

Cannabinoid Receptors Differentially Modulate Potassium A and D Currents in Hippocampal Neurons in Culture¹

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ABSTRACT

Cannabinoid (CB₁) receptor activation produced differential effects on voltage-gated outward potassium currents in whole-cell recordings from cultured (7–15 days) rat hippocampal neurons. Voltage-dependent potassium currents A (I_A) and D (I_D) were isolated from a composite tetraethylammonium-insensitive current (I_{comp}) by blockade with either 4-aminopyridine (500 μ M) or dendrotoxin (2 μ M) and subtraction of the residual I_A from I_{comp} to reveal I_D. The time constants of inactivation (τ) of I_A and I_D as determined in this manner were found to be quite different. The CB₁ agonist WIN 55,212-2 produced a 15- to 20-mV positive shift in voltage-dependent inactivation of I_A and a simultaneous voltage-independent reduction in the amplitude of I_D in the same neurons. The EC₅₀ value for the effect of WIN 55,212-2 on I_D amplitude (13.9 nM) was slightly lower than the

EC₅₀ value for its effect on I_A voltage dependence (20.6 nM). Pretreatment with either the CB₁ antagonist SR141716A or pertussis toxin completely blocked the differential effects of WIN 55,212-2 on I_A and I_D, whereas cellular dialysis with guanosine-5'-O-(3-thio)triphosphate mimicked the action of cannabinoids but blocked the action of simultaneously administered cannabinoid receptor ligands. Finally, the differential effects of cannabinoids on I_A and I_D were both shown to be mediated via the well documented cannabinoid receptor inhibition of adenylyl cyclase and subsequent modulation of cAMP and protein kinase. These actions are considered in terms of cAMP-mediated phosphorylation of separate I_A and I_D channels and the contribution of each to composite voltage-gated potassium currents in these cells.

Activation of the cannabinoid receptor in cultured neurons inhibits adenylyl cyclase (Little and Martin, 1989; Bidaut-Russell et al., 1990) and enhances an outward potassium current in cultured hippocampal neurons (Deadwyler et al., 1993, 1995a; Hampson et al., 1995). The mechanism of cannabinoid receptor modulation of this potassium current has been previously described (Deadwyler et al., 1995b). This current has been identified as similar to A current (I_A) in other reports on the basis of activation and inactivation time constants (5 and 50 ms, respectively), steady-state inactivation and activation voltage ranges ($V_{1/2}$ = -70 to -75 and -20 to -15, respectively), refractory period (~200 ms), insensitivity to tetraethylammonium (TEA; 25 mM), and sensitivity to high concentrations of 4-aminopyridine (4-AP; 5 mM; Storm, 1990). Several G_{i/o} protein-linked receptor systems, such as γ -aminobutyric acid_B (Gage, 1992), serotonin_{1A} (Segal, 1980), and adenosine A₁ (Mei et al., 1995), enhance I_A

by producing a positive shift in steady-state inactivation of the channel, resulting in fewer inactivated channels and hence increased current at membrane potentials between -80 and -50 mV. The cannabinoid receptor agonist WIN 55,212-2 produces a 15- to 25-mV positive shift in I_A through a similar second messenger cascade (Hampson et al., 1995; Mu et al., 1996) and can be blocked by SR141716A (Mu et al., 1995), a competitive antagonist of the CB₁ cannabinoid receptor (Rinaldi-Carmona et al., 1994).

Initial descriptions of the cannabinoid receptor modulation of I_A in hippocampal neurons focused on the cannabinoid produced shift in voltage dependence of steady-state inactivation of I_A. However, an additional effect on this current was observed in which the inactivation time constant (τ) was also markedly reduced from 50 to 25 ms after cannabinoid exposure (Mu et al., 1997). This effect was observed only with higher concentrations (>300 μ M) of 4-AP. Although voltage-dependent outward potassium currents with both 25 and 50 ms τ values have been previously observed (Storm, 1990; Sheng et al., 1993), it is likely that a composite of two differ-

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ABBREVIATIONS: I_A, voltage-sensitive potassium A current; I_D, voltage-sensitive potassium D current; I_{comp}, composite tetraethylammonium-insensitive voltage-sensitive potassium current; GTP γ S, guanosine-5'-O-(3-thio)triphosphate; PTX, pertussis toxin; PKA, protein kinase A; TTX, tetrodotoxin; 8-br-cAMP, 8-bromo-cAMP; TEA, tetraethylammonium; Rp-cAMPS, (Rp)-diastereomer of cAMP; Cat.S., catalytic subunit of PKA; OA, okadaic acid; 4-AP, 4-aminopyridine; DTX, dendrotoxin; $V_{1/2}$, half-inactivation voltage of steady-state inactivation (of I_A and I_D); τ , time constant of inactivation of voltage-sensitive potassium current.

ent potassium currents (only one of which was I_A) with overlapping voltage dependencies really comprised this TEA-insensitive "control" current reported in those studies.

A likely candidate for the second current was potassium I_D or delay current (I_D) on the basis of the inactivation τ , insensitivity to TEA, and high sensitivity to 4-AP (100–500 μ M; Storm, 1990; Wu and Barish, 1992; Locke and Nerbonne, 1997a). I_D or I_D -like currents have been characterized in several different mammalian neurons, including cultured hippocampal neurons (Wu and Barish, 1992; Luthi et al., 1996). It differs from I_A in that I_D is steady-state inactivated and activated at approximately 30 mV more positive membrane potentials than I_A (Storm, 1990; Wu and Barish, 1992). I_D is also slower to activate ($\tau = 20$ ms) and inactivate ($\tau = 100$ ms) with a refractory period of up to 20 s (Storm, 1990). In addition, I_D is blocked by low concentrations of 4-AP (<1 mM) and by dendrotoxin (DTX; 2 μ M), whereas I_A is insensitive to DTX and requires much higher concentrations of 4-AP (5 mM) for blockade.

There have been no prior reports of the sensitivity of I_D to cannabinoid receptor modulation in any type of central nervous system neuron. In the present experiments, we therefore investigated two main issues: 1) whether the second current contributing to the composite outward potassium current was I_D , and 2) if so, whether that current also was modulated by cannabinoid receptor activation.

Materials and Methods

Cell Culture. The preparation of hippocampal neurons in culture was similar to that described in several previous reports (Deadwyler et al., 1993, 1995a; Hampson et al., 1995). Hippocampi from fetal (E-18) rats (Zivic-Miller) were incubated with neutral protease (2 U/ml Dispase 1; Boehringer Mannheim Biochemica, Mannheim, Germany) for 40 to 50 min at 37°C. After stopping the enzymatic reaction with 1.0 mM NaEDTA, cells were dissociated by gentle trituration via two flame-polished Pasteur glass pipettes and plated at a density of 3 to 4 $\times 10^5$ cells/35-mm dish. The plating medium consisted of 59% Dulbecco's modified Eagle's medium (1 $\times$), 19.5% Ham's F-12 Nutrient Mixture (1 $\times$), 10% FBS, 10% horse serum, and 1% L-glutamine (200 mM) (all from GIBCO BRL, Gaithersburg, MD). Cultures were grown at 37°C in a humidified 5% CO₂ incubator. After 48 h, half of the medium was replaced by "feeding" medium, which consisted of 98% neurobasal medium, 2% B-27 supplement, 0.25% L-glutamine (200 mM), 0.1% 2-mercaptoethanol (all purchased from GIBCO BRL), and 25 mM KCl. At 72 h after plating, half the medium was again replaced with feeding medium, and cultures were treated with 0.75 μ M cytosine- β -D-arabinofuranoside (Sigma Chemical Co., St. Louis, MO) to prevent proliferation of glia. The culture medium was then changed every 3 days for the remainder of the experiment. Experiments were performed on cultured cells between days 7 and 15.

