SUPPRESSION OF OUTWARD K⁺ CURRENTS BY WIN55212-2 IN RAT RETINAL GANGLION CELLS IS INDEPENDENT OF CB1/CB2 RECEPTORS

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Abstract—Cannabinoid CB1 receptor (CB1R) signaling system is extensively distributed in the vertebrate retina. Activation of CB1Rs regulates a variety of functions of retinal neurons through modulating different ion channels. In the present work we studied effects of this receptor signaling on K⁺ channels in retinal ganglion cells by patch-clamp techniques. The CB1R agonist WIN55212-2 (WIN) suppressed outward K⁺ currents in acutely isolated rat retinal ganglion cells in a dose-dependent manner, with an IC₅₀ of 4.7 μM. We further showed that WIN mainly suppressed the tetraethylammonium (TEA)-sensitive K⁺ current component. While CB1Rs were expressed in rat retinal ganglion cells, the WIN effect on K⁺ currents was not blocked by either AM251/SR141716, specific CB1R antagonists, or AM630, a selective CB2R antagonist. Consistently, cAMP-protein kinase A (PKA) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways were unlikely involved in the WIN-induced suppression of the K⁺ currents because both PKA inhibitors H-89/Rp-cAMP and MAPK/ERK1/2 inhibitor U0126 failed to block the WIN effects. WIN-induced suppression of the K⁺ currents was not observed when WIN was intracellularly applied. Furthermore, an endogenous ligand of the cannabinoid receptor anandamide, the specific CB1R agonist ACEA and the selective CB2R agonist CB65 also suppressed the K⁺ currents, and the effects were not blocked by AM251/SR141716 or AM630 respectively. All these results suggest that the WIN-induced suppression of the outward K⁺ currents in rat retinal ganglion cells, thereby regulating the cell excitability, were not through CB1R/CB2R signaling pathways. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cannabinoid receptor, CB1/CB2 receptor agonist, TEA-sensitive K⁺ current, anandamide, patch-clamp.

INTRODUCTION

Endocannabinoid (eCB) signaling is involved in regulating multiple neuronal functions in the central nervous system (CNS), by activating G-protein-coupled CB1 receptors (CB1Rs) (Howlett and Fleming, 1984; Howlett and Mukhopadhyay, 2000; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Chevaleyre et al., 2006; Wang et al., 2006; Galve-Poperth et al., 2007; Hashimotodani et al., 2007). Growing evidence has shown that CB1R signaling is also extensively distributed in the vertebrate retina (Yazulla, 2008; Zabouri et al., 2011). CB1Rs and eCBs, such as anandamide (AEA) and 2-Arachidonoylglycerol (2-AG), as well as the eCB degradative enzyme, fatty acid amide hydrolase (FAAH), are present in a variety of retinal cell populations and in the inner plexiform layer (IPL) (Straiker et al., 1999; Yazulla et al., 1999, 2000; Porcella et al., 2000; Lalonde et al., 2006). These results suggest that eCBs may regulate the functions of retinal neurons, thus being involved in visual information processing conducted by multiple circuits (Straiker et al., 1999; Yazulla et al., 1999, 2000). Activation of CB1Rs modulates various ion channels, particularly voltage-gated K⁺ and Ca²⁺ channels in the retina (Yazulla, 2008 for review). In these studies, WIN55212-2 (WIN), a cannabinoid receptor agonist, has been extensively used. Neuronal K⁺ channels are key factors in determining the resting membrane potential and modulating the cell excitability (Hille, 2001). In the retina, WIN suppresses K⁺ currents in tiger salamander rods and cones (Straiker and Sullivan, 2003), but exhibits a dose-dependent biphasic modulation of K⁺ current in goldfish cones (Fan and Yazulla, 2003). It was also reported that WIN inhibited delayed rectifying K⁺ channels in goldfish retinal bipolar cells (BCs) (Yazulla et al., 2000). All these effects were mediated by the activation of CB1Rs (Yazulla et al., 2000; Fan and Yazulla, 2003).
Retinal ganglion cells, output neurons of the retina, express many types of voltage-gated K⁺ channels (Lipton and Tauck, 1987; Ettaiche et al., 2001; Clark et al., 2009; Fohlmeister et al., 2010; Koebeler et al., 2010). While CB1Rs are present in retinal ganglion cells (Straiker et al., 1999; Zabouri et al., 2011), there is no evidence concerning effects of CB1R signaling on the K⁺ channels in these cells. In the present work we showed, using whole-cell patch-clamp techniques, that WIN suppressed outward K⁺ currents in acutely isolated rat retinal ganglion cells in a dose-dependent manner. We further showed that the WIN effect was not mediated by activating CB1Rs and/or CB2 receptors (CB2Rs).

