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

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ABSTRACT

Cannabinoid 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 µM) or dendrotoxin (2 µM) and subtraction of the residual I_A from I_comp to reveal I_O. The time constants of inactivation (τ) of I_A and I_O 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_O in the same neurons. The EC₅₀ value for the effect of WIN 55,212-2 on I_O 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_O, 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_O 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_O 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 (V₅₀ = −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 G₂₅₅ protein-linked receptor systems, such as γ-aminobutyric acidB (Gage, 1992), serotonin₁A (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 µM) 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-

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; V₅₀, 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 IA) with overlapping voltage dependencies really comprised this TEA-insensitive "control" current reported in those studies.

A likely candidate for the second current was potassium D or delay current (ID) on the basis of the inactivation \( \tau \), insensitivity to TEA, and high sensitivity to 4-AP (100–500 \( \mu \)M; Storm, 1990; Wu and Barish, 1992; Locke and Nerbhonne, 1997a). ID or ID-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 IA in that ID is steady-state inactivated and activated at approximately 30 mV more positive membrane potentials than IA (Storm, 1990; Wu and Barish, 1992). ID is also slower to activate \( (\tau = 20 \text{ ms}) \) and inactivate \( (\tau = 100 \text{ ms}) \) with a refractory period of up to 20 s (Storm, 1990). In addition, ID is blocked by low concentrations of 4-AP (<1 \( \mu \)M) and by dendrotoxin (DTX; 2 \( \mu \)M), whereas IA 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{sub 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{sub 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 protose (2 U/ml Displace 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{sup 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% CO{sub 2} incubator.

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 then replaced with plating medium, and cultures were treated with 0.75 \( \mu \)M cytosine-\( b \)-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-\( \mu \)M (2–5 M\( \Omega \)) tip openings. Electrodes were filled by suction and backfilling with a standard intracellular solution of 140 mM KCl, 11 mM EGTA, 1 mM CaCl{sub 2}, 2 mM MgCl{sub 2}, 2 mM ATP, 200 \( \mu \)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 CaCl{sub 2}, 2 mM MgCl{sub 2}, 10 mM glucose, and 20 mM HEPES, with 1 \( \mu \)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 IA and ID (Storm, 1990; Wu and Barish, 1992). Cultured cells were heated (37°C) and perfused with oxygenated (95% O{sub 2}/5% CO{sub 2}) bathing medium throughout the experiment. The osmolality of the bath was 320 ± 10 mOsm, and the pipette solution was 280 ± 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 CLAMP 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\( \Omega \), 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 IA 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 IA. Activation of IA and ID 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 IA, respectively. Because the activation and inactivation voltages for IA and D were overlapped (−60 mV activation, <−70 mV inactivation for IA; >−70 mV inactivation, <−40 mV inactivation for ID), it was possible to record IA in the absence of IB by using the IA inactivation protocol and pharmacologically blocking IB with 4-AP or DTX. IA could be recorded in the absence of IB by using a −40-mV, 50-msec prepulse that steady-state inactivated most IA 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-\( \mu \)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 applications to separate neurons, as well as pre- and post-drug...
measurements of $I_{h}$ and $I_D$ within the same WIN 55,212-2-treated neurons. DTX (Sigma Chemical Co.) was used to selectively block $I_D$ (Storm, 1990; Wu and Barish, 1992) was dissolved directly in bathing medium to a concentration of 2 µM. 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 µg/ml) was added to the culture medium 18 h before recording and to the pipette solution for dialyzation 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 µM guanosine-5’-O-(3-thio)triphosphate (GTP$\gamma$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$\gamma$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-h-cAMP; 10 µM) 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 (CB$_1$-specific) antagonist SR141716A (provided by Sanofi Reserche, 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 ($\tau$) 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 IA or ID generated by the inactivation and activation protocols (see Deadwyler et al., 1993). Peak current amplitude ($I_{\text{max}}$) 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. $I_{\text{max}}$ was the same or very near the same for all treatment conditions in a given cell. For each combination of prepulse and step voltage, $I_{h}$ or $I_D$ ($U_{\text{max}}$) was converted to relative conductance $G/G_{\text{max}}$ 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 $G/G_{\text{max}}$ and conditioning prepulse voltages ($V_{\text{pp}}$) via the following formula: $G/G_{\text{max}} = 1/(1 + \exp(-V_{\text{pp}} - V_{1/2}/k))$, where $V_{1/2}$ is the conditioning prepulse potential for inactivation; $V_{1/2}$ is the voltage at which $G/G_{\text{max}}$ = 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 ± S.E. $G/G_{\text{max}}$. Changes in $I_{h}$ 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 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 ($I_{\text{comp}}$) 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 $I_{\text{comp}}$ displays fast activation time (<5 ms) and an inactivation time constant (~50 ms) of 50 mV. 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. $I_{\text{comp}}$ 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 µM) or moderate concentrations of 4-AP (500 µM) reduced the mean $\tau$ of $I_{\text{comp}}$ from 50 to 25 ms (Fig. 1, $I_{h}$). Mean ± S.E. $I_{\text{comp}}$ amplitudes and $\tau$ values are given in Table 1 for the indicated number of cells tested. A significant ($F_{1,05} = 12.2, p < .001$) reduction in $I_{\text{comp}}$ $\tau$ was obtained in all cells exposed to 4-AP or DTX. The remaining
current profile of $I_{\text{comp}}$ 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 $I_{\text{comp}}$ curve (Fig. 1, bottom). The recovered current trace has a much slower activation time (~20 ms) and inactivation $\tau$ (100 ms) and meets the characteristics stipulated above for $I_A$ (Storm, 1987, 1990; Wu and Barish, 1992; Locke and Nerbonne, 1997b). Thus, $I_{\text{comp}}$ was successfully partitioned into two components: $I_A$ and $I_D$.

