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Cannabinoid CB_1 receptor-mediated inhibition of glutamate release from rat hippocampal synaptosomes

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Abstract

Cannabinoid receptors are widely expressed in the brain and have been shown to regulate synaptic transmission through a presynaptic mechanism. Using synaptosomal preparation, I show here that 2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)-pyrrolo-1,4-benzoxazin-6-yl-1-naphthalenylmethanone (WIN 55212-2) strongly depressed 4-aminopyridine-evoked glutamate release in a concentration-dependent manner, and this effect was reversed by the selective cannabinoid CB_1 receptor antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM 281). The inhibitory modulation by WIN 55212-2 was not due to a decrease in synaptosomal excitability or a direct effect on the release machinery because WIN 55212-2 did not alter 4-aminopyridine-mediated depolarization and ionomycin-induced glutamate release. In addition, the WIN 55212-2-mediated inhibition of glutamate release was blocked by the G_i/G_o protein inhibitor pertussis toxin, but not by the protein kinase A inhibitor 2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo-benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720). Furthermore, this inhibitory effect was associated with a decrease in 4-aminopyridine-evoked Ca^{2+} influx, which could be completely prevented in synaptosomes pretreated with the N- and P/Q-type Ca^{2+} channel blockers. Together, these observations indicate that activation of cannabinoid CB_1 receptors inhibit 4-aminopyridie-evoked glutamate release from hippocampal synaptosomes through a inhibitory G protein to suppress N- and P/Q-type Ca^{2+} channel activity.

Keywords: Cannabinoid; Exocytotic release; Hippocampus; Nerve terminal; Ca^{2+} channel

1. Introduction

Marijuana, besides its well-known psychoactive properties, also has a broad range of potential medical benefits, including analgesic, antiemetic and appetite stimulant effects, in patients with cancer and acquired immunodeficiency syndrome (AIDS) (Hoelett, 1995; Ameri, 1999). Cannabinoids, the major psychoactive components of marijuana, also impair some cognitive processes including learning and memory (Ameri, 1999; Sullivan, 2000). These effects are presumably mediated via the activation of G_i/G_o protein-coupled receptors (Pertwee, 1997). To date, two subtypes of cannabinoid receptors, known as CB_1 and CB_2 cannabinoid receptors, have been identified. The cannabinoid CB_1 receptor is distributed widely throughout the central nervous system (CNS), whereas the cannabinoid CB_2 receptor is expressed primarily in immune cells (Herkenham et al., 1990, 1991; Galiegue et al., 1995).

Activation of cannabinoid CB_1 receptors has been shown to inhibit adenylate cyclase, inhibit N- and P/Q-type Ca^{2+} channels, activate mitogen-activated protein kinase, and enhance inwardly rectifying K^+ channels (Deadwyler et al., 1993; Mackie et al., 1995; Twitchell et al., 1997; Shen and Thayer, 1998; Sullivan, 1999; Schweitzer, 2000). Several studies have reported that cannabinoid CB_1 receptor activation inhibits glutamatergic synaptic transmission in hippocampal neurons through a presynaptic site of action (Shen and Thayer, 1999; Shen et al., 1996), which implies that the presynaptic inhibition of neurotransmitter release is a major mode of action of cannabinoids. Although previous studies have implicated cannabinoid CB_1 receptors in the modulation of glutamate release, the mechanisms by which the effects of cannabinoid are mediated has not been examined directly in nerve terminal preparations.

Since cannabinoid CB_1 receptors are present at high density on the presynaptic terminals of hippocampal glutamatergic synapses (Twitchell et al., 1997), and their activa-

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tion is associated with a reduction in glutamate release (Piomelli et al., 2000), the aim of the present study was to investigate the effects of the selective cannabinoid CB_1 receptor agonist WIN 55212-2 on 4-aminopyridine-evoked glutamate release from hippocampal nerve terminals. Using hippocampal synaptosomes, I found that WIN 55212-2 inhibited 4-aminopyridine-evoked glutamate release through the activation of presynaptic cannabinoid CB_1 receptors in response to a pertussis toxin-sensitive G protein-coupled modulation of presynaptic N- and P/Q-type Ca^{2+} channels.

2. Materials and methods

2.1. Preparation of synaptosomes

Synaptosomes were prepared from the hippocampus of 2-month-old male Sprague–Dawley rats as previously described (Sihra, 1997). The final synaptosomal pellet was resuspended in 2 ml HEPES-buffered incubation medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2{\cdot}6H_2O$, 1.2 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES and 1 mg/ml bovine serum albumin (pH 7.4). Protein concentration was determined with the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets containing 0.3 mg protein. Synaptosomal pellets were stored on ice and used within 2–3 h.

