

Desensitization of Cannabinoid-Mediated Presynaptic Inhibition of Neurotransmission Between Rat Hippocampal Neurons in Culture

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ABSTRACT

Prolonged exposure to cannabinoids results in tolerance in vivo and desensitization of cannabinoid receptors in vitro. We show here that cannabinoid-induced presynaptic inhibition of glutamatergic neurotransmission desensitized after prolonged exposure to the cannabinoid receptor agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate (Win55,212-2). Synaptic activity between hippocampal neurons in culture was determined from network-driven increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$, spikes) and excitatory postsynaptic currents. Win55,212-2-induced (100 nM) inhibition partially desensitized after 2 h and completely desensitized after 18- to 24-h exposure. The desensitization could be overcome by higher concentrations of agonist as indicated by a parallel rightward shift of the concentration response curve from an EC_{50} of 2.7 ± 0.3 nM to 320 ± 147 nM for inhibition of $[\text{Ca}^{2+}]_i$, spiking and from 43 ± 17

nM to 4505 ± 403 nM for inhibition of synaptic currents, suggesting that this phenomenon may underlie tolerance. Presynaptic expression of dominant negative G-protein-coupled-receptor kinase (GRK2-Lys220Arg) or β -arrestin (319-418) reduced the desensitization produced by 18- to 24-h pretreatment with 100 nM, Win55,212-2 suggesting that desensitization followed the prototypical pathway for G-protein-coupled receptors. Prolonged treatment with Win55,212-2 produced a modest increase in the EC_{50} for adenosine inhibition of synaptic transmission and pretreatment with cyclopentyladenosine produced a slight increase in the EC_{50} for Win55,212-2, suggesting a reciprocal ability to produce heterologous desensitization. The long-term changes in synaptic function that accompany chronic cannabinoid exposure will be an important factor in evaluating the therapeutic potential of these drugs and will provide insight into the role of the endocannabinoid system.

Cannabinoids are responsible for the psychoactive effects of marijuana (Abood and Martin, 1992) and also produce effects that are of potential therapeutic benefit (Pertwee, 2000). Thus, a number of cannabinoid receptor agonists, such as Win55,212-2, have been synthesized (Huffman, 1999). Cannabimimetic drugs interact with receptors that are part of an endogenous signaling system that seems to participate in the control of movement, appetite, pain, and memory (Pertwee, 2000; Piomelli et al., 2000). These central effects probably result from the modulation of synaptic transmission. Cannabinoids act presynaptically to inhibit glutamatergic (Shen et al., 1996; Levenes et al., 1998; Sullivan, 1999) and

GABA-ergic (Chan et al., 1998; Katona et al., 1999; Hoffman and Lupica, 2000) neurotransmission. The endogenous ligands such as 2-arachidonylglycerol and arachidonyl ethanolamide act as retrograde transmitters (Maejima et al., 2001). Prolonged exposure to cannabinoid receptor agonists may affect the modulation of synaptic function by endogenous and exogenous cannabinoids.

Animals treated chronically with cannabinoid receptor agonists develop tolerance to the behavioral effects of these drugs (Pertwee et al., 1993). CB1 receptors from animals made tolerant to Δ^9 -THC seem to have desensitized as indicated by reduced agonist stimulated $\text{GTP}\gamma\text{S}$ binding (Sim et al., 1996). Cannabinoid-induced inhibition of adenylate cyclase desensitized after exposure to cannabinoids for 24 h (Dill and Howlett, 1988). Cannabinoid CB1 receptors desensitize and internalize via a pathway similar to that described

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ABBREVIATIONS: GABA, γ -aminobutyric acid; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; Win55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate; SR141716, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; Win55,212-3, (*S*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; HHSS, HEPES-buffered Hank's salt solution; EPSC, excitatory postsynaptic current; DMEM, Dulbecco's modified Eagle's media; GRK, G protein-coupled-receptor kinase; NMDA, *N*-methyl-D-aspartate.

for the β adrenergic receptor (Lefkowitz, 1998). Receptor activation leads to liberation of G $\beta\gamma$ and subsequent receptor phosphorylation by a G protein-coupled receptor kinase (Jin et al., 1999). β -Arrestin binds to the receptor enabling internalization via clathrin-coated pits (Hsieh et al., 1999). The internalization of the receptor also occurs in hippocampal neurons grown in primary culture, although at a slower rate than that observed when receptors and signaling components were expressed at high levels in heterologous systems (Coutts et al., 2001). What remains unclear is how the changes in receptor localization and coupling to G proteins produced by prolonged exposure to cannabinoids affect synaptic transmission.

We studied the effects of prolonged exposure to cannabinoid receptor agonists on synaptic transmission between rat hippocampal neurons in culture. Prolonged exposure to Win55,212-2 rendered the culture less sensitive to synaptic inhibition by the agonist. CB1 receptor desensitization will have significant consequences for therapeutic application of cannabimimetic drugs and may have profound effects on the endogenous cannabinoid system.

Materials and Methods

Materials were obtained from the following sources: β -arrestin (319–418) and GRK2 (Lys220Arg) expression vectors (pcDNA3) were kindly provided by Dr. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA); SR141716, National Institute on Drug Abuse drug supply system (Bethesda, MD); indo-1 acetoxymethyl ester, Molecular Probes (Eugene, OR); Win55,212-2, Win55,212-3, CNQX, adenosine, cyclopentyladenosine, and all other reagents (Sigma/RBI, Natick, MA).

Rat hippocampal neurons were grown in primary culture as described previously (Wang et al., 1994) with minor modifications. Neurons dissociated from hippocampi of embryonic day 17 rats were plated as a droplet onto glass coverslips at an approximate density of 5×10^4 cells/well for $[\text{Ca}^{2+}]_i$ recordings and at 3×10^4 cells/well for recording synaptic currents. Cultures were grown without mitotic inhibitors for a minimum of 12 days before use.

$[\text{Ca}^{2+}]_i$ was measured in single hippocampal neurons using indo-1-based microfluorometry as described previously (Werth and Thayer, 1994). Hippocampal neurons were loaded with $2 \mu\text{M}$ indo-1 acetoxymethyl ester for 45 min at 37°C in 0.5% bovine serum albumin in HHSS. Experiments were performed at room temperature in a recording chamber that was continuously perfused (2 ml/min) with HHSS composed of the following: 20 mM HEPES, 137 mM NaCl, 1.3 mM CaCl_2 , 0.1 mM MgCl_2 , 5.0 mM KCl, 0.4 mM KH_2PO_4 , 0.6 mM Na_2HPO_4 , 3.0 mM NaHCO_3 , 5.6 mM glucose, and 0.01 mM glycine, pH 7.45. $[\text{Ca}^{2+}]_i$ spiking was evoked by reducing $[\text{Mg}^{2+}]_o$ to 0.1 mM as described previously (McLeod et al., 1998).

