Cannabinoids Inhibit N- and P/Q-Type Calcium Channels in Cultured Rat Hippocampal Neurons

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Twitchell, W., S. Brown, and K. Mackie. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J. Neurophysiol.* 78: 43–50, 1997. Cannabinoids and their analogues have been found to inhibit N- and P/Q-type Ca^{2+} currents in cell lines and sympathetic neurons transfected with cannabinoid CB1 receptor. However, the effects of cannabinoids on Ca^{2+} currents in the CNS are largely unexplored. In this study we investigated whether these compounds inhibit Ca^{2+} channels in cultured rat hippocampal neurons. With the use of antibodies directed against the amino-terminus of the CB1 receptor, we found that in 5-day cultures pyramidally shaped neurons expressed somatic CB1 receptors, whereas in 4-wk cultures the receptor was predomintely located on neurites. In early cultures, the cannabimimetic WIN 55,212-2 reversibly inhibited whole cell Ba^{2+} current in a concentration-dependent ($K_{1/2} = 21$ nM) and pertussis-toxin-sensitive fashion. Inhibition was reduced by the CB1 antagonist SR141716. The current was unaffected by the nonpsychoactive enantiomer WIN 55,212-3. Maximal inhibition by the nonclassical cannabinoid agonist CP 55,940 and by an endogenous cannabinoid, anandamide, were similar to that seen with maximal concentrations of WIN 55,212-2. The Ba^{2+} current modulated by cannabinoids was carried by N-type (ω-conotoxin-GVIA-sensitive) and P/Q-type (ω-conotoxin-MVIIC-sensitive) channels. These results demonstrate cannabinoid-receptor-mediated inhibition of distinct Ca^{2+} channels in central neurons. Because the channels that underlie these currents are chiefly located presynaptically, and are required for evoked neurotransmitter release, our results suggest a major role for cannabinoids (endogenous and exogenous) in the modulation of synaptic transmission at CNS synapses.

INTRODUCTION

Cannabis extracts have been used medicinally and socially for millenia. The primary active constituents of *Cannabis* extracts are Δ^9-tetrahydrocannabinol and related cannabinoids. The behavioral effects following cannabinoid administration are distinctive for this family of compounds and include difficulty in concentration, impairment of memory, an enhancement of sensory perception, and mild euphoria (Dewey 1986; Hollister 1986; Mechoulam 1986). These effects suggest that cannaboids mimic endogenous compounds that play important roles in the processing of neural signals for memory, mood, and higher cognitive functions. This hypothesis is supported by the identification and cloning of a G protein-coupled receptor that specifically binds cannabinoids (CB1) (Matsuda et al. 1990), and the discovery in the brain of a family of eicosanoids whose synthesis is tightly regulated and that bind with high affinity to the cannabinoid receptor, eliciting classical cannabinoid effects (Devane et al. 1992; Di Marzo et al. 1994). Furthermore, the receptor and its mRNA are highly enriched in the brain regions most likely involved in the psychoactive effects of cannabinoids (Herkenham et al. 1991c; Matsuda et al. 1993).

We are interested in determining the intracellular signaling pathways used by the CB1 receptor. The first action demonstrated was the inhibition of adenylyl cyclase mediated by pertussis-toxin (PTX)-sensitive G protein(s). This effect is seen in cell lines, membrane fractions from brain, and neuronal cell cultures (Bidaut-Russell et al. 1990; Howlett et al. 1986; Pacheco et al. 1991). One consequence of the inhibition of adenylyl cyclase that will affect neuronal excitability is a slowing of inactivation of the A-type potassium current at high concentrations of cannabinoid (Deadwyler et al. 1995). The second well-characterized messenger system of the CB1 receptor is the stimulation of the mitogen-activated protein kinase (Bouaboula et al. 1995; Wartmann et al. 1995). Thus far, activation of the mitogen-activated protein kinase pathway by the CB1 receptor has only been found in cell lines; therefore its role in the CNS is at present unclear. The third major action of cannabinoids is the direct G protein-mediated modulation of ion channels. Cannabinoids have been found to inhibit N- and P/Q-type voltage-dependent calcium currents potently in several cell lines (Caulfield and Brown 1992; Mackie and Hille 1992; Mackie et al. 1995). More recently, modulation of N-type calcium currents in cultured rat superior cervical sympathetic ganglion (SCG) neurons by cannabinoids was found after injection of these neurons with mRNA encoding the CB1 receptor (Pan et al. 1996). However, the actions of cannabinoids on calcium currents in central neurons are unexplored. In addition to inhibition of calcium currents by cannabinoids, they also activate inwardly rectifying potassium currents (Henry and Chavkin 1995; Mackie et al. 1995).

Cannabinoid modulation of the voltage-dependent calcium channels involved in neurotransmitter release (Luebke et al. 1993; Wheeler et al. 1994; Wu and Saggau 1994) suggests that inhibition of neurotransmitter release might be a major mode of action of cannabinoids. In the peripheral nervous system there is ample evidence that cannabinoids inhibit neurotransmitter release (Pertwee 1993; Roth 1978). Recently, cannabinoids have been found to inhibit glutamatergic synaptic transmission in hippocampal cultures (Shen et al. 1996). Thus we were interested in determining whether cannabinoids inhibit voltage-dependent calcium channels in cultured rat hippocampal neurons. With the use of cultures of neonatal hippocampus we have found that N- and P/Q-type calcium channels are potently and stereospe-

cifically inhibited by cannabinoids acting at the CB1 receptor via a PTX-sensitive G protein.