2.2. Glutamate release assay

Glutamate release was assayed by on-line fluorimetry as described previously (Nicholls and Sihra, 1986). Synaptosomal pellets were resuspended in HEPES-buffered incubation medium and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter. $NADP^+$ (1 mM), glutamate dehydrogenase (50 U/ml) and $CaCl_2$ (1 mM) were added after 5 min. After a further 10 min of incubation, 3 mM 4-aminopyridine was added to depolarize the synaptosomes. Glutamate release was monitored by measuring the increase in fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) due to NADPH being produced by the oxidative deamination of released glutamate by glutamate dehydrogenase. Data were collected at 2-s intervals. Exogenous glutamate (5 nmol) was added as standard at the end of each experiment and the fluorescence change produced by the standard addition was used to calculate the released glutamate as nmol glutamate/mg synaptosomal protein. Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was done with two-tailed Student's *t*-tests.

2.3. Membrane potential measurement using $DiSC_3(5)$

Synaptosomes were resuspended in 2 ml HEPES-buffered incubation medium and incubated in a stirred and thermostatted cuvette at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter. After a 3-min incubation, 4 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min. 4AP was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively, and data were collected at 2-s intervals. Cumulative data were analysed using MicroCal Origin and results are expressed in fluorescence units. Statistical analysis was done with two-tailed Student's *t*-tests.

2.4. Cytosolic Ca^{2+} measurements using Fura-2

Synaptosomes (0.3 mg/ml) were preincubated in incubation medium containing 5 μM Fura-2-acetoxymethyl ester and 0.1 mM $CaCl_2$ for 30 min at 37 °C in a stirred test tube. After Fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 10000 g. The synaptosomal pellets were resuspended in incubation buffer at 37 °C and the synaptosomal suspension was stirred in a thermostatted cuvette in a Perkin-Elmer LS-50B spectrofluorimeter. $CaCl_2$ (1 mM) was added after 3 min and further additions were made after an additional 5 min, as described in the legends to the figures. Fura-2-Ca fluorescence was measured at excitation wavelengths of 340 nm and 380 nm (emission wavelength 505 nm) at 7.5-s intervals. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$, nM) was calculated by using calibration procedures and equations described previously (Sihra et al., 1992). Cumulative data were analysed using Lotus 1-2-3.and MicroCal Origin. Statistical analysis was done with two-tailed Student's *t*-tests.

2.5. Materials

2,3-Dihydro-5-methyl-3-(4-morpholinyl-methyl)-pyrrolo-1,4-benzoxazin-6-yl-1-naphthalenylmethanone (WIN 55212-2), 2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo-benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720) and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM 281) were obtained from Tocris Cookson (Bristol, USA). Fura-2-acetoxymethyl ester and $DiSC_3(5)$ were obtained from Molecular Probes (Eugene, OR, USA). Glutamate dehydrogenase and all other reagents were obtained from Sigma (Poole, UK) or Merck (Poole, UK).

3. Results

3.1. Cannabinoid receptor activation inhibits the 4-aminopyridine-evoked glutamate release

To examine the effects of cannabinoid receptor activation on glutamate release evoked by 4-aminopyridine, which

opens voltage-gated Ca^{2+} channels and induces the release of vesicular glutamate by blocking presynaptic K^+ channels (Tibbs et al., 1989), the selective and potent cannabinoid CB_1 receptor agonist 2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)-pyrrolo-1,4-benzoxazin-6-yl-1-naphthalenyl-methanone (WIN55212-2) was used. Under control conditions, 4-aminopyridine (3 mM) evoked glutamate release of 24.3 ± 0.8 nmol glutamate per mg synaptosomal protein after 4 min of depolarization. Preincubation of synaptosomes with WIN 55212-2 (5 μM) before 4-aminopyridine addition inhibited glutamate release to 12.3 ± 0.4 nmol per mg per 4 min ($n = 8$; $P < 0.001$; Fig. 1A), and the effect of WIN 55212-2 was concentration-dependent (Fig. 1B). A robust depression of 4-aminopyridine-evoked glutamate release was seen with 5 μM WIN 55212-2 (49 ± 3% compared with control; Fig. 1A). Because this response was on the linear part of the concentration–response curve (Fig. 1B), 5 μM WIN 55212-2 was used in subsequent experiments to evaluate the cellular mechanisms underlying the inhibitory effect of cannabinoid CB_1 receptor activation on evoked glutamate release from cerebrocortical nerve terminals. The IC_{50} value for WIN 55212-2-induced inhibition of 4-aminopyridine-evoked glutamate release, derived from a log dose–response curve, was 2.7 μM. Additionally, the endogenous cannabinoid anandamide (10 μM) also effectively inhibited 4-aminopyridine (3 mM)-evoked