EXPERIMENTAL PROCEDURES

Animals
Male Sprague–Dawley rats, weighing 100–150 g and obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China), maintained under a 12-h light/ dark cycle for at least 1 week before they were used for experiments. All experimental procedures dealing with the animals in the present work were in accordance with the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and the guidelines of the Fudan University on the ethical use of animals. All animal care and procedures in this work were approved by the Institutes of Brain Science, Institute of Neurobiology and the State Key Laboratory of Medical Neurobiology of the Fudan University, Shanghai, China, and all efforts were made to minimize the number of animals used and their suffering.

Retrograde labeling of retinal ganglion cells
Retrograde labeling of retinal ganglion cells was previously described in detail (Zhao et al., 2010; Ji et al., 2011). Briefly, after the rats were deeply anesthetized with 40 mg/ml sodium pentobarbital (0.1 ml/100 g), 4% formaldehyde (1 ml/100 g), 40 mg/ml sodium pentobarbital (0.1 ml/100 g), 4% formaldehyde (1 ml/100 g), and 2.0 mm lateral to the bregma and 4–5 mm deep into the superior colliculus bilaterally (6.0 mm posterior to the midline), they were anesthetized with 40 mg/ml sodium pentobarbital (0.1 ml/100 g), 4% formaldehyde (1 ml/100 g), and 2.0 mm lateral to the bregma and 4–5 mm deep into the superior colliculus bilaterally (6.0 mm posterior to the midline), they were then fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h. The eyes were removed, the optical tissue was dissected, and the retina was then isolated and incubated in oxygenated Hank’s solution containing the following (in mM): NaCl 140, NaHCO₃ 0.5, Na₂HPO₄ 1, KCl 3, CaCl₂ 2, MgSO₄ 1, HEPES 20, sodium pyruvate 1 and glucose 16 adjusted to pH 7.4 with NaOH, and then digested in 1.6 U/ml papain (Worthington Biochemical, Freehold, NJ, USA) containing Hank’s solution, supplemented with 0.2 mg/ml l-cysteine and 0.2 mg/ml bovine serum albumin for 30 min at 35.5–36.5 °C. Mechanical dissociation of retinal neurons was conducted using fire-polished Pasteur pipettes and cell suspension was plated onto a culture dish mounted on an inverted microscope (IX 70; Olympus Optical, Tokyo, Japan). RITC-labeled retinal ganglion cells, showing red fluorescence, were chosen for whole-cell patch-clamp recording within 2–3 h after dissociation.

Whole-cell recording
Membrane currents were recorded by whole-cell voltage-clamp techniques (Zhao et al., 2010; Yang et al., 2011; Ji et al., 2012). Patch pipettes used in the present work had a resistance of 2–5 MΩ after filling with an internal solution consisting of (in mM): KCl 140, NaCl 9, MgCl₂ 1, EGTA 0.2, ATP-Mg 2, GTP-Na 0.25, HEPES 10, and adjusted to pH 7.2 with KOH and to 290–300 mosm/L. To record voltage-gated K⁺ currents, the dissociated cells were superfused continuously with the extracellular solution of the following composition (in mM): NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 1, HEPES 10, and glucose 20, with 0.4 μM tetrodotoxin (TTX) and 100 μM CdCl₂, pH adjusted to 7.4 with NaOH and to 290–300 mosm/L. Whole-cell membrane currents were recorded by a patch amplifier (Axonpatch 200B), Digidata 1322A data acquisition board, and Clampex 8.0 software (Molecular Devices, Foster City, CA, USA) at a sample rate of 10 kHz, filtered at 1 kHz. Fast capacitance was fully canceled and cell capacitance was partially canceled as much as possible by the amplifier circuits. Seventy percent of the series resistance of the recording electrode was compensated. Leakage currents were subtracted on-line using a P/4 subtraction procedure.

