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Δ^9-Tetrahydrocannabinol and Endogenous Cannabinoid Anandamide Directly Potentiate the Function of Glycine Receptors

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ABSTRACT

Anandamide (AEA) and Δ^9-tetrahydrocannabinol (THC) are endogenous and exogenous ligands, respectively, for cannabinoid receptors. Whereas most of the pharmacological actions of cannabinoids are mediated by CB1 receptors, there is also evidence that these compounds can produce effects that are not mediated by the activation of identified cannabinoid receptors. Here, we report that THC and AEA, in a CB1 receptor-independent manner, cause a significant potentiation of the amplitudes of glycine-activated currents (I_{Gly}) in acutely isolated neurons from rat ventral tegmental area (VTA) and in *Xenopus laevis* oocytes expressing human homomeric (α1) and heteromeric ($\alpha1\beta1$) subunits of glycine receptors (GlyRs). The potentiation of I_{Gly} by THC and AEA is concentration-dependent, with respective EC_{50} values of 86 $\pm$ 9 and 319 $\pm$ 31 nM for α1 homomeric receptors, 73 $\pm$ 8 and 318 $\pm$ 24 nM for $\alpha1\beta1$ heteromeric receptors, and 115 $\pm$ 13 and 230 $\pm$ 29 nM for native GlyRs in VTA neurons. The effects of THC and AEA are selective for I_{Gly}, because GABA-activated current in VTA neurons or in *X. laevis* oocytes expressing $\alpha2\beta3\gamma2$ $GABA_A$ receptor subunits were unaffected by these compounds. The maximal potentiation by THC and AEA was observed at the lowest concentration of glycine; with increasing concentrations of glycine, the potentiation significantly decreased. The site for THC and AEA seems to be distinct from that of the alcohol and volatile anesthetics. The results indicate that THC and AEA, in pharmacologically relevant concentrations, directly potentiate the function of GlyRs through an allosteric mechanism.

Cannabis is the most commonly used illicit psychotropic substance in the world. The psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), and endogenously produced cannabinoid anandamide (AEA) have widespread actions in the brain (Howlett et al., 2002): in the hippocampus, it influences learning and memory (Gerdeman et al., 2002; Howlett et al., 2002); in the basal ganglia and hypothalamus, it modulates locomotor activity, food intake, and reward pathways (Gerdeman et al., 2002; Tanda and Goldberg, 2003); and in the spinal cord and supra spinal sites, it has antinociceptive effects (Walker and Huang, 2002; Cravatt and Lichtman, 2004). In the nervous system, these effects of cannabinoids are mediated primarily by the activation of the G-protein-coupled cannabinoid CB1 receptors (Gerdeman et al., 2002; Howlett et al., 2002). However, several recent studies indicate that these compounds can produce effects that are not mediated by the activation of identified cannabinoid receptors (Venance et al., 1995; Poling et al., 1996; Oz et al., 2000, 2002; Maingret et al., 2001; Barann et al., 2002; Di Marzo et al., 2002; Oz et al., 2003, 2006; Oliver et al., 2004). For example, it has been demonstrated that THC inhibits the function of serotonin type 3 ($5\text{-}HT_3$) receptor in a cannabinoid receptor-independent manner (Barann et al., 2002). Likewise, AEA can inhibit the function of gap junctions (Venance et al., 1995), voltage-dependent Ca^{2+} channels (Oz et al., 2000), various types of K^+ channels (Munro et al., 1993; Poling et al., 1996; Oliver et al., 2004), $5\text{-}HT_3$ receptor (Fan, 1995), and nicotinic acetylcholine (nACh) receptors (Oz et al., 2003). This suggests that addi-

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ABBREVIATIONS: THC, Δ^9-tetrahydrocannabinol; I_{Gly}, glycine-activated current; VTA, ventral tegmental area; AEA, anandamide; GlyR, glycine receptor; $5\text{-}HT_3$, serotonin type 3; nACh, nicotinic acetylcholine; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM404, *N*-(4-hydroxyphenyl) arachidonoylamide.

tional molecular targets for cannabinoids exist in the central nervous system and that these targets may represent important sites for cannabinoids to alter neuronal function.

The glycine receptors (GlyRs) belong to the cys-loop superfamily, which comprises both cationic receptors such as nACh and 5-HT_3 receptors and anionic receptors such as GABA type A ($GABA_A$) GlyRs (Lester et al., 2004; Lynch, 2004). The GlyR consists of α and β subunits, which combine to form a pentameric receptor complex. Whereas the α1 subunit is expressed mainly in the spinal cord, the α2 subunit was found in several brain regions, including layer VI of the cerebral cortex, hippocampus, and ventral tegmental areas (VTA) (Betz et al., 1999). The anatomical locations of GlyRs and the potential involvement of GlyRs in pain transmission (Betz et al., 1999; Lynch, 2004) and dopamine release from VTA neurons (Molander and Soderpalm, 2005a,b) suggest that these receptors can play a role in analgesia and drug addiction.