Recording Methods. The procedure for whole-cell recording was similar to that reported previously (Deadwyler et al., 1993). Briefly, patch electrodes were prepared from 1.5-mm o.d./1.1-mm i.d. borosilicate glass capillaries to produce 1- to 2- μ m (2–5 M Ω) tip openings. Electrodes were filled by suction and backfilling with a standard intracellular solution of 140 mM KCl, 11 mM EGTA, 1 mM CaCl₂, 2 mM MgCl₂, 2 mM ATP, 200 μ M GTP, and 20 mM HEPES buffer (Sigma Chemical Co.). Hippocampal cells in primary culture (7–15 days) were washed and constantly perfused with extracellular medium consisting of 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 20 mM HEPES, with 1 μ M tetrodotoxin (TTX; Sigma Chemical Co.) added to block voltage-gated sodium channels. Slowly activating/noninactivating potassium currents

were blocked by 35 mM TEA, leaving only the transient I_A and I_D (Storm, 1990; Wu and Barish, 1992). Cultured cells were heated (37°C) and perfused with oxygenated (95% O₂/5% CO₂) bathing medium throughout the experiment. The osmolality of the bath was 320 $\pm$ 10 mOsm, and the pipette solution was 280 $\pm$ 10 mOsm. Osmolality was adjusted to prevent cell shrinkage or swelling resulting from dialysis with the recording pipette solution. Cells were covered to a depth of 2 ml with bathing medium and placed in a Leiden microincubator (Medical Systems, Inc., Greenvale, NY) on a Nikon Diaphot inverted microscope. Positive pressure was applied to the recording pipette as it was lowered into the medium and approached the cell membrane. Constant negative pressure was applied to form the seal. A sharp pulse of negative pressure opened the cell membrane for whole-cell recording (Hamill et al., 1981).

Voltage-clamp recordings and command voltage steps were performed with an AxoPatch 1D amplifier and TL-1-LabMaster controller (Axon Instruments, Burlingame, CA) connected to a PC-compatible computer. Whole-cell records were acquired and stored on magnetic disk using pClamp CLAMPEX software (Axon Instruments). Pipette tip junction potentials were continuously monitored and compensated as necessary at the amplifier before breakage of the seal. Access resistance was typically 2 to 5 M Ω , and series resistance compensation was not usually necessary because of low resistance of the pipette. Leakage correction and capacitance compensation (typically 10–30 pF) used dialed-in compensation adjustment at the amplifier, as well as a P/4 subtraction procedure within the acquisition program. Cells were voltage-clamped and held near resting membrane potential (usually –50 mV). An indication that the cells were adequately space-clamped was that sodium currents in non-TTX-treated cells were not delayed relative to the onset of depolarizing voltage steps, and peak I_A in TTX-treated cells did not vary over a range of 5.0 ms with any of the protocols used. A standard steady-state inactivation protocol using a single depolarizing pulse (+50 mV) preceded by series of hyperpolarizing prepulses (–120 to –50 mV) was used to elicit the I_A . Activation of I_A and I_D used a protocol consisting of a multiple depolarizing pulses (–30 to +40 mV) preceded by a single prepulse step of either –120 or –40 mV that either enhanced or inactivated I_A , respectively. Because the activation and inactivation voltages for I_A and I_D overlapped (>–60 mV activation, <–70 mV inactivation for I_A , >–70 mV activation, <–40 mV inactivation for I_D), it was possible to record I_A in the absence of I_D by using the I_A inactivation protocol and pharmacologically blocking I_D with 4-AP or DTX. I_D could be recorded in the absence of I_A by using a –40-mV, 50-ms prepulse that steady-state inactivated most I_A channels (Storm, 1990; Wu and Barish, 1992). The measurement of current amplitudes and time constants was made with the pClamp software.

Drug Preparation. The cannabinoid drug was applied via a pressure pipette (10- to 50- μ m tip opening) controlled by a solenoid valve (Picospritzer II; General Valve Co., Fairfield, NJ) modified to eject a steady stream of drug-containing media over the surface of the cell. WIN 55,212-2 (Sterling Drug Co., Malvern, PA) was prepared daily from a 10 mM stock solution in ethanol, diluted with extracellular bathing medium, and the ethanol evaporated under a constant stream of nitrogen (Deadwyler et al., 1993). Equivalent bath concentrations corresponding to the pressure pipette concentrations of WIN 55,212-2 are reported in the text. The drug solution was titrated to the same osmolality as the extracellular bathing medium. Due to the lipophilicity of the drug, a 30-s ejection via the application pipette was followed by a washout period of at least 2 min. Previous studies demonstrated that the effects of pressure pipette applications of WIN55,212-2 were rapid (~10-s onset) and were fully reversed after 2-min perfusion of bathing medium after the application; therefore, all current traces were obtained during this 30-s application period (Deadwyler et al., 1993). No effect was observed on whole-cell currents with the application of vehicle-only solution via the same procedure. Controls consisted of vehicle-only applications to separate neurons, as well as pre- and post-drug

measurements of I_A and I_D within the same WIN 55,212-2-treated neurons. DTX (Sigma Chemical Co.) used to selectively block I_D (Storm, 1990; Wu and Barish, 1992) was dissolved directly in bathing medium to a concentration of 2 μ M. Controls consisted of recordings in normal medium before perfusion with DTX-containing medium. In experiments designed to inhibit cannabinoid receptor coupling to G proteins, cultured neurons (6–15 days) were pretreated with pertussis toxin (PTX; islet-activating protein; Sigma Chemical Co.; Deadwyler et al., 1993). PTX (10 μ g/ml) was added to the culture medium 18 h before recording and to the pipette solution for dialysis into the cell during recording. Control cells were from the same batch of culture plates with no PTX added. To irreversibly activate $G_{i/o}$ proteins, 600 μ M guanosine-5'-O-(3-thio)triphosphate (GTP γ S), a non-hydrolyzable GTP analog, was added to the pipette solution. Controls were conducted with neurons in the same culture plate not exposed to GTP γ S. All results were from cells exposed to only one of the above conditions unless otherwise specified. The water-soluble cAMP analog 8-bromo-cAMP (8-br-cAMP; 10 μ M) was applied to the cells via the bathing medium. The protein kinase A inhibitors IP-20 ("Walsh" inhibitory peptide, Sigma Chemical Co.) and Rp-cAMPS [(Rp)-diastereomer of cAMP; Sigma Chemical Co.] were dialyzed into the cell through the recording solution. The cannabinoid receptor (CB₁-specific) antagonist SR141716A (provided by Sanofi Recherche, Montpellier, France) was prepared as a 1 mM stock solution in ethanol and diluted in bathing medium to 300 to 500 nM, and the ethanol was removed by evaporation under nitrogen.

Analysis. Time constants (τ) for inactivation of potassium currents were calculated by curve-fitting to the inactivating portion of the outward current trace evoked by a depolarizing voltage step using the CLAMPEX and CLAMPAN patch-clamp acquisition and analysis programs (Axon Instruments). The separation of currents from the composite outward potassium current was also performed using the CLAMPAN program. Measurement of observed changes in voltage dependence of the A and D currents was accomplished by fitting Boltzmann functions to the isolated I_A or I_D generated by the inactivation and activation protocols (see Deadwyler et al., 1993). Peak current amplitude (I_{max}) in the inactivation protocol was calculated using a -120 -mV hyperpolarizing prepulse, whereas peak activation amplitude was calculated using a depolarizing step to $+50$ mV. I_{max} was the same or very near the same for all treatment conditions in a given cell. For each combination of prepulse and step voltage, I_A or I_D (I/I_{max}) was converted to relative conductance [G/G_{max} ; because $G_A = I_A/(V_m - E_K)$, with E_K determined to be approximately -100 mV in cultured hippocampal neurons] and plotted to compare changes in voltage dependence across different drug conditions and different cells (Saint et al., 1990). Boltzmann functions were fitted to mean data points of G/G_{max} and conditioning prepulse voltages (V_{pp}) via the following formula: $G/G_{max} = 1/[1 + \exp(-(V_{pp} - V_{1/2})/k)]$, where V_{pp} is the conditioning prepulse potential for inactivation; $V_{1/2}$ is the voltage at which $G/G_{max} =$ half-maximum conductance, and k is a slope factor. The coefficients ($V_{1/2}$ and k) from the above Boltzmann equations were calculated via nonlinear regression (PC-SAS NLIN).

The Boltzmann coefficients were then used to plot a Boltzmann curve over all cells with data points shown as mean $\pm$ S.E. G/G_{max} . Changes in I_A and I_D within each protocol were subjected to direct statistical analyses by casting the mean $V_{1/2}$ values into either two-factor (drug versus voltage step) or three-factor (drug versus voltage step versus concentration) ANOVA designs. Only mean differences in these conductance measures calculated across all cells tested and reaching a $p < .01$ level of significance (adjusted simple effects contrasts) were considered.