Whole-cell currents were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Union City, CA) and the BASIC-FASTLAB interface system (Indec Systems, Capitola, CA). For recording EPSCs, pipettes (3–5 M Ω resistance) were pulled from borosilicate glass (Narashige, Greenvale, NY) and filled with a solution containing: 130 mM K-gluconate, 10 mM KCl, 10 mM NaCl, 10 mM BAPTA, 10 mM HEPES, 10 mM glucose, 5 mM MgATP, and 0.3 mM Na_2GTP , 300 mOsm/kg, adjusted to pH 7.2. The postsynaptic cell was voltage clamped at -70 mV. The extracellular solution was composed of 140 mM NaCl, 5 mM KCl, 9 mM CaCl_2 , 6 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH and to 315 mOsm/kg with sucrose. EPSCs were elicited every 20 s by a 0.1-ms pulse delivered by a concentric-bipolar stimulating electrode (FHC, Inc., Bowdoinham, MA) positioned near the cell body of a presynaptic neuron. The high $[\text{Mg}^{2+}]_o$ reduced polysynaptic responses and isolated the non-NMDA component of the synaptic re-

sponse. In some experiments, the external solution contained $10 \mu\text{M}$ bicuculline methochloride; results were similar with or without bicuculline in the bath, so the data were pooled. Recorded currents were sampled every 100 μs , filtered at 2 kHz, and were not corrected for leak currents.

Hippocampal neurons were transfected between 11 to 15 days in vitro using a modification of a protocol described previously (Xia et al., 1996). Briefly, hippocampal cultures were incubated for at least 20 min in DMEM supplemented with 1 mM sodium kynurenate, 20 mM MgCl_2 , and 5 mM HEPES, to reduce neurotoxicity. The DNA/calcium phosphate precipitate was prepared, allowed to form for 30 min at room temperature and added to the culture. Total plasmid DNA (3–4 μg) was used for each 30-mm diameter well of a six-well plate. After 50 min of incubation, cells were “shocked” with 2% dimethyl sulfoxide in HHSS with 1 mM sodium kynurenate, 10 mM MgCl_2 , and 5 mM HEPES for 2 min. Then cells were washed twice with DMEM supplemented with sodium kynurenate, MgCl_2 and HEPES, once with regular DMEM and then returned to conditioned media, saved at the beginning of the procedure. For desensitization experiments, 24 h after the transfection, the media was supplemented with 100 nM Win55,212-2 and used for experiments 18 to 24 h later.

Data are presented as mean \pm S.E.M. Statistical comparisons were made by Student's *t* test and analysis of variance with Bonferroni's post-test. Concentration-response curves were fit by a logistic equation of the form % Inhibition = $I_{\text{max}} / [1 + (X/\text{EC}_{50})^b]$, where *X* is the drug concentration, I_{max} is the percentage of inhibition calculated for an “infinite” concentration, and *b* is a slope factor that determines the steepness of the curve. EC_{50} values were calculated by a nonlinear, least-squares curve-fitting algorithm using Origin Version 4.1 (Microcal, Northampton, MA), and are expressed as mean \pm S.E.M.

Results

Cannabinoid Inhibition of Glutamatergic Synaptic Transmission Partially Desensitizes over 2 h. Glutamatergic synaptic transmission was studied optically using the Ca^{2+} sensitive dye indo-1 to monitor synaptically driven $[\text{Ca}^{2+}]_i$ spiking in cultures of rat hippocampal neurons. Reducing the $[\text{Mg}^{2+}]_o$ from 0.9 mM to 0.1 mM evoked repetitive $[\text{Ca}^{2+}]_i$ spikes (Fig. 1A) that were driven by glutamatergic synaptic transmission as they were blocked by both NMDA (10 μM (\pm)-2-amino-5-phosphonopentanoic acid) and non-NMDA (10 μM CNQX) receptor antagonists (McLeod et al., 1998). This model provides a noninvasive approach to study the effects of prolonged exposure to cannabinoids on glutamatergic synaptic transmission (Shen et al., 1996). The frequency of $[\text{Ca}^{2+}]_i$ spikes was stable during superfusion with low $[\text{Mg}^{2+}]_o$ solution (Fig. 1A) and after 2 h it was $80 \pm 5\%$ of the initial frequency (Fig. 1D, \circ ; *n* = 8).

Superfusion with 100 nM Win55,212-2, a potent cannabinoid receptor agonist, completely blocked $[\text{Ca}^{2+}]_i$ spiking activity initially. However, in the continuous presence of agonist $[\text{Ca}^{2+}]_i$ spiking partially recovered (Fig. 1B). After 120 min of exposure to Win55,212-2, the $[\text{Ca}^{2+}]_i$ spiking frequency recovered to $46 \pm 13\%$ of the initial frequency, suggesting that the inhibitory response had desensitized (Fig. 1D, \bullet , *n* = 14). Superfusion with 3 nM Win55,212-2 (Fig. 1C), a concentration approximating the EC_{50} in this system (Shen et al., 1996), produced a smaller initial decrease in the $[\text{Ca}^{2+}]_i$ spiking frequency ($67 \pm 5\%$, *n* = 4) that failed to desensitize during the 120-min exposure. After 120 min in 3 nM Win55,212-2, the $[\text{Ca}^{2+}]_i$ spiking frequency was $56 \pm 8\%$ of the initial value (Fig. 1D, \blacklozenge). These data suggest that the