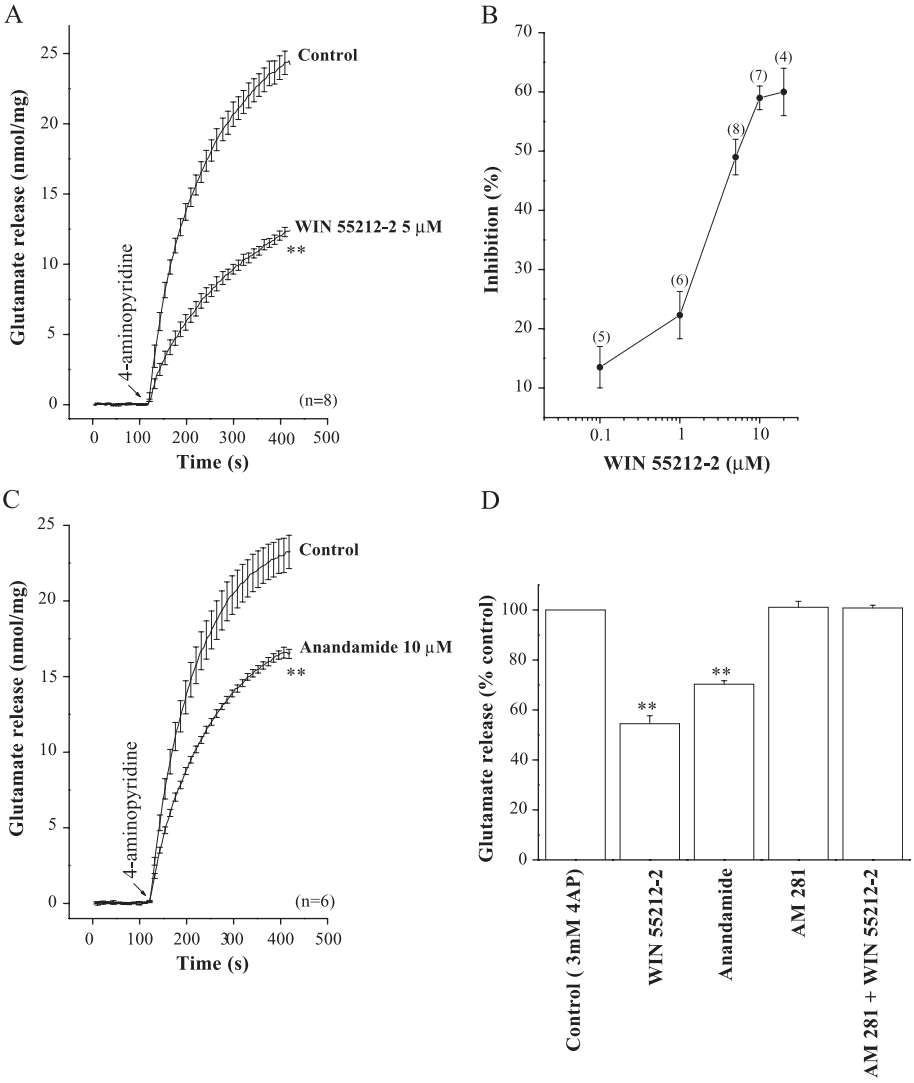

Fig. 1. WIN 55212-2 inhibits 4AP-evoked glutamate release from hippocampal synaptosomes by activation of cannabinoid CB_1 receptors. (A and C) Glutamate release was evoked by the addition of 4-aminopyridine (3 mM) in the absence (Control) and in the presence of WIN 55212-2 (5 μM) or anandamide (10 μM). (B) Concentration-dependent inhibition of glutamate release by WIN 55212-2. (D) The bar diagrams show glutamate release after 4 min of depolarization with 3 mM 4-aminopyridine in the presence of WIN 55212-2 (5 μM), anandamide (10 μM), or AM 281 (5 μM) + WIN 55212-2 (5 μM). Data represent means ± S.E.M. from independent experiments carried out with individual synaptosomal preparations from the indicated number of animals (n). Mean ± S.E.M. was obtained for each data point (2 s), but error bars are only shown every 10 s for visual clarity. Neurotransmitter release in the presence of WIN 55212-2 or anandamide was significantly different from that in control (**$P < 0.001$).

release from 23.2 ± 1.1 nmol per mg per 4 min to 16.5 ± 0.4 nmol per mg per 4 min ($n = 6$; $P < 0.001$; Fig. 1C). To determine whether the WIN 55212-2 effect was mediated via activation of cannabinoid CB_1 receptors, the selective cannabinoid CB_1 receptor antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM 281) was used. Control 4-aminopyridine (3 mM)-evoked glutamate release was 19.8 ± 1.2 nmol per mg per 4 min, and application of AM281 (5 ìM) alone did not produce a measurable effect on 4-aminopyridine-evoked release (20.3 ± 1.4 nmol per mg per 4 min). However, the WIN 55212-2-mediated inhibition of release was completely blocked in the presence of 5 μM AM281 (20.1 ± 0.9 nmol per mg per 4 min; $n = 7$; Fig. 1D), indicating that the effect of WIN 55212-2 is mediated by cannabinoid CB_1 receptors.