**Effects of Cannabinoids on $I_{\text{comp}}$.** Figure 2 shows the effect of exposure to the potent cannabinoid WIN 55,212 (40 nM) on $I_{\text{comp}}$ elicited by the above steady-state inactivation protocol (see Materials and Methods). These effects were compared with those of DTX (2 μM) on $I_{\text{comp}}$ using the same inactivation protocol. In both cases, mean $\tau$ was significantly reduced from control $I_{\text{comp}}$ levels (Table 1; $F_{1,9} > 7.00$, $p < .001$). However, an effect not mimicked by DTX was the increase in $I_{\text{comp}}$ amplitude and change in slope of Boltzmann curve (4-AP values were used as baseline for pharmacological subtraction).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>$I_{\text{comp}}$ (Inactivation)</th>
<th>$I_D$ (Activation)</th>
<th>$I_{\text{comp}}$ (Inactivation)</th>
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<tr>
<td></td>
<td>Peak $I_A$</td>
<td>$\tau$</td>
<td>$V_{1/2}$</td>
</tr>
<tr>
<td><strong>Control-comp.</strong></td>
<td>1.24 ± 0.10</td>
<td>51.2 ± 2.9</td>
<td>-73.1 ± 3.1</td>
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<td>4-AP</td>
<td>1.15 ± 0.08</td>
<td>24.4 ± 3.1</td>
<td>-71.5 ± 3.7</td>
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<tr>
<td>DTX</td>
<td>1.13 ± 0.07</td>
<td>25.4 ± 4.9</td>
<td>-72.1 ± 4.8</td>
</tr>
<tr>
<td>WIN</td>
<td>1.29 ± 0.08</td>
<td>21.2 ± 3.4</td>
<td>-54.3 ± 4.4</td>
</tr>
<tr>
<td>WIN + DTX</td>
<td>1.27 ± 0.06</td>
<td>18.4 ± 5.2</td>
<td>-53.2 ± 2.4</td>
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<tr>
<td>PTX</td>
<td>1.22 ± 0.09</td>
<td>50.4 ± 1.6</td>
<td>-70.9 ± 3.8</td>
</tr>
<tr>
<td>PTX + WIN</td>
<td>1.23 ± 0.07</td>
<td>51.1 ± 4.1</td>
<td>-72.1 ± 2.3</td>
</tr>
<tr>
<td>PTX + DTX</td>
<td>1.19 ± 0.08</td>
<td>24.1 ± 3.7</td>
<td>-70.9 ± 2.9</td>
</tr>
<tr>
<td>GTPγS + WIN</td>
<td>1.32 ± 0.04</td>
<td>22.5 ± 1.5</td>
<td>-58.5 ± 2.9</td>
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<tr>
<td>8-br-cAMP + WIN</td>
<td>1.04 ± 0.09</td>
<td>65.3 ± 2.4</td>
<td>-85.3 ± 4.2</td>
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<tr>
<td>cAMP + WIN</td>
<td>1.05 ± 0.08</td>
<td>64.2 ± 2.1</td>
<td>-85.3 ± 3.6</td>
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<td>IP-20</td>
<td>1.27 ± 0.09</td>
<td>28.9 ± 3.2</td>
<td>-58.2 ± 4.7</td>
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<tr>
<td>IP-20 + WIN</td>
<td>1.30 ± 0.11</td>
<td>26.3 ± 2.7</td>
<td>-55.9 ± 3.4</td>
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<td>Rp-cAMPS</td>
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<td>34.7 ± 3.9</td>
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<td>22.8 ± 4.3</td>
<td>-55.8 ± 3.3</td>
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<tr>
<td>Cat.S. + WIN</td>
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<td>58.9 ± 2.7</td>
<td>-81.3 ± 3.2</td>
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<td>SR</td>
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<td>50.6 ± 3.3</td>
<td>-70.9 ± 3.1</td>
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<tr>
<td>SR + WIN</td>
<td>1.23 ± 0.07</td>
<td>51.5 ± 2.9</td>
<td>-72.2 ± 4.5</td>
</tr>
</tbody>
</table>