METHODS

Materials

WIN 55,212-2 and WIN 55,212-3 were obtained from Sterling Winthrop, Rensselaer, NY; 2-chloroadenosine (2-CA) and anandamide from RBI, Natick MA; CP 55,940 from Pfizer, Groton, CT; PTX from List Biological Laboratories, Campbell, CA; media and sera from GIBCO Labs, Grand Island, NY; ω-conotoxin-GVIA (ω-CTX-GVIA) from Bachem; SNX-230 (ω-conotoxin-MVIIC) as a gift from Neurex; papain from Worthington, Freehold NJ; MITO+Serum Extender from Collaborative Research, Bedford MA; and glass coverslips from Fisher Scientific. All other chemicals were obtained from Sigma, St. Louis, MO.

Cell culture

Hippocampal cells were isolated and cultured with the use of a modification of a published protocol (Bekkers and Stevens 1991). Briefly, cells were obtained from the CA1 and CA3 regions of the hippocampi of 1- to 4-day postnatal rats. Tissue was digested in a 20-U/ml papain solution and plated on rat tail collagen/poly-D-lysine-coated glass coverslips in a culture medium consisting of Minimal Essential Medium, 5% fetal bovine serum, 0.1% MITO+Serum Extender, 0.2% B-27, and 1:200 penicillin/streptomycin. Cytosine arabinoside (5 μM) was added after 5 days in culture to suppress the growth of dividing cells. Recordings were made between 2 and 10 days in culture. Pyramidal neurons were identified on the basis of their distinct morphology (large triangular or bipolar soma, long neuritic process).

Immunocytochemistry

Polyclonal antiserum to a glutathione-S transferase (GST) fusion protein containing the first 77 residues of the CB1 receptor (GST-CB1:1–77) was raised in rabbits with the use of conventional techniques. A fraction highly enriched in antibodies directed against the CB1 receptor was prepared by affinity purfication of the immune serum over a glutathione Sepharose column (to remove GST antibodies) followed by purification over a GST-CB1:1–77 affinity column. Antibodies purified in this fashion showed little or no cross-reactivity toward GST (data not shown). To determine the specficity of the antibodies, Western blots were performed on either 10 μg of bacterial lysate or 50 μg of hippocampal membrane preparation (per lane), as indicated in the figure legends, and bound antibody was detected with the use of enhanced chemiluminescense (ECL, Amersham). Blocking experiments were performed by preincubation of the antibody with 2 μg of the appropriate protein for 30 min at room temperature.

For immunocytochemistry, cultures were stained after growing for 3–28 days. Briefly, cells were washed three times with sodium phosphate buffer (PB; 0.1 M, pH 7.4), fixed in 4% paraformaldehyde in PB for 20 min, and washed twice with PB and three times with PB + 150 mM NaCl (PBS). Cells were blocked with 10% nonfat dry milk in PBS for 30 min, incubated with primary antibody (1:500 in PBS + milk) for 1–2 h, and washed five times with PBS, and bound antibody was detected with fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit immunoglobulin G (1:150, Zymed) in PBS + milk. Finally, cells were washed three times with PBS, twice with PB, and twice with water, air dried, and mounted with Vectashield (Vector Labs). Images were collected with a Biorad MRC600 confocal microscope with the use of an FITC cube and processed with the use of Adobe Photoshop.

Electrical measurements

Whole cell currents were recorded with the use of an Axopatch 200A patch-clamp amplifier (Axon Instruments), filtered at 4 kHz with a low-pass Bessel filter, and digitized at the appropriate frequency. Analog compensation was used to attenuate capacitive transients and to determine whole cell capacitance. Data acquisition and analysis were performed with Pulse software (version 8.02, Instrutech, Great Neck, NY and HEKA Elektronik, Lambrecht, Germany) running on an Apple Power Macintosh 8100/100 computer. The extracellular solution consisted of (in mM) 138 NaCl, 4 KCl, 1 $MgCl_2$, 2.5 $BaCl_2$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 glucose, pH adjusted to 7.4 with NaOH. Fatty-acid-free bovine serum albumin (3 μM) was added to decrease adsorbtion of cannabinoids, and tetrodotoxin (100 nM) was added to block voltage-activated sodium currents. The pipette solution contained (in mM) 100 CsCl, 1 $MgCl_2$, 3 MgATP, 0.3 Na_2GTP, 10 HEPES, 0.5 $CaCl_2$ (free $Ca^{2+} \sim$ 3.5 nM), 20 tetraethylammonium chloride, and 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH adjusted to 7.4 with NaOH. Whole cell patch pipettes had resistances of 3–5 MΩ, and the pipette solution was allowed to equilibrate with the cell interior for $\geq$3–5 min before the beginning of data acquisition. Except as noted, high-voltage-activated currents were evoked every 8 s by 30-ms depolarizing pulses to 0 mV from a holding potential of −70 mV. Currents were not leak subtracted, but the Cd^{2+}-resistant current was subtracted off-line in all experiments. To control for variations in response between cultures, control recordings were interleaved with experimental recordings. All the experiments were carried out at room temperature (21–23°C).

Drug application

Compounds were applied via a multibarrel pipette positioned near the cell. Drug application was controlled by solenoid valves and flowed under gravity. The bath chamber was constantly perfused with artificial cerebrospinal fluid (composition, in mM: 138 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 HEPES, and 15 glucose) while the multibarrel pipette was used to apply external recording solutions. Interruptions in drug application could easily be detected by the reversal of tetrodotoxin blockade of voltage-activated Na^+ current and the appearance of large sodium currents.