Reagents and drug application
WIN, AEA, AM630, AM251, N-(2-Chloroethyl)-5Z, 8Z,11Z,14Z-eicosa-5,8,11,14Z-eicosa tetraenamide (ACEA) and N-Cyclohexyl-7-chloro-1-[2-(4-morpholino)ethyl]-quinolin-4(1H)-one-3-carboxamide (CB65) were obtained from Tocris Bioscience (Ellisville, MO, USA), and SR141716 was from Cayman Chemicals (Ann Arbor, Michigan, MI, USA). All the other chemicals were from Sigma (Sigma–Aldrich, Inc., St. Louis, MO, USA). WIN, AEA, AM251, SR141716, ACEA, CB65 and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were first dissolved in dimethyl sulfoxide (DMSO) and then added to the extracellular or internal solution, with the final concentration of DMSO being less than 0.1%. The other chemicals were freshly dissolved in the extracellular solution. Drugs were delivered by a superfusion drug application system (DAD-8VCSP, ALA Scientific Instruments, Westbury, NY, USA), which has eight pressurized 5 ml reservoirs, each with its own control valve to feed fluid through an eight-to-one tubing manifold (500 μm inner diameter; ALA Scientific Instruments). The open/close switch of each valve was manually controlled. Once the valve was open, the solution in the corresponding reservoir was pressure ejected by nitrogen gas along the pipes. With the large...
flow pipes, 10–90% whole-cell solution exchanges were achieved in less than 2.5 ms.

**Data analysis**

The steady-state K⁺ current amplitudes were measured at 350 ms of the voltage pulses (400-ms duration). The dose–response curve was fitted by a sigmoidal dose–response equation in Graphpad Prism software (version 4.0; Graphpad Software, San Diego, CA, USA). The data analysis was performed by using Clampfit 8.0 (Molecular Devices, Foster City, CA, USA) and Igor 4.0 (WaveMetrics, Lake Oswego, OR, USA). Data are all presented as mean ± SEM. Student’s t test or one-way analysis of variance (ANOVA) was used for statistical analysis.

**RESULTS**

**Suppression of outward K⁺ currents by WIN**

Effects of WIN on outward K⁺ currents of retinal ganglion cells, induced by a series of 400-ms depolarizing voltage pulses from a holding potential of −70 mV to +30 mV in increments of 10 mV, were first examined. Perfusion of 2 μM WIN significantly and reversibly suppressed the currents (Fig. 1A). For example, at +30 mV test potential the steady-state current amplitude was reduced to 1.00 nA from the control value of 1.47 nA, and following 5-min washout the current recovered to 1.44 nA. Similar results were obtained from the other seven cells, with the average current amplitude at +30 mV being reduced to 69.6 ± 9.6% of control (n = 8, P < 0.001). The effect was reversible and the average current amplitude returned to 96.2 ± 7.3% of control by 5-min washout (P > 0.05) (Fig. 1A). WIN suppressed the outward K⁺ currents in a concentration-dependent manner, with an IC₅₀ of 4.7 μM (Fig. 1B). Extracellular application of 10 μM AEA showed a similar effect (Fig. 1C, left panel), and the average current amplitude at +30 mV was reduced to 55.6 ± 9.0% of control (1.08 ± 0.19 nA vs 1.92 ± 0.04 nA) (n = 4, P < 0.001) (Fig. 1C, right panel).

