, and vice versa (five experiments).

Intracellular GDP-β-S or PTX pretreatment abolished the modulation of the Ca^{2+} current by the rat brain CB_1 cannabinoid receptor. Dialysis of neurons with pipette solutions containing GDP-β-S, a nonhydrolyzable analogue of GDP, has been shown to abolish the G protein-mediated effects of neurotransmitters (22). Intracellular dialysis of SCG neurons injected with rat brain cannabinoid receptor cRNA with an internal solution containing 2 mM GDP-β-S completely abolished the effect of WIN 55,212–2 on the Ca^{2+} current. WIN 55,212–2 (0.1 μM) was applied 5 min after the whole-cell patch was formed to allow sufficient time

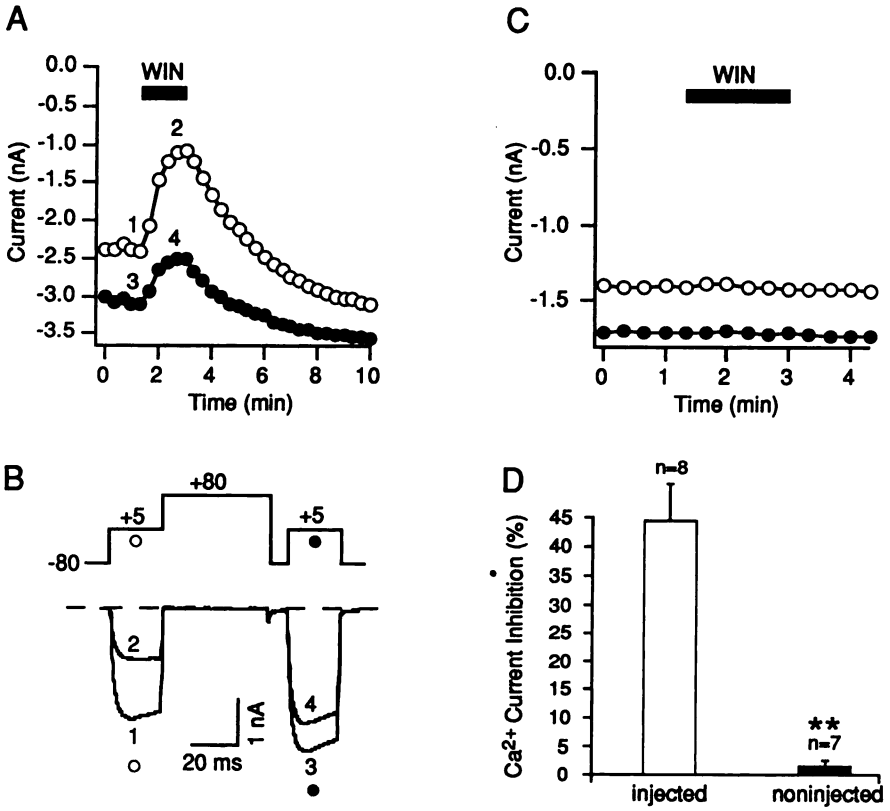

Fig. 2. WIN 55,212–2 (*WIN*) inhibits the Ca^{2+} current in a voltage-dependent manner in SCG neurons injected with rat brain CB_1 cannabinoid receptor cRNA but not in uninjected neurons. A, A prepulse depolarization to +80 mV partially relieves the inhibition of the Ca^{2+} current induced by activation of the cannabinoid receptor. A double-pulse protocol (as shown in B) was used to elicit control (○) and facilitated (●) Ca^{2+} currents in an SCG neuron injected with rat brain CB_1 cannabinoid receptor cRNA. The double-pulse protocol was repeated every 20 sec, and the current amplitudes are 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 3*). Application of 0.1 μM WIN 55,212–2 (*shaded bar*) resulted in an inhibition of the control Ca^{2+} current (*point 2*), which was partially relieved by a depolarizing prepulse (*point 4*). B, The double-pulse protocol (*top*) was used to elicit control (○) and facilitated (●) Ca^{2+} currents. Superimposed current traces (*bottom*) elicited by the double-pulse paradigm are shown in the absence (*1 and 3*) and presence (*2 and 4*) of 0.1 μM WIN 55,212–2 at the time points indicated in A. C, SCG neurons lack endogenous cannabinoid receptors. Ca^{2+} currents were elicited by the double-pulse protocol in a noninjected SCG neuron. Application of 0.1 μM WIN 55,212–2 (*shaded bar*) had no effect on the amplitude of the Ca^{2+} currents. D, WIN 55,212–2 significantly inhibited (**, $p < 0.001$) the Ca^{2+} currents in neurons injected with cannabinoid receptor cRNA (*open column*; 44.2 ± 5.3%) compared with noninjected neurons (*filled column*; 1.4 ± 0.9%). Number of neurons tested is indicated above columns.