To date, although direct effects of THC and AEA have been demonstrated on cationic (nACh and 5-HT_3) receptors (Fan, 1995; Barann et al., 2002; Oz et al., 2002, 2003), such an interaction between these cannabinoids and anionic ($GABA_A$ and glycine) receptors has not been described. Here, we report that THC and AEA in pharmacologically relevant concentrations directly potentiate the function of native GlyRs in rat VTA neurons and recombinant human GlyRs expressed in *Xenopus laevis* oocytes.

Materials and Methods

Site-Directed Mutagenesis. Point mutations of a cloned human glycine α1 subunit were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The authenticity of the DNA sequence through the mutation sites was confirmed by double-strand DNA sequencing using an ABI Prism 377 Automatic DNA Sequencer (Applied Biosystems, Foster City, CA).

Preparation of cRNAs and Expression of Receptors. cRNAs were synthesized in vitro from linearized template cDNAs with a mMESSAGE mMACHINE RNA transcription kit (Ambion, Austin, TX). The quality and sizes of synthesized cRNAs were confirmed by denatured RNA agarose gels. Mature female *X. laevis* frogs were anesthetized by submersion in 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis MO), and oocytes were surgically excised. Oocytes were separated, and the follicular cell layer was removed by treatment with type I collagenase (Boehringer Mannheim, Indianapolis, IN) for 2 h at room temperature. Each oocyte was injected with 20 ng of cRNA in 20 nl of diethylpyrocarbonate-treated water and was incubated at 19°C in modified Barth's solution: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 10 mM HEPES, pH 7.4.

Recording from *X. laevis* Oocytes. After incubation for 2 to 5 days, oocytes were studied at room temperature (20–22°C) in a 90-μl chamber. The oocytes were superfused with modified Barth's solution at a rate of 6 ml/min. Agonists and chemical agents were diluted in the bath solution and applied to the oocytes for a specified time using a solenoid valve controlled superfusion system. Agonists and other chemical agents were diluted either directly in the bath solution or dissolved in ethanol before dilution. The final ethanol concentration was less than 0.7 mM. Membrane currents were recorded by two-electrode voltage clamp at a holding potential of -70 mV using a Gene Clamp 500 amplifier (Axon Instruments Inc., Burlingame, CA). The recording microelectrodes were filled with 3 M KCl and had electrical resistances of 0.5 to 3.0 MΩ. Data were routinely recorded on a chart recorder (Gould 2300S; Gould Inc., Cleveland, OH). Average values are expressed as means $\pm$ S.E.

Neuron Dissociation. Neurons were freshly isolated from the VTA of Sprague-Dawley rats aged between 4 and 15 postnatal days. The VTA neurons were prepared as described previously (Ye et al., 2005). In brief, rats were decapitated, and the midbrain was isolated and transversely sliced (400 μm) with a Vibroslice (Campden Instruments, Leicester, UK) or a VF-100 slicer (Precisionary Instruments, Greenville, NC) while kept in ice-cold artificial cerebrospinal fluid saturated with 95% O_2/5% CO_2 containing 126 mM NaCl, 1.6 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose. Slices were transferred to the standard external solution (see below for components) saturated with O_2 containing 1 mg pronase/6 ml and incubated (31°C) for 20 min. After an additional 20-min incubation in 1 mg thermolysin/6 ml, the VTA was identified under a dissecting microscope as the region medial to the accessory optic tract and lateral to the fasciculus retroflexus. Micropunches of the VTA were isolated and transferred to a 35-mm culture dish. Mild trituration of these tissue punches through heat-polished pipettes of progressively smaller-tip diameters dissociated single neurons. Isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments 20 min after the trituration.

Electrophysiological Recording. Whole-cell patch-clamp techniques were used to record electrical activity with an Axopatch 200B amplifier, a Digidata 1320A analog-to-digital converter, and Clampex 9 (all from Axon Instruments). Data were sampled at 5 kHz and filtered at 1 kHz. All currents were recorded with standard external solution containing 140 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.4 with 1 N NaOH. The junction potential between the pipette and the bath solutions was nullified just before gigaseal formation. The patch electrodes had a resistance of 3 to 5 MΩ, when filled with the following: 140 mM CsCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM GTP, the pH was adjusted to 7.2 with KOH, and the osmolarity was adjusted to 280 to 300 mOsm with sucrose. All recordings were performed at room temperature (22–24°C).