Outward K⁺ currents recorded from rat retinal ganglion cells may contain the contributions from different K⁺ channels, such as large-conductance Ca²⁺-activated K⁺ channels (BKCa), delayed rectifying K⁺ channels, and ATP-sensitive K⁺ (KATP) channels (Lipton and Tauck, 1987; Ettaiche et al., 2001; Clark et al., 2009; Fohlineimer et al., 2010; Koebeler et al., 2010). Under our experimental conditions three major components may be tetraethylammonium (TEA)-, 4-aminopyridine (4-AP)- and ATP-sensitive ones because the co-application of TEA (10 mM), 4-AP (5 mM) and glybenclamide (Gb,10 μM), a KATP blocker, suppressed most of the outward K⁺ currents (73.5 ± 7.4% of control, n = 11) (Fig. 2A). The remaining current component in the presence of TEA, 4-AP and Gb may be mainly mediated by inward-rectifying K⁺ channels (Scher and Lipton, 1992; Guenther et al., 1999; Reiff and Guenther, 1999). To determine the identity of the K⁺ channel(s) suppressed by WIN, we examined effects of WIN on three major components. As shown in Fig. 2B, co-application of TEA (10 mM) and 4-AP (5 mM) reduced the current amplitudes to 49.0 ± 4.8% of control (n = 7, P < 0.001). In the presence of TEA and 4-AP the currents were hardly changed by WIN (2 μM) (41.3 ± 7.8% of control, n = 7, P > 0.05). Similarly, WIN did not affect the 4-AP-sensitive current component recorded in the presence of TEA (10 mM) and Gb (10 μM) (24.7 ± 2.3% of control with WIN vs 26.5 ± 2.3% of control without WIN, n = 11, P > 0.05) (Fig. 2C). In contrast, following co-application of 4-AP (5 mM) and Gb (10 μM), which suppressed the currents by 22.9% (± 3.6%) (n = 5), the remaining currents were further reduced by 16.1% (± 4.4%) (n = 5), suggesting a suppression of the TEA-sensitive component by WIN (Fig. 2D). These data demonstrated that the WIN-induced suppression of the TEA-sensitive K⁺ channels was largely responsible for the WIN effect on the outward K⁺ currents of rat retinal ganglion cells.

Further experiments showed that the WIN effect on K⁺ currents could not be reversed by CB1R antagonists. A representative result is shown in Fig. 3A. In this experiment, K⁺ currents were evoked by a depolarizing voltage pulse from −70 mV to +30 mV. Application of AM251 (2 μM), a selective CB1R antagonist, did not change the K⁺ current amplitude. In the presence of AM251, addition of 2 μM WIN persisted to suppress the K⁺ current amplitude in a reversible way. The average current amplitude at +30 mV obtained from eight cells was unchanged (96.4 ± 2.0% of control, P > 0.05) when AM251 was applied alone, but reduced to 79.7 ± 7.5% of control by adding WIN (P < 0.001) (Fig. 3A, right panel). The WIN-induced suppression extent in the presence of 2 μM AM251 was not significantly different from that obtained in normal bath solution (69.6 ± 9.6%, n = 8, P > 0.05). Similarly, SR141716 (1 μM), another CB1R antagonist, also failed to block the WIN effect on K⁺ currents (Fig. 3B, left panel). The average current amplitude was reduced to 76.3 ± 4.8% of control by the application of 2 μM WIN in the presence of 1 μM SR141716 (n = 5, P < 0.001) (Fig. 3B, right panel), a suppression extent that was comparable to that obtained in normal bath solution.

CB2Rs are also expressed in rat retinal ganglion cells (Buckley et al., 1998; Lu et al., 2000; López et al., 2011). While WIN has a highly selectivity for CB1Rs, WIN also exhibits a modest degree of selectivity for CB2Rs (Pertwee, 1997; Luca et al., 2009; Ndong et al., 2011). We examined whether the WIN-induced suppression on the K⁺ currents may be mediated by activating CB2Rs. Fig. 3C (left panel) shows that perfusion of 100 nM AM630, a CB2R selective antagonist, did not change the K⁺ current amplitude in a retinal ganglion cell, but the addition of WIN (2 μM) persisted to reduce the current amplitude. Data obtained from five cells revealed that the average K⁺ current amplitude was unchanged following the application of AM630 (97.9 ± 0.7% of control, P > 0.05), but reduced to 72.9 ± 2.9% of control when WIN was added (P < 0.001) (Fig. 3C, right panel). Similar results were obtained when the concentration of AM630 was
Fig. 1. CB1R agonists induced suppression of outward K⁺ currents in rat retinal ganglion cells. (A) Representative outward K⁺ currents recorded from a retinal ganglion cell, showing that extracellular application of WIN55212-2 (WIN, 2 μM) reversibly reduced the current amplitudes. The currents were evoked by a series of voltage pulses (from −70 mV to +30 mV in increments of 10 mV). Bar chart summarizing the changes of K⁺ current amplitudes at +30 mV. n = 8, ***P < 0.001 vs control. (B) Concentration-dependent suppression of outward K⁺ current amplitudes by WIN (n = 3–7 for each WIN concentration). (C) Representative outward K⁺ currents recorded from another retinal ganglion cell, showing anandamide (AEA), an eCB, induced suppression of the current (left panel). The current was induced by a voltage step from −70 mV to +30 mV. Bar chart summarizing the changes in K⁺ current amplitudes at +30 mV after application of AEA (right panel). n = 4, ***P < 0.001 vs control. All data are normalized to control and presented as mean ± S.E.M.
increased to 1 μM (data not shown). These results suggest that CB1Rs/CB2Rs are unlikely involved in the WIN-induced suppression of K⁺ currents.