Data analysis and presentation

Decreases in current are reported as percent decrease from control current. All measurements are given as means ± SE. When data are plotted as bar graphs, the number above the bar indicates the number of cells tested. Effects were statistically compared with control inhibition by a two-tailed, nonpaired Student's t-test at a 0.05 level of significance.

To determine the mean effective concentration (EC_{50}; $K_{1/2}$) of the effect of WIN 55,212-2 (WIN) on the whole cell Ba^{2+} current, we used the equation

$$I_{WIN} = I_{WIN(m)}/\{1 + (K_{1/2}/[WIN]^n\}. \qquad (1)$$

Where I_{WIN} denotes inhibition of the current in the presence of WIN, $I_{WIN(m)}$ is the maximum inhibition of Ba^{2+} current by WIN, [WIN] is the WIN concentration, $K_{1/2}$ is the WIN concentration producing half-maximal inhibition of Ba^{2+} current, and n is the Hill coefficient.

RESULTS

Hippocampal neurons express CB1 receptor

We generated an antibody directed against the amino-terminus of the rat cannabinoid receptor to determine its

expression in cultured hippocampal neurons. Western blotting was used to characterize the antibody. Figure 1a shows that an antibody to residues 1–77 of the CB1 receptor recognizes one major and four minor protein species in rat cortical membranes. These are likely all to be related to the cannabinoid receptor (the larger, 70-kDa species might reflect differential glycosylation and the lower M_r species may represent proteolytic fragments), because they are blocked by preincubation of the antibody with the immunizing peptide (Fig. 1a). It is unlikely that any of these bands represent either the CB1 splice variant, CB1A (Shire et al. 1995), or the CB2 receptor (Munro et al. 1993), because these latter proteins have no sequence similarity to the first 77 residues of CB1. The additional bands seen in the blocking experiment with cortical membranes are likely due to low-affinity interactions between the secondary antibody and proteins on the blot.

Rat hippocampal pyramidal neurons express low to moderate levels of CB1 receptor mRNA (Matsuda et al. 1993). We wished to determine whether cultured hippocampal neurons also expressed CB1 protein. Figure 1b shows that this is the case. Most cells with a pyramidal morphology were labeled, whereas cells likely to be glia were not (data not shown). Hippocampal cultures stained after 3–4 wk of growth showed intense, punctate staining along processes, with little staining over cell bodies (Fig. 1c); for this reason recordings were performed on cultures grown for ≤10 days. In both cases, staining was eliminated by preincubation of the antibody with 10 μg/ml of the immunizing protein (data not shown).

WIN 55,212-2 potently inhibits I_{Ba}

We found that application of WIN 55,212-2 (100 nM) reversibly inhibited Ba^{2+} current (I_{Ba}) between 18% and 35% (Fig. 2A). Inhibition was stereoselective, because application of the nonpsychoactive enantiomer WIN 55,212-3 (100 nM) was ineffective in modulating I_{Ba} (Figs. 2, A, B, and D, and 3A). WIN 55,212-2 inhibited I_{Ba} at all voltages tested (Fig. 2C). WIN 55,212-2 inhibition of whole cell barium currents in pyramidal cells was concentration dependent and showed little coopertivity, being half-maximal at 21 nM and having a Hill coefficient of 1.4 (Fig. 2D).
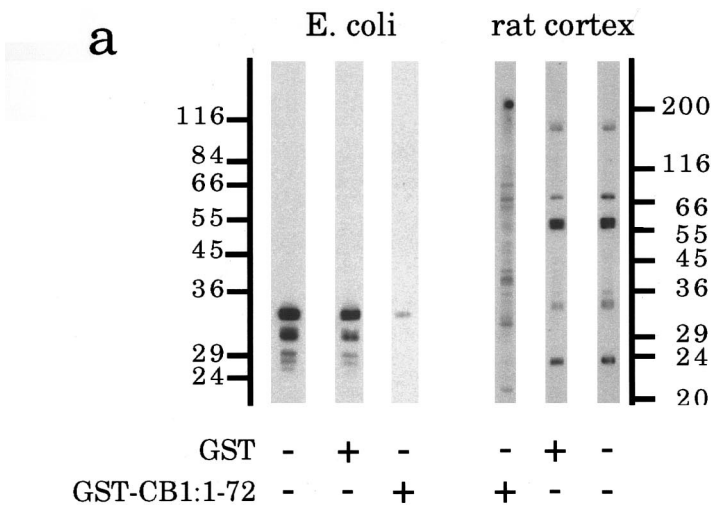