Data Analysis. Statistical analysis of concentration-response data were performed with the use of the nonlinear curve-fitting program ALLFIT. Data were fitted to the logistic equation $Y = \{(E_{max} - E_{min})/[1 + (X/EC_{50})^{nH}]\} + E_{min}$, where X and Y are concentration and response, respectively; E_{max} and E_{min} are the maximal and minimal responses, respectively, EC_{50} is the half-maximal concentration; and n_H is the slope (apparent Hill coefficient). Data were statistically compared by the paired t test or analysis of variance, as noted. Average values are expressed as mean $\pm$ S.E.

Chemicals and Application. Most of the chemicals, including glycine and GABA, were from Sigma. Solutions were prepared on the day of the experiment and applied to neurons using a "Y- tube" perfusion system (Ye et al., 2005). A perfusion pipette with a diameter of 50 μm was placed 50 to 100 μm away from the neuron. With this perfusion system, solutions in the vicinity of a neuron can be completely exchanged within 20 ms without a loss of mechanical stability.

Results

Although the α subunit alone is sufficient to form a homomeric functional channel, native GlyRs are believed to consist of both α and β subunits (Lynch, 2004). In this regard, we expressed both homomeric (α1 subunit alone) and heteromeric (α1 with β1 subunits) GlyRs in *X. laevis* oocytes. The EC_{50} and slope values for glycine were 85 $\pm$ 8 μM and 1.2 $\pm$ 0.3 for the α1 homomeric receptors and 141 $\pm$ 18 μM and 1.1 $\pm$ 0.2 for the $\alpha1\beta1$ heteromeric receptors, respectively. Consistent with previous observations (Betz et al., 1999; Lynch, 2004), the heteromeric GlyRs were less sensitive to picrotoxin, a chloride-channel blocker. Picrotoxin at 100 μM

inhibited 75 ± 3% of I_{Gly} activated by glycine at EC_{50} values in cells expressing the α1 homomers and 25 ± 2% of I_{Gly} activated by glycine at EC_{50} values in cells expressing the $\alpha1\beta1$ heteromers.

Application of AEA (300 nM) or THC (300 nM) for 15 s did not alter baseline holding currents in oocytes expressing homomeric or heteromeric GlyRs, suggesting that, under control conditions, these compounds do not alter endogenously expressed ionic conductances in oocytes, and they do not have an agonistic activity on GlyRs. In the first series of experiments, we have investigated the effects of THC (300 nM) or AEA (300 nM) on glycine-activated currents (I_{Gly}). The peak amplitudes of currents activated by 3 μM glycine were markedly increased when glycine was coapplied with THC or AEA in oocytes expressing α1 homomers (Fig. 1A) and $\alpha1\beta1$ heteromers (Fig. 1B). In the continued presence of glycine, I_{Gly} reached steady-state levels after the peak amplitude (Fig. 1C). During this plateau phase, the addition of THC or AEA increased the amplitudes of I_{Gly} in oocytes expressing homomeric and heteromeric GlyRs (Fig. 1, C and D). Both THC and AEA increased the amplitude of I_{Gly} in a reversible and concentration-dependent manner. The EC_{50} values for THC and AEA were 86 ± 9 and 319 ± 31 nM, respectively, for the α1 homomers (Fig. 2A) and 73 ± 8 and 318 ± 24 nM, respectively, for the $\alpha1\beta1$ heteromers (Fig. 2B).

To exclude the possibility that the THC and AEA-induced potentiation of GlyRs was mediated by CB1 receptors, we tested the effects of the CB1 receptor antagonist SR141716A on the THC and AEA potentiation of GlyR-mediated currents (Fig. 3). In cells expressing α1 homomeric GlyRs, applications of SR141716A alone at 1 μM did not significantly alter the amplitude of I_{Gly} induced by 3 μM glycine (5 ± 7% of control; $P > 0.1$, unpaired t test, $n = 5$). Likewise, the pretreatment with SR141716A at 1 μM did not significantly alter the AEA- and THC-induced potentiation of I_{Gly} in cells expressing α1 homomeric GlyRs (Fig. 3; $P > 0.1$, unpaired t test, $n = 5$). In addition, we tested the effect of AM404, an anandamide membrane transport inhibitor, on the THC and AEA potentiation of I_{Gly}. Application of 1 μM AM404 did not significantly affect the magnitude of THC- and AEA-induced potentiating effects on I_{Gly}.