Activation of CB1Rs/CB2Rs by WIN is negatively coupled to adenylate cyclase (AC), thus down-regulating the cAMP-protein kinase A (PKA) signaling pathway (Howlett and Fleming, 1984; Howlett and Mukhopadhyay, 2000). If the effect of WIN on the K⁺ currents is not mediated by activating CB1Rs/CB2Rs, the intracellular cAMP-PKA signaling pathway should not modify the WIN effect. This was experimentally confirmed. In the result of such an experiment shown in Fig. 3D (left panel), following 20-min pre-incubation of H-89 (2 μM), a PKA inhibitor, the K⁺ current was first recorded as control. Extracellular perfusion of WIN (2 μM) reduced the current amplitude to 0.80 nA from 1.15 nA. Cumulative data showed that the average current amplitude was reduced to 72.3 ± 2.9% of Fig. 2.
control \((n = 6, P < 0.001)\) in the presence of \(2 \mu M\) WIN, and then returned to \(102 \pm 4.6\%\) of control \((P > 0.05\) vs control) by washout (Fig. 3D, right panel). Similarly, Rp-cAMP \((10 \mu M)\), another PKA inhibitor, also failed to block the WIN-induced suppression of the retinal ganglion cell \(K^+\) current (Fig. 3E).

That is, following 2-min application of \(2 \mu M\) WIN, the average current amplitude was reduced to \(72.6 \pm 1.8\%\) of control \((n = 6, P < 0.001)\) in the presence of \(10 \mu M\) Rp-cAMP (Fig. 3E, right panel), which was comparable to that obtained in the absence of Rp-cAMP \((69.6 \pm 9.6\%)\).

Stimulation of cannabinoid receptors by WIN may also lead to the activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (Turu and Hunyady, 2010; Gomez et al., 2011; Hwangpo et al., 2012; Franklin and Carrasco, 2013; Sun et al., 2013). We examined if this signaling pathway may be involved in the WIN-induced suppression of \(K^+\) currents in retinal ganglion cells. As shown in Fig. 3F (left panel), the cell was first pre-incubated with \(U0126\) \((10 \mu M)\), a selective inhibitor of the MAPK/ERK1/2 signaling pathway (Favata et al., 1998; Compagnucci et al., 2013), for 20 min, then the \(K^+\) currents were recorded (control). Extracellular perfusion of WIN \((2 \mu M)\) still reversibly suppressed the \(K^+\) currents in the presence of \(U0126\) \((10 \mu M)\), a MAPK/ERK signaling pathway inhibitor (left panel). Bar chart summarizing the changes of \(K^+\) current amplitudes at \(+30\) mV after WIN application in the presence of \(U0126\) \((n = 9)\) (right panel). All data are normalized to control and presented as mean ± S.E.M. \(*P < 0.01\) and \(* * * P < 0.001\) vs control.