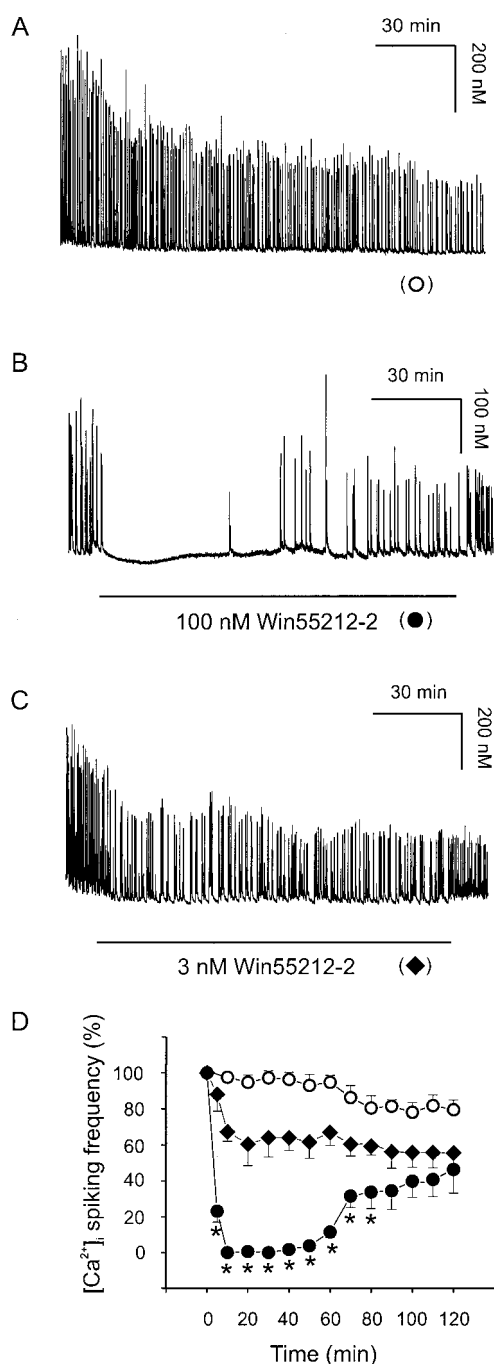


Fig. 1. Desensitization of cannabinoid inhibition of $[Ca^{2+}]_i$ spiking activity. $[Ca^{2+}]_i$ spiking was elicited by bathing hippocampal neurons in 0.1 mM $[Mg^{2+}]_o$ and recorded with indo-1-based photometry as described under *Materials and Methods*. The horizontal bars indicate changes in the composition of the continuously flowing superfusion solution. A, the frequency of low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spiking was stable during 2 h of continuous recording. The amplitude of $[Ca^{2+}]_i$ spikes was less stable than frequency; thus, frequency was used to quantify synaptic activity. B, superfusion with 100 nM Win55,212-2 completely blocked $[Ca^{2+}]_i$ spiking. Spiking partially recovered during the 2-h recording in the maintained presence of drug. C, superfusion with 3 nM Win55,212-2 produced a partial inhibition of $[Ca^{2+}]_i$ spiking that failed to desensitize during the 2-h recording. D, plot summarizes the results of recordings represented by the traces in A to C. The $[Ca^{2+}]_i$ spiking frequency was normalized to the starting frequency and plotted versus time from control cells (open circles, $n = 8$), cells treated with 100 nM Win55,212-2 (●, $n = 14$) and cells treated with 3 nM Win55,212-2 (◆, $n = 4$). Data presented are means \pm S.E. *, $p < 0.05$, 3 nM Win55,212-2-treated versus 100 nM Win55,212-2-treated, analysis of variance with Bonferroni's post test.

degree of desensitization was dependent on the level of receptor activation.

Treatment with Win55,212-2 for 24 h Markedly Desensitizes Inhibition of Excitatory Synaptic Transmission by Cannabinomimetic Drugs. Acute treatment with Win55,212-2 (100 nM) completely inhibited 0.1 mM $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spiking activity in hippocampal neuronal networks. This inhibition was readily reversed during a 10-min washout period (Fig. 2A). This effect was concentration dependent with an EC_{50} of 2.7 ± 0.3 nM in good agreement with our previous studies (Shen et al., 1996). Cultures treated for 18 to 24 h with 100 nM Win55,212-2 were considerably less sensitive to cannabinoid receptor agonists. After 24-h treatment with Win55,212-2 and a 20-min washout of the agonist, reapplication of 100 nM Win55,212-2 no longer inhibited $[Ca^{2+}]_i$ spiking (Fig. 2B). This loss of activity was manifest as a decrease in potency as indicated by the approximately 100-fold rightward shift in the concentration response curve (Fig. 2C). The EC_{50} for Win55,212-2 in cells pretreated with agonist was 320 ± 147 nM.

To confirm that the modulation of network driven $[Ca^{2+}]_i$ spiking activity accurately reflects modulation of glutamatergic synaptic transmission, we examined the effects of prolonged exposure to Win55,212-2 on excitatory postsynaptic currents (EPSCs). An extracellular stimulating electrode was placed 10 to 20 μ m from the soma of a cell that was 40 to 140 μ m from the postsynaptic cell held at -70 mV in the whole-cell configuration of the patch clamp. Monosynaptic EPSCs ($n = 36$) were coupled in a strictly one-to-one fashion with the stimulus, had a smooth rise and decay, a short rise time, and a latency (3.8 ± 0.2 ms from stimulus artifact to onset of the EPSC) that did not fluctuate between trials. Superfusion of 10 μ M CNQX completely blocked the EPSC ($n = 36$). In a previous study using the same hippocampal culture preparation, we found that cannabinoid receptor agonists increased the coefficient of variation, increased the number of synaptic failures, and failed to affect currents evoked by direct application of kainate, consistent with a presynaptic site of action (Shen et al., 1996). An example of cannabinoid inhibition of EPSC amplitude is shown in Fig. 2D. Win55,212-2 (100 nM) inhibited EPSC amplitude by $64 \pm 5\%$ (Fig. 2D, $n = 8$). 300 nM Win55,212-2 produced complete inhibition of the EPSCs. The EC_{50} for Win55,212-2 inhibition of EPSCs was 43 ± 17 nM. Thus, Win55,212-2 displayed an approximately 10-fold lower potency for inhibition of synaptic currents relative to inhibition of network driven $[Ca^{2+}]_i$ spiking. However, a similar shift in potency was observed after 24-h pretreatment with Win55,212-2 (Fig. 2, D and E). 100 nM Win55,212-2 no longer inhibited EPSCs (Fig. 2E). The concentration-response curve was shifted approximately 100-fold to the right after 18- to 24-h treatment with Win55,212-2, yielding an EC_{50} of 4505 ± 403 nM. Clearly, prolonged (18–24 h) exposure to the full cannabinoid receptor agonist Win55,212-2 functionally desensitized the CB1-mediated responses whether $[Ca^{2+}]_i$ spiking activity (Fig. 2C) or synaptic currents (Fig. 2F) were used to assess function. The slope factors for all four curves in Fig. 2, C and F, approximated 1, suggesting that in both biological assays, Win55,212-2 activated a single class of noninteracting binding sites (De Lean et al., 1978).

The Degree of Functional Desensitization Depends on the Duration of Exposure to Cannabinoid Receptor Agonist. In the next set of experiments we compared the completeness of functional desensitization and the recovery

from agonist effects after short (2–6 h) and prolonged (18–24 h) application of cannabinoid receptor agonist. After 2 to 6 h of exposure to 100 nM Win55,212-2 the $[Ca^{2+}]_i$ spiking frequency was inhibited by $56 \pm 7\%$ ($n = 23$) relative to the mean $[Ca^{2+}]_i$ spiking frequency in naïve cells. Thus, the $[Ca^{2+}]_i$ spiking frequency in Win55,212-2 was suppressed as shown in the representative trace in Fig. 3A (at the time indicated by the solid bar in Fig. 3, A and C, $p < 0.001$). Washout of drug produced an immediate and highly variable rebound in $[Ca^{2+}]_i$ spiking that we did not quantify because the spikes typically fused producing an elevated basal $[Ca^{2+}]_i$. After a 20-min wash period, $[Ca^{2+}]_i$ spiking frequency stabilized at a frequency $61 \pm 5\%$ greater than that in drug (Fig. 3, A and C, ▨) to approximate the control frequency of 25 ± 4 spikes/10 min observed in untreated cells ($n = 21$; dashed line in Fig. 3C). Subsequent reapplication of Win55,212-2 (100 nM) inhibited spike frequency by $50 \pm 7\%$ (Fig. 3, A and C, □). Washout of Win55,212-2 returned $[Ca^{2+}]_i$ spiking to control frequency (Fig. 3, A and C, ▩).