Results

Characterization of Composite Outward Current in Cultured Hippocampal Neurons. Figure 1 (top) shows the nondifferentiated whole-cell current elicited by the voltage

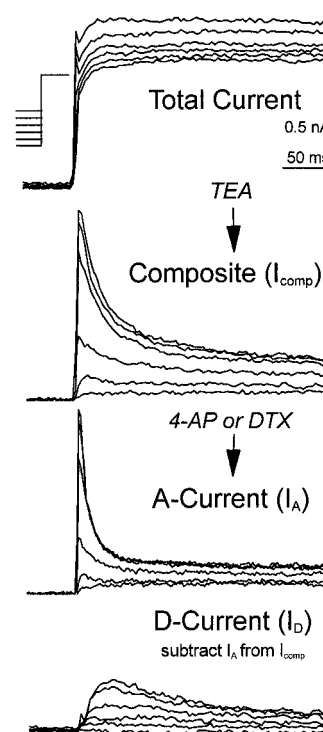


Fig. 1. Separation and identification of voltage-dependent I_A and I_D . Total current, whole-cell voltage-gated potassium current elicited by the inactivation protocol shown at the top. The holding potential was -50 mV; prepulses were stepped from -90 to -20 mV in 15-mV increments and then followed by a 375-ms, $+50$ -mV pulse (protocol inset). TTX (0.5 μ M) was present in the bath solution for block of voltage-gated sodium current and presynaptic activity. Composite (I_{comp}), after blocking the noninactivating I_K with TEA (25 mM), TEA-insensitive outward potassium current shows time-dependent inactivation. I_A , residual inactivating current after the addition of 4-AP (500 μ M) or DTX (2 μ M) to block I_D . The remaining current fits the criteria for I_A . I_D was calculated by subtraction of the 4-AP- and DTX-insensitive I_A from I_{comp} . Amplitude and inactivation time constant (τ) of subtracted or "recovered" I_D are within the same range as determined by direct activation (see Fig. 3). Note differences in inactivation τ and peak current amplitude for I_A and I_D .

step protocol for outward potassium currents in hippocampal cells (Saint et al., 1990; Storm, 1990; Wu and Barish, 1992; Deadwyler et al., 1993). The "composite" current (I_{comp}) was recorded after the addition of TEA (25 mM) to the external medium, which abolished the noninactivating delayed rectifier, I_K , and other noninactivating outward potassium currents elicited by this protocol (Storm, 1990). The residual I_{comp} displays fast activation time (<5 ms) and an inactivation time constant (τ) of 50 ms. The current has a steady-state voltage-dependent activation between -50 and 0 mV and steady-state inactivation between -90 and -50 mV, with a refractory period of approximately 200 ms. I_{comp} is blocked by high concentrations (>1 mM) of 4-AP (cf. Saint et al., 1990; Wu and Barish, 1992; Deadwyler et al., 1993) and has been extensively characterized in prior studies of cannabinoid receptor modulation (Deadwyler et al., 1993, 1995a,b; Hampson et al., 1995).

The application of DTX (2 μ M) or moderate concentrations of 4-AP (500 μ M) reduced the mean τ of I_{comp} from 50 to 25 ms (Fig. 1, I_A). Mean $\pm$ S.E. I_{comp} amplitudes and τ values are given in Table 1 for the indicated number of cells tested. A significant ($F_{1,95} = 12.2$, $p < .001$) reduction in I_{comp} τ was obtained in all cells exposed to 4-AP or DTX. The remaining

TABLE 1

Effects of drug treatments on I_{comp} and I_{D} currents

	I_{comp} (Inactivation)			I_{D} (Activation)	I_{D} (Inactivation)		N
	Peak I_{A}	τ	$V_{1/2}$	Peak I_{D}	τ	$V_{1/2}$	
	nA	ms	mV	nA	ms	mV	
Control-comp.	1.24 ± 0.10	51.2 ± 2.9	-71.3 ± 3.1	0.64 ± 0.09	98.2 ± 7.4	-39.9 ± 3.5	43
4-AP	1.15 ± 0.08	24.4 ± 3.1^a	-71.5 ± 3.7	0.24 ± 0.06^a	b	b	21
DTX	1.13 ± 0.07	25.4 ± 4.9^a	-72.1 ± 4.8	0.22 ± 0.05^a	NC	NC	8
WIN	1.29 ± 0.08	21.2 ± 3.4^a	-54.3 ± 4.4^a	0.25 ± 0.03^a	NC	NC	12
WIN + DTX	1.27 ± 0.06	18.4 ± 5.2^a	-53.2 ± 2.4^a	0.21 ± 0.03^a	NC	NC	7
PTX	1.22 ± 0.09	50.4 ± 1.6	-70.9 ± 3.8	0.60 ± 0.05	96.1 ± 6.5	-40.9 ± 4.8	9
PTX + WIN	1.23 ± 0.07	51.1 ± 4.1	-71.2 ± 2.3	0.59 ± 0.08	99.2 ± 8.3	-38.2 ± 3.9	9
PTX + DTX	1.19 ± 0.08	24.1 ± 3.7^a	-70.9 ± 2.9	0.24 ± 0.04^a	NC	NC	4
GTP γ S	1.29 ± 0.09	28.3 ± 4.0^a	-60.1 ± 4.0^a	0.30 ± 0.08^a	NC	NC	8
GTP + WIN	1.32 ± 0.04^a	22.5 ± 1.5^a	-58.5 ± 2.9^a	0.31 ± 0.07^a	NC	NC	8
8-br-cAMP	1.04 ± 0.09^a	65.3 ± 2.4^a	-85.3 ± 4.2^a	0.81 ± 0.08^a	NC	-33.7 ± 4.6	7
cAMP + WIN	1.05 ± 0.08^a	64.2 ± 2.1^a	-85.1 ± 3.6^a	0.80 ± 0.06^a	NC	-34.6 ± 3.9	7
IP-20	1.27 ± 0.09	28.9 ± 3.2^a	-58.2 ± 4.7^a	0.28 ± 0.09^a	NC	NC	6
IP-20 + WIN	1.30 ± 0.11	26.3 ± 2.7^a	-55.9 ± 3.4^a	0.24 ± 0.07^a	NC	NC	6
Rp-cAMPS	1.29 ± 0.06	34.7 ± 3.9	-63.2 ± 2.1^a	0.41 ± 0.05^a	NC	NC	6
Rp + WIN	1.31 ± 0.07^a	22.8 ± 4.3^a	-55.8 ± 3.3^a	0.27 ± 0.07^a	NC	NC	6
Cat.S.	1.08 ± 0.06^a	58.9 ± 2.7^a	-81.3 ± 3.2^a	0.77 ± 0.10^a	101.3 ± 8.8	-33.2 ± 4.9	4
Cat.S. + WIN	1.06 ± 0.07^a	56.2 ± 3.3	-80.7 ± 2.8^a	0.80 ± 0.09^a	98.8 ± 7.5	-34.3 ± 3.8	4
OA	1.05 ± 0.07^a	61.3 ± 3.5^a	-83.0 ± 2.9^a	0.79 ± 0.07^a	99.2 ± 8.1	-32.8 ± 4.3	4
OA + WIN	1.07 ± 0.06^a	60.2 ± 2.9^a	-82.1 ± 3.0^a	0.77 ± 0.06^a	94.5 ± 7.2	-33.3 ± 5.3	4
SR	1.22 ± 0.09	50.6 ± 3.3	-70.9 ± 3.1	0.59 ± 0.09	99.3 ± 8.2	-39.1 ± 3.9	8
SR + WIN	1.23 ± 0.07	51.5 ± 2.9	-72.2 ± 4.5	0.58 ± 0.06	96.2 ± 7.7	-40.6 ± 4.1	8

^a Measures were significantly different from control, $F_{1,95} > 7.00$, $p < .01$.^b NC, τ and $V_{1/2}$ could not be calculated due to reduction in I_{D} amplitude and change in slope of Boltzmann curve; 4-AP values were used as baseline for pharmacological subtraction.

current profile of I_{comp} after 4-AP or DTX exposure meets the characteristics of I_{A} as reported by several investigators (Segal and Barker, 1984; Saint et al., 1990; Deadwyler et al., 1993; Hoffman and Johnston, 1998). The current that was eliminated by 4-AP and DTX was subsequently "recovered" by subtracting the latter I_{A} from the original I_{comp} curve (Fig. 1, bottom). The recovered current trace has a much slower activation time (~ 20 ms) and inactivation τ (100 ms) and meets the characteristics stipulated above for I_{D} (Storm, 1987, 1990; Wu and Barish, 1992; Locke and Nerbonne, 1997b). Thus, I_{comp} was successfully partitioned into two components: I_{A} and I_{D} .