We next examined whether THC and AEA could potentiate I_{Gly} in acutely dissociated rat VTA neurons. As demonstrated in Fig. 4A, SR141716A at 1 μM alone did not change the holding current of VTA neurons, nor did it alter the amplitude of currents activated by 5 μM glycine. After 15-s preincubation with SR141716A, 300 nM THC continued to potentiate I_{Gly} induced by 5 μM glycine (Fig. 4B). THC-induced potentiation was fully reversible upon cessation of THC application and remained unaltered in the absence (60 ± 15%) and presence of 1 μM SR141716A (53 ± 14%; $n = 5$, $P = 0.38$). These results indicate that THC-induced potentiation of I_{Gly} is independent of CB1 receptors. AEA at 300 nM also increased I_{Gly} of a VTA neuron pretreated with SR141716A

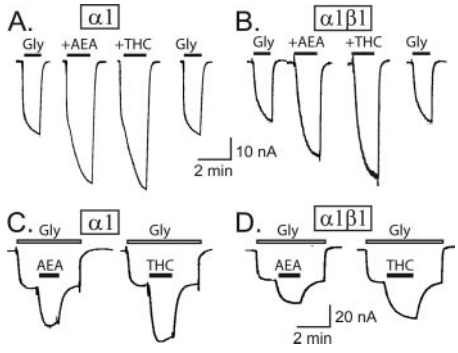

Fig. 1. THC and AEA potentiation of human recombinant GlyRs. A, trace records showing THC and AEA (300 nM) enhancement of amplitude of currents activated by 3 μM glycine in oocytes expressing the α1 subunits. B, trace records showing THC and AEA (300 nM) enhancement of amplitude of currents activated by 5 μM glycine in oocytes expressing the $\alpha1\beta1$ subunits. C, trace records showing THC and AEA (300 nM) enhancement of amplitude of steady-state current activated by 3 μM glycine in oocytes expressing the α1 subunits. D, trace records showing THC and AEA (300 nM) enhancement of steady state of currents activated by 5 μM glycine in oocytes expressing the $\alpha1\beta1$ subunits. The open bar on the top of each record indicates the time of glycine application.

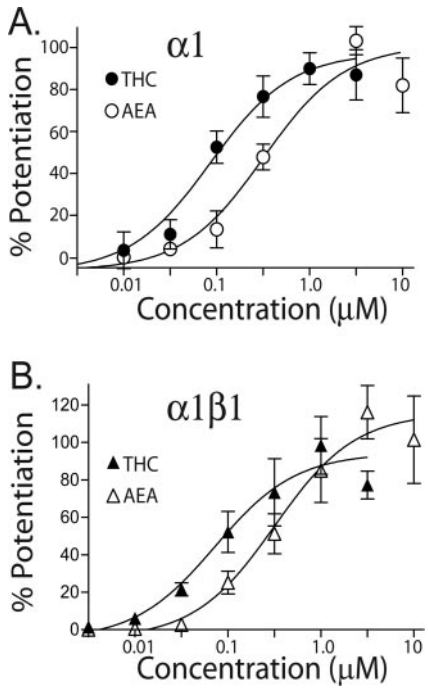

Fig. 2. The concentration-response curves of THC and AEA potentiation of the α1 and $\alpha1\beta1$ subunits. A and B, graph plotting the average percentage of potentiation by THC and AEA as a function of THC and AEA concentration. Each data point represents the average of 5 to 10 oocytes. The curves shown are the best fits of the data using the Hill equation described under *Materials and Methods*. Error bars not visible are smaller than the size of the symbols.

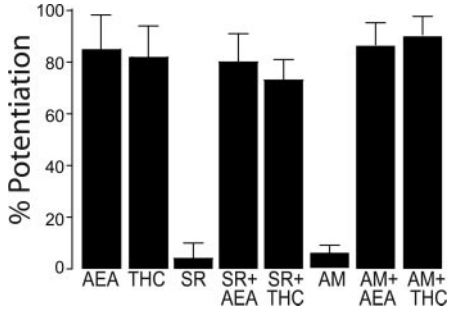

Fig. 3. Effects of SR141716A and AM404 on THC and AEA potentiation of I_{Gly}. The solid bar graphs shown plot the average percentage of potentiation by 300 nM AEA and THC of I_{Gly} activated by 3 μM glycine in the presence and absence of 1 μM SR141716A. The oocytes expressing the α1 subunits were preincubated with SR141716A or AM404 for 15 s before exposure to a mixture of THC plus glycine or AEA plus glycine. Each data point represents mean ± S.E. of five to nine oocytes.