3.2. Effects of cannabinoid CB_1 receptor activation on the synaptosomal membrane potential

The mechanism by which cannabinoid CB_1 receptor activation mediates the inhibition of glutamate release was addressed by assessing the effects of WIN 55212-2 on synaptosamal plasma membrane potential. The synaptosomal membrane potential was determined with the membrane potential sensitive dye $DiSC_3(5)$. 4-Aminopyridine (3 mM) caused an increase in $DiSC_3(5)$ fluorescence of 2.6 ± 0.2 fluorescence units per 3 min. Preincubation of synaptosomes with WIN 55212-2 (5 μM) did not alter the resting plasma membrane potential (data not shown) and produced no significant change in the 4-aminopyridine-mediated increase in $DiSC_3(5)$ fluorescence (2.58 ± 0.17 fluorescence units per 3 min; $n = 6$; $P > 0.05$; Fig. 2). These experiments indicate that the inhibition of evoked glutamate release by WIN 55212-2 is unlikely to be due to a hyperpolarizing effect of the agonist on the synaptosomal plasma membrane potential or to an attenuation of depolarization produced by 4-aminopyridine.

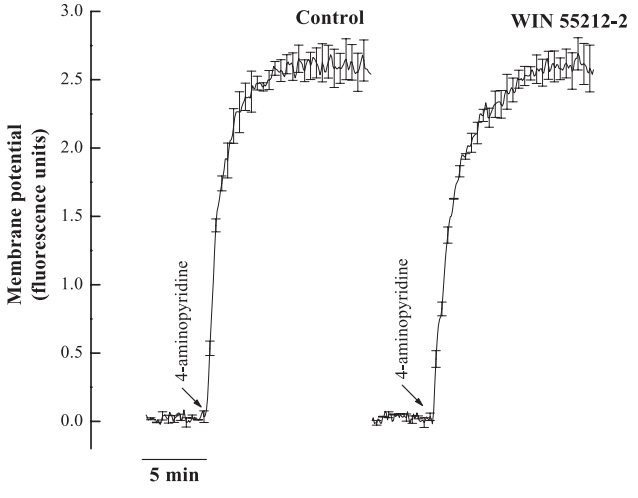

Fig. 2. WIN 55212-2 effect on synaptosomal membrane potential. Synaptosomal membrane potential, monitored with $DiSC_3(5)$, after depolarization with 4-aminopyridine (3 mM) in the absence (Control) or presence of 5 μM WIN 55212-2 added 2 min before depolarization. Each trace is the mean ± S.E.M. of independent experiments, using synaptosomal preparations from six animals. The S.E.M. was computed for each point (2-s interval), but error bars are only shown every 10 s for clarity.

3.3. Cannabinoid receptor activation inhibits the evoked release via a pertussis toxin-sensitive Gi/Go protein independently of the cAMP-protein kinase A mechanism

Most actions of cannabinoids mediated by the cannabinoid CB_1 receptor are transduced by pertussis toxin-sensitive G proteins, presumably G_i or G_o. To determine whether the inhibitory G_i/G_o proteins were involved in the coupling of presynaptic cannabinoid CB_1 receptors to the metabotropic effects, culminating in the inhibition of evoked release of glutamate, synaptosomes were incubated for 4 h in the absence or presence of pertussis toxin (2 μg/ml). Control 4-aminopyridine (3 mM)-evoked glutamate release was 23.2 ± 0.6 nmol per mg 4 min, and pertussis toxin treatment of synaptosomes did not significantly alter glutamate release (23.1 ± 0.4 nmol per mg per 4 min; $n = 5$; $P > 0.05$; Fig. 3A). Additionally, the inhibitory effect of WIN 55212-2 (5 μM) was fully blocked in the pertussis toxin-treated synaptosomes (22.9 ± 0.8 nmol per mg per 4 min; $n = 5$; Fig. 3A). On average, WIN 55212-2 (5 μM) produced a 0.9 ± 1.2% decrease in 4-aminopyridine-evoked release after treatment with pertussis toxin, which was significantly different from the inhibition produced by WIN 55212-2 alone (49 ± 3%; $n = 7$; $P < 0.001$; Fig. 3C). These results suggest that the WIN 55212-2-induced inhibition of glutamate release is mediated via a pertussis toxin-sensitive inhibitory G protein.

Previous work has shown that the activation of cannabinoid CB_1 receptors inhibits adenylate cyclase via an inhibitory G protein (Childers and Deadwyler, 1996; Pertwee, 1997). If the observed WIN 55212-2-mediated inhibition is indeed the result of inhibition of adenylate cyclase, leading to a reduced level of cyclic adenosine monophosphate and protein kinase A, then it should be possible to disrupt this process by blocking protein kinase A activation. To test this possibility, a membrane-permeable protein kinase A inhibitor 2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo-benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720) was examined. Preincubation of synaptosomes with KT 5720 (100 μM), however, had no significant effect on either 4-aminopyridine (3 mM)-evoked release of glutamate or the inhibition produced by WIN 55212-2 (5 μM) (Fig. 3B). In seven synaptosomes tested, WIN 55212-2 (5 μM) produced a 41 ± 2.7% decrease in the 4-aminopyridine-evoked glutamate release after the application KT 5720, which was not significantly different from the inhibition produced by WIN 55212-2 alone (49 ± 3%; $P > 0.05$; Fig. 3C). These results indicate that a cAMP-