Effects of Cannabinoids on I_{comp} . Figure 2 shows the effect of exposure to the potent cannabinoid WIN 55,212 (40 nM) on I_{comp} elicited by the above steady-state inactivation protocol (see *Materials and Methods*). These effects were compared with those of DTX (2 μ M) on I_{comp} using the same inactivation protocol. In both cases, mean τ was significantly reduced from control I_{comp} levels (Table 1; $F_{1,95} = 17.75$, $p < .001$). However, an effect not mimicked by DTX was for WIN 55,212-2 to increase the amplitude of I_{comp} (now I_{A}) at all voltage steps (see intermediate traces shown in Fig. 2, bottom left), resulting in a positive shift in the voltage dependence of steady-state inactivation. This is shown graphically by the Boltzmann curves at the right in Fig. 2. As reported elsewhere (Deadwyler et al., 1993, 1995a; Hampson et al., 1995), both steady-state inactivation (circles) and activation (squares) of I_{comp} were positively shifted by exposure to WIN 55,212-2 (mean I_{comp} inactivation $V_{1/2} = -71.3 \pm 3.1$ mV, mean I_{comp} + WIN 55,212-2 inactivation $V_{1/2} = -54.3 \pm 4.4$ mV, $F_{1,95} = 12.57$, $p < .001$; mean I_{comp} activation $V_{1/2} = -20.1 \pm 2.6$ mV, mean I_{comp} + WIN 55,212-2 activation $V_{1/2} = -3.5 \pm 2.4$ mV, $F_{1,95} = 14.2$, $p < .001$). The positive shift in the Boltzmann curves for I_{comp} produced by WIN 55,212-2 was blocked by simultaneous exposure to

SR141716A, the CB₁ receptor antagonist (Table 1). As stated above, this shift in the voltage dependence of I_{comp} steady-state inactivation did not occur after exposure to DTX (cf. Fig. 1; mean I_{comp} + DTX inactivation $V_{1/2} = -72.1 \pm 4.8$; DTX versus WIN 55,212-2, $F_{1,95} = 0.56$, $p = .45$) or to WIN 55,212-2 + DTX (Fig. 2; mean I_{comp} + WIN 55,212-2 + DTX inactivation $V_{1/2} = -53.2 \pm 2.4$, $F_{1,95} = .38$, $p = .54$).

The Boltzmann curves in Fig. 2 suggest that the changes in voltage dependence of I_{comp} produced by WIN 55,212-2 can be explained by a change in voltage dependence of I_{A} because they are occur in DTX-containing media. However, the reduction in I_{comp} τ cannot result from effects on I_{A} because I_{A} inactivation τ is not affected by cannabinoid exposure (Deadwyler et al., 1993). The τ reduction in I_{comp} can be explained appropriately by selective removal of I_{D} from I_{comp} , leaving only the τ (25 ms) for I_{A} . Thus, cannabinoids produced two independent changes in I_{comp} , a positive shift in $V_{1/2}$ of I_{A} , which increased peak amplitude of I_{comp} and reduced τ suggesting decreased contribution of I_{D} .

Effects of Cannabinoids on I_{D} . Figure 3 shows the mean Boltzmann curves for steady-state inactivation and activation of I_{D} recorded in cultured hippocampal neurons ($n = 12$). The insets show individual currents developed by the respective steady-state inactivation and activation protocols (see *Materials and Methods*). Because there is no pharmacological means of blocking I_{A} independent of I_{D} and the inactivation protocol includes voltages within a range that will also affect inactivation of I_{A} , the steady-state inactivation curve for I_{D} (Fig. 3, circles) was constructed by subtracting I_{A} from I_{comp} . To reconstruct I_{D} , I_{comp} was recorded at each voltage step in the presence and absence of 4-AP (500 μ M) to isolate I_{A} . The latter current (I_{A}) was then subtracted from the untreated I_{comp} to provide the curve for I_{D} . The $V_{1/2}$ for I_{D} steady-state inactivation was determined to be -39.9 ± 3.5 as shown in Table 1, 30 mV more positive than I_{A} (Deadwyler et al.,

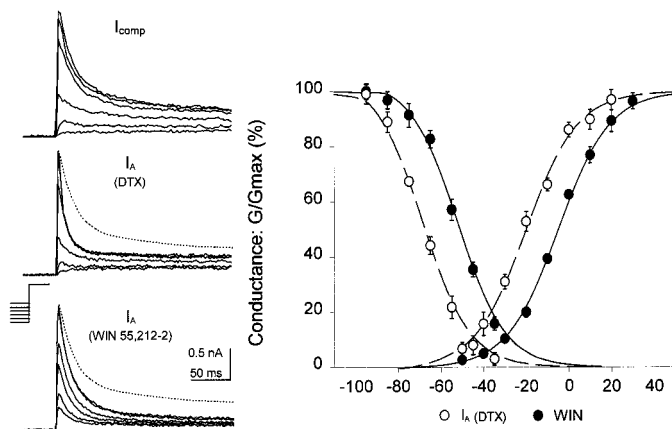


Fig. 2. Comparison of the effects of DTX and cannabinoid receptor agonist WIN 55,212-2 on I_{comp} with cannabinoid enhancement of I_A . Left, I_{comp} elicited with steady-state inactivation protocol under control conditions (top) and after exposure to DTX (2 μ M, middle) or WIN 55,212-2 (40 nM, bottom). Both DTX and WIN 55,212-2 produced a similar reduction in inactivation τ values (compare with dashed trace for I_{comp}), leaving a residual current characteristic in both cases of I_A . WIN 55,212-2 also produced an increase in peak amplitudes at intermediate voltage steps, indicating a shift in the voltage dependence of steady-state inactivation of I_A . Scale bars: 50 ms, 0.5 nA. Right, Boltzmann curves for steady-state inactivation and activation of I_A . I_A inactivation (● and ○). Peak I_A amplitude (I_{max}) was measured for -120 mV prepulse with depolarizing step to +50 mV. Hyperpolarizing prepulses were then varied in 15-mV steps (positive), and the ratio of I_A to I_{max} was computed. The resulting relative current (I_A/I_{max}) was converted to relative conductance (G/G_{max} ; see text) and fitted by nonlinear regression to the Boltzmann function: $G/G_{max} = 1/[1 + \exp(-(V_{pp} - V_{1/2})/k)]$. Symbols indicate mean $\pm$ S.E.M. conductance (G/G_{max}) across different cells (DTX: $n = 8$; WIN 55,212-2, $n = 12$). ○, Boltzmann curve for I_A (DTX treated). ●, Boltzmann curve and conductance for WIN 55,212-2-treated I_A and reflect a 15-mV positive shift in the steady-state inactivation of I_A from the DTX-treated condition. I_A activation (■ and □). Peak I_A amplitude (I_{max}) was measured for -120-mV prepulse and depolarizing step to +50 mV. Depolarizing prepulse was then varied in 10-mV steps (negative); I_A/I_{max} was computed, converted to conductance, and fitted to the Boltzmann equation: $G/G_{max} = 1/[1 + \exp(-(V_{pp} - V_{1/2})/k)]$.

1993). The curve for steady-state activation of I_D was constructed with an activation protocol and membrane holding potential at -40 mV (Fig. 3, squares). The $V_{1/2}$ for steady-state activation of I_D was $+2.6 \pm 2.7$ mV (Table 1). Either method (subtraction or direct activation) produced comparable current profiles (insets, Fig. 3) respect to both τ and amplitude of I_D . The voltage dependence for both steady-state inactivation and activation were consistent with several previous reports of I_D characteristics (Storm, 1987, 1990; Wu and Barish, 1992).