(Fig. 4C). The magnitude of the AEA-induced potentiation of I_{Gly} was similar in the absence and presence of SR141716A (17 ± 8 vs. 20 ± 4%, $n = 4–8$, $P = 0.13$). Both THC and AEA potentiated I_{Gly} in a concentration-dependent manner over a concentration range of 10 to 3000 nM (Fig. 4D). However, THC was more potent than AEA to potentiate I_{Gly}. The EC_{50} values for THC and AEA potentiation were 115 ± 13 and 230 ± 29 nM, respectively. The maximal potentiation by THC and AEA were 55 ± 5 and 20 ± 4%, respectively. These values are significantly different ($n = 5–6$, $P < 0.001$).

$GABA_A$ receptors are the major inhibitory neurotransmitter-gated ion channels in the brain, and they are functionally and structurally similar to GlyRs (Lynch, 2004). For this reason, we next investigated whether THC could also modulate $GABA_A$ receptor function. Neither THC nor AEA at 300 nM significantly altered the amplitude of currents activated by 3 μM GABA in *X. laevis* oocytes expressing $\alpha2\beta3\gamma2$ subunits of $GABA_A$ receptor (Fig. 5A). We also observed that THC at 300 nM did not affect currents activated by various submaximal concentrations of GABA I_{GABA} in isolated VTA neurons (Fig. 5B). In the presence of THC, the magnitudes of GABA-activated currents were 97 ± 2% of control at 1 μM GABA ($n = 3$), 99 ± 2% of control at 3 μM GABA ($n = 5$), 97 ± 2% of control at 10 μM GABA ($n = 4$), and 98 ± 2% of control at 30 μM GABA ($n = 4$). These values are not significantly different ($P > 0.1$).

Next, we examined the effects of increasing glycine concentration on the THC and AEA potentiation of I_{Gly}. In *X. laevis* oocytes expressing the α1 GlyR homomers, the magnitude of THC- and AEA-induced potentiation of I_{Gly} decreased significantly with increasing concentrations of glycine (Fig. 6A). The magnitudes of potentiation by 300 nM THC and AEA were 76 ± 9 and 48 ± 6% at 3 μM glycine, respectively, and 3 ± 1 and 7 ± 8% at 30 μM glycine, respectively, in *X. laevis* oocytes expressing the α1 subunits (Fig. 6B; $P < 0.01$, unpaired t test, $n = 5$). The dependence on agonist concentration was also evident in THC potentiation of I_{Gly} in VTA neurons. A fixed concentration of THC (300 nM) was applied with increasing glycine concentrations (from 5 to 30 μM). As illustrated in Fig. 6C, whereas the currents induced by 5 μM glycine were markedly enhanced by 300 nM THC, the I_{Gly} level induced by 30 μM glycine remained unchanged in the presence of THC (Fig. 6C). On average, 300 nM THC potentiated I_{Gly} induced by 5, 10, and 30 μM glycine by 51 ± 5%

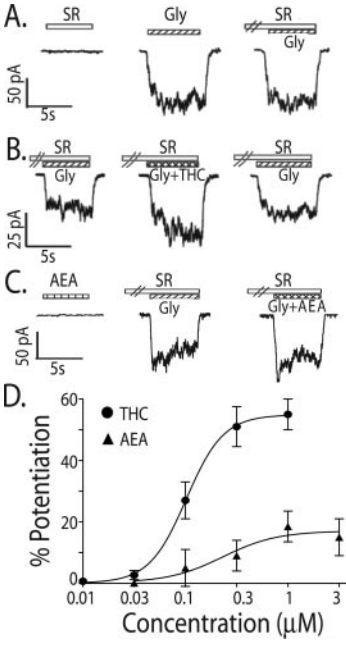

Fig. 4. THC and AEA potentiation of glycine-activated current in isolated VTA neurons. A, membrane currents recorded after 1 μM SR141716A (SR) application (left) after 5 μM glycine was applied alone and after coapplication of SR and glycine. B, I_{Gly} (induced by 5 μM glycine) recorded before (left), during (middle), and after (right) the application of 300 nM THC in the presence of 1 μM SR. The SR was applied 15 s before the application of the mixture of glycine + THC + SR. C, I_{Gly} (induced by 5 μM glycine) recorded before (left), during (middle), and after (right) the application of 300 nM AEA in the presence of 1 μM SR. The SR was applied 15 s before the application of the mixture of glycine + AEA + SR. D, concentration-response curves of the THC and AEA potentiation of I_{Gly} (activated by 5 μM glycine) in the presence of 1 μM SR. The error bars not visible are smaller than the size of the symbols.