FIG. 1. Characterization of CB1 antibodies and detection of CB1 receptor in cultured hippocampal neurons. a, left: affinity purified antiserum (1:30,000) detects the glutathione-S transferase (GST)–CB1 fusion protein in bacterial lysates. Binding is not affected by preincubation of the antibody with purified GST (2 μg), but is markedly decreased by preincubation with the GST-CB1 fusion protein (2 μg). a, right: affinity-purified antiserum (1:500) recognizes a major band of M_r 60 kDa and less intense bands at ∼23, ∼72, and ∼180 kDa in cortical membranes. Binding at these four bands is unaffected by preincubation of the antibody with purified GST (2 μg) but is eliminated by preincubation with the GST-CB1 fusion protein (2 μg). b: 5-day-old hippocampal cultures contain cells that are labeled by the CB1 antibody and have the morphology expected of pyramidal cells (×600). c: 4-week-old hippocampal cultures show profuse, intense punctate staining over many processes, with little staining of cell bodies (×600).
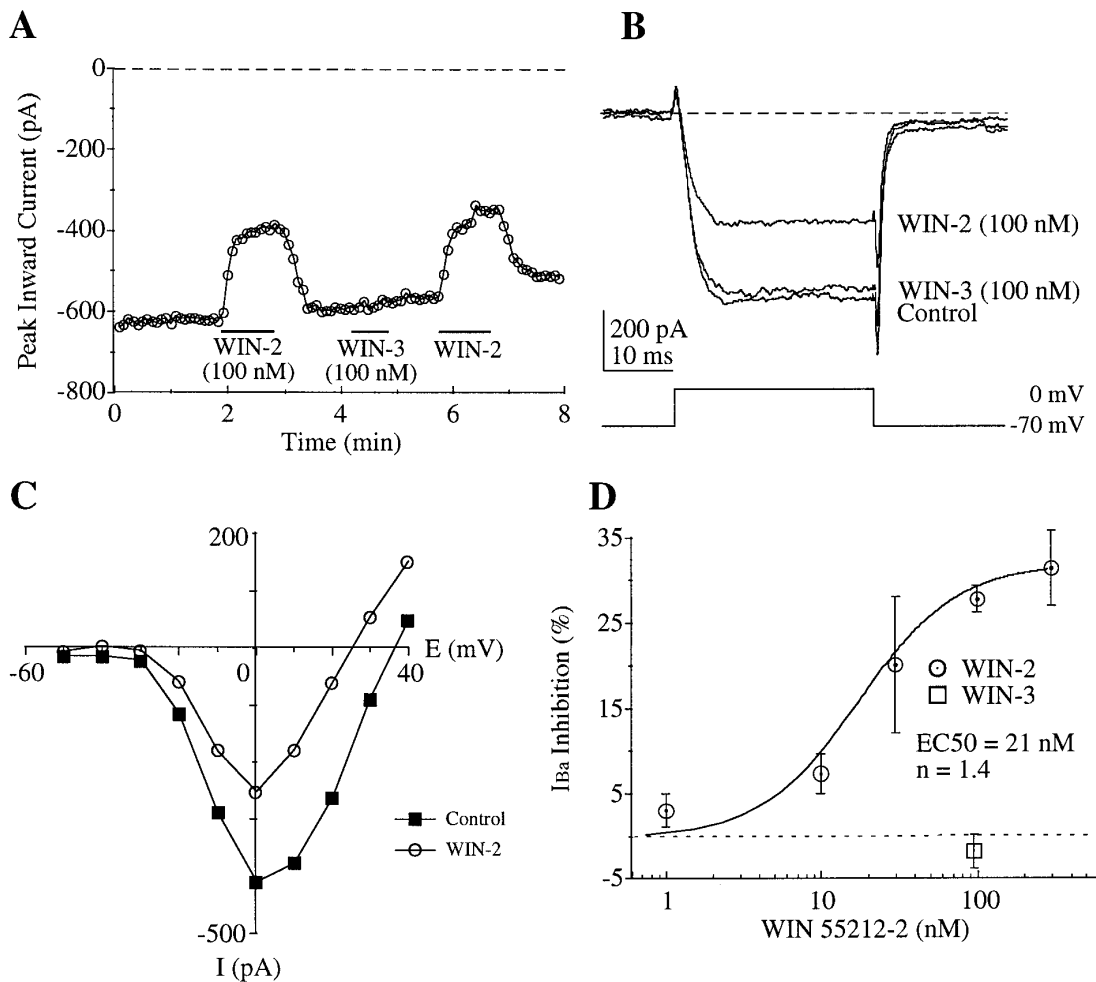
A

B

C

D
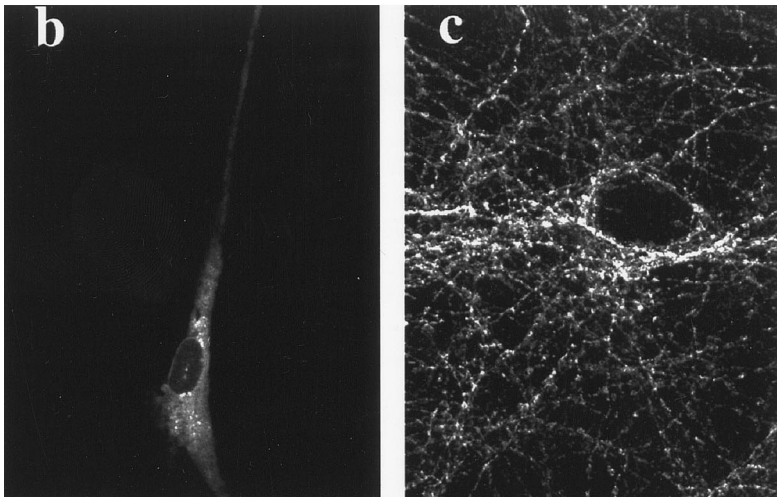
FIG. 2. WIN 55,212-2 inhibits Ba^{2+} current in rat hippocampal neurons. a: currents were elicited by stepping to 0 mV from a holding potential of -70 mV for 25 ms. Mean current from the latter half of the depolarization was plotted vs. time. WIN 55,212-2 (100 nM) reversibly inhibits whole cell Ba^{2+} current (I_{Ba}), whereas the inactive enatiomer 100 nM WIN 55,212-3 has little effect. B: individual current traces from (A), showing prominent inhibition by WIN 55,212-2 and little inhibition by the inactive enantiomer WIN 55,212-3 (100 nM). C: current-voltage relationship of I_{Ba} in a hippocampal neuron. No low-voltage-activated I_{Ba} is apparent. Leftward shift of reversal potential following WIN 55,212-2 was unusually large in this cell; usually it was <5 mV. D: concentration response for WIN 55,212-2 inhibition of I_{Ba} reveals a half-maximal inhibition at 21 nM with a Hill coeffcient of 1.4. WIN 55,212-3 (100 nM) has a negligible effect on the currents. Each point is the mean $\pm$ SE of 3–7 cells.