for GDP-β-S to dialyze the cell. Controls without GDP-β-S in the patch pipette were run on alternate dishes of neurons. WIN 55,212–2 inhibited the Ca^{2+} current in the absence of GDP-β-S by 44.2 ± 5.3% (six experiments) (Fig. 4) and in the presence of GDP-β-S by 1.4 ± 1.0% (eight experiments) (Fig. 4). These results suggest that the heterologously expressed rat brain cannabinoid receptor inhibits Ca^{2+} currents in SCG neurons through a G protein-dependent coupling process. To test whether this G protein was sensitive to PTX, SCG neurons injected with the rat brain cannabinoid receptor cRNA were treated overnight with 500 ng/ml PTX. PTX pretreatment abolished the effect of WIN 55,212–2 in neurons injected with the rat brain cannabinoid receptor cRNA. Controls without PTX were run on alternate dishes of neurons. WIN 55,212–2 (0.1 μM) inhibited the Ca^{2+} current by 44.3 ± 6.4% (eight experiments) in control neurons (Fig. 4) and by 2.6 ± 1.7% (eight experiments) in neurons pretreated with PTX (Fig. 4). These results demonstrate that the rat brain CB_1 cannabinoid receptor inhibits Ca^{2+} currents through PTX-sensitive G proteins in SCG neurons.

Agonist activation of the rat brain CB_1 cannabinoid receptor inhibits primarily ω-CgTX GVIA-sensitive N-type Ca^{2+} currents in SCG neurons. The Ca^{2+} channel target of cannabinoid receptor modulation was identified using the N-type Ca^{2+} channel antagonist ω-CgTX GVIA. Fig. 5A illustrates the time course of an experiment on an SCG neuron injected with rat brain CB_1 cannabinoid receptor cRNA and challenged with WIN 55,212–2 before and after treatment with 10 μM ω-CgTX GVIA. Application of 0.1 μM WIN 55,212–2 before the application of ω-CgTX GVIA decreased the Ca^{2+} current amplitude. After washout of WIN 55,212–2, the amplitude of the Ca^{2+} current overrecovered. Application of 10 μM ω-CgTX GVIA produced a rapid and irreversible reduction in the Ca^{2+} current amplitude. A subsequent application of 0.1 μM WIN 55,212–2 had only a small effect on the remaining Ca^{2+} current. When Ca^{2+} current inhibition was normalized to the control value before ω-CgTX GVIA, the average current inhibition by 0.1 μM WIN 55,212–2 was 40.9 ± 5.8% before ω-CgTX GVIA (eight experiments) (Fig. 5C) and was 6.3 ± 1.2% after ω-CgTX GVIA (eight experiments) (Fig. 5C). The effect of WIN 55,212–2 was significantly reduced after ω-CgTX GVIA application. These results suggest that agonist activation of the rat brain CB_1 cannabinoid receptor inhibits primarily ω-CgTX GVIA-

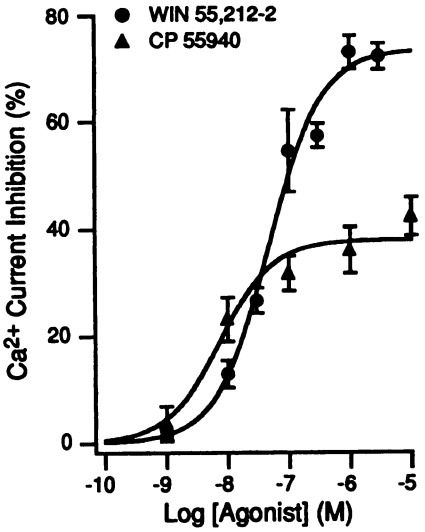

Fig. 3. WIN 55,212–2 and CP 55,940 inhibit the Ca^{2+} current in a concentration-dependent manner. Ca^{2+} currents were half-maximally inhibited (IC_{50}) by 47 nM WIN 55,212–2 and 7 nM CP 55,940. The maximal block of the Ca^{2+} current for WIN 55,212–2 and CP 55,940 was 73% and 38%, respectively.