Neurons treated with Win55,212-2 (100 nM) for 18 to 24 h ($n = 10$) exhibited a higher spiking frequency in the presence of agonist (Fig. 3, B and C, ■) relative to cells treated for 2 to 6 h and demonstrated less pronounced facilitation of $[Ca^{2+}]_i$ spiking upon agonist washout (cross-hatched bar in Fig. 3, B and C; $34 \pm 5\%$, $p < 0.01$). The amplitude of EPSCs increased $54 \pm 15\%$ ($n = 6$) after a 20-min drug-free wash period ($p < 0.01$) (data not shown) using a similar protocol (18–24 h

Win55,212-2). Re-application of 100 nM Win55,212-2 (Fig. 3, B and C, □) did not significantly inhibit $[Ca^{2+}]_i$ spiking frequency. $[Ca^{2+}]_i$ spiking remained at control frequency after washout of Win55,212-2 (Fig. 3, B and C, ▩).

These data indicate that after a 2- to 6-h exposure to 100 nM Win55,212-2, although significant desensitization has occurred (Fig. 3A), the drug continues to exert significant inhibition of synaptic activity ($p < 0.001$; Fig. 3C, left, ■). After 24 h, the desensitization was more complete and the residual inhibitory effect of the agonist was not significantly different from control (Fig. 3C, right, ■), although a significant increase in spiking frequency was observed upon washout of the drug ($p < 0.01$).

Desensitization of Cannabinoid-Mediated Inhibition of Synaptic Activity Is Receptor Mediated. To address the specificity of Win55,212-2-induced desensitization, we initially used the CB1 receptor antagonist SR141716, which we have shown prevents cannabinoid inhibition of $[Ca^{2+}]_i$ spiking (Shen and Thayer, 1999). Unfortunately, we were unable to fully reverse the effects of this high-affinity antagonist in our system. Even after a 4-h wash to remove SR141716, application of 100 Win55,212-2 produced only $51 \pm 1\%$ ($n = 3$) of spiking inhibition. Thus, we took advantage of the stereoselective interaction of the enantiomers of Win55,212 with the cannabinoid receptor.

After 24-h treatment with Win55,212-2 (100 nM), reapplication of the agonist produced only $19 \pm 6\%$ inhibition of

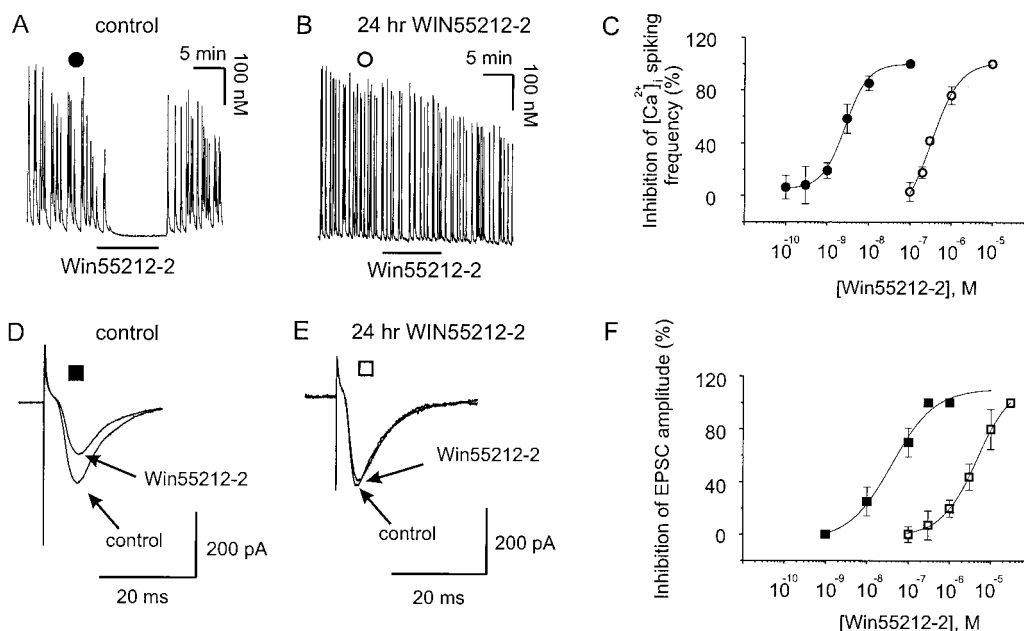


Fig. 2. Treatment (24 h) with Win55,212-2 desensitizes cannabinimetic inhibition of $[Ca^{2+}]_i$ spiking and synaptic currents. A to C, low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spiking was recorded from control cultures (●) or cultures treated for 18 to 24 h with 100 nM Win55,212-2 (○) as described under *Materials and Methods*. A, representative trace shows inhibition of $[Ca^{2+}]_i$ spiking produced by a 10-min application of 100 nM Win55,212-2 applied to a control cell. Drug was applied at the time indicated by the horizontal bar and the cell was superfused with 0.1 mM $[Mg^{2+}]_o$ throughout the recording. B, trace shows an experiment similar to A except that the cell was treated with 100 nM Win55,212-2 for 24 h and washed for 20 min before starting the recording. C, concentration response curves summarize inhibition of $[Ca^{2+}]_i$ spiking frequency from experiments such as those shown in A and B ($n \geq 3$). Curve fits were performed as described under *Materials and Methods*. Both curves produced 100% maximal inhibition and had EC_{50} values and slope factors of 2.7 ± 0.3 nM and 1.6 ± 0.3 for control and 320 ± 147 nM and 1.3 ± 0.5 for treated cells, respectively. D to F, whole-cell recordings were performed as described under *Materials and Methods*. Traces are mean EPSCs of 8 to 15 sweeps recorded during the 2 min before drug application (control) and 10 min after application of 100 nM Win55,212-2. EPSCs were recorded from control cultures (■) or cultures treated for 18 to 24 h with 100 nM Win55,212-2 (□). D, representative trace shows inhibition of EPSCs produced by application of 100 nM Win55,212-2 to a control cell. E, trace shows an experiment similar to A except that the cell was treated with 100 nM Win55,212-2 for 24 h and washed for 20 min before starting the recording. F, concentration response curves summarize inhibition of EPSC amplitude from experiments such as those shown in D and E ($n \geq 3$). Curve fits were performed as described under *Materials and Methods*. Both curves produced 100% maximal inhibition and had EC_{50} values and slope factors of 43 ± 17 nM and 1.0 ± 0.2 for control and 4505 ± 403 nM and 1.1 ± 0.1 for treated cells, respectively.