Fig. 3. WIN-induced suppression of outward \(K^+\) currents was independent of CB1R/CB2R. (A, B) Sample current traces show that AM251 \((2 \mu M)\) (A) and SR141716 \((SR, 1 \mu M)\) (B), selective CB1R antagonists, failed to block the WIN effects on \(K^+\) currents recorded from two different retinal ganglion cells (left panel). Cumulative data \((n = 8, for AM251; n = 5, for SR)\) are shown in right panel. (C) Sample current traces show that AM630 \((100 \text{nM})\), a selective CB2R antagonist, did not block the WIN \((2 \mu M)\)-induced suppression of \(K^+\) current recorded in a retinal ganglion cell (left panel). Bar chart summarizing the changes of \(K^+\) current amplitudes at \(+30\) mV caused by AM630 and AM630 plus WIN \((n = 5)\) (right panel). (D) Sample current traces recorded from a retinal ganglion cell show that WIN \((2 \mu M)\) reversibly suppressed the \(K^+\) currents in the presence of H-89 \((2 \mu M)\), a PKA inhibitor (left panel). Bar chart summarizing the changes of \(K^+\) current amplitudes at \(+30\) mV \((n = 6)\) (right panel). (E) Representative current traces recorded from a retinal ganglion cell, showing that WIN \((2 \mu M)\) suppressed the current amplitude in the presence of Rp-cAMP \((10 \mu M)\), another PKA inhibitor (left panel). Bar chart summarizing the changes of \(K^+\) current amplitudes at \(+30\) mV after WIN application in the presence of Rp-cAMP \((n = 4)\) (right panel). (F) Sample current traces recorded from a retinal ganglion cell show that WIN \((2 \mu M)\) reversibly suppressed the \(K^+\) currents in the presence of \(U0126\) \((10 \mu M)\), a MAPK/ERK signaling pathway inhibitor (left panel). Bar chart summarizing the changes of \(K^+\) current amplitudes at \(+30\) mV \((n = 9)\) (right panel). All data are normalized to control and presented as mean ± S.E.M. \(*P < 0.01\) and \(* * * P < 0.001\) vs control.
Fig. 4. AEA-induced suppression of outward K⁺ currents was independent of CB1R/CB2R. (A) Sample current traces show that AM251 (2 μM), a selective CB1R antagonists, failed to block the AEA (10 μM) effects on K⁺ currents recorded from a retinal ganglion cell (left panel). Bar chart summarizing the changes of K⁺ current amplitudes at +30 mV caused by AM251 applied alone or along with AEA (n = 5) (right panel). (B) Sample current traces show that AM630 (100 nM), a selective CB2R antagonists, failed to block the AEA (10 μM) effects on K⁺ currents recorded from another retinal ganglion cell (left panel). Bar chart summarizing the changes of K⁺ current amplitudes at +30 mV caused by AM630 alone or along with AEA (n = 6) (right panel). (C) Sample current traces show that SR141716 (SR, 1 μM) failed to block the ACEA (1 μM) effects on K⁺ currents recorded from a retinal ganglion cell (left panel). Bar chart summarizing the changes of K⁺ current amplitudes at +30 mV caused by SR applied alone or along with ACEA (n = 5) (right panel). (D) Sample current traces show that AM630 (100 nM) failed to block the CB65 (500 nM) effects on K⁺ currents recorded from a retinal ganglion cell (left panel). Bar chart summarizing the changes of K⁺ current amplitudes at +30 mV caused by AM630 applied alone or along with CB65 (n = 7) (right panel). All data are normalized to control and presented as mean ± S.E.M. **P < 0.01 and ***P < 0.001 vs control.
We also tested the effect of AEA, an endogenous ligand of CB1R/CB2R, on the K⁺ currents in the presence of CB1R and CB2R antagonists. As shown in Fig. 4A, co-application of AM251 (2 μM) and AEA (10 μM) still suppressed the K⁺ current, with an average current amplitude at +30 mV being 54.1 ± 7.3% of control (n = 5, P < 0.01) (Fig. 4A, right panel), which was comparable to that obtained in the absence of AM251 (Fig. 1C). Similarly, AM630 also failed to block the AEA effect on K⁺ currents (Fig. 4B, left panel). In the presence of AM630 (100 nM), the average current amplitude at +30 mV was reduced to 60.9 ± 10.7% of control (n = 6, P < 0.01) by adding 10 μM AEA (Fig. 4B, right panel).