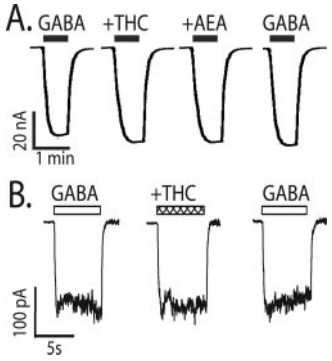

Fig. 5. THC and AEA do not affect I_{GABA}. A, trace records of currents activated by 3 μM GABA in the absence and presence of 300 nM THC and AEA in *X. laevis* oocytes expressing the α1 subunits. The solid bar on the top of each record indicates the time of drug application. B, traces of currents activated by 3 μM GABA in the absence and presence of 300 nM THC in a VTA neuron. The cell was preincubated with 1 μM SR141716A for 15 s.

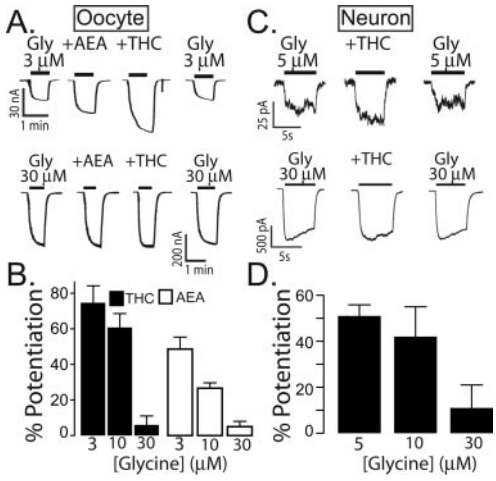

Fig. 6. Glycine concentration-dependence of THC and AEA potentiation. A, trace records showing THC (300 nM) and AEA (300 nM) enhancement of currents activated by 3 and 30 μM glycine in oocytes expressing the α1 subunits. B, average potentiation by THC and AEA of I_{Gly} activated by various concentrations of glycine in oocytes expressing the α1 subunits. Each bar represents the average of four to five oocytes. C, trace records showing THC enhancement of amplitude of currents activated by 5 and 30 μM glycine in VTA neurons. D, potentiation by THC of I_{Gly} by various concentrations of glycine in VTA neurons. Each bar represents the average of three to five cells.

($n = 8, P = 0.001$), 42 ± 13% ($n = 5, P = 0.005$), and 11 ± 10% ($n = 7, P = 0.2$), respectively (Fig. 6D).

The above observations have suggested that AEA and THC are likely to directly interact with glycine receptors. Previous studies have identified single amino acid residue at 267 in the transmembrane-2 domain of glycine receptor α subunits as a distinct site for a variety of volatile anesthetics, *n*-alcohols, and commonly abused inhalants (Mihic et al., 1997; Wick et al., 1998). Given that both THC and AEA are hydrophobic substance with massive volume, we proposed that the potentiation of I_{Gly} induced by AEA and THC may share a molecular basis similar to that of alcohol/anesthetics. If so, a point mutation at Ser267 should reduce AEA- and THC-induced potentiating effects of I_{Gly}. To test this hypothesis, we substituted a serine at 267 with an asparagine (glutamine) and injected cRNA into the oocytes. The S267Q mutation did not significantly alter the receptor's sensitivity to glycine (72 ± 9 versus 85 ± 8 μM; $n = 5, P = 0.5$). Consistent with previous reports (Mihic et al., 1997), whereas 100 mM EtOH significantly potentiated I_{Gly} by 91% (Fig. 7A), the S267Q mutation completely abolished the potentiating effect induced by 100 mM ethanol (Fig. 7, B and C), respectively (Mihic et al., 1997). However, the S267Q mutation did not significantly alter the potentiation induced by THC and AEA (Fig. 7, B and C; $n = 5, P = 0.2$).

Discussion

In this study, we have shown that THC and AEA induced a concentration-dependent potentiation of currents mediated by α1 homomeric and $\alpha1\beta1$ heteromeric GlyRs expressed in *X. laevis* oocytes or by native GlyRs in acutely isolated VTA neurons. THC seems to be more potent than AEA on both recombinant and native GlyRs. The EC_{50} values for the effect of THC on heteromeric GlyRs (73 nM) and native GlyRs (115 nM) are in the pharmacological ranges that induce psychotropic and antinociceptive effects in humans. For example, the plasma THC concentration was found to be 162 nM after 1 h of low-dose cannabis smoking (Huestis and Cone, 2004). Likewise, the EC_{50} values for the AEA potentiation of recombinant and native GlyRs are in the range of 230 to 318 nM, which is comparable with AEA's affinity (89–300 nM) for CB1 receptor (Howlett et al., 2002).