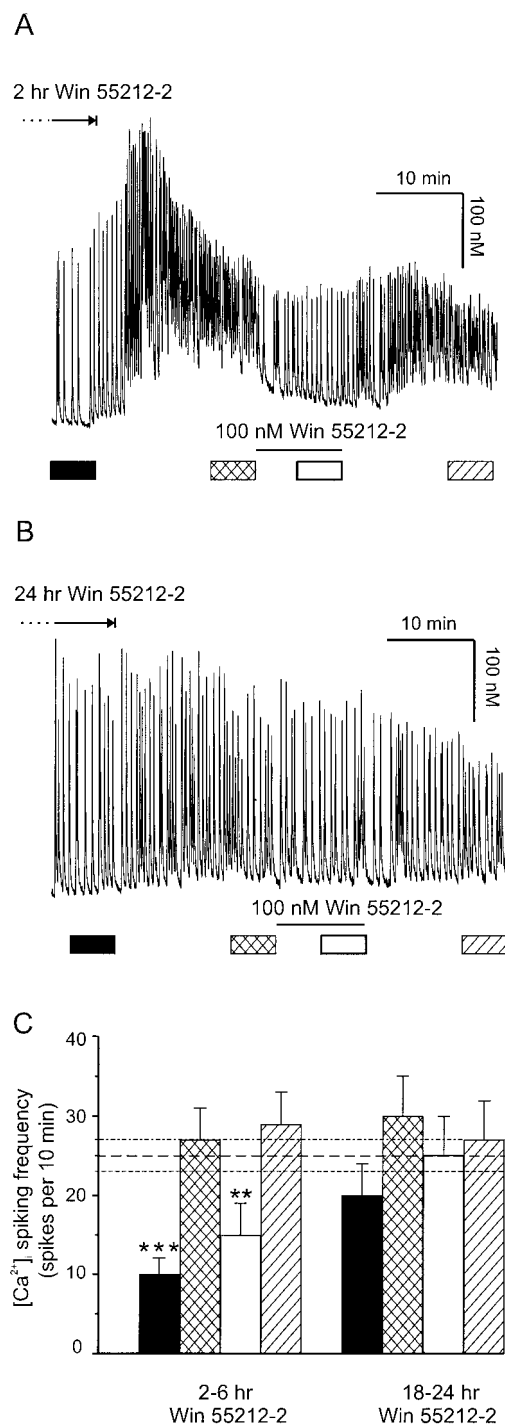


Fig. 3. Comparison of functional desensitization of cannabinoid inhibition of $[Ca^{2+}]_i$ spiking frequency produced by 2- to 6-h versus 18- to 24-h exposure to Win55,212-2. Low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spiking was elicited from cultures treated with 100 nM Win55,212-2 for either 2 to 6 or 18 to 24 h as described under *Materials and Methods*. A and B, representative traces from a culture treated for 2 h (A) or 24 h (B) with Win55,212-2. C, bar graph summarizes the $[Ca^{2+}]_i$ spiking frequency from experiments such as those shown in A and B. Frequencies were measured at the times indicated by the horizontal bars in A and B and displayed with the corresponding fill in C. Reported frequencies are in the presence of pretreatment (■), 20 min after washout of drug (▨), after reapplication of 100 nM Win55,212-2 (□) and 20 min after washout of acute drug application (▩). Dashed horizontal line indicates mean $[Ca^{2+}]_i$ spiking frequency of 25 ± 4 spikes/10 min in untreated control cells ($n = 21$). *, $p < 0.05$; ***, $p < 0.001$ significantly different from untreated control, Student's t test.

$[Ca^{2+}]_i$ spiking (Fig. 4A; $n = 10$). As shown in Fig. 4B, after 24-h pretreatment with Win55,212-3 (100 nM), the less active enantiomer of Win55,212, the culture was still fully sensitive to the active enantiomer Win55,212-2 ($n = 3$). In summary, the full agonist Win55,212-2 produced significantly greater inhibition of excitatory synaptic activity after treatment with the inactive enantiomer relative to that observed after treatment with the full agonist Win55,212-2 ($p < 0.001$, Fig. 4C). Desensitization evidently required receptor activation and thus, was only observed after treatment with the active enantiomer.

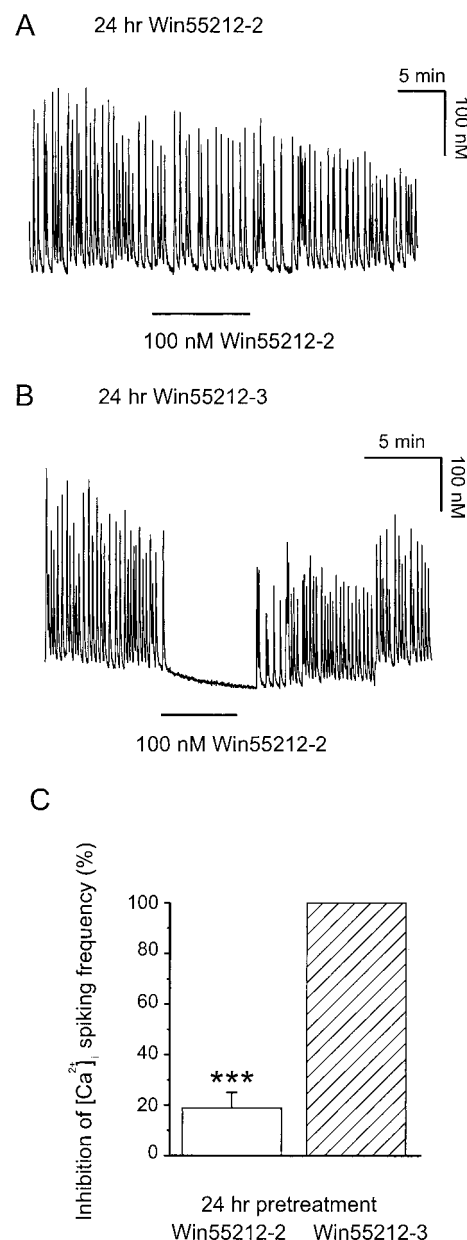


Fig. 4. Prolonged exposure to the inactive enantiomer Win55,212-3 did not affect sensitivity to Win55,212-2. $[Ca^{2+}]_i$ spiking induced by 0.1 mM $[Mg^{2+}]_o$ was elicited from cultures as described under *Materials and Methods*. A and B, representative traces from cultures treated for 24 h with 100 nM Win55,212-2 (A) or 100 nM Win55,212-3 (B). Recordings were initiated after a 20-min washout of drug. C, histogram summarizes the $[Ca^{2+}]_i$ spiking frequency from experiments such as those shown in A and B. ***, $p < 0.001$ significantly different from Win55212-3-treated cells, Student's t test.