The activation protocol was therefore used to determine the effects of WIN 55,212-2 (10 and 40 nM) and DTX (2 μ M) on I_D . The traces in Fig. 4 show the effects of WIN 55,212-2 (10 and 40 nM) on I_D recorded in TEA. The effects of DTX (2 μ M) are shown below for comparison. Exposure to WIN 55,212-2 (40 nM) reduced the peak I_D amplitude by 60% (Table 1, $n = 12$ cells, mean I_D amplitude = 0.64 ± 0.09 , mean I_D + WIN 55,212-2 amplitude = 0.25 ± 0.03 , $F_{1,95} = 16.53$, $p < .001$) relative to the TEA control amplitude. The addition of DTX either alone or in combination with WIN 55,212-2 also produced a 70% reduction in I_D amplitude relative to control (mean I_D + DTX amplitude = 0.22 ± 0.05 nA, mean I_D + WIN 55,212-2 + DTX amplitude = 0.21 ± 0.03 ; all $F_{1,95} > 7.01$, $p < .01$). The reduction of I_D amplitude by WIN 55,212-2 was blocked by the cannabinoid receptor antagonist SR141716A (Table 1).

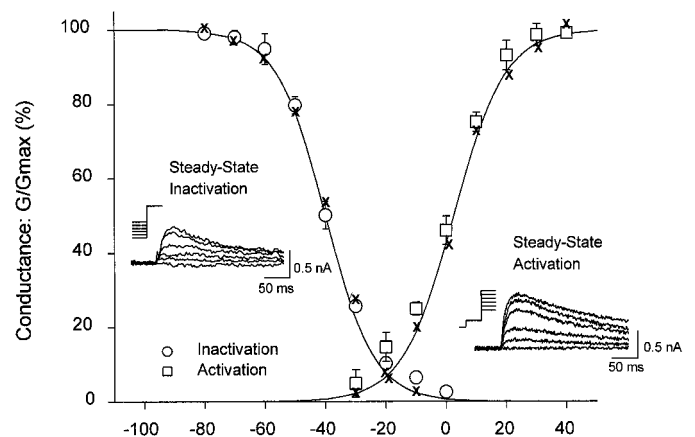


Fig. 3. Steady-state inactivation and activation of I_D . Peak I_D amplitude (I_{max}) was derived and measured using the same inactivation and activation protocols as in Figs. 1 and 2. I_A was pharmacologically separated from I_{comp} in the steady-state inactivation protocol and then subtracted from I_{comp} to produce I_D . I_A (-40-mV prepulse) was eliminated in the activation protocol (-50-mV prepulse, depolarizing step to +50 mV). For each combination of prepulse (inactivation) or step voltage (activation), Boltzmann curves for steady-state inactivation and activation were fitted as in Fig. 2. Points are mean $\pm$ S.E. conductance elicited for each voltage ($n = 21$ cells). Inset traces, isolated I_D currents elicited by the inactivation and activation protocols. ×, on the Boltzmann curves, single-cell conductance measurements obtained from the traces shown. Scale bars: 50 ms, 0.5 nA.

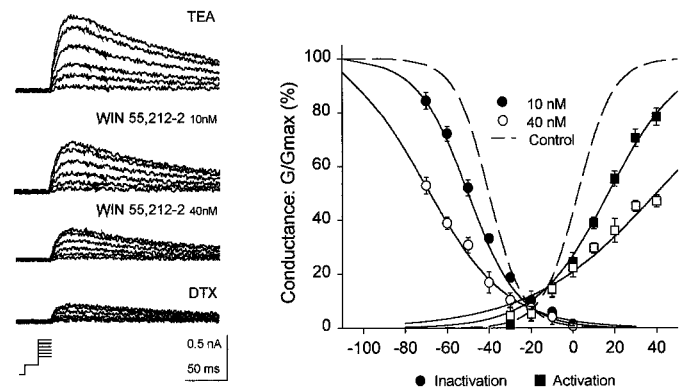


Fig. 4. Cannabinoid effects on I_D . Left, WIN 55,212-2 effects on I_D activation (steady-state activation protocol). TEA, activation of TEA-insensitive I_D in normal media with 25 mM TEA added. Steady-state inactivation of I_A is effected by a step to -40 mV before depolarizing steps to 0 to +50 mV (inset, I_D activation protocol). WIN 55,212-2, exposure to 10 and 40 nM WIN 55,212-2 (in TEA) produces a dose-dependent decrease in I_D amplitude with no change in τ . DTX, exposure to DTX (2 μ M) produced a slightly greater reduction in I_D . Scale bar: 50 ms, 0.5 nA. Right, Boltzmann curves for steady-state inactivation and activation of I_D . Exposure to WIN 55,212-2 (solid lines) produced an apparent shift in voltage dependence of inactivation and activation compared with control (dashed line); however, the shifts were accompanied by symmetric decreases in slope for activation and inactivation (see text).

The reduction of I_D amplitude by WIN 55,212-2 was not accompanied by a shift in voltage dependence of steady-state inactivation of I_D . This is suggested by the consistency of the relative amplitudes at intermediate voltage steps in the traces in Fig. 4 (left), which show a decrease in the I_D amplitude for each trace. This voltage-independent decrease in amplitude is reflected by the altered slope of the Boltzmann curves in Fig. 4 after exposure to WIN 55,212-2 (10 nM, filled symbols; 40 nM, open symbols). The change in slope was responsible for the apparent shift in $V_{1/2}$ for the same Boltzmann curves and therefore did not reflect a change in volt-

age dependence of I_D . Figure 4 shows that the effect of cannabinoids on I_D peak amplitude was also dose dependent but not as effective as DTX (see below).

Relative Potency and Efficacy of Cannabinoids on I_A and I_D . Figure 5 shows the concentration-effect (2.5–40 nM) curves for the WIN 55,212-2 modulation of I_D amplitude (filled circles) and shift in $V_{1/2}$ for steady-state inactivation of I_A (open squares). A comparison of the EC_{50} values for each effect revealed that the cannabinoid reduction in mean I_D amplitude occurred at lower concentrations (13.9 ± 1.6 nM) than the mean $V_{1/2}$ shift in I_A voltage dependence (20.6 ± 1.9 nM; $T_{14} = 2.69$, $p < .01$). Thus, I_D was 34% more sensitive to the influence of cannabinoids than I_A , a further indication that cannabinoid alteration of I_{comp} was produced by the above demonstrated independent effects on I_A and I_D .

Cannabinoid Effects on I_A and I_D Are Mediated by $G_{i/o}$ Proteins, cAMP, and Protein Kinase A (PKA). It has been firmly established that cannabinoid receptor inhibition of adenylyl cyclase is mediated through PTX-sensitive G proteins (Howlett et al., 1986; Bidaut-Russell et al., 1990; Pacheco et al., 1991). Consistent with this effect of cannabinoid receptor activation, previous reports demonstrated that the effects of cannabinoids on I_A were G protein (Deadwyler et al., 1993) and cAMP dependent (Deadwyler et al., 1995a). In those studies, increased levels of cAMP (8-br-cAMP), PKA, protein kinase inhibitors (IP-20 and Rp-cAMPS), and WIN 55,212-2 were all used (Deadwyler et al., 1993, 1995a; Hampson et al., 1995). The results of similar treatments with respect to effects on I_A were replicated in the present study and are summarized in Table 1 and Fig. 6, A and B. In general, manipulations that increased cAMP levels produced a negative shift in the voltage dependence of I_A but not the slope of the steady-state activation and inactivation curves (Deadwyler et al., 1995a; Hampson et al., 1995). Treatments that decreased cAMP levels or blocked PKA produced a positive shift in I_A activation and inactivation with no change in