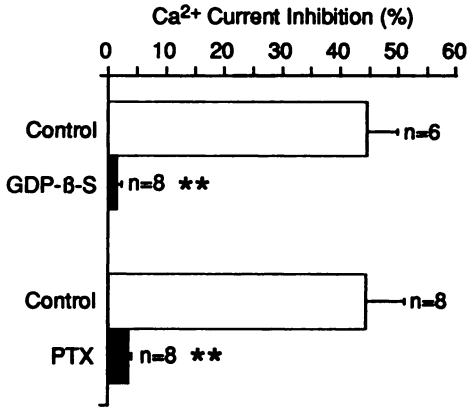

Fig. 4. The rat CB_1 cannabinoid receptor inhibits the Ca^{2+} current by coupling to G proteins that are PTX sensitive. Inclusion of 2 mM GDP-β-S in the patch pipette significantly reduced (**, $p < 0.001$) the inhibition of the Ca^{2+} current by WIN 55,212–2. In SCG neurons injected with CB_1 cRNA, 0.1 μM WIN 55,212–2 inhibited the Ca^{2+} current by 44.2 ± 5.3% (*top open bar*) compared with 1.4 ± 1.0% with GDP-β-S in the patch pipette (*top filled bar*). Overnight incubation of neurons injected with CB_1 cRNA with PTX (500 ng/ml) significantly inhibited (**, $p < 0.001$) the effect of WIN 55,212–2 on the Ca^{2+} current. The Ca^{2+} current was inhibited by 44.3 ± 6.4% in control neurons (*bottom open bar*) but by only 2.6 ± 1.7% in neurons pretreated with PTX (*bottom filled bar*). Number of neurons tested is indicated beside columns.

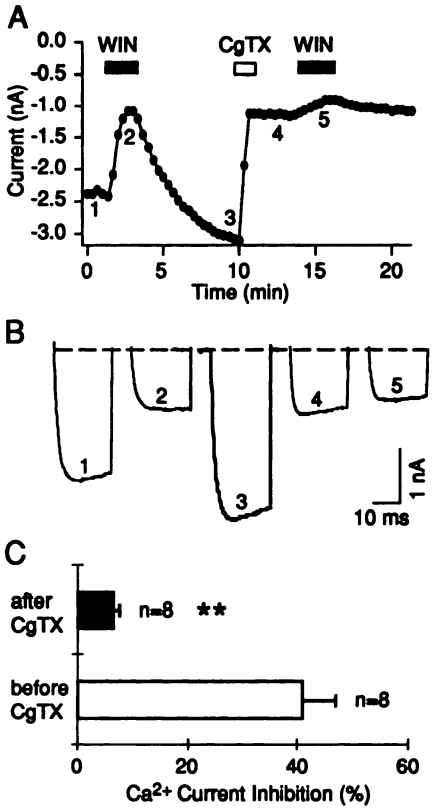

Fig. 5. The rat brain CB_1 cannabinoid receptor inhibits the ω-CgTX GVIA-sensitive N-type Ca^{2+} channel. A, A double-pulse protocol (as shown in Fig. 2B) was used to elicit Ca^{2+} currents in an SCG neuron injected with cannabinoid receptor cRNA. The current amplitudes of the first pulse are plotted over the time course of the experiment. Application of 0.1 μM WIN 55,212–2 (*WIN, shaded bar*) decreased the Ca^{2+} current amplitude, which overrecovered after washout. Application of 10 μM ω-CgTX GVIA (*CgTX, open bar*) irreversibly decreased the Ca^{2+} current amplitude. A subsequent application of 0.1 μM WIN 55,212–2 had only a small effect on the Ca^{2+} current. B, Current traces labeled with numbers corresponding to the time points indicated in A. C, The percentage of the total Ca^{2+} current inhibited by 0.1 μM WIN 55,212–2 before application of ω-CgTX GVIA (*open bar*; 40.9 ± 5.8%) was significantly decreased (**, $p < 0.001$) after application of 10 μM ω-CgTX GVIA (*filled bar*; 6.3 ± 1.2%). Data were normalized to the control value before ω-CgTX application. Number of neurons tested is indicated above columns.