GRK and β -Arrestin Mediate the Desensitization of Cannabinoid Receptor-Induced Presynaptic Inhibition of Glutamatergic Neurotransmission. Desensitization of G protein-coupled receptors after agonist activation can be divided into two components. The first requires receptor phosphorylation and is mediated by $G\beta\gamma$ activation of GRK. The second component involves receptor internalization and requires β -arrestin binding to the receptor as well as binding to clathrin. In heterologous expression systems, GRK3 and β -arrestin participate in rapid desensitization and internalization of CB1 cannabinoid receptors (Hsieh et al., 1999; Jin et al., 1999). We hypothesized that this pathway would mediate functional desensitization of CB1 receptors in primary neurons as well. We employed an approach used successfully to study the pathway leading to desensitization of other G protein-coupled receptors based on expression of dominant negative inhibitors of the pathway. GRK2 (Lys220Arg), which binds heptahelical receptors but fails to phosphorylate them and β -arrestin (319–418), which competes with receptor bound β -arrestin for binding to clathrin, were used as dominant negative inhibitors of desensitization (Kong et al., 1994; Krupnick et al., 1997).

Hippocampal neurons were cotransfected with GFP and either empty vector, GRK2 (Lys220Arg) expression vector, or β -arrestin (319–418) expression vector. As shown in Fig. 5A, we placed a stimulating electrode adjacent to a GFP-positive cell and voltage-clamped a nontransfected postsynaptic cell in the whole-cell configuration. Cultures were pretreated for 24 h with 100 nM Win55,212-2. Transfection with empty vector had no effect on the characteristics of the EPSC and did not affect the desensitization produced by 24 h exposure to Win55,212-2. As shown in Fig. 5B, the cells had desensitized and were thus, insensitive to 100 nM Win55,212-2. In contrast, when the presynaptic cell was transfected with the GRK2 (Lys220Arg) expression vector, EPSCs were completely blocked by reapplication of 100 nM Win55,212-2 (Fig. 5C). Indeed, Win55,212-2 produced a significantly greater inhibition of EPSC amplitude in the cells expressing GRK2(Lys220Arg) relative to cells transfected with empty vector ($p < 0.001$). Thus, inhibiting phosphorylation of the cannabinoid receptor by a GRK prevented functional desensitization. Transfection of the presynaptic cell with the β -arrestin (319–418) expression vector produced an intermediate response (Fig. 5D). Win55,212-2 (100 nM) produced $75 \pm 7\%$ inhibition of the EPSC amplitude, comparable with that seen in untreated cells and significantly greater than that seen in cells transfected with empty vector ($p < 0.001$). Thus, inhibition of β -arrestin-mediated receptor internalization reduced functional desensitization of cannabinoid receptors.

Cross Talk between Cannabinoid CB1 and Adenosine A_1 Receptors. Adenosine type A_1 receptors are heptahelical receptors that, like CB1 receptors, signal through inhibitory G-proteins and produce presynaptic inhibition in the hippocampus (Brundage and Dunwiddie, 1996). Thus, we decided to investigate the potential for cross talk between cannabinoid and adenosine signaling pathways. As shown in Fig. 6A adenosine produces concentration-dependent inhibition of $[Ca^{2+}]_i$ spiking frequency with an EC_{50} of 57 ± 8 nM (Fig. 6, \blacktriangle). The A_1 receptor specific agonist cyclopentyladenosine also potently inhibited excitatory synaptic transmission ($EC_{50} = 3.2 \pm 0.4$ nM, data not shown). Pretreating the hippocampal culture for 24 h with cyclopentyladenosine (100

nM) shifted the concentration response curve for adenosine approximately 10-fold to the right ($EC_{50} = 725 \pm 24$ nM; Fig. 6, \triangle). Efficacy was reduced to $70.3 \pm 0.9\%$ of maximum without changing the slope of the concentration-response curve. Prolonged exposure (24 h) to 100 nM Win55,212-2

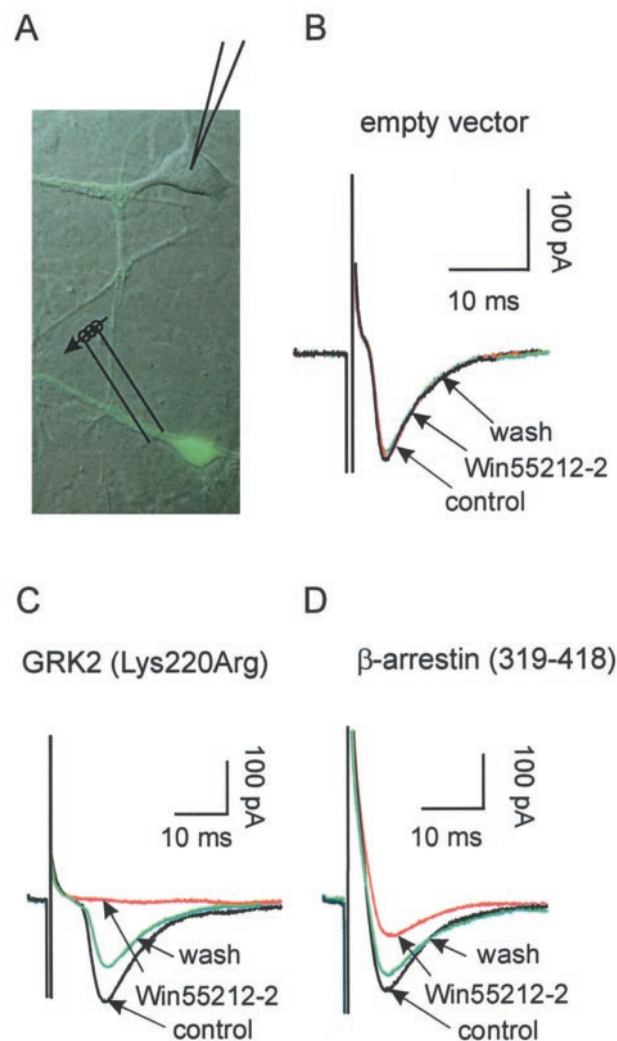


Fig. 5. Attenuation of cannabinoid receptor desensitization in synaptically coupled hippocampal neurons transfected with dominant-negative GRK2 or β -arrestin. Hippocampal cultures were transfected between day 11 and 15 in vitro and 24 h after transfection treated with 100 nM Win55,212-2. After 24 h exposure to Win55,212-2, the cells were placed in the recording chamber, washed in drug-free solution for 20 min, and then synaptic currents recorded. A, micrograph shows GFP fluorescence overlaid on a differential interference contrast image of a pair of synaptically coupled hippocampal neurons in culture. The GFP positive cell was stimulated with a bipolar electrode (note the stimulus electrode) and monosynaptic EPSCs were recorded from a nontransfected cell (note the patch pipette). B to D, whole-cell recordings were performed as described under *Materials and Methods*. Traces are mean EPSCs of 8 to 15 sweeps recorded during the 2 min before drug application (control), 10 min after application of 100 nM Win55,212-2, or 30 min after washout of drug (wash). B, transfection of the presynaptic cell with pcDNA3 (empty vector) did not affect EPSCs ($n = 3$). Representative recording shows that Win55,212-2 (100 nM) failed to affect EPSC amplitude consistent with the desensitization described for nontransfected cells in Fig. 2E. C, transfection of the presynaptic cell with the GRK2 (Lys220Arg) expression vector prevented desensitization. Representative recording shows that 100 nM Win55,212-2 completely inhibited the EPSC ($n = 3$). D, transfection of the presynaptic cell with the β -arrestin (319–418) expression vector reduced desensitization. Representative recording shows that 100 nM Win55,212-2 inhibited the EPSC ($n = 4$).