To further confirm the cannabinoid receptor-independent modulation of the K⁺ currents in rat retinal ganglion cells, we examined the effects of highly specific CB1R agonist ACEA and CB2R agonist CB65 on the K⁺ currents. Fig. 4C (left panel) shows that in the presence of SR141716 (1 μM), extracellular perfusion of ACEA (1 μM) persisted to reduce the K⁺ current amplitude. The average current amplitude at +30 mV was 96.0 ± 5.5% of control (n = 5, P > 0.05) in the presence of SR141716, and reduced to 54.4 ± 6.1% of control (n = 5, P < 0.001) by adding ACEA (Fig. 4C, right panel). Similarly, perfusion of CB65 (500 nM) also suppressed the K⁺ current in the presence of AM630 (100 nM) (Fig. 4D, left panel), with the average being reduced from 98.7 ± 3.7% of control (n = 7, P > 0.05) in the presence of AM630 to 68.7 ± 7.0% of control (n = 7, P < 0.001) by adding CB65 (Fig. 4D, right panel).

**Intracellular dialysis of WIN did not influence outward K⁺ currents**

Since WIN is liposoluble, there is a possibility that WIN exerted its effect on the K⁺ currents by modulating intracellular pathways, associated with these channels. To examine this possibility, outward K⁺ currents of retinal ganglion cells were monitored when WIN (2 μM) was dialyzed into these cells through recording pipettes. In normal bath solution, immediately following the formation of whole-cell recording configuration in retinal ganglion cells, the outward K⁺ currents recorded tended to decrease in amplitudes slightly in the first 2 min (93.0 ± 3.9% of control in 2 min, n = 8), but did not show further decrease (Fig. 5A). In WIN-dialyzed cells, similar changes in K⁺ currents were observed (Fig. 5A, open circles), which were not significantly different from those obtained in normal retinal ganglion cells. It should be mentioned that extracellular application of WIN (2 μM) did suppress the outward K⁺ currents in the WIN-dialyzed cells (n = 8), as shown in Fig. 5B.

**DISCUSSION**

WIN-induced suppression of outward K⁺ currents of rat retinal ganglion cells is not mediated by CB1R/CB2R

While WIN was reported to inhibit voltage-gated Ca²⁺ channels in the purified culture of rat retinal ganglion cells (Lalonde et al., 2006), the present work is the first one to show that WIN suppresses outward K⁺ currents in a dose-dependent manner, with an IC₅₀ of 4.7 μM. We further demonstrated that in the three major components contributing to the outward K⁺ currents of rat retinal ganglion cells (Lipton and Tauck, 1987; Ettaiche et al., 2001; Fohlmeister et al., 2010; Koebel et al., 2010; and Fig. 3) it was the TEA-sensitive one, that was mainly suppressed by WIN. Under our experimental conditions, TEA-sensitive current component may be mediated by BKCa (Lipton and Tauck, 1987; Reiff and Guenther, 1999). We could not rule out a possibility that WIN may also affect other current components. It has been indeed reported that WIN may suppress the delayed rectifier K⁺ and TASK-1 currents in a CB1R/CB2R-independent manner in smooth muscle cells (Van den Bossche and Vanheel, 2000; Maingret et al., 2001).

A most significant finding in this work is that the WIN-induced suppression of outward K⁺ currents of rat retinal ganglion cells was not blocked by CB1R and CB2R antagonists (Fig. 3A–C), strongly suggesting that the WIN effect was not mediated by the activation of CB1Rs and CB2Rs, even though these two receptors are indeed expressed in these cells. Consistent with this, inhibition of the cAMP-PKA signaling pathway by H-89 and Rp-cAMP, or inhibition of MAPK/ERK signaling pathway by U0126, failed to block the WIN effect. Moreover, AEA, ACEA and CB65 also suppressed the K⁺ currents in the presence of CB1R/CB2R antagonists (Fig. 4A–D), further suggesting that eCBs may modulate the K⁺ channels in rat retinal ganglion cells in a CB1R/CB2R-independent manner.