CP 55,940 and anandamide are full agonists as inhibitors of I_{Ba}

Comparing the relative efficacies of structurally dissimilar cannabinoid agonists in modulating excitable properties is complicated. The endogenous cannabinoid agonist anandamide has a lower intrinsic efficacy than CP 55,940 or WIN 55,212-2 in inhibiting N-type calcium channels in N18 neuroblastoma cells, where it acts as a partial agonist (Mackie et al. 1993). Furthermore, in SCG neurons heterologously expressing CB1, CP 55,940 was less efficacious than WIN 55,212-2 and anandamide had small and variable effects (Pan et al. 1996). Finally, compared with WIN 55,212-2 and anandamide, CP 55,940 acted as a partial agonist in inhibiting hippocampal synaptic transmission (Shen et al. 1995). Thus it was of interest to compare WIN 55,212-2, CP 55,940, and anandamide inhibition of calcium channels in cultured hippocampal pyramidal cells. Interestingly, in these cells CP 55,940 and anandamide were as efficacious

as WIN 55,212-2. They inhibited I_{Ba} by 25.5 $\pm$ 2.4% (mean $\pm$ SE; $n = 5$), 28.6 $\pm$ 1.9% ($n = 4$), and 29.5 $\pm$ 1.8% ($n = 13$), respectively (Fig. 3A). This is demonstrated more rigorously by application of CP 55,940 in the presence of WIN 55,212-2. In this experiment WIN 55,212-2 inhibition was 29.5 $\pm$ 1.8% ($n = 13$), compared with a 25.2 $\pm$ 2.2% ($n = 4$) inhibition when CP 55,940 and WIN 55,212-2 were coapplied. This lack of a significant difference in I_{Ba} inhibition suggests that CP 55,940 acts as a full agonist when inhibiting somatic calcium channels.

WIN 55,212-2 inhibits I_{Ba} via the CB1 receptor and a PTX-sensitive G protein

The compound SR141716 appears to be a specific CB1 antagonist (Rinaldi-Carmona et al. 1994). Treatment of the neurons with 200 nM SR141716 reduced WIN 55,212-2 inhibition of I_{Ba} from 31.5 $\pm$ 2.5% ($n = 8$) to 7.9 $\pm$ 1.5% ($n = 9$), whereas it did not significantly affect the inhibition
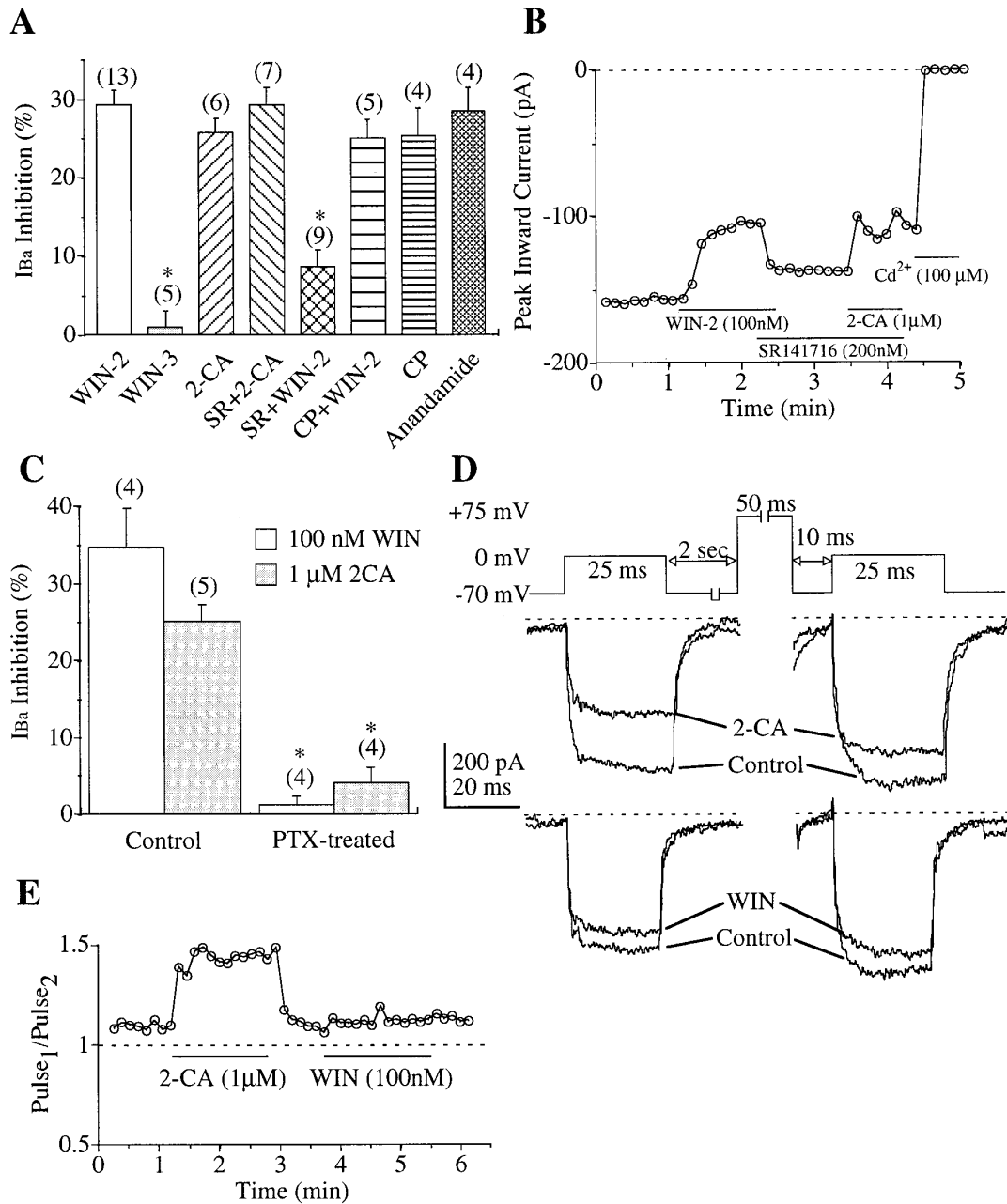
FIG. 3. Pharmacology and G protein dependence of I_{Ba} inhibition. A: ability of various cannabinoids to inhibit I_{Ba} was compared. WIN 55,212-2 (WIN-2, 100 nM), 300 nM anandamide, and 100 nM CP 55,940 (CP) were all similarly efficacious in inhibiting I_{Ba}. The CB1 antagonist SR141716 (SR, 200 nM), reduced inhibition by 100 nM WIN 55,212-2, without affecting inhibition by the adenosine A1 agonist 2-chloroadenosine (2-CA). B: experiment performed as in Fig. 2a shows that SR141716 reversed WIN 55,212-2 inhibition of I_{Ba} without reducing inhibition of I_{Ba} by 2-CA. C: CB1 inhibition of I_{Ba} is mediated by a pertussis-toxin (PTX)-sensitive G protein. Pretreatment of hippocampal cultures with 200 ng/ml PTX for 15 h eliminated inhibition by 100 nM WIN 55,212-2 and 1 μM 2-CA. Parallel cultures were treated with 200 ng/ml heat-inactivated (95°C for 15 min) PTX. Numbers in parentheses: number of cells tested for each condition. Asterisk: $P <$ 0.05. D: inhibition of I_{Ba} by WIN 55,212-2 was not voltage dependent, whereas inhibition by 2-CA was. Voltage dependence was assessed by applying a control depolarization to 0 mV, followed after a 2-s pause by a strong depolarization to +70 mV, and then a 2nd depolarization to 0 mV (see figure for pulse protocol). E: facilitation ratio (peak of 2nd pulse divided by 1st pulse) plotted vs. time for the same cell as in D.