$a$ Measures were significantly different from control, $F_{1,9} > 7.9$, $p < .01$.

$b$ NC, $\tau$ 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.

SR141716A, the CB1 receptor antagonist (Table 1). As stated above, this shift in the voltage dependence of $I_{\text{comp}}$ steady-state inactivation did not occur after exposure to DTX (cf. Fig. 1; mean $I_{\text{comp}} +$ DTX inactivation $V_{1/2} = -72.1 ± 4.8$; DTX versus WIN 55,212-2, $F_{1,9} = 0.56$, $p = .45$) or to WIN 55,212-2 + DTX (Fig. 2; mean $I_{\text{comp}} +$ WIN 55,212-2 + DTX inactivation $V_{1/2} = -53.2 ± 2.4$, $F_{1,9} = 0.38$, $p = .54$).

The Boltzmann curves in Fig. 2 suggest that the changes in voltage dependence of $I_{\text{comp}}$ produced by WIN 55,212-2 can be explained by a change in voltage dependence of $I_A$ because they are occur during DTX-containing media. However, the reduction in $I_{\text{comp}}$ $\tau$ cannot result from effects on $I_A$ because $I_A$ inactivation $\tau$ is not affected by cannabinoid exposure (Deadwyler et al., 1993). The $\tau$ reduction in $I_{\text{comp}}$ can be explained appropriately by selective removal of $I_{\text{comp}}$ leaving only the $\tau$ (25 ms) for $I_A$. Thus, cannabinoids produced two independent changes in $I_{\text{comp}}$, a positive shift in $V_{1/2}$ of $I_A$, which increased peak amplitude of $I_{\text{comp}}$ and reduced $\tau$ suggesting decreased contribution of $I_D$.
Fig. 2. Comparison of the effects of DTX and cannabinoid receptor agonist WIN 55,212-2 on \(I_{\text{comp}}\) with cannabinoid enhancement of \(I_{\text{A}}\). Left, \(I_{\text{comp}}\) elicited with steady-state inactivation protocol under control conditions (top) and after exposure to DTX (2 \(\mu\)M, middle) or WIN 55,212-2 (40 \(nM\), bottom). Both DTX and WIN 55,212-2 produced a similar reductions in inactivation \(\tau\) values (compare with dashed trace for \(I_{\text{comp}}\), leaving a residual current characteristic in both cases of \(I_{\text{A}}\). 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 \(I_{\text{A}}\). Scale bars: 50 ms, 0.5 nA. Right, Boltzmann curves for steady-state inactivation and activation of \(I_{\text{A}}\). \(I_{\text{A}}\) inactivation (● and ○). Peak \(I_{\text{A}}\) amplitude (\(I_{\text{max}}\)) 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 \(I_{\text{A}}\) to \(I_{\text{max}}\) was computed. The resulting relative current (\(I_{\text{A}}/I_{\text{max}}\)) was converted to relative conductance (\(G/\text{G}_{\text{max}}\); see text) and fitted by nonlinear regression to the Boltzmann function: \(G/\text{G}_{\text{max}} = 1/(1 + \exp(-(V_{\text{pp}} - V_{1/2})/k))\). Symbols indicate mean ± S.E.M. conductance (\(G/\text{G}_{\text{max}}\)) across different cells (DTX: \(n = 8\); WIN 55,212-2: \(n = 12\)). ○, Boltzmann curve for \(I_{\text{A}}\) (DTX treated). ●, Boltzmann curve and conductance for WIN 55,212-2-treated \(I_{\text{A}}\) and reflect a 15-mV positive shift in the steady-state inactivation of \(I_{\text{A}}\) from the DTX-treated condition. \(I_{\text{A}}\) activation (● and ○). Peak \(I_{\text{A}}\) amplitude (\(I_{\text{max}}\)) was measured for \(-120\) mV prepulse and depolarizing step to \(+50\) mV. Depolarizing prepulse was then varied in 10-mV steps (negative); \(I_{\text{A}}/I_{\text{max}}\) was computed, converted to conductance, and fitted to the Boltzmann equation: \(G/\text{G}_{\text{max}} = 1/(1 + \exp(-(V_{\text{pp}} - V_{1/2})/k))\).