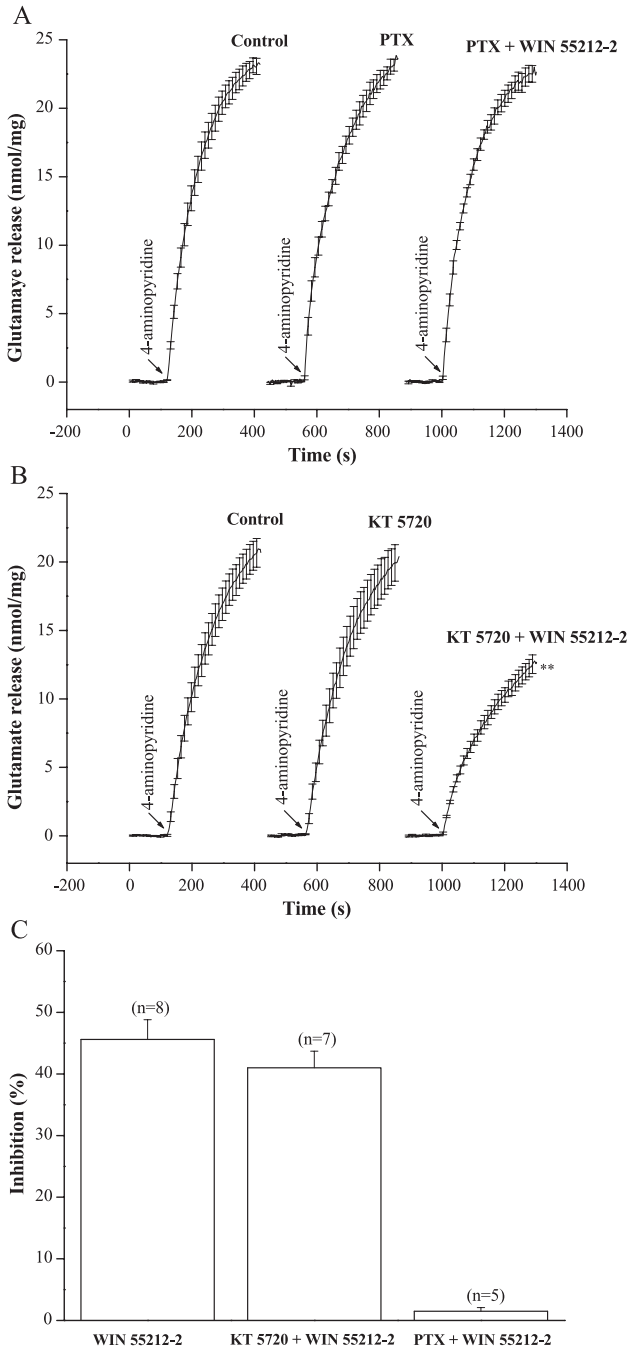

Fig. 3. WIN 55212-2-mediated inhibition of glutamate release is blocked by pertussis toxin pretreatment but not by protein kinase A inhibitor. (A) The synaptosomes were incubated with pertussis toxin (2 μg/ml) for 4 h, and the release of glutamate evoked by 3 mM 4-aminopyridine was determined in the absence (control) and in the presence of WIN 55212-2 (5 μM) added 5 min before depolarization. (B) Glutamate release evoked by 4-aminopyridine (3 mM) in the absence or presence of protein kinase A inhibitor KT 5720 (100 μM) added 3 min prior to the addition of WIN 55212-2 (5 μM). (C) Bar plots showing the average percent inhibition of evoked release produced by WIN 55212-2 (5 μM) in synaptosomes from control, pertussis toxin-treated groups and KT 5720-treated groups. Each trace is the mean ± S.E.M. of independent experiments, using synaptosomal preparations from seven animals. The S.E.M. was computed for each point (2-s interval), but error bars are only shown every 10 s for clarity.

dependent pathway is not involved in the observed effects of WIN 55212-2 on 4-aminopyridine-evoked glutamate release.

3.4. The effects of cannabinoid CB_1 receptor activation on exocytotic machinery and Ca^{2+} influx

To determine whether cannabinoid CB_1 receptor activation impinged on the exocytotic machinery itself, downstream of Ca^{2+} entry, the effects of WIN 55212-2 on glutamate release evoked by the Ca^{2+} ionophore ionomycin was examined. Ionomycin (5 μM) evoked the release of 13.6 ± 0.7 nmol glutamate per mg per 4 min. Preincubation of synaptosomes with WIN 55212-2 (5 μM) did not significantly affect ionomycin-induced release of glutamate (13.8 ± 0.6 nmol per mg per 4 min; $n=8$; $P>0.05$; Fig. 4A). These results suggest that a major site of cannabinoid action lies upstream of Ca^{2+} entry, potentially at the level of Ca^{2+} channel activity. To assess further whether WIN 55212-2 reduces the influx of Ca^{2+}, I determined the $[Ca^{2+}]_c$ using the Ca^{2+} indicator fura-2. As illustrated in Fig. 4B, 4-aminopyridine (3 mM) caused a rise in $[Ca^{2+}]_c$ to a plateau level of 253.8 ± 10.9 nM. Preincubation of synaptosomes with WIN 55212-2 (5 μM) reduced the 4AP-evoked $[Ca^{2+}]_c$ increase by 85.3 nM (168.5 ± 7.1 nM; $n=5$; $P<0.001$).