of I_{Ba} by the adenosine A1 agonist 2-CA (25.8 ± 1.7%, $n =$ 6 vs. 29.4 ± 2.2%, $n = 7$) in the absence and presence of SR141716, respectively (Fig. 3, A and B). These results indicate that the effect of WIN 55,212-2 is mediated via a CB1 cannabinoid receptor.

Most actions of cannabinoids mediated by the CB1 receptor are transduced by PTX-sensitive G proteins, presumably G_i or G_o. We treated the cultures with either PTX or boiled PTX (both at 200 ng/ml) for 15 h before recording. We found that cultures treated with PTX, but not boiled PTX,

failed to respond to either WIN 55,212-2 ($-1.2 \pm 1.1\%$, $n = 4$ vs. $34.7 \pm 5.2\%$, $n = 4$) or 2-CA ($-4.0 \pm 2.2\%$, $n = 4$ vs. $26.3 \pm 1.4\%$, $n = 4$; Fig. 3*C*). These data strongly suggest that the CB1 receptor couples to $G_{i/o}$ proteins in rat hippocampal neurons as in other cells. Interestingly, unlike the cannabinoid-receptor-mediated inhibition of calcium currents in other cells (Mackie et al. 1993, 1995), cannabinoid inhibition of I_{Ba} in cultured rat hippocampal neurons was not voltage dependent, despite inhibition by 2-CA being voltage dependent (Fig. 3, *D* and *E*). In control cells, facilitation, defined as the amplitude of the second pulse divided by that of the first pulse, was 1.13 ± 0.02 ($n = 5$). In WIN 55,212-2-treated cells, facilitation was 1.11 ± 0.05 ($n = 4$; $P > 0.3$ vs. control), whereas in 2-CA-treated cells it was 1.42 ± 0.09 ($n = 5$; $P < 0.02$ vs. both control and WIN 55,212-2). In these experiments the average inhibition by WIN 55,212-2 was $22 \pm 2\%$ ($n = 4$), whereas for 2-CA it was $25 \pm 1\%$ ($n = 5$).

WIN 55,212-2 modulates N- and P/Q-type currents

Hippocampal pyramidal neurons express several pharmacologically distinct subtypes of high-voltage-activated calcium channels (Ishibashi and Akaike 1995; Scholz and Miller 1995). To determine the specific subtype of current inhibited by cannabinoids, we used a strategy of successively applying channel blockers. The dihydropyridine 2 μM isradipine was used to block L-type calcium channels. Its application blocked about a third of the I_{Ba} (Fig. 4*A*). Cytochrome c (Cyto c) was used as a carrier for the peptide toxins ω-CTX-GVIA and ω-conotoxin-MVIIC (SNX-230), which were used to block N- and P/Q-type channels, respectively. Cyto c had little effect on I_{Ba} ($2.0 \pm 1.4\%$ inhibition, $n = 7$) in the presence of isradipine (Fig. 4*A*). In the presence of isradipine, 1 μM ω-CTX-GVIA inhibited $31 \pm 4.5\%$ of the residual I_{Ba}, whereas in the presence of both isradipine and ω-CTX-GVIA, 5 μM ω-conotoxin-MVIIC inhibited the residual I_{Ba} by $26 \pm 7\%$ (Fig. 4*A*).