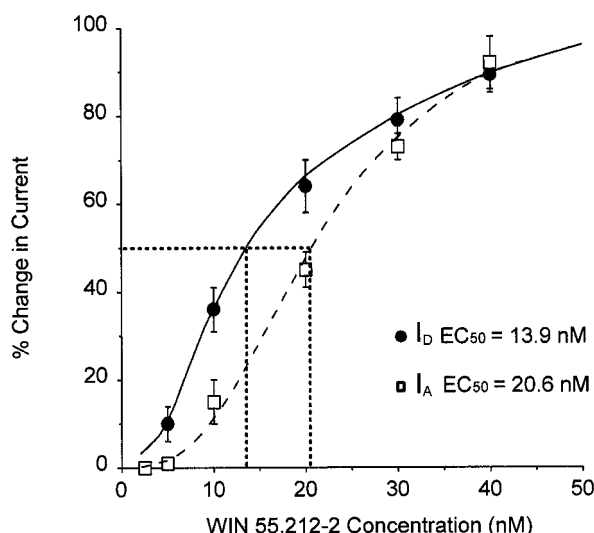


Fig. 5. Concentration-dependent effects of WIN 55,212-2 on I_A and I_D (range, 2.5–100 nM). Dose-effect curves for WIN 55,212-2 effect on reduction of I_D amplitude (●). Half-maximal effect (EC_{50}) was 13.9 nM, and the maximal effect was obtained at 70 nM (not shown). The dashed line shows the effects of the same concentrations of WIN 55,212-2 on the shift in voltage dependence ($V_{1/2}$) for steady-state inactivation of I_A (□). The EC_{50} value for cannabinoid effects on I_A was 20.6 nM, and the maximal effect was obtained at 80 nM (at least 4 cells/point; range, 4–21).

slope of the Boltzmann curves (Fig. 6, A and B). Because the effects on PKA presumably altered phosphorylation of membrane channel proteins, the catalytic subunit of PKA (Cat.S.; Table 1) and the phosphatase inhibitor okadaic acid (OA; Table 1) were also tested. Both Cat.S. and OA induced a negative shift in I_A voltage dependence that was similar to increased cAMP levels (8-br-cAMP). Table 1 shows that the effects of WIN 55,212-2 on I_A and I_{comp} were similar in direction and magnitude to decreased cAMP levels or PKA inhibition (especially IP-20) and opposite in effect to increased cAMP levels and enhanced PKA-dependent phosphorylation by other agents. These results indicate that the differential shifts in I_A voltage dependence were regulated by the phosphorylation status of the I_A channel protein. Furthermore, because exposure to 8-br-cAMP, Cat.S., or OA blocked cannabinoid effects on I_A (Table 1) and IP-20 or Rp-cAMPS plus WIN 55,212-2 were not additive, it is therefore likely that the same cAMP/PKA cascade was involved for all of the above influences on I_A .

Table 1 also indicates similar tests of G protein and cAMP involvement conducted on activation and inactivation of I_D . The reduction by WIN 55,212-2 of I_D amplitude was blocked by PTX (mean PTX amplitude = 0.60 ± 0.05 nA; mean PTX + WIN 55,212-2 amplitude = 0.59 ± 0.08 nA; $F_{1,95} = 0.19$, $p = .65$), but importantly, PTX did not alter the ability of DTX to reduce I_D amplitude (mean PTX + DTX amplitude = 0.24 ± 0.04 nA, $F_{1,95} = 6.47$; $p < .01$, Table 1). Cells ($n = 8$) dialyzed with the G protein activator GTP γ S (600 μ M) also exhibited reduced I_D amplitude (mean GTP γ S amplitude = 0.30 ± 0.08 ; $F_{1,95} = 6.11$, $p < .01$) while blocking the effect of WIN 55,212-2 (mean GTP γ S + WIN 55,212-2 amplitude = 0.31 ± 0.07 nA, $F_{1,95} = 0.18$, $p = .67$, Table 1).

Because cannabinoid receptor effects on I_D were shown to be dependent on linkage to $G_{i/o}$ proteins, further tests of dependence on the cAMP/PKA cascade were performed. Figure 6D (inset) shows that exposure to 8-br-cAMP (10 μ M) significantly increased I_D amplitude by 25% (mean 8-br-cAMP amplitude = 0.81 ± 0.08 nA, mean control amplitude = 0.64 ± 0.09 nA, $F_{1,95} = 6.29$, $p < .01$, Table 1), whereas IP-20, markedly reduced I_D amplitude by 63% (mean IP-20 amplitude = 0.24 ± 0.09 nA, $F_{1,95} = 6.51$, $p < .01$). Boltzmann curves for steady-state inactivation and activation of I_D are shown in Fig. 6, C and D, for the same treatment conditions shown in Fig. 6, A and B. Several differences are immediately apparent. First, the effects of 8-br-cAMP on I_D were not equivalent for steady-state inactivation versus activation of I_D . The shift in voltage dependence in I_D produced by 8-br-cAMP was unchanged for steady-state inactivation of I_D relative to control conditions (Fig. 6C) but shifted significantly negative for steady-state activation of I_D (Fig. 6D, squares). Second, PKA reduction (Rp-cAMPS) produced a small but significant negative shift in steady-state inactivation of I_D (Fig. 6C). In all other instances, a change in slope of the Boltzmann curve for either steady-state inactivation and/or activation of I_D was obtained (Fig. 6, C and D).

The change in slope in the I_D Boltzmann curves in Fig. 6, C and D, indicates a change in the kinetics of the I_D channel. One possible source of that change is a shift in sensitivity of voltage dependence “outside” the range of the protocol used, which would have reduced maximum current (I_{max} ; see *Materials and Methods*) capable of being evoked by the protocol. If so, different I_{max} values would be obtained depending on

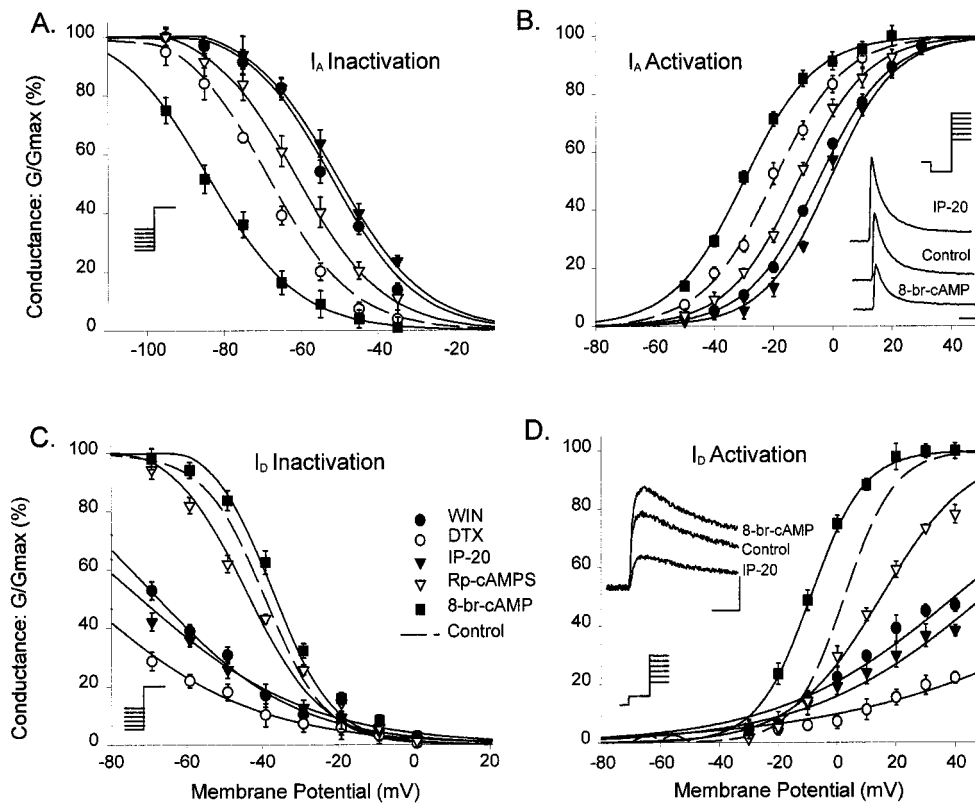


Fig. 6. cAMP/PKA modulation of I_A and I_D steady-state inactivation and activation. **A**, Boltzmann curves (see Fig. 2) for steady-state inactivation of I_A (see Protocol inset) calculated for currents recorded in 8-br-cAMP (10 μ M), WIN 55,212-2 (WIN 55,212-2; 40 nM), or DTX (2 μ M) in the bathing solution or after dialyzing the cell, via the recording pipette solution, with the PKA inhibitory peptide IP-20 (6 μ M) or partial PKA inhibitor Rp-cAMPS (2 μ M). Symbols reflect mean $\pm$ S.E. conductance across all cells for each prepulse voltage (see Table 1 for $V_{1/2}$ and n values). **B**, activation of I_A . Treatment conditions were the same as above for Inactivation. Symbols reflect mean $\pm$ S.E. conductance across cells for each depolarizing step voltage shown in Protocol inset. Inset, current traces show change in peak I_A (activation) after exposure to IP-20 and 8-br-cAMP and under control conditions. **C**, I_D inactivation. Boltzmann curves for steady-state inactivation of I_D (by subtraction of I_A from I_{comp}) were calculated by subtraction of I_A from I_{comp} (Fig. 2, see also Table 1 for $V_{1/2}$ and n values). **D**, Boltzmann curves for activation of I_D . Treatment conditions and symbols are the same as in **B**. Inset, current traces elicited by the I_D steady-state activation protocol for 8-br-cAMP, IP-20, and control. Scale bar: 50 ms, 0.5 nA