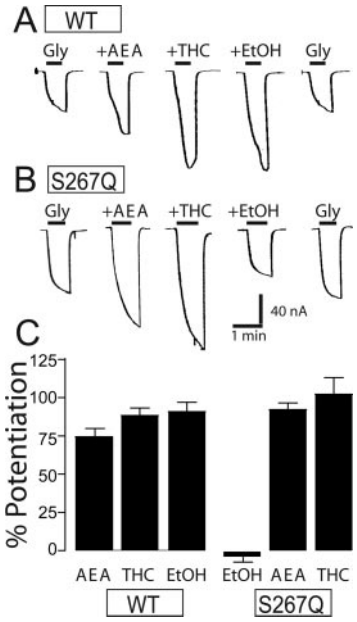

Fig. 7. The S267Q mutation abolished the potentiation by EtOH but not AEA and THC. A, trace records of current activated by 1 μM glycine in the presence of AEA (300 nM), THC (300 nM), and EtOH (300 mM) in oocytes expressing the wild-type α1 subunits. B, trace records of current activated by 1 μM glycine in the presence of THC, AEA, and EtOH in oocytes expressing the mutant S267Q subunits. C, average potentiation by AEA, THC, and EtOH of I_{Gly} in oocytes expressing the wild-type and mutant α1 subunits. Each bar represents the average of four to five oocytes.

AEA and THC, at the concentration range used in this study, have been shown to activate native cannabinoid receptors (Howlett et al., 2002). However, earlier studies indicate that the cloned cannabinoid receptors CB1 and CB2 are not expressed in *X. laevis* oocytes (Henry and Chavkin, 1995; Ho et al., 1999; Jin et al., 1999). Furthermore, SR141716A, a CB1 receptor antagonist, did not prevent the AEA or THC potentiation of I_{Gly} in both *X. laevis* oocytes and VTA neurons, suggesting that the potentiating effects induced by both THC and AEA are not mediated by CB1 receptors. We did not directly examine the ability of CB2 antagonists to block THC and AEA potentiation of I_{Gly} in VTA neurons, because CB2 receptors are not found in the brain (Howlett et al., 2002; Walker and Huang, 2002). Based on our data, we conclude that the ability of AEA and THC to potentiate GlyR-mediated currents does not result from the activation of identified cannabinoid receptors.

Our findings in VTA neurons are consistent with a recent study which reported that GlyR-mediated synaptic currents in hypoglossal motoneurons were potentiated directly by conditions that promote Ca^{2+}-dependent endocannabinoid production (Diana and Bregestovski, 2005). This result indicates that in native neurons, production of endocannabinoids potentiates the function of GlyRs in a CB1 receptor-independent mechanism. Likewise, another recent study has reported that endogenous cannabinoids modulated I_{Gly} in hippocampal neurons through a CB1-independent mechanism (Lozovaya et al., 2005). However, the authors of this article observed that AEA directly produced inhibition rather than potentiation of I_{Gly} in the presence of CB1/CB3 receptor antagonists. There are a number of reasons to explain the discrepancy between our and their observations, such as different compositions of glycine receptor subunits and different signal transduction pathways in presynaptic and postsynaptic neurons. In addition, the inhibition of I_{Gly} seemed to occur at high concentrations of glycine (100 μM) (Lozovaya et al., 2005), whereas THC and AEA potentiation reported in our study only occurred at low concentrations of glycine less than 5 μM. Collectively, these findings suggest that, under physiological conditions in which the activation of cannabinoid receptors cause a strong presynaptic inhibition, endocannabinoids can induce dual modulatory effects on neuronal excitability [i.e., presynaptic inhibition of the glycinergic synaptic transmission and the potentiation of postsynaptic (both synaptically and extrasynaptically located) GlyRs].

It is worth mentioning that neurons in the VTA contain vanilloid receptors, which are another possible noncannabinoid site of action for AEA (Di Marzo et al., 2002; Kim et al., 2005). However, it is unlikely that modulation of GlyR by THC and AEA observed in this study is mediated by endogenous vanilloid receptors. First, the EC_{50} value of THC- and

AEA-induced potentiating effects on GlyR are found to be at nanomolar concentrations, whereas AEA action on the vanilloid receptor is observed at submicromolar concentrations. Second, application of THC and AEA alone did not induce any detectable inward currents in cells voltage-clamped at −60 mV. Third, *X. laevis* oocytes have been widely used to express and study the function of recombinant vanilloid receptors, because these cells do not have endogenous vanilloid receptors (Premkumar and Ahern, 2000; Schumacher et al., 2000).