Discussion

Cannabinoid-induced presynaptic inhibition of glutamatergic neurotransmission desensitized after prolonged exposure to a cannabinoid receptor agonist. Win55,212-2 (100 nM) produced partial functional desensitization after 2 h that increased to $97 \pm 7\%$ after 18- to 24-h exposure. The desensitization could be overcome by higher concentrations of agonist as indicated by a parallel rightward shift of the concentration response curve, suggesting that this phenomenon may underlie tolerance. The presynaptic expression of dominant negative GRK or β -arrestin reduced the desensitization indicating that the prototypical pathway for desensitization of G-protein-coupled receptors mediated the process (Lefkowitz, 1998).

The changes in sensitivity to cannabinoids after prolonged exposure to Win55,212-2 were consistent with studies performed on heterologously expressed receptors but exhibited some differences as well. The principle signaling pathway seems similar, namely agonist-induced liberation of $\beta\gamma$ subunits from inhibitory G proteins activating GRK with subsequent binding of β -arrestin to the receptor. Because G protein-coupled receptors interact with multiple GRKs, inhibition by dominant negative GRK2 (Lys220Arg) does not necessarily identify the GRK subtype active in the nerve terminal (Freedman et al., 1995). Similarly, β -arrestin (319–418) binds to clathrin but not to G-protein-coupled receptors and thus prevents internalization of many G protein-coupled receptors that traffic via clathrin-mediated endocytosis (Krupnick et al., 1997). The functional desensitization of cannabinoid inhibition of synaptic transmission developed more slowly than the desensitization of CB1 activation of G-protein-gated, inwardly rectifying K^+ channels observed in *Xenopus laevis* oocytes or the receptor internalization observed in AtT20 cells expressing CB1, GRK3, and β -arrestin 2 (Hsieh et al., 1999; Jin et al., 1999). Interestingly, Jin et al. (1999) found that receptors in which the putative GRK phosphorylation sites had been mutated still desensitized, suggesting that β -arrestin-mediated internalization does not require phosphorylation of the receptor. We found that expression of a dominant negative GRK prevented desensitization at the synapse. Our inability to detect a GRK-independent component of desensitization might result from differences in the levels of signaling proteins expressed in nerve terminals versus the AtT20 expression system. For example, overexpression of β -arrestin enabled internalization of β -adrenergic receptors lacking GRK phosphorylation sites (Ferguson et al., 1996). Alternatively, considerable receptor internalization may be required at the nerve terminal before a functional effect on transmitter release is detected. When dominant negative β -arrestin was expressed, desensitization at the synapse was reduced. This observation is in good agreement with the reduced tolerance to morphine observed in β -arrestin 2 knockout animals (Bohn et al., 2000). Interestingly, these animals still developed physical dependence to morphine, separating the development of tolerance from physical dependence, a finding that is consistent with the mild withdrawal symptoms observed in humans tolerant to cannabinoids (Abood and Martin, 1992) and the need to administer an antagonist to tolerant animals to precipitate robust withdrawal symptoms (Aceto et al., 1995; Tsou et al., 1995). Removal of Win55,212-2 from cells treated for 18 to

produced a small but significant decrease in the potency of adenosine relative to that seen in control cells (\circ ; $EC_{50} = 135 \pm 22$ nM, $p < 0.05$).

Concentration-response curves for Win55,212-2-inhibition of $[Ca^{2+}]_i$ spiking before and after 24-h exposure to agonist are plotted in Fig. 6B (\bullet and \circ , respectively). Prolonged exposure to the adenosine agonist cyclopentyladenosine (100 nM) produced a slight desensitization of the Win55,212-2 induced inhibition (Fig. 6B, Δ). 24 h treatment with cyclopentyladenosine resulted in a small but significant decrease in the potency of the cannabinoid agonist ($EC_{50} = 16 \pm 2$ nM versus $EC_{50} = 4.0 \pm 0.3$ nM in control, $p < 0.05$). Thus, treatments with cannabinoid and adenosine receptor agonists that produced strong homologous desensitization also produced a more modest heterologous desensitization.

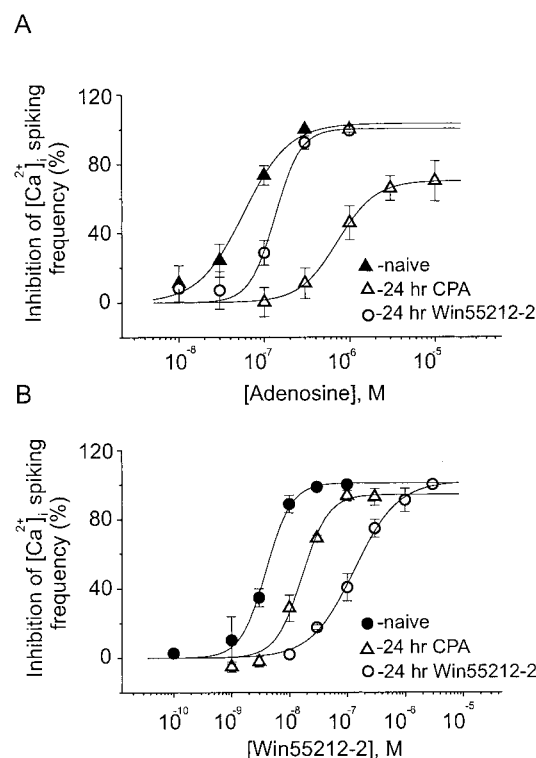


Fig. 6. Cannabinoid and adenosine receptor cross-talk. Low $[Mg^{2+}]_o$ -induced $[Ca^{2+}]_i$ spiking was recorded as described under *Materials and Methods*. Concentration response curves summarize inhibition of $[Ca^{2+}]_i$ spiking frequency ($n \geq 3$). Curve fits were performed as described under *Materials and Methods*. A, adenosine inhibited $[Ca^{2+}]_i$ spiking in a concentration dependent manner. Cultures were naive (\blacktriangle), pretreated for 24 h with cyclopentyladenosine (Δ) or pretreated for 24 h with 100 nM Win55,212-2 (\circ). Adenosine produced 100% maximal inhibition in naive and Win55,212-2-treated cells, whereas maximum inhibition in cyclopentyladenosine-treated cells was $70.3 \pm 0.9\%$. EC_{50} values and slope factors were 58 ± 8 nM and 1.6 ± 0.3 for control, 725 ± 24 nM and 2.0 ± 0.1 for cyclopentyladenosine-treated cells, and 135 ± 22 nM and 3 ± 1 for Win55,212-2-treated cells, respectively. B, Win55,212-2 inhibited $[Ca^{2+}]_i$ spiking in a concentration-dependent manner. Cultures were naive (\bullet), pretreated for 24 h with cyclopentyladenosine (Δ) or pretreated for 24 h with 100 nM Win55,212-2 (\circ). Win55,212-2 produced 100% maximal inhibition in all three cultures and EC_{50} values and slope factors were 4.0 ± 0.3 nM and 2.0 ± 0.3 for control, 17 ± 2 nM and 1.9 ± 0.4 for cyclopentyladenosine-treated cells, and 136 ± 14 nM and 1.1 ± 0.1 for Win55,212-2-treated cells, respectively. Data presented in this figure were taken from matched cell cultures and thus differ slightly from those presented in Fig. 2C.