the range of voltages used in the protocols. Alternatively, the change in slope of the Boltzmann curves may have resulted from total inactivation (lack of conductance) of a subpopulation of I_D channels (Hille, 1992), also resulting in a decrease in I_{max} . In the latter case, I_{max} would be relatively unaffected by changes in voltage range. Figure 7 shows the maximum (i.e., peak) I_D amplitude recorded using the same activation protocol at a higher voltage (+80 mV depolarization) but

preceded by one of three different levels of hyperpolarizing prepulses (−120, −80, or −40 mV) to maximally activate I_D . The three conditions provided a greater range of depolarization to assess whether maximum I_D amplitudes were differentially altered by the indicated cAMP/PKA treatments. The bar graph in Fig. 7 shows no significant difference in current amplitude recorded as a function of the three protocols; this indicates that the reduction in I_D , and hence the change in slope of the Boltzmann curves in Fig. 6, C and D, produced by cAMP/PKA inhibitors, did not result from a shift in voltage dependence but rather a decrease in the maximum current that could be evoked regardless of voltage. Thus, in contrast to effects on I_A , manipulations of the cAMP/PKA cascade and consequent phosphorylation status of channel proteins altered the conductance or availability of I_D channels and not the voltage dependence of those channels.

Contributions of I_A and I_D to I_{comp} . Figure 8 shows the net changes in I_{comp} with different manipulations of cAMP. The solid traces show I_{comp} recorded from a single neuron under control conditions (i.e., “resting” levels of cAMP) and after exposure to 8-br-cAMP (10 μ M) or the cannabinoid agonist WIN 55,212-2 (40 nM). The horizontal lines at the top in Fig. 8 show that increased cAMP levels (8-br-cAMP) reduced the peak amplitude of I_{comp} by 16% relative to control (long vertical arrow); in contrast, cannabinoid exposure increased peak I_{comp} amplitude by only 4% (short vertical arrow). However, at the same time, increased cAMP levels (8-br-cAMP) caused a 27% increase in I_{comp} τ relative to control, whereas cannabinoids decreased the I_{comp} τ by 58%, almost twice the change produced by cAMP. The dashed line in Fig. 8 (8-br-cAMP and Cannabinoid) indicates the degree of change in time course of I_{comp} under both conditions as indicated by the direction of the arrow. Thus, the maximum

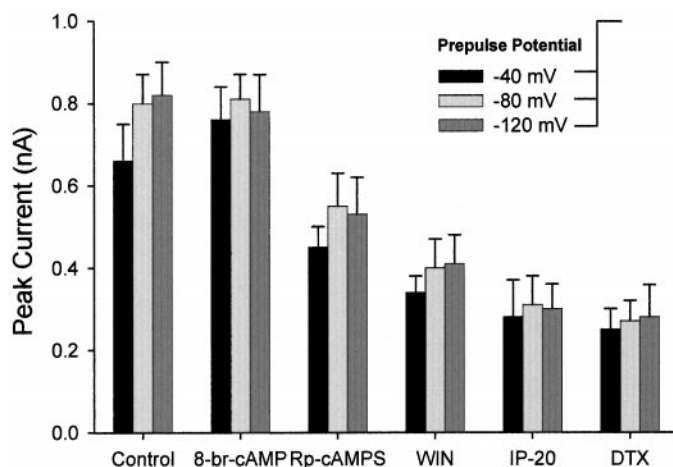


Fig. 7. Maximum I_D activation. I_D was recorded using three combinations of prepulse (holding) voltage (−120, −80, and −40 mV) with a +80-mV depolarizing voltage in the protocol indicated. Voltages were selected to exceed the range used to elicit maximum currents in Fig. 6, C and D, to test whether the slope changes resulted from a shift in voltage dependence or decreased maximal conductance. Peak current was not significantly different for each protocol (Control) but was greatly reduced after exposure to PKA inhibitors, WIN 55,212-2 and DTX. Rp-cAMPS, thio-cAMP stereoisomer, WIN 55,212-2, cannabinoid agonist WIN 55,212-2; and IP-20, PKA inhibitory peptide (20 amino acid).

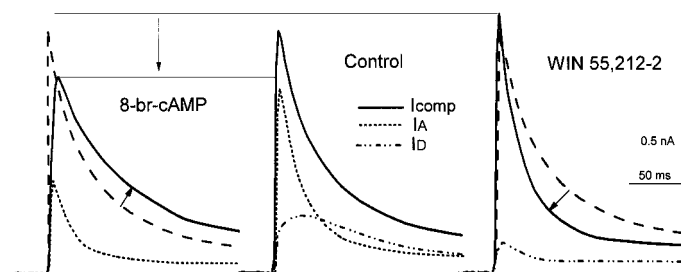


Fig. 8. Effects of differential modulation of I_A and I_D by cAMP on I_{comp} . The three sets of single-cell voltage-clamp current traces show I_{comp} under control (center), 8-br-cAMP (10 μ M, left), and WIN 55,212-2 (40 nM, right) recording conditions. Solid current traces indicate I_{comp} recorded under each of the three conditions. Dashed current traces at left (8-br-cAMP) and right (WIN 55,212-2) are superimposed control I_{comp} (center) traces. Bold arrows show differential change in τ from control I_{comp} . Horizontal lines and arrow at top depict change in I_{comp} amplitude (right to left) produced by increasing levels of cAMP. Dotted traces (middle and left) depict decrease in I_A as cAMP levels increase (8-br-cAMP). Dash-dotted traces (middle and right) show decrease from maximum I_D (8-br-cAMP), through control (middle), to minimum I_D after exposure to WIN 55,212-2 (right).

range in I_{comp} τ from increased cAMP levels (Fig. 8, 8-br-cAMP) to decreased cAMP levels produced by cannabinoid receptor activation (Fig. 8, Cannabinoid) was 68%. The dotted traces in Fig. 8 depict I_A (8-br-cAMP and Control) and show that I_{comp} amplitude was reduced in conjunction with I_A amplitude after exposure to increased levels of cAMP. Conversely, the dash-dotted traces (Control and Cannabinoid) show the reciprocal change in amplitude and τ of I_D as a function of decreased cAMP levels produced by cannabinoid exposure. In each case, it is clear that an increase in cAMP produced an increase in the duration of I_{comp} but at the expense of a marked reduction in amplitude. Decreasing cAMP levels via cannabinoid receptor activation resulted in a marked decrease in the duration of I_{comp} , with a relatively insignificant increase in amplitude. This dual modulation of I_{comp} shown in Fig. 8 is cAMP dependent; however, the differential modulation of I_A and I_D must occur “downstream” at the level of PKA-dependent phosphorylation, as indicated by the differential effects of IP-20 and Cat.S. on both currents (see Table 1).

Discussion

The above results clearly demonstrate that a major source of voltage-dependent potassium current (I_{comp}) in hippocampal cells is selectively modulated by cannabinoid receptor occupancy. I_A and I_D , which contribute to I_{comp} in cultured hippocampal neurons, are voltage-dependent outward currents with overlapping activation ranges; both are differentially sensitive to 4-AP, and both are TEA resistant. Because the two currents, I_A and I_D , inactivate at different τ values ($I_A \approx 25$ ms; $I_D \approx 100$ ms), have different voltage dependencies for steady-state inactivation ($V_{1/2}$: $I_A \approx -70$ mV; $I_D \approx -40$ mV), and can be totally differentiated pharmacologically with DTX (2 μ M) or low-to-moderate concentrations of 4-AP (500 μ M), the cannabinoid receptor modulation of each current was examined independent of the other.

