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

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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 (Iₐ) and D (I₆) were isolated from a composite tetraethylammonium-insensitive current (I₃comp) by blockade with either 4-aminopyridine (500 μM) or dendrotoxin (2 μM) and subtraction of the residual Iₐ from I₃comp to reveal I₉. The time constants of inactivation (τ) of Iₐ and I₆ 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 Iₐ and a simultaneous voltage-independent reduction in the amplitude of I₆ in the same neurons. The EC₅₀ value for the effect of WIN 55,212-2 on I₆ amplitude (13.9 nM) was slightly lower than the EC₅₀ value for its effect on Iₐ 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 Iₐ and I₆, 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 Iₐ and I₆ were both shown to be mediated via the well documented cannabinoid receptor inhibition of adenyl cyclase and subsequent modulation of cAMP and protein kinase. These actions are considered in terms of cAMP-mediated phosphorylation of separate Iₐ and I₆ channels and the contribution of each to composite voltage-gated potassium currents in these cells.

Activation of the cannabinoid receptor in cultured neurons inhibits adenyl cyclase (Little and Martin, 1989; Bidaut-Russell et al., 1990) and enhances an outward potassium current in cultured hippocampal neurons (Deadwyler et al., 1995b). 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 (Iₐ) in other reports on the basis of activation and inactivation time constants (5 and 50 ms, respectively), steady-state inactivation and activation voltage ranges (V₁/₂ = −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 Gₛ protein-linked receptor systems, such as γ-aminobutyric acidB (Gage, 1992), serotonin₁A (Segal, 1980), and adenosine A₁ (Mei et al., 1995), enhance Iₐ 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 Iₐ 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 Iₐ in hippocampal neurons focused on the cannabinoid produced shift in voltage dependence of steady-state inactivation of Iₐ. 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-

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ABBREVIATIONS: Iₐ, voltage-sensitive potassium A current; I₆, voltage-sensitive potassium D current; I₃comp, composite tetraethylammonium-insensitive voltage-sensitive potassium current; GTPγS, 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; V₁/₂, half-inactivation voltage of steady-state inactivation (of Iₐ and I₆); 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, insensitivity to TEA, and high sensitivity to 4-AP (100–500 μM; Storm, 1990; Wu and Barish, 1992; Locke and Nerbonne, 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 (τ = 20 ms) and inactivate (τ = 100 ms) with a refractory period of up to 20 s (Storm, 1990). In addition, ID is blocked by low concentrations of 4-AP (<1 mM) and by dendrotoxin (DTX; 2 μM), 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 ID 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 ID, 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 × 10^4 cells/35-mm dish. The plating medium consisted of 59% Dulbecco’s modified Eagle’s medium (1×), 19.5% Ham’s F-12 Nutrient Mixture (1×), 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. Due to the lipophilicity of the drug, a 30-s ejection via the pressure pipette (10- to 50-μm 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 55212-2 (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 55212-2 were 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 WIN55212-2 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.

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measurements of \( I_A \) 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 \( \mu \)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 \( \mu \)g/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 \( G_{io} \) proteins, 600 \( \mu \)M guanosine-5'-O-(3-thio)triphosphate (GTP\(_S\)), 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\(_S\). 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; Sigma Chemical Co.) was dialyzed into the cell 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 a concentration of 2 \( \times \)10\(^{-9}\) M or DTX (2 \( \mu \)M) to block \( I_D \).

**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 \( I_{(comp)} \) 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 (\(\tau\)) 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 \( \mu \)M) or moderate concentrations of 4-AP (500 \( \mu \)M) reduced the mean \( \tau \) of \( I_{(comp)} \) from 50 to 25 ms (Fig. 1, \( I_A \)). Mean \( \pm \) S.E. \( I_{(comp)} \) amplitudes and \( \tau \) values are given in Table 1 for the indicated number of cells tested. A significant \( F_{1, 95} = 12.2, p < 0.001 \) reduction in \( I_{(comp)} \) \( \tau \) 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 55,212-2 (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 $\mu$M) 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; $F_{1,96} = 17.75, p < .001$). However, an effect not mimicked by DTX was for WIN 55,212-2 to increase the amplitude of $I_{\text{comp}}$ (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 $I_{\text{comp}}$ were positively shifted by exposure to WIN 55,212-2 (mean $I_{\text{comp}}$ inactivation $V_{1/2} = -71.3 \pm 3.1$ mV, mean $I_{\text{comp}} + \text{WIN} 55,212-2$ inactivation $V_{1/2} = -54.3 \pm 4.4$ mV, $F_{1,95} = 12.57, p < .001$; mean $I_{\text{comp}}$ activation $V_{1/2} = -20.1 \pm 2.6$ mV, mean $I_{\text{comp}} + \text{WIN} 55,212-2$ 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 $I_{\text{comp}}$ 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 $I_{\text{comp}}$ steady-state inactivation did not occur after exposure to DTX (cf. Fig. 1; mean $I_{\text{comp}} + \text{DTX}$ 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 $I_{\text{comp}} + \text{WIN} 55,212-2 + \text{DTX}$ 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 $I_{\text{comp}}$ 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 $I_{\text{comp}}$ $\tau$ 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 $I_A$ from $I_{\text{comp}}$, 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 $I_D$.