24 h occasionally produced a rebound increase in $[Ca^{2+}]_i$ spiking activity. While the idea that a rapid increase in synaptic transmission was precipitated by drug withdrawal is intriguing, we could not quantify this effect because it was highly variable, and attempts to precipitate the increase by application of a CB1 antagonist did not improve reproducibility.

The functional desensitization described here was in good agreement with the receptor internalization described for CB1 receptors on GABA-ergic terminals in cultures from postnatal rat hippocampus (the culture used here was derived from embryonic day 17 rats) (Coutts et al., 2001). Exposure to 100 nM Win55,212-2 for 16 h reduced CB1 receptors on the cell surface by approximately half (Coutts et al., 2001), a treatment that in our study shifted the EC_{50} by more than 100-fold but did not affect the maximal inhibition of neurotransmitter release, suggesting a large pool of spare receptors in control cultures. The 100-fold shift in the EC_{50} described here is comparable with the decreased inhibition of adenylyl cyclase by cannabinoids in N18TG2 cells after 24-h drug exposure (Dill and Howlett, 1988).

In animals tolerant to Δ^9 -THC, cannabinoid-induced GTP γ S binding, a measure of receptor activation, was reduced (Breivogel et al., 1999). This loss of function correlated with receptor down-regulation and developed most dramatically over the first 3 days. Our data suggest that this down-regulation might result in decreased presynaptic inhibition by cannabinoids. Chronic exposure to Δ^9 -THC in vivo decreased the response to Win55,212-2 by approximately 70% in hippocampus (Sim et al., 1996), a level of desensitization in reasonable agreement with the less than 20% inhibition of $[Ca^{2+}]_i$ spiking frequency that remained after 18- to 24-h treatment with Win55,212-2 in our study. Injection of cannabinomimetic drugs into the substantia nigra increases firing by inhibiting GABA release from striatonigral projections; this effect also desensitized as indicated by diminished response frequencies resulting from repeated drug administration (Tersigni and Rosenberg, 1996). In contrast, the increase in dopaminergic drive after application of a cannabinoid receptor agonist to the ventral tegmental slice preparation was not suppressed by repeated agonist application (Cheer et al., 2000).

Quantitative comparisons between various studies on chronic exposure to cannabinoids are complicated by several factors. When measuring function as opposed to receptor affinity or localization, several amplification steps are present. In addition to the receptor to G protein amplification, in this study it is important to consider the power function that relates Ca^{2+} influx to neurotransmitter release. Furthermore, the difference in EC_{50} for Win55,212-2 inhibition of $[Ca^{2+}]_i$ spiking relative to inhibition of EPSCs suggests that there is further amplification at the level of the synaptic network. Multiple synaptic layers with many recurrent excitatory synapses probably drive network spiking. Thus, a modest inhibition at any single synapse would be amplified by the reduction in recurrent excitation. The conditions for recording spiking differed from those used to record EPSCs. Spiking conditions promoted excitatory polysynaptic activity and EPSCs were recorded under conditions designed to evoke monosynaptic responses. Thus, spiking was recorded in low $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ from high-density cultures, whereas EPSCs were recorded in high

external divalent cations from low-density cultures. Other complicating factors include the levels of receptor and signaling components expressed in various brain regions and in primary tissue versus heterologous expression systems. Finally, it is obvious that in vivo experiments will differ from in vitro experiments because of pharmacokinetic issues, the role of endocannabinoids, and synaptic plasticity that may not be expressed in vitro. Thus, despite common mechanisms, a number of factors might result in differences in the time course and degree of desensitization when cannabinoids are evaluated in various systems.

Prolonged treatment with Win55,212-2 produced a modest increase in the EC_{50} for adenosine inhibition of synaptic transmission and pretreatment with cyclopentyladenosine produced a slight increase in the EC_{50} for Win55,212-2, suggesting a reciprocal ability to produce heterologous desensitization. Adenosine A1 and cannabinoid CB1 receptors are both present in hippocampus and produce presynaptic inhibition in cell culture models (Thompson et al., 1992; Shen et al., 1996). This cross talk might result from GRK-mediated phosphorylation of multiple receptors (Freedman et al., 1995). Alternatively, protein kinase C will disrupt both A1 and CB1 receptor coupling to ion channels (Thompson et al., 1992; Garcia et al., 1998) and $G\beta\gamma$ can activate phospholipase $C\beta$ in neuronal cells (Yoon et al., 1999) that might lead to phosphorylation of both receptors. Finally, the synaptic network may compensate for prolonged inhibition reducing the sensitivity to multiple forms of synaptic inhibition.

Cannabinoid-induced desensitization of presynaptic inhibition probably accounts for tolerance observed in vivo. Thus, heavy social use or chronic therapeutic use will result in reduced sensitivity to cannabinoids at the synaptic level. Because little inhibition of neurotransmission by Win55,212-2 remained after 24 h exposure, it seems likely that chronic cannabinoid exposure will significantly affect the endogenous cannabinoid signaling system. Di Marzo et al. (2000) found changes in the levels of arachidonylethanolamide, *N*-arachidonoylphosphatidylethanolamide, and 2-arachidonoylglycerol after chronic treatment with Δ^9 -THC, although a uniform picture was not apparent (Di Marzo et al., 2000). It will be interesting to learn whether the depolarization-induced synthesis of endocannabinoids will compensate for reduced receptor sensitivity. Cannabinoids affect synaptic plasticity in vitro (Kim and Thayer, 2001) and chronic exposure may alter long-term changes in synaptic strength.

In summary, we have shown that the presynaptic inhibition produced by cannabinoid receptor agonists desensitizes. Because the central effects of cannabinoids are mediated through modulation of synaptic transmission, these changes are probably the cellular mechanism that in vivo produces tolerance. The desensitization described here provides a foundation for understanding how the endocannabinoid system compensates for lost sensitivity in processes such as depolarization-induced synaptic inhibition in which endocannabinoids act on CB1 receptors to modulate synaptic transmission. The long-term changes in synaptic function that accompany chronic cannabinoid exposure will probably be an important factor in evaluating the therapeutic potential of these drugs and will provide insight into the role of the endocannabinoid system.

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