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Research report

Activation of CB1 cannabinoid receptors inhibits neurotransmitter release from identified synaptic sites in rat hippocampal cultures

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Abstract

The effects of cannabinoids on synaptic transmission were measured optically in rat hippocampal cultures. Synaptic release sites were labeled with the fluorescent dye FM1-43 in a stimulus-dependent manner. Action potential-induced release of FM1-43 required extracellular Ca^{2+} and was inhibited 65 ± 3% by blockade of high-threshold voltage-gated Ca^{2+} channels with ω-grammotoxin SIA (300 nM). The cannabimimetic drug, Win 55212-2 (300 nM), inhibited FM1-43 release by 51 ± 3%. The inhibition produced by Win55212-2 was blocked by the CB1 cannabinoid receptor antagonist, SR141716 (1 μM). The intensity of FM1-43 labeled puncta ranged 4-fold, although the inhibition produced by Win55212-2 was distributed normally across synaptic sites of various labeling intensities. The FM1-43-based optical method appears promising for the study of the effects of cannabinoids and other drugs on synaptic networks. These results indicate that cannabimimetics act presynaptically to inhibit the release of neurotransmitter and that this inhibition is observed uniformly at boutons of varied activity levels.

Keywords: Cannabinoid; Δ^9-tetrahydrocannabinol; Glutamate neurotransmission; FM1-43; Presynaptic inhibition; Neurotransmitter release

1. Introduction

Cannabinoids, including the principal psychoactive ingredient in marijuana Δ^9-tetrahydrocannabinol, produce euphoria, sedation, hypoactivity, hypothermia, hypotension and bradycardia [1,10]. These effects are mediated by cannabinoid receptors [14] that couple to inhibitory G proteins [4] to activate K^+ channels [5,7,13] and inhibit Ca^{2+} channels [12,22,26]. The activation of these receptors by cannabimimetic drugs attenuates glutamatergic neurotransmission between cultured hippocampal neurons [21] and GABAergic neurotransmission at striatonigral synapses [3].

Cannabinoid receptors are present at high density on the presynaptic terminals of glutamatergic synapses in hippocampal cultures [26]. Several lines of physiological evidence suggest that these receptors, when activated, inhibit glutamate release by a presynaptic mechanism. Cannabinoid receptor agonists increase the coefficient of variation of excitatory postsynaptic currents, increase the number of synaptic failures, and fail to influence currents evoked by direct application of *N*-methyl-D-aspartate (NMDA) or kainate [21].

We used the fluorescent dye FM1-43 to label synaptic vesicles as a means to measure neurotransmitter release optically. Our objectives were to measure the effects of cannabinoids on individual release sites, to simultaneously monitor many release sights in order to determine the effects of these drugs on the synaptic network as a whole, and to test the conclusions reached from electrophysiological studies with a different approach. We found that cannabimimetic drugs acting via the CB1 receptor inhibited the release of FM1-43. While release sites varied widely in their rate of vesicle cycling, the inhibition produced by cannabimimetic drugs displayed a normal frequency distribution.

2. Materials and methods

2.1. Materials

Materials were obtained from the following companies: FM1-43, Molecular Probes, Eugene, OR; Win55212-2

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(*R* enantiomer, (+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl] pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-napthalenyl)methanone monomethanesulfonate)), RBI, Natick, MA; SR141716 (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) Sanofi Recherche, Montpellier Cedex, France; TTX (tetrodotoxin) and all other reagents, Sigma, St. Louis, MO.

2.2. Cell culture

Rat hippocampal neurons were grown in primary culture as previously described [27] 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. Cultures were grown without mitotic inhibitors for a minimum of 12 days before use.

2.3. Experimental procedure

Experiments were performed at room temperature in a recording chamber [25] that was continuously perfused with HEPES buffered Hanks' salt solution (HHSS) composed of the following (in mM): HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgCl_2$, 0.1; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; glucose, 5.6; and glycine, 0.01; pH 7.45. Drugs were applied by superfusion of solutions selected from several reservoirs with a multiport valve. Action potentials were evoked by applying electric field potentials (1 ms, 30 V) across two platinum stimulating electrodes with a Grass S44 stimulator [28].

Presynaptic release sites were labeled with FM1-43 by evoking trains of action potentials in the presence of the fluorescent styryl dye FM1-43 (5 μM). FM1-43 was added to the superfusing solution, and action potentials were evoked at 10 to 15 Hz for 20 s. Cells were left in the