3.5. Cannabinoid receptor activation depresses evoked glutamate release by inhibiting presynaptic N- and P/Q-type Ca^{2+} channels

Because cannabinoid receptor activation inhibits N- and/or P/Q-type Ca^{2+} channels (Twitchell et al., 1997; Shen and Thayer, 1998; Sullivan, 1999), and these channels are principally responsible for trigging the presynaptic release of glutamate (Wheeler et al., 1994; Wu and Saggau, 1997), inhibition of these Ca^{2+} channels is a candidate mechanism for the WIN 55212-2-mediated inhibition of glutamate release. I therefore examined the effects of WIN 55212-2 on 4-aminopyridine-evoked glutamate release before and after selective blockade of each of these Ca^{2+} channel subtypes. As shown in Fig. 5, ω-conotoxin-GVIA (2 μM), a selective N-type Ca^{2+} channel blocker, reduced 4-aminopyridine-evoked glutamate release by 27.9 ± 4.3% ($n=7$; $P<0.01$). In synaptosomes pretreated with ω-conotoxin-GVIA (2 μM), WIN 55212-2 (5 μM) reduced glutamate release by 46.2 ± 2.4%, which was not significantly different from the inhibition produced by WIN 55212-2 alone (49 ± 3.2%; $n=8$; $P>0.1$). Application of the P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA (200 nM) reduced 4-aminopyridine-evoked glutamate release by 50.8 ± 4.2% ($n=6$; $P<0.001$), but did not significantly prevent the action of WIN 55212-2. After application of ω-agatoxin IVA (200 nM), WIN 55212-2 (5 mM) was still able to reduce glutamate release by 66.1 ± 2% ($n=6$; $P<0.001$). To test the

possibility that WIN 55212-2 mediates its effects through the combined inhibition of N- and P/Q-type Ca^{2+} channels, the effects of WIN 55212-2 were tested before and after the combined application of ω-conotoxin-GVIA and ω-agatoxin IVA. Combined application of ω-conotoxin-GVIA (2 μM) and ω-agatoxin IVA (200 nM) reduced 4AP-evoked glutamate release by 69.8 ± 3.9% ($n = 5$; $P < 0.001$). In the combined presence of ω-conotoxin-GVIA and ω-agatoxin IVA, application of WIN 55212-2 (5 μM) reduced glutamate release by only 6.8 ± 3.4% ($n = 5$), which was significantly less than that when WIN 55212-2 was applied alone (49 ± 3.2%; $n = 8$; $P < 0.001$) (Fig. 5). These results indicate that, in hippocampal synaptosomes, WIN 55212-2 acts by inhibiting both N- and P/Q type Ca^{2+} channels to reduce the release of glutamate.