It is clear from the above data that the previously reported effects of cannabinoids (Deadwyler et al., 1993, 1995a; Hampson et al., 1995) on I_A in cultured hippocampal neurons can now be identified with respect to the contributions of I_A

and I_D to I_{comp} (Figs. 2, 6, and 8). Hence the positive shift in voltage dependence of I_{comp} resulted from cannabinoid-mediated enhancement of I_A , whereas the reduction in τ of I_{comp} was derived from decreased I_D amplitude. The addition of the selective channel blocker DTX produced a reduction in the I_{comp} τ similar to WIN 55,212-2 (Figs. 1 and 2) and blocked any further actions of WIN 55,212-2 (Table 1). Exposure to WIN 55,212-2 also produced a reduction in I_D amplitude in both the steady-state inactivation and activation protocols (Fig. 4). DTX, however, did not provoke a change in the voltage dependence of I_A (Fig. 2). Further evidence that both the positive shift in I_A voltage dependence and the reduction in I_D amplitude were cannabinoid receptor mediated was provided by blockade by the CB₁ receptor antagonist SR141716A (Table 1). Although a potential alternative interpretation for the dual effects of cannabinoids on I_{comp} is that the shift in voltage dependence of I_A (Fig. 2) could have resulted directly from the elimination or marked reduction of I_D (Fig. 4), the removal of a current with a more positive Boltzmann curve ($V_{1/2}$) than I_A (as was the case for I_D ; see Fig. 6) would have produced a negative (not a positive) shift in the voltage dependence of I_{comp} (Fig. 2). The predicted negative shift would result only if both currents were activated with the same time constant (cf. Fig. 6). The slower activation time constant for I_D (20 ms) requires that the initial peak of I_{comp} (5-ms duration) consists almost entirely of I_A ; thus, the positive shift in I_{comp} voltage dependence was strictly the result of the cannabinoid receptor activation on I_A , not I_D . Similarly, the reduction in I_{comp} τ resulted from direct cannabinoid effects on I_D amplitude because I_A inactivation τ was not altered by cannabinoids (Table 1).

Prior reports have established that cannabinoid receptor modulation of I_A voltage dependence can be attributed ultimately to PKA-dependent phosphorylation (Table 1; Hampson et al., 1995; Mu et al., 1996). Several manipulations of the cAMP/PKA cascade, including direct application of Cat.S (Table 1), confirmed that the reduction in I_D amplitude produced by cannabinoid receptor activation was consistent with a decrease in PKA dependent phosphorylation (presumably of I_D channel proteins) in these cells (Fig. 6, Table 1). Figure 8 further demonstrates that the effect of increased cAMP on I_D steady-state inactivation was reciprocal to modulation by the cannabinoid receptor of I_A . The fact that the control (untreated) measures of I_D amplitude and I_A $V_{1/2}$ were between these two extremes shows that a tonic level of cAMP is normally present and active, allowing both I_A and I_D to be only partially “expressed” under normal, control conditions (Control trace in Fig. 1). Because there was a change in slope in the Boltzmann curves for I_D , it was not possible to determine whether the voltage dependence of either activation or inactivation of I_D was altered by cannabinoids and other agents that modulate cAMP levels (Fig. 6). However, there was a cAMP-dependent decrease in I_D amplitude, presumably due to total blockade of the channel as occurs with DTX (Storm, 1990; Wu and Barish, 1992). The fact that the EC₅₀ value was lower for cannabinoid reduction in I_D amplitude than for shifting the voltage dependence of I_A suggests a slight bias toward decreasing the τ of I_{comp} .

Mechanism of Cannabinoid Modulation of I_A and I_D .

The above results provide evidence that a reciprocal relationship exists between I_A and I_D with respect to cannabinoid receptor modulation of levels of cAMP. Modulation of voltage-

gated potassium channel currents (I_{comp}) can play a major role in altering the temporal and amplitude characteristics of action potentials in hippocampal neurons (Segal et al., 1984). Such reciprocity is likely explained by differences in the subtypes of K^+ channels producing I_A and I_D . If the two currents were produced by different subtypes (i.e., *shaker*-type Kv1 versus *shal*-type Kv4 channels or even Kv4.2 versus Kv4.3 channels), then the common factor of cannabinoid receptor-mediated inhibition of adenylyl cyclase, PKA, and subsequent protein phosphorylation could be translated into opposite actions on I_A and I_D . The I_A channel described in these studies fits the description of the Kv4.2 or Kv4.3 subtypes, which have been shown to be present in hippocampal pyramidal cells and interneurons (Villarroel and Schwarz, 1996; Serodio and Rudy, 1998). Although the profile of I_A is also satisfied by Kv1.4, this can be ruled out on the basis of a lack of effect of H_2O_2 on either I_A or I_{comp} (Mu et al., 1997). Different inactivation mechanisms have been demonstrated for different types of potassium channels (Levitan, 1994). When phosphorylated, the Kv4.2- and Kv4.3-type homomultimers have low conductances that change drastically toward maximum under conditions of dephosphorylation (Aiello et al., 1995). Thus, for this putative I_A channel, cannabinoid receptor-mediated decreases in PKA phosphorylation would lead to a positive shift in the inactivation voltage of the I_A channel (Deadwyler et al., 1993; Hampson et al., 1995).

The same cannabinoid-induced decrease in cAMP and consequent decrease in PKA dependent phosphorylation were also associated with a decreased amplitude of I_D (Figs. 4, 6, and 7). Decreased phosphorylation in Kv3.3- or Kv3.4-type K^+ channels (possible candidates for I_D) results in a decrease in channel conductance (Massengill et al., 1997). Because amplitude reduction in I_D may occur without a shift in voltage dependence, the phosphorylation site on the I_D channel may not be associated with an inactivation or activation process as it is in I_A (Fig. 6). Thus, in the I_D -type channel, cAMP-dependent reduction in current amplitude may reflect a more complex interaction, involving multiple phosphorylation sites (Fadool and Levitan, 1998). Such a mechanism could be responsible for the altered slope in the Boltzmann curves in Fig. 6, showing that peak conductance of I_D at all voltage levels was markedly reduced by decreases in cAMP and/or PKA inhibition. Hence, the effects of cannabinoid drugs on I_A and I_D are coupled through the cannabinoid receptor/cAMP cascade. Receptor occupancy thus appears to have opposite functional consequences, presumably due to different conductance states of the two channels with respect to PKA-dependent phosphorylation (Levitan, 1994).

The reciprocal nature of cannabinoid receptor-mediated effects on I_A and I_D provides insight into prior reports that did not entirely distinguish among I_{comp} , I_A , and I_D in central nervous system neurons (Brew and Forsythe, 1995; Zhang and McBain, 1995; Keros and McBain, 1997). I_A and I_D were distinguished on the basis of changes in synaptic and action potential profiles as a function of sensitivity to low versus high concentrations of 4-AP (Storm, 1987, 1990; Hamon et al., 1995; Inokuchi et al., 1997). Low concentrations of 4-AP (30–500 μM) enhanced excitatory postsynaptic potentials (Southan and Owen, 1997), increased action potential duration (Storm, 1987, 1987), and decreased interspike intervals (Brew and Forsythe, 1995; Locke and Nerbonne, 1997a), presumably as the result of a selective blockade of I_D (Fig. 8,

Cannabinoid, I_D). High concentrations of 4-AP were shown to enhance these effects as would be predicted by eliminating the residual I_{comp} shown in Fig. 8 (Cannabinoid, solid line), which is mostly I_A . In contrast, cannabinoids reduce I_D amplitude while simultaneously producing a positive voltage shift in inactivation and activation of I_A (Figs. 2, 6, and 8), thereby counteracting the spike-broadening effects (Storm, 1987; Locke and Nerbonne, 1997a) without influencing the decrease in interspike interval produced by blockade of I_D (Locke and Nerbonne, 1997a). Therefore, the positive voltage shift in I_A produced by cannabinoid receptor inhibition of adenylyl cyclase and subsequent blockade of the cAMP/PKA cascade would increase the number of action potentials resulting from the decrease in I_{comp} τ (Fig. 8, Cannabinoid, I_D) while limiting calcium influx during the action potential (Storm, 1987; Locke and Nerbonne, 1997a). These results suggest the role of cannabinoids is to “fine-tune” the activity and excitability of neurons through the modulation of voltage-dependent potassium channels.

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