It has been reported that eCBs, at pharmacological concentrations, may regulate cell functional properties through modulating various K⁺ channels in a receptor-independent manner (Poling et al., 1996; Van den Bossche and Vanheel, 2000; Maingret et al., 2001; Oz, 2006a,b; Mato et al., 2009; Amorós et al., 2010; Barana et al., 2010). Specifically, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), a phytocannabinoid, AEA and 2-AG, endogenous cannabinoid receptor agonists, inhibited Shaker-related Kv1.2, human cardiac Kv1.5 and Kv4.3 channels expressed in the cell lines, and the effects could not be blocked by CB1R antagonists SR141716A and rimonabant, and CB2R antagonist AM630 (Poling et al., 1996; Amorós et al., 2010; Barana et al., 2010). It is of interest that in those studies the IC₅₀ values (2.7 μM for the AEA effect and 2.4 μM for the Δ⁹-THC effect) are comparable to that for the WIN effect on outward K⁺ currents reported in the present work (4.7 μM). Moreover, in smooth muscle cells of rat aorta and cerebellar granule neurons, AEA, methanandamide and WIN were demonstrated to suppress the delayed rectifier K⁺ and TASK-1 currents in a CB1R/CB2R-independent manner (Van den Bossche and Vanheel, 2000; Maingret et al., 2001). Nevertheless, our finding raises two interesting points. First, both receptor-dependent and -independent modulation of the same channel by cannabinoids/CB1R agonists was previously reported in other cells. For instance, in rat sympathetic
neurons injected with cDNA encoding a human CB1R. AEA inhibits Ca\(^{2+}\) currents in both CB1R-dependent and -independent mechanisms (Guo and Ikeda, 2004). In oligodendrocytes cannabinoids inhibit depolarization-evoked Ca\(^{2+}\) transients via CB1R-independent and -dependent mechanisms that involve the activation of pertussis toxin (PTX)-sensitive \(G_{i/o}\) proteins and the blockade of Kir channels (Mato et al., 2009). However, in this work we demonstrated that cannabinoids modulate K\(^+\) channels in rat retinal ganglion cells via CB1R-independent mechanism, unlike cannabinoid-induced modulation of Ca\(^{2+}\) channels, which is CB1R-dependent (Lalonde et al., 2006). Secondly, mechanisms underlying WIN-induced modulation of K\(^+\) channels may be distinct among different types of retinal neurons. In both photoreceptors and BCs modulation by WIN of K\(^+\) currents was CB1R-dependent (Yazulla et al., 2000; Fan and Yazulla, 2003). In rat retinal ganglion cells, however, WIN-induced suppression was independent of CB1R/CB2R. Therefore, it should be cautious to generalize the results concerning WIN-induced effects obtained in a specific neuronal type, even in the same tissue.

**Possible mechanisms of WIN-induced suppression of K\(^+\) currents**

Like eCBs and other cannabinoid receptor agonists, WIN is a polyunsaturated fatty acid-based lipid molecule. Due to similarities in their chemical structures and biophysical properties, WIN may share similar mechanisms with those lipid molecules for its direct action on the K\(^+\) channels of rat retinal ganglion cells. WIN may enter to cytoplasm and directly inhibit the K\(^+\) channels, or non-specifically modulate intracellular signaling pathways, thus inhibiting the K\(^+\) channels (Poling et al., 1996; Wang et al., 2012). This possibility seems unlikely since the inhibitory effect of WIN on the K\(^+\) currents was quickly eliminated by normal bath solution washout and intracellular dialysis of WIN did not suppress the K\(^+\) currents (Fig. 5). Therefore, we speculate that WIN may bind to extracellular sites of the
K+ channels and allosterically change the gating properties of the channels. It was previously reported that AEA (IC50, 2.7 μM) blocked Kv1.2 channels expressed in murine fibroblasts (B82 cell line) (Poling et al., 1996). Moreover, AEA suppressed the Kv3.2 channels in smooth muscle cells of the rat aorta (Van den Bossche and Vanheel, 2000) and expressed in Xenopus oocytes (Oliver et al., 2004). All these effects of AEA have been shown to be independent of CB1Rs and thought to be an allosteric action of this agonist on the gating properties of these channels from the extracellular side.

In summary, the present work demonstrates that WIN suppresses the TEA-sensitive outward K+ current component of rat retinal ganglion cells, which is independent of CB1Rs/CB2Rs. Such modulation may be involved in regulation of the retinal ganglion cell excitability, thus influencing the output signals of these cells.

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