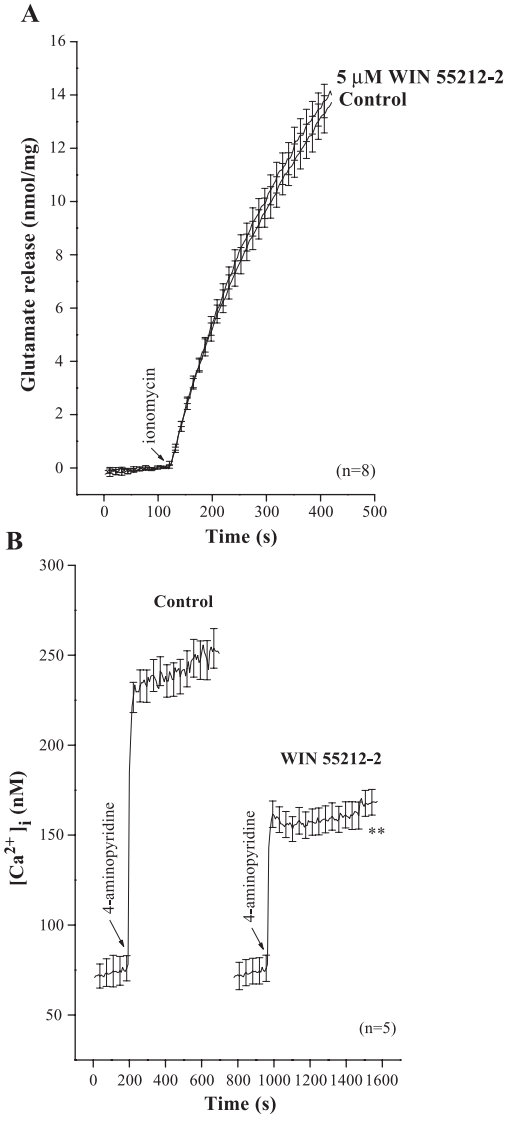

Fig. 4. WIN 55212-2 effects on ionomycin-induced glutamate release and Ca^{2+} influx. (A) Glutamate release was induced by ionomycin (5 μM) in the absence (Control) or presence of WIN 55212-2 (5 μM). Data represent means ± S.E.M. for seven to eight independent synaptosomal preparations. Error bars are shown every 10 s for clarity. Glutamate release in the presence of WIN 55212-2 5 μM was not significantly different from that in control. (B) Synaptosomes (0.3 mg/ml) were incubated as described in Methods, and $[Ca^{2+}]_C$ was monitored using fura-2. Voltage-dependent Ca^{2+} influx was evoked by 4-aminopyridine in the absence or presence of WIN 55212-2 (5 μM). Data represent means ± S.E.M. of five independent synaptosomal preparations. Error bars are shown every 10 s for clarity. $[Ca^{2+}]_C$ in the presence of WIN 55212-2 was significantly different from control (**$P < 0.01$).

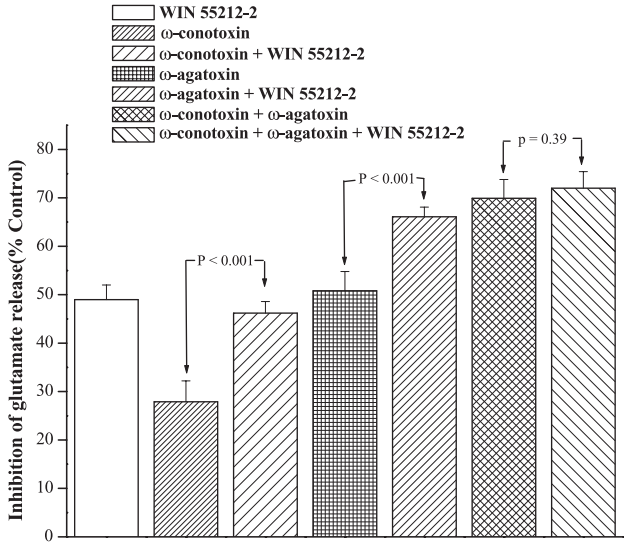

Fig. 5. Blockade of N- and P/Q-type Ca^{2+} channels abolishes WIN 55212-2 inhibition of glutamate exocytosis. The bar diagrams showing the release of glutamate evoked by 4-aminopyridine (3 mM) in the absence (control) or presence of ω-conotoxin GVIA (2 μM), ω-conotoxin GVIA (2 μM) + WIN 55212-2 (5 μM), ω-agatoxin VIA (200 nM), ω-agatoxin VIA (200 nM) + WIN 55212-2 (5 μM), ω-conotoxin GVIA (2 μM) + ω-agatoxin VIA (200 nM) and ω-conotoxin GVIA (2 μM) + ω-agatoxin VIA (200 nM) + WIN 55212-2 (5 μM). Data represent means ± S.E.M. of five to seven independent synaptosomal preparations.

4. Discussion

Although electrophysiological studies have suggested that cannabinoids produce presynaptic inhibition of glutamate transmission by impinging on cannabinoid CB_1 receptors on the excitatory terminals (Shen et al., 1996; Sullivan, 1999; Huang et al., 2001), the presynaptic cannabinoid CB_1 receptor effects on transmitter release were monitored indirectly, by looking at changes in postsynaptic potentials. The use of isolated nerve terminals (synaptosomes) allows a more direct evaluation of presynaptic cannabinoid receptor regulation of the steps leading to exocytosis. Here, I used the synaptosome model to assess the characteristics and mechanism of action of cannabinoid CB_1 receptors involved in regulating glutamate exocytosis in the hippocampus. The primary finding of this study is that the activation of cannabinoid receptors

by WIN 55212-2 depresses glutamate release evoked by 4-aminopyridine through an inhibitory G protein-coupled suppression of presynaptic N- and P/Q-type Ca^{2+} channel activity.

4.1. Mechanism of presynaptic inhibition of glutamate release by WIN 55212-2

In synaptic terminals, neurotransmitter release is a complex phenomenon and is modulated at several putative sites, including Na^{+} channels, K^{+} channels, Ca^{2+} channels, as well as the release process itself. Previous studies have been shown that cannabinoid activation of K^{+} channels may produce a presynaptic hyperpolarization that contributes to the inhibition of glutamate release (Deadwyler et al., 1993; Henry and Chavkin, 1995). In marked contrast, I found that the inhibition of 4-aminopyridine-evoked glutamate release by WIN 55212-2 is not due an indirect effect through modulation of membrane potential changes because I observed no effect of WIN 55212-2 on 4-aminopyridine-evoked membrane potential depolarization measured using a membrane potential dye, $DiSC_3(5)$.