We next determined which calcium channels were inhibited by cannabinoids by first blocking a subset of the calcium channels and then applying 100 nM WIN 55,212-2. The barium current inhibited by WIN 55,212-2 was then compared with the barium current present before channel blockade and the percent inhibition was calculated. Blockade of L-type calcium channels by isradipine did not affect the amount of current inhibited by 100 nM WIN 55,212-2 (Fig. 4*B*). Similarly, Cyto c had little effect on the inhibition of I_{Ba} by WIN 55,212-2 in the presence of isradipine ($58.9 \pm 4.1\%$, $n = 8$ vs. $59.7 \pm 5.2\%$, $n = 8$; Fig. 4*B*). However, 1 μM ω-CTX-GVIA significantly reduced WIN 55,212-2 inhibition to $21.1 \pm 2\%$ ($n = 3$), compared with $59.7 \pm 5.2\%$ ($n = 8$) in isradipine-treated cells. In the combined presence of isradipine and ω-CTX-GVIA, 5 μM ω-conotoxin-MVIIC (SNX-230) significantly reduced WIN 55,212-2 inhibition to $3.6 \pm 1\%$ ($n = 5$), compared with $59.7 \pm 5.2\%$ ($n = 8$) in isradipine-treated control (Fig. 4, *b* and *c*). The conclusion from this series of experiments is that these cultured hippocampal neurons express L-, N-, P/Q-, and R-type calcium channels. Of these channels, ~70% of the current inhibited by cannabinoids is carried by N-type calcium channels, whereas the balance is carried by P/Q-type calcium channels.

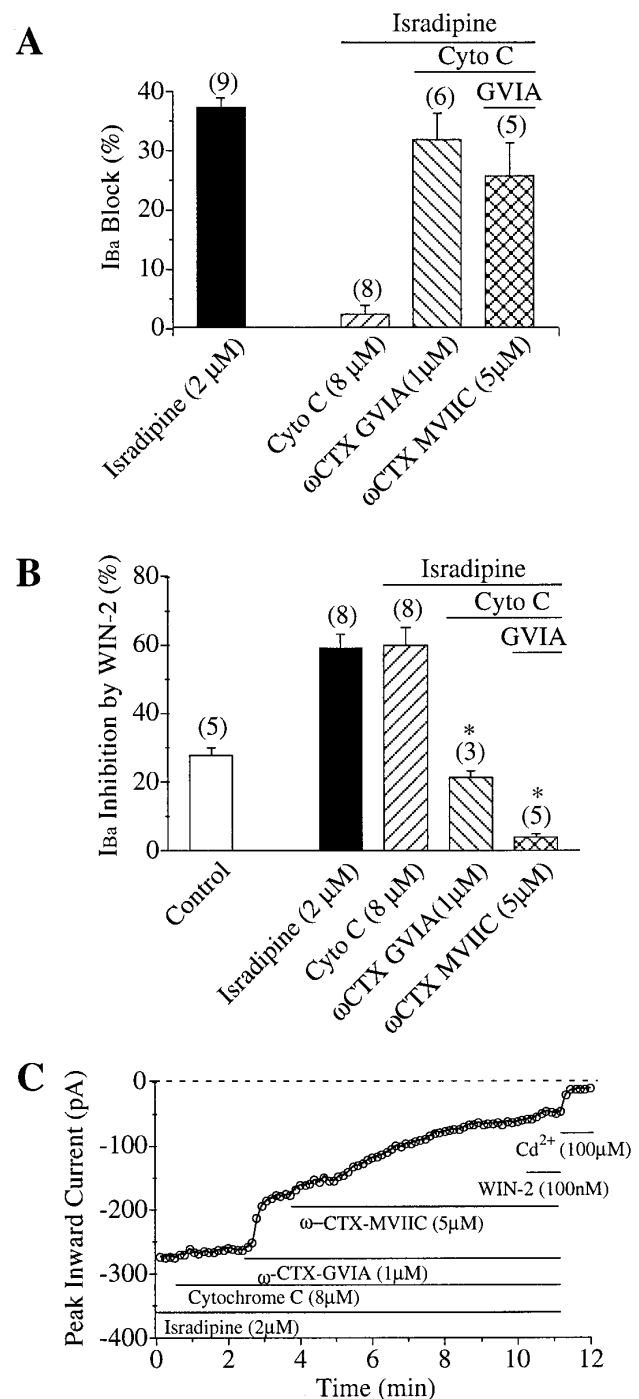FIG. 4. Identification of calcium channel subtypes present in hippocampal cultures and inhibited by WIN 55,212-2. *A*: hippocampal cultures express L-, N-, P/Q-, and R-type calcium channels. Blockade of I_{Ba} by 2 μM isradipine, 8 μM cytochrome c, 1 μM ω-CTX-GVIA (in the presence of isradipine), and 5 μM ω-conotoxin-MVIIC (SNX-230; in the presence of isradipine and 1 μM ω-CTX-GVIA). *B*: WIN 55,212-2 inhibits N- and P/Q-type calcium channels while sparing L- and R-type calcium channels. Inhibition refers to % of current inhibited relative to the current in the presence of 2 μM isradipine. *C*: time course showing the composition of whole cell Ba^{2+} current by selective Ca^{2+} channel blockers in a single cell. Numbers in parentheses: number of cells tested for each condition. Asterisk: $P < 0.05$.