The curve for steady-state activation of \(I_{\text{D}}\) 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 \(I_{\text{D}}\) 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 \(\tau\) and amplitude of \(I_{\text{D}}\). The voltage dependence for both steady-state inactivation and activation were consistent with several previous reports of \(I_{\text{D}}\) 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 \(\mu\)M) on \(I_{\text{D}}\). The traces in Fig. 4 show the effects of WIN 55,212-2 (10 and 40 nM) on \(I_{\text{D}}\) recorded in TEA. The effects of DTX (2 \(\mu\)M) are shown below for comparison. Exposure to WIN 55,212-2 (40 nM) reduced the peak \(I_{\text{D}}\) amplitude by 60% (Table 1, \(n = 12\) cells, mean \(I_{\text{D}}\) amplitude = \(0.64 \pm 0.09\), mean \(I_{\text{D}}\) + WIN 55,212-2 amplitude = \(0.25 \pm 0.03\), \(F_{1,95} = 16.53\), \(p < .001\)) 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 \(I_{\text{D}}\) amplitude relative to control (mean \(I_{\text{D}} + \text{DTX} \) amplitude = \(0.22 \pm 0.05\) nA, mean \(I_{\text{D}} + \text{WIN 55,212-2 + DTX} \) amplitude = \(0.21 \pm 0.03\); all \(F_{1,95} > 7.01\), \(p < .01\)). The reduction of \(I_{\text{D}}\) amplitude by WIN 55,212-2 was blocked by the cannabinoid receptor antagonist SR141716A (Table 1).

The reduction of \(I_{\text{D}}\) amplitude by WIN 55,212-2 was not accompanied by a shift in voltage dependence of steady-state inactivation of \(I_{\text{D}}\). 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 \(I_{\text{D}}\) amplitude for each trace. This voltage-independent decrease in amplitudes 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 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 $I_D$ 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; $F_{5,19} = 0.19$, $p = .65$), but importantly, PTX did not alter the ability of DTX to reduce $I_D$ amplitude (mean PTX + DTX amplitude = 0.24 ± 0.04 nA; $F_{5,19} = 6.47$, $p < .01$, Table 1). Cells ($n = 8$) dialyzed with the G protein activator GTP-$S$ (600 μM) also exhibited reduced $I_D$ amplitude (mean GTP-$S$ amplitude = 0.30 ± 0.08; $F_{5,19} = 6.11$, $p < .01$) while blocking the effect of WIN 55,212-2 (mean GTP-$S$ + WIN 55,212-2 amplitude = 0.31 ± 0.07 nA; $F_{5,19} = 0.18$, $p = .67$, 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 μM) 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; $F_{5,19} = 6.29$, $p < .01$, Table 1), whereas IP-20, markedly reduced $I_D$ amplitude by 63% (mean IP-20 amplitude = 0.24 ± 0.09 nA; $F_{5,19} = 6.51$, $p < .01$). 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 of $I_D$ 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 the 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 ($I_{\text{max}}$; see Materials and Methods) capable of being evoked by the protocol. If so, different $I_{\text{max}}$ values would be obtained depending on
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 ID 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_A, 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 μM) 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-AMP) 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
Conversely, the dash-dotted traces (Control and Cannabi-
Dashed current traces at left (8-br-cAMP) and right (WIN 55,212-2) are superimposed control Icomp (center) traces. Bold arrows show differential change in τ from control Icomp. Horizontal lines and arrow at top depict change in Icomp amplitude (right to left) produced by increasing levels of cAMP. Dotted traces (middle and left) depict decrease in Icomp as cAMP levels increase (8-br-cAMP). Dash-dotted traces (middle and right) show decrease from maximum Icomp (8-br-cAMP), through control (middle), to minimum Icomp after exposure to WIN 55,212-2 (right).