sensitive N-type Ca^{2+} currents in SCG neurons. ω-CgTX MVIIC has been shown to inhibit a Q-type Ca^{2+} channel in cerebellar granule neurons (23). To determine whether the small block by WIN 55,212–2 in the presence of ω-CgTX GVIA could be due to inhibition of a Q-type Ca^{2+} channel in SCG neurons, 5 μM ω-CgTX MVIIC was applied after ω-CgTX GVIA. ω-CgTX MVIIC had no effect on the Ca^{2+} current and WIN 55,212–2 still produced a small block in two cells.

To determine whether the cannabinoid receptor could affect the L-type Ca^{2+} current present in SCG neurons, tail currents were studied in the presence of a dihydropyridine agonist. Ca^{2+} currents were evoked with a 30-msec test pulse to +5 mV from a holding potential of −80 mV followed by a repolarizing pulse to −40 mV. The prolonged tail current induced by 2 μM (+)-202–791, a dihydropyridine Ca^{2+} channel agonist, was studied before and after application of 0.1 μM WIN 55,212–2 in SCG neurons injected with rat brain CB_1 cannabinoid receptor cRNA. There was no significant change in the tail current amplitudes between applications of (+)-202–791 alone and the subsequent application of both (+)-202–791 and WIN 55,212–2, whereas the step current was inhibited by WIN 55,212–2 (six experiments). This indicates that activation of cannabinoid receptors has no significant effect on the L-type, dihydropyridine-sensitive Ca^{2+} current in SCG neurons.

The endogenous cannabinoid receptor agonist anandamide had an inconsistent effect on the Ca^{2+} current. Anandamide (arachidonyl ethanolamide) is an endogenous ligand for the cannabinoid receptor (24) and acts as a partial agonist to inhibit the Ca^{2+} current in N18 cells (25). Therefore, the ability of anandamide to inhibit the Ca^{2+} current

was tested on the cloned rat brain cannabinoid receptor. Of 33 SCG neurons injected with the rat brain CB_1 cannabinoid receptor cRNA, anandamide (0.1 μM) decreased the Ca^{2+} current in 2 cells by 40% and in 6 cells by ~10%; however, in 25 cells, anandamide had no effect. Compared with WIN 55,212–2, which always produced a reliable Ca^{2+} current inhibition, anandamide did not change the current amplitude in the majority of neurons. We tested whether the inconsistency of the effect of anandamide on the Ca^{2+} current could be due to the purity of anandamide. The purity of anandamide was checked with the use of thin layer chromatography. Anandamide from two different suppliers migrated as a single spot with an R_F value of ~0.5, indicating that these samples were pure. Another possibility for the inconsistency of the effect of anandamide may be due to degradation of anandamide. To prevent degradation of anandamide, the serine protease inhibitor phenylmethylsulfonyl fluoride (50 μM) was added to the culture medium and to the external recording solution. In the presence of phenylmethylsulfonyl fluoride, anandamide (0.1 μM) still had no effect on the Ca^{2+} current in seven neurons injected with the rat brain CB_1 cannabinoid receptor cRNA.

The cannabinoid receptor had no effect on the M-type K^+ current or inactivation of the K^+ A current. To test whether the cannabinoid receptor can modulate K^+ channels, the effect of WIN 55,212–2 was tested on both the M current and the A current, two types of K^+ currents found in SCG neurons. WIN 55,212–2 had no effect on the M current in SCG neurons injected with the rat brain CB_1 cannabinoid receptor cRNA. The amplitude of the M current in the presence of WIN 55,212–2 decreased by 0.8 ± 6.0% (four experiments) compared with a decrease of 84.3 ± 9.1% (three experiments) induced by the activation of endogenous muscarinic acetylcholine receptors by 10 μM muscarine.