**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 $I_{\text{comp}}$. To reconstruct $I_D$, $I_{\text{comp}}$ was recorded at each voltage step in the presence and absence of 4-AP (500 $\mu$M) to isolate $I_A$. The latter current ($I_A$) was then subtracted from the untreated $I_{\text{comp}}$ to provide the curve for $I_D$. The $V_{1/2}$ for $I_D$ steady-state inactivation was determined to be $-39.9 \pm 3.5$ as shown in Table 1, 30 mV more positive than $I_A$ (Deadwyler et al.,

### Table 1: Effects of drug treatments on $I_{\text{comp}}$ and $I_D$ currents

<table>
<thead>
<tr>
<th>Drug</th>
<th>$I_{\text{comp}}$ (Inactivation)</th>
<th>$I_D$ (Activation)</th>
<th>$I_{\text{comp}}$ (Inactivation)</th>
<th>$I_D$ (Activation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak $I_{\text{comp}}$ (nA)</td>
<td>$V_{1/2}$ (mV)</td>
<td>$\tau$ (ms)</td>
<td>Peak $I_D$ (nA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-comp.</td>
<td>1.24 ± 0.10</td>
<td>-71.3 ± 3.1</td>
<td>0.64 ± 0.09</td>
<td>98.2 ± 7.4</td>
</tr>
<tr>
<td>4-AP</td>
<td>1.15 ± 0.08</td>
<td>-71.5 ± 3.7</td>
<td>0.24 ± 0.06</td>
<td>98.2 ± 7.4</td>
</tr>
<tr>
<td>DTX</td>
<td>1.13 ± 0.07</td>
<td>-72.1 ± 4.8</td>
<td>0.22 ± 0.05</td>
<td>NC</td>
</tr>
<tr>
<td>WIN</td>
<td>1.29 ± 0.08</td>
<td>-54.3 ± 4.4</td>
<td>0.25 ± 0.03</td>
<td>NC</td>
</tr>
<tr>
<td>WIN + DTX</td>
<td>1.27 ± 0.06</td>
<td>-53.2 ± 2.4</td>
<td>0.21 ± 0.03</td>
<td>NC</td>
</tr>
<tr>
<td>PTX</td>
<td>1.22 ± 0.09</td>
<td>-70.9 ± 3.8</td>
<td>0.60 ± 0.05</td>
<td>NC</td>
</tr>
<tr>
<td>PTX + WIN</td>
<td>1.23 ± 0.07</td>
<td>-71.2 ± 2.3</td>
<td>0.59 ± 0.08</td>
<td>NC</td>
</tr>
<tr>
<td>PTX + DTX</td>
<td>1.19 ± 0.08</td>
<td>-70.9 ± 2.9</td>
<td>0.24 ± 0.04</td>
<td>NC</td>
</tr>
<tr>
<td>8-br-cAMP</td>
<td>1.32 ± 0.04</td>
<td>-58.5 ± 2.9</td>
<td>0.51 ± 0.07</td>
<td>NC</td>
</tr>
<tr>
<td>cAMP + WIN</td>
<td>1.05 ± 0.08</td>
<td>-85.3 ± 4.2</td>
<td>0.81 ± 0.08</td>
<td>NC</td>
</tr>
<tr>
<td>IP-20</td>
<td>1.27 ± 0.09</td>
<td>-85.1 ± 3.6</td>
<td>0.80 ± 0.06</td>
<td>NC</td>
</tr>
<tr>
<td>IP-20 + WIN</td>
<td>1.30 ± 0.11</td>
<td>-58.2 ± 4.7</td>
<td>0.28 ± 0.09</td>
<td>NC</td>
</tr>
<tr>
<td>Rp-cAMPS</td>
<td>1.30 ± 0.06</td>
<td>-55.9 ± 3.4</td>
<td>0.24 ± 0.07</td>
<td>NC</td>
</tr>
<tr>
<td>Cat.S. + WIN</td>
<td>1.31 ± 0.07</td>
<td>-63.2 ± 2.1</td>
<td>0.41 ± 0.05</td>
<td>NC</td>
</tr>
<tr>
<td>OA</td>
<td>1.10 ± 0.08</td>
<td>-58.5 ± 3.3</td>
<td>0.27 ± 0.07</td>
<td>NC</td>
</tr>
<tr>
<td>OA + WIN</td>
<td>1.05 ± 0.07</td>
<td>-80.7 ± 2.8</td>
<td>0.80 ± 0.09</td>
<td>NC</td>
</tr>
<tr>
<td>NR</td>
<td>1.05 ± 0.07</td>
<td>-83.0 ± 2.9</td>
<td>0.79 ± 0.07</td>
<td>NC</td>
</tr>
<tr>
<td>SR</td>
<td>1.05 ± 0.07</td>
<td>-82.1 ± 3.0</td>
<td>0.77 ± 0.06</td>
<td>NC</td>
</tr>
<tr>
<td>SR + WIN</td>
<td>1.05 ± 0.07</td>
<td>-72.2 ± 4.5</td>
<td>0.58 ± 0.06</td>
<td>NC</td>
</tr>
</tbody>
</table>