DISCUSSION

The primary finding of this study is that cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat

hippocampal neurons. Thus this study extends earlier work from cell lines and transfected cells to CNS neurons. The inhibition we have found shares many of the characteristics of the inhibition in cell lines: in particular, it is potent, with an EC_{50} of ~20 nM, mediated by a PTX-sensitive G protein, blocked by a CB1 antagonist, mimicked by an endogenous cannabinoid, anandamide, and reversible. As in cell lines, L-type and ω-CTX-GVIA- and ω-conotoxin-MVIIC-insensitive channels (denoted here as R-type) were not modulated by cannabinoids in hippocampal neurons. The lack of voltage dependence is surprising for G_i/G_o-mediated calcium channel inhibition (Bean 1989; Hille 1994).

Because N- and P/Q-type channels are presynaptically located (Westenbroek et al. 1992, 1993), and required for evoked neurotransmitter release in the CNS (Takahashi and Momiyama 1993; Turner et al. 1993; Wheeler et al. 1994), inhibition of these channels by cannabinoids likely reduces neurotransmitter release from CB1-expresssing presynaptic terminals. Indeed this seems to be the case. Cannabinoids are well known to inhibit neurotransmitter release from presynaptic terminals in the ileum and vas deferens (Pertwee 1993; Roth 1978). More recently cannabinoids have also been found to inhibit glutamatergic synaptic transmission in hippocampal cultures (Shen et al. 1996). The inhibition of N- and P/Q-type calcium channels we observed in the current study would be a sufficient mechanism to account for the strong suppression of synaptic transmission found in the previously mentioned studies, although other mechanisms might contribute (Capogna et al. 1996; Dittman and Regeher 1996).

We chose to study cultures grown for a short period for two reasons. First, our immunocytochemistry experiments demonstrated that cells from younger cultures (5 days in vitro) had a high density of somatic cannabinoid receptors (i.e., where we can measure the calcium currents), whereas in more mature cultures (3–4 wk in vitro) the receptors were more distally localized (Fig. 1c). This result in culture parallels the situation in mature brain, where cannabinoid receptors were greatly enriched along axons and dendrites and on presynaptic terminals (Herkenham et al. 1991a–c). The second reason that young cultures were used was that voltage clamp was better in these cultures, before extensive processes had developed. Consistent with a more distant location of cannabinoid receptors, modulation of somatic calcium currents by cannabinoids in mature cultures was smaller and more variable (data not shown). Although we were measuring modulation of somatic calcium currents, it is likely that similar modulation takes place in the presynaptic terminals because both the receptor (Fig. 1c) and channels (Scholz and Miller 1995) are found in the processes of more mature neurons.

The intrinsic efficacy of the three major classes of cannabinoid receptor (CB1) agonists appears variable as it relates to modulation of calcium currents and neurotransmitter release (Caulfield and Brown 1992; Mackie and Hille 1992; Mackie et al. 1993, 1995; Pan et al. 1996; Shen et al. 1996). The aminoalkylindole WIN 55,212-2 appears to be the most efficacious in inhibiting calcium channels in all cells studied (N18 cells, NG108-15 cells, AtT20 cells expressing CB1, SCG neurons expressing CB1, and hippocampal neurons) (Mackie and Hille 1992; Mackie et al. 1993, 1995; Pan et

al. 1996; Shen et al. 1996). The nonclassical cannabinoid CP 55,940 is as efficacious as WIN 55,212-2 in inhibiting calcium channels in some cells (N18 cells, NG108-15 cells, and hippocampal neurons) (Mackie and Hille 1992; Mackie et al. 1993; present study), but is less efficacious than WIN 55,212-2 in SCG neurons expressing CB1 (Pan et al. 1996) and in inhibiting release from hippocampal neurons (Shen et al. 1996). Anandamide is as efficacious as WIN 55,212-2 in some cells [AtT20 cells expressing CB1 and hippocampal neurons (inhibition of both calcium channels and neurotransmitter release)] (Mackie et al. 1995; Shen et al. 1996; present study) but is a partial agonist in N18 cells (Mackie et al. 1993) and weakly active in SCG neurons expressing CB1 (Pan et al. 1996).

The explanation for these diverse results may in part be due to the distinct but overlapping binding sites for WIN 55,212-2, CP 55,940, and anandamide in the CB1 receptor (Song and Bonner 1996); these may favor receptor conformations that alter the profile or extent of G protein activation. Another explanation could be differing receptor densities and stoichiometries of the downstream signaling components in the various model systems used. An additional explanation for the lower efficacy of CP 55,940 in inhibiting release compared with calcium channel modulation is that WIN 55,212-2 and anandamide might inhibit events that occur following calcium influx into presynaptic terminals (Capogna et al. 1996; Dittman and Regeher 1996), thus augmenting the inhibition of neurotransmitter release. Still another possible explanation would be the involvement of as yet undetected cannabinoid receptor isoforms.

In summary, we have found potent inhibition of N- and P/Q-type calcium currents by cannabinoids acting through the CB1 cannabinoid receptor in cultured rat hippocampal neurons. It is likely that inhibition of these calcium channels causes the decrease in neurotransmitter release mediated by these compounds contributing to the well-known psychoactive actions of cannabinoids.

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