A voltage-dependent depolarizing shift in the inactivation of the A current in rat hippocampal neurons by cannabinoid agonists has been reported (26). To test whether the A current in rat SCG neurons could be similarly modulated, inactivation of the A current was studied in SCG neurons injected with rat brain CB_1 cannabinoid receptor cRNA. A series of 1000-msec hyperpolarizing prepulses from −140 mV to −50 mV were followed by a 60-msec step to a test potential of −30 mV. The voltage step to −30 mV elicited a transient outward current that was half-inactivated by a prepulse potential of −87.3 ± 4.3 mV (six experiments), similar to that previously reported (27). In the presence of 0.1 μM WIN 55,212–2, the potential of half-inactivation was −89.4 ± 3.5 mV (six experiments), which was not significantly different from control. A concentration of 1 μM WIN 55,212–2 was also tested on two cells. This higher concentration of WIN 55,212–2 also did not cause a depolarizing shift in the half-inactivation potential. However, the cannabinoid agonist enhanced the transient outward current amplitude by 13.6 ± 7.3% in 6 of 11 neurons measured at a prepulse potential of −120 mV in the presence of 0.1 μM WIN 55,212–2.

Discussion

The physiological effects of the cloned rat brain CB_1 cannabinoid receptor were studied using a neuronal expression system. N-type Ca^{2+} channels were the effector target of the cloned cannabinoid receptor and were inhibited by the cannabimimetic WIN 55,212–2 in a concentration-dependent and reversible manner. The heterologously expressed cannabinoid receptor coupled to a PTX-sensitive G protein. These results on the identified rat brain CB_1 cannabinoid receptor are similar to inhibition of N-type Ca^{2+} channels by cannabinoids in NG108–15 cells (13). WIN 55,212–2 inhibited the Ca^{2+} current with an IC_{50} value of 10 nM in NG108–15 cells (13) and of 47 nM in SCG neurons expressing the rat brain CB_1 cannabinoid receptor. The potency of CB_1 cannabinoid receptor-mediated Ca^{2+} current inhibition was similar to the inhibition of forskolin-stimulated cAMP accumulation by WIN 55,212–2 (IC_{50} = 24 nM) in Chinese hamster ovary cells transfected with the rat brain CB_1 cannabinoid receptor (28). The synthetic cannabinoid analogue CP 55,940 also inhibited Ca^{2+} currents in SCG neurons expressing the rat brain CB_1 cannabinoid receptor, with a maximal inhibition of 38% and an IC_{50} value of 7 nM. The maximal effect of CP 55,940 was approximately half of the maximal effect of 73% induced by WIN 55,212–2 in the SCG neurons. CP 55,940 was also reported to have a lower efficacy compared with WIN 55,212–2 in the inhibition of glutamatergic synaptic transmission between cultured hippocampal neurons (29).

A rebound overrecovery of the Ca^{2+} currents was seen in SCG neurons injected with rat brain CB_1 cannabinoid receptor on washout of WIN 55,212–2 in approximately half of the neurons. Washout of opiate agonist has also been reported to enhance the Ca^{2+} current amplitude in NG108–15 cells (30). This phenomenon was termed rebound facilitation, and Kasai (30) suggested that it was caused by suppression of tonic inhibition of the Ca^{2+} current. If this were the case, the current amplitude of the control test pulse should approach the amplitude of the facilitated test pulse that relieves tonic inhibition. This was not the case for the cannabinoid receptor. Both the control and facilitated Ca^{2+} current amplitudes were enhanced during overrecovery, indicating that this phenomenon is not simply a reversal of tonic G protein-dependent Ca^{2+} current inhibition.

The endogenous cannabinoid receptor ligand anandamide was inconsistently effective as a cannabinoid agonist compared with WIN 55,212–2. WIN 55,212–2 was always effective in inhibiting the Ca^{2+} current in SCG neurons expressing the rat brain CB_1 cannabinoid receptor. Anandamide, on the other hand, was not as reliable; 75% of cells injected with cannabinoid receptor cRNA did not respond to anandamide. Mackie *et al.* (25) reported that anandamide is a partial agonist. The effect of a partial agonist depends on the number and state of the receptors. If receptor numbers are low, then anandamide might not activate enough receptors to produce a full physiological effect. Perhaps the absence of an anandamide effect in some experiments is due to low levels of receptor expression. Full agonists may still produce maximal effects, even in cells with low numbers of receptors. Because the density of cannabinoid receptors varies among cells of different brain regions (4, 9), the effects of partial agonist could vary depending on the number and state of these receptors.