* Measures were significantly different from control, $F_{1,96} > 7.90, p < .01.$

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.
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 μM, middle) or WIN 55,212-2 (40 nM, bottom). Both DTX and WIN 55,212-2 produced a similar reductions 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/I_{max}) was converted to relative conductance (G/G_{max}; see text) and fitted by nonlinear regression to the Boltzmann function: $G/G_{max}=1/(1+exp(-(Vpp-V_{1/2})/k))$. Symbols indicate mean ± 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/I_{max} was computed, converted to conductance, and fitted to the Boltzmann equation: $G/G_{max}=1/(1+exp(-(Vpp-V_{1/2})/k))$. 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_A was +2.6 ± 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 μM) 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 μM) 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).

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-
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<sub>50</sub> 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; $F_{1,4} = 2.69, p < .01$). 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<sub>i/o</sub>, 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 $I_A$ 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 GTPgammaS (600 µM) also exhibited reduced $I_D$ amplitude (mean GTPgammaS amplitude = 0.30 ± 0.08; $F_{1,95} = 6.11, p < .01$) while blocking the effect of WIN 55,212-2 (mean GTPgammaS + 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<sub>i/o</sub> 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; $F_{1,95} = 6.29, p < .01$, 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

![Figure 5](image-url)
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 \( I_{\text{max}} \). In the latter case, \( I_{\text{max}} \) 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 (\( +80 \, \text{mV} \) depolarization) but preceded by one of three different levels of hyperpolarizing prepulses (\( -120, -80, \) or \( -40 \, \text{mV} \)) 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 \( I_{\text{comp}} \)** Figure 8 shows the net changes in \( I_{\text{comp}} \) with different manipulations of cAMP. The solid traces show \( I_{\text{comp}} \) recorded from a single neuron under control conditions (i.e., “resting” levels of cAMP) and after exposure to 8-br-cAMP (10 \( \mu \text{M} \)) and 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 \( I_{\text{comp}} \) by 16% relative to control (long vertical arrow); in contrast, cannabinoid exposure increased peak \( I_{\text{comp}} \) amplitude by only 4% (short vertical arrow). However, at the same time, increased cAMP levels (8-br-AMP) caused a 27% increase in \( I_{\text{comp}} \) relative to control, whereas cannabinoids decreased the \( I_{\text{comp}} \) 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 \( I_{\text{comp}} \) under both conditions as indicated by the direction of the arrow. Thus, the maximum

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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 \( \mu \text{M} \)), WIN 55,212-2 (WIN 55,212-2; 40 nM), or DTX (2 \( \mu \text{M} \)) in the bathing solution or after dialyzing the cell, via the recording pipette solution, with the PKA inhibitory peptide IP-20 (6 \( \mu \text{M} \)) or partial PKA inhibitor Rp-cAMPS (2 \( \mu \text{M} \)). 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 \( I_{\text{comp}} \)) were calculated by subtraction of \( I_A \) from \( I_{\text{comp}} \) (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 three sets of single-cell voltage-clamp current traces show $I_{comp}$ under control (center), 8-br-cAMP (10 μM, 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 $\tau$ 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).

**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 $\tau$ 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 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 $I_A$ 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 $\tau$ of $I_{comp}$ was derived from decreased $I_D$. The addition of the selective channel blocker DTX produced a reduction in the $I_{comp}$, $\tau$ 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 $I_{comp}$ $\tau$ resulted from direct cannabinoid effects on $I_D$ amplitude because $I_D$ inactivation $\tau$ 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 (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 $\tau$ 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 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 (Villarreal 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 Forstythe, 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 (Soutain and Owen, 1997), increased action potential duration (Storm, 1987, 1997), and decreased interspike intervals (Brew and Forstythe, 1995; Locke and Nerbbonne, 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_D (Figs. 2, 6, and 8), thereby counteracting the spike-broadening effects (Storm, 1987; Locke and Nerbbonne, 1997a) without influencing the decrease in interspike interval produced by blockade of I_D (Locke and Nerbbonne, 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} τ (Fig. 8, Cannabinoid, I_D) while limiting calcium influx during the action potential (Storm, 1987; Locke and Nerbbonne, 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


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