In terms of receptor–effector coupling, there is some consensus that cannabinoid CB_1 receptor responses are coupled through pertussis toxin-sensitive G proteins (Matsuda et al., 1990; Pacheco et al., 1993; Hoelett, 1995). Pertussis toxin treatment previously was found to block cannabinoid receptor-mediated inhibition of synaptic transmission in cultured hippocampal neurons (Shen et al., 1996; Sullivan, 1999), a result confirmed here by the finding that pertussis toxin treatment eliminated the effects of WIN 55212-2 on 4-aminopyridine-evoked glutamate release. This suggests that activation of a pertussis toxin-sensitive inhibitory G protein is involved in the action of WIN 55212-2. In addition, like many other receptors coupled to pertussis toxin-sensitive G protein, cannabinoid CB_1 receptor actions include inhibition of adenylate cyclase. The inhibitory action of WIN 55212-2 on the release evoked by 4-aminopyridine observed here may represent a negative coupling of cannabinoid CB_1 receptors to adenylate cyclase activity to cause a decrease in cyclic AMP levels. This lowering of protein kinase A activity may then indirectly lead to attenuation of glutamate release. Consistent with this, WIN 55212-2-mediated inhibition of synaptic transmission in striatal neurons is antagonized by the adenylate cyclase activator forskolin and the cAMP analogue Sp-cAMP (Huang et al., 2002). I found here that a protein kinase A inhibitor, KT 5720, did not reverse the inhibitory effects of WIN 55212-2 on 4-aminopyridine-evoked glutamate release. This implies that, in common with some other presynaptic metabotropic receptors (Herrero et al., 1996; Perkinton and Sihra, 1998), the observed inhibition of glutamate release by CB_1 cannabinoid receptor activation is not mediated by a cAMP-dependent mechanism. This hypothesis is supported by electrophysiological studies showing that cannabinoid CB_1 receptor-mediated presynaptic inhibition of glutamatergic transmission is independent of the cAMP-protein kinase A cascade in the nucleus accumbens (Robbe et al., 2001).

Using fura-2, I demonstrated that WIN 55212-2 significantly inhibited voltage-dependent Ca^{2+} entry stimulated by 4-aminopyridine. This then provides a causal link between cannabinoid CB_1 receptor-mediated changes in $[Ca^{2+}]_c$ and the inhibition of glutamate release, because N- and P/Q-type Ca^{2+} channels control the release of glutamate in the hippocampus (Luebke et al., 1993; Wheeler et al., 1994; Scholz and Miller, 1995), and activation of the cannabinoid receptor has been found to inhibit these Ca^{2+} channels (Shen and Thayer, 1998; Twitchell et al., 1997; Sullivan, 1999). Thus, it is likely that cannabinoids inhibit excitatory neurotransmission by modulating the influx of Ca^{2+} into the nerve terminal. In the present study, the observation that the WIN 55212-2-induced inhibition of glutamate release persisted after blockade of N- or P/Q-type Ca^{2+} channels indicates either that WIN 55212-2 does not exert its effects through these channels, or that WIN 55212-2 exerts its effects through the combined inhibition of these channels. Consistent with this interpretation, the present study demonstrated that the combined application of N- and P/Q-type Ca^{2+} channel blockers completely blocked the inhibitory effect of WIN 55212-2 on the glutamate release evoked by 4-aminopyridine. This result supports the hypothesis that inhibition of both N- and P/Q-type Ca^{2+} channel activity is the primary mechanism of the cannabinoid CB_1 receptor-mediated decrease in glutamate release from hippocampal nerve terminals.

Although the inhibition of presynaptic Ca^{2+} channels represents a likely mechanism for the cannabinoid CB_1 receptor-mediated inhibition of glutamate release, these experiments did not eliminate the possibility that WIN 55212-2 may also act on the exocytotic machinery itself, downstream of Ca^{2+} entry. This mechanism was important to evaluate because the release machinery has been implicated as a target of other presynaptic receptors involved in neurotransmitter release (Scanziani et al., 1992; Sanchez-Prieto et al., 1996; Wu and Saggau, 1997). Therefore, I examined this possibility by using ionomycin, which induces glutamate release by a direct increase in intrasynaptosomal Ca^{2+} levels, without previous depolarization and Ca^{2+} channel activation (Sihra et al., 1992). I found that WIN 55212-2 had no effect on the ionomycin-induced release of glutamate, suggesting therefore that cannabinoid CB_1 receptor-mediated inhibition of glutamate release is not due to a direct effect on the release machinery.

In conclusion, the present experiments demonstrate that activation of cannabinoid CB_1 receptors inhibit evoked glutamate release from hippocampal synaptosomes through a suppression of N- and P/Q-type Ca^{2+} channel activity, rather than by an indirect effect on neuronal

excitability or a direct effect on the release machinery. Because glutamate is the predominant excitatory neurotransmitter in the brain, and its abnormal release has been implicated in neurotoxicity (Meldrum and Garthwaite, 1990; Beal, 1992; Obrenovitch and Urenjak, 1997), it is possible that inhibition of presynaptic Ca^{2+} channels causes the decrease in glutamate release mediated by cannabinoids, contributing to the potential therapeutic benefits of cannabinoids.

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