Recent studies using exogenously supplied glycine and specific inhibitors of glycine transporters have revealed that the glycine-binding site associated with *N*-methyl-D-aspartate receptors (which has 100-fold higher glycine affinity than GlyRs) is not saturated at the synapse (Gomeza et al., 2003; Bradaia et al., 2004; Eulenburg et al., 2005). Extracellular glycine concentrations in rat spinal cord were determined to be in the range of 2 to 3 μM, and the glycine content of cerebrospinal fluid was determined to be 6 μM (Whitehead et al., 2001). These findings indicate that synaptic and extrasynaptic glycine is often present at concentrations that produce only low occupancy of GlyRs. Therefore, cannabinoid-induced potentiation of GlyRs at low occupancy is likely to have physiological roles in neuronal excitability.

Neither AEA nor THC affected $GABA_A$ receptor-mediated responses, even though these receptors share structural and functional similarities with GlyRs (Lynch, 2004). Given that the $GABA_A$ $\alpha2\beta3\gamma2$ receptors are abundantly expressed in the central nervous system (Sigel and Kannenberg, 1996), the observation reported in this study suggests that direct actions of AEA and THC are relatively specific for GlyR. However, other cannabinoid receptor ligands, such as AM251 (1 μM), a CB1 receptor antagonist, caused significant (31%) inhibition of exogenous GABA-activated currents in basolateral amygdala neurons (Zhu and Lovinger, 2005). Thus, the effects of other cannabinoid receptor ligands on different $GABA_A$ receptor subunit combinations expressed in other brain regions cannot be excluded.

The role of GlyRs in pain transmission (Lynch, 2004) suggests another potential role for cannabinoid and endocannabinoid effects on the receptor. For instance, whereas THC-induced analgesic effects are completely abolished in the hot plate and formalin tests in the CB1 knockout mouse, THC-induced analgesia in the tail-flick test remains intact (Zimmer et al., 1999). On the other hand, AEA still exerts a cannabimimetic activity in all three analgesic tests in CB1 knockout mice, suggesting that not all cannabinoid and endocannabinoid-induced analgesic effects are mediated by CB1 receptors (Di Marzo et al., 2000). Future studies should address whether enhancement of GlyR function by THC and AEA could contribute to their non-CB1-mediated antinociceptive effects.

The VTA is believed to be a potential target region for the direct actions of cannabinoids on the GlyR, and it is the origin of mesolimbic dopamine system that mediates the reinforcing properties of cannabinoids (Gerdeman et al., 2002). Increased dopamine release by exogenous glycine application has also been shown (Molander and Soderpalm, 2005a,b). In this regard, we tentatively predict that the potentiation of the GlyR function by THC and AEA may have a modulatory role in CB1 receptor-dependent release of dopamine in the mesolimbic system.

The precise molecular mechanisms underlying THC and AEA potentiation of GlyR-mediated responses are currently unknown. The potentiation by THC and AEA exhibited fast onset and offset times (Fig. 2). Similar to ethanol potentiation of 5-HT_3 receptor-meditated currents (Lovinger and Zhou, 1998; Zhang et al., 2002), the cannabinoid potentiation of GlyRs decreased significantly with increasing agonist concentrations. Our earlier studies have shown that the agonist-concentration dependence of ethanol actions resulted from direct modulatory actions of ethanol on the gating properties of 5-HT_3 receptor-channel complex (Zhang et al., 2002). It is important to note that whereas cannabinoids, endocannabinoids, and ethanol can potentiate GlyR, these effects seem to be mediated by different molecular determinants. The amino acid residue at position 267 or the equivalent position of GlyR has been shown to be critical for alcohol and volatile anesthetic-induced modulation of GlyR function (Mihic et al., 1997; Ye et al., 2001; Tao and Ye, 2002). The similar case was also found to be true for $GABA_A$ receptor (Ueno et al., 2000). However, point mutation at Ser267 did not affect THC- and AEA-induced potentiation of GlyR. This finding also excludes the possibility that the potentiation of GlyR by THC and AEA is due to a contamination from ethanol-induced effects, because we dissolved both THC and AEA in ethanol in stock solutions. It is likely that THC and AEA may still exert their effects by modulating the gating properties of GlyRs through allosteric mechanisms. Further studies are needed to identify molecular determinants of the actions of cannabinoids on GlyRs. It should also be interesting to determine whether low concentrations of ethanol and THC or AEA may each enhance the other's potentiating effects on GlyR because such an interaction between alcohol and THC has been reported in animal and human studies (Doty et al., 1992; Chait and Perry, 1994; Liguori et al., 2002; Oz et al., 2005).

In conclusion, we have shown that THC and AEA potentiate the function of GlyRs in a CB1 receptor-independent manner. The effects of THC and AEA are mediated by a direct interaction between cannabinoids and GlyRs. Collectively, the results suggest that GlyRs represent a novel target in mediating the pharmacological actions of endogenous and exogenous cannabinoids.

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