ω-CgTX GVIA, an irreversible blocker of the N-type Ca^{2+} channel, occluded the majority of Ca^{2+} current inhibition by WIN 55,212–2, suggesting that the primary target of the rat brain CB_1 cannabinoid receptor heterologously expressed in SCG neurons is the N-type Ca^{2+} channel. The N-type Ca^{2+} channel was also found to be the target of endogenous can-

nabinoid receptors in neuroblastoma cells (12, 13). Because the N-type channel plays a role in neurotransmitter release from many neurons (31–33), cannabinoids may function to inhibit neurotransmitter release. It has been suggested that inhibition of neurotransmitter release may contribute to the psychoactive effects of cannabinoids (34). In agreement with this hypothesis, cannabinoid receptors have been located on presynaptic neurons (4, 35), and cannabinoids attenuate the electrically evoked twitch contraction of vas deferens prejunctionally (36–38).

In SCG neurons, the N-type Ca^{2+} channels carry the majority of the Ca^{2+} current, with a small contribution from the L-type channel (39). T- and P-type channels are not found in SCG neurons (16, 40), and no evidence of a Q-type channel sensitive to ω-CgTX MVIIC after block of the N-type channel with ω-CgTX GVIA was found in this study. Mackie *et al.* (41) reported that the CB_1 cannabinoid receptor can inhibit an ω-CgTX MVIIC-sensitive Q-type Ca^{2+} current in transfected AtT-20 cells. Thus, the cannabinoid receptor can inhibit both N- and Q-type Ca^{2+} channels. Inhibition of these two types of voltage-gated Ca^{2+} channels may block neurotransmitter release in specific neurons in which both receptor and channels are expressed.

In cultured rat hippocampal neurons, activation of the endogenous cannabinoid receptor was shown to enhance the rapidly inactivating K^+ A current via a shift in the voltage dependence of channel inactivation (26). Activation of the rat brain CB_1 cannabinoid receptor heterologously expressed in rat SCG neurons did not shift the voltage- dependence of inactivation of a rapidly inactivating K^+ A current found in these neurons. We did observe, however, a small increase in the A current amplitude in 6 of 11 neurons. The differences in the effects of cannabinoids on the A current may arise from differences in the subtypes of A current expressed in hippocampal and SCG neurons. Also, because the cannabinoid effects in hippocampal neurons are mediated through a cAMP-dependent second messenger pathway (42), the conditions of our experiments may not have been optimal for the effective operation of this second messenger system.

In summary, we report the expression and functional coupling of the cloned rat brain cannabinoid receptor in a fully differentiated, mammalian neuron. Expression of the rat brain CB_1 cannabinoid receptor in rat SCG neurons was both rapid and highly reproducible. Receptor expression was detected in all neurons at 16–25 hr after injection of receptor cRNA. Activation of the rat brain CB_1 cannabinoid receptor resulted in a voltage-dependent inhibition of N-type Ca^{2+} channels through a PTX-sensitive G protein similar to the activation of the endogenous cannabinoid receptors in neuroblastoma cells (13). Washout of the cannabinoid receptor agonist resulted in an overrecovery of the Ca^{2+} current amplitude in approximately half of the cells tested and was not simply a reversal of tonic Ca^{2+} current inhibition. Overrecovery of the Ca^{2+} current on washout could also contribute to the physiological effects mediated by cannabinoid receptors. Ca^{2+} channels were specific effector targets of the rat brain CB_1 cannabinoid receptor as two types of K^+ current, the M-type K^+ current and the rapidly inactivating A current, were not affected. The ability to express identified cannabinoid receptors, splice variants (43), and receptor mutants in mature neurons will facilitate the identification of ion channel targets, cellular second messenger pathways, and structural studies of the cannabinoid receptor.

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