range in Icomp τ from increased cAMP levels (Fig. 8). 8-br-cAMP to decreased cAMP levels produced by cannabinoid receptor activation (Fig. 8, Cannabinoi) was 68%. The dotted traces in Fig. 8 depict Icomp (8-br-cAMP and Control) and show that Icomp amplitude was reduced in conjunction with Icomp 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 Icomp 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 Icomp 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 Icomp with a relatively insignificant increase in amplitude. The dual modulation of Icomp shown in Fig. 8 is cAMP dependent; however, the differential modulation of Icomp and Icomp 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 (Icomp) in hippocampal cells is selectively modulated by cannabinoid receptor occupancy. Icomp and Icomp, which contribute to Icomp 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, Icomp and Icomp, inactivate at different τ values (Icomp = 25 ms; Icomp = 100 ms), have different voltage dependencies for steady-state inactivation (V1/2: Icomp = –70 mV; Icomp = –40 mV), and can be totally differentiated pharmacologically with DTX (2 μM) or low-to-moderate concentrations of 4-AP (500 μM), 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 Icomp in cultured hippocampal neurons can now be identified with respect to the contributions of Icomp and Icomp to Icomp (Figs. 2, 6, and 8). Hence the positive shift in voltage dependence of Icomp resulted from cannabinoid-mediated enhancement of Icomp, whereas the reduction in τ of Icomp was derived from decreased Icomp amplitude. The addition of the selective channel blocker DTX produced a reduction in the Icomp 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 Icomp amplitude in both the steady-state inactivation and activation protocols (Fig. 4). DTX, however, did not provoke a change in the voltage dependence of Icomp (Fig. 2). Further evidence that both the positive shift in Icomp voltage dependence and the reduction in Icomp 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 Icomp is that the shift in voltage dependence of Icomp (Fig. 2) could have resulted directly from the elimination or marked reduction of Icomp (Fig. 4), the removal of a current with a more positive Boltzmann curve (V1/2) than Icomp (as was the case for Icomp; see Fig. 6) would have produced a negative (not a positive) shift in the voltage dependence of Icomp (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 Icomp (20 ms) requires that the initial peak of Icomp (5-ms duration) consists almost entirely of Icomp; thus, the positive shift in voltage dependence was strictly the result of the cannabinoid receptor activation on Icomp, not Icomp. Similarly, the reduction in Icomp τ resulted from direct cannabinoid effects on Icomp amplitude because Icomp inactivation τ was not altered by cannabinoids (Table 1).

Prior reports have established that cannabinoid receptor modulation of Icomp 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 Icomp amplitude produced by cannabinoid receptor activation was consistent with a decrease in PKA dependent phosphorylation (presumably of Icomp channel proteins) in these cells (Fig. 6, Table 1). Figure 8 further demonstrates that the effect of increased cAMP on Icomp was not only partially “expressed” under normal, control conditions (Control trace in Fig. 1). Because there was a change in slope in the Boltzmann curves for Icomp, it was not possible to determine whether the voltage dependence of either activation or inactivation of Icomp was altered by cannabinoids and other agents that modulate cAMP levels (Fig. 6). However, there was a cAMP-dependent decrease in Icomp 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 Icomp amplitude than for shifting the voltage dependence of Icomp suggests a slight bias toward decreasing the τ of Icomp.

Mechanism of Cannabinoid Modulation of Icomp and Icomp

The above results provide evidence that a reciprocal relationship exists between Icomp and Icomp 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 adenyl 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$_2$O$_2$ 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 Forstyhe, 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 $I_P$ 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 adenyl cyclase and subsequent blockade of the cAMP/PKA cascade would increase the number of action potentials resulting from the decrease in $I_{comp}$ (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.

References