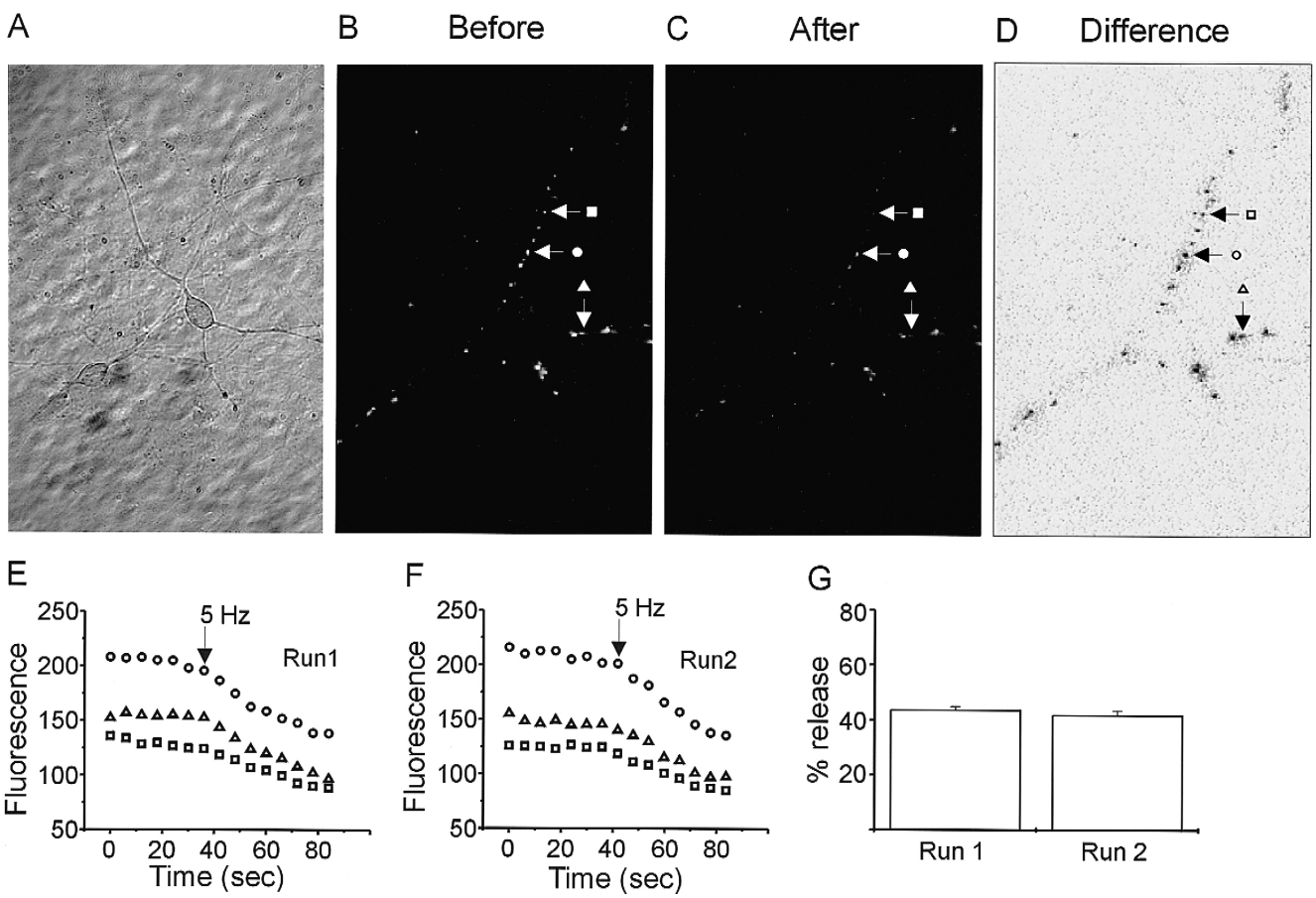

Fig. 1. Loading and unloading of synaptic release sites with FM1-43. (A) Bright field image of two hippocampal neurons making putative synaptic contacts. (B) The same field showing punctate FM1-43 fluorescence staining of synaptic release sites. FM1-43 was loaded by applying 15 Hz electric field stimulation for 20 s in the presence of dye (5 μM). (C) Fluorescence image after 5 Hz electric field stimulation for 42 s in the absence of FM1-43. Images B and C are displayed on the same gray scale. Punctate spots that responded to electrical stimulation were identified by subtracting image C from image B, resulting in the difference image D. Image D was inverted to highlight releasable puncta. (E) The change in FM1-43 fluorescence during 5 Hz stimulation (initiated at arrow) is plotted for the sites indicated by white arrows in B (run 1). (F) The same experiment shown in E was repeated after a second loading with FM1-43 (run 2). (G) Histogram summarizes the amount of dye released during electrical stimulation (5 Hz for 42 s) of 61 release sites from three experiments.

FM1-43-containing solution for at least 1 min to ensure complete endocytosis of dye labeled synaptic vesicles, and then washed for 5 min in saline containing TTX (300 nM).

The recording chamber was mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot) and the cultures imaged through a 70× objective (Leitz 1.3 n.a.) with a cooled charged couple device camera (384 × 576 pixels, Photometrics, Tucson, AZ). FM1-43 was excited at 470 (20) nm and fluorescence detected at 540 (40) nm. Fluorescent puncta with intensities greater than 100 photoelectron counts (12-bit scale) were selected for analysis. They were identified as synaptic release sites if electrical stimulation produced a 15% decrease in intensity. Images were acquired every 6 s.

Data are presented as mean ± S.E.M. Statistical comparisons were made by Student's *t*-test and ANOVA with Bonferoni's post-test.

3. Results

3.1. Stimulus-dependent labeling and release of FM1-43 from synaptic sites

Rat hippocampal neurons were grown in culture for 12–14 days during which time they established an extensive synaptic network. Field stimulation of these cultures elicits action potentials and synaptic transmission [17]. Betz et al. [2] have shown that incubating nerve endings in the anionic fluorescent dye FM1-43 during neurotransmission will trap the dye within synaptic vesicles. FM1-43 binds to plasma membranes and is endocytosed during the recycling of synaptic vesicles. Subsequent removal of the extracellular dye leaves synaptic sites labeled with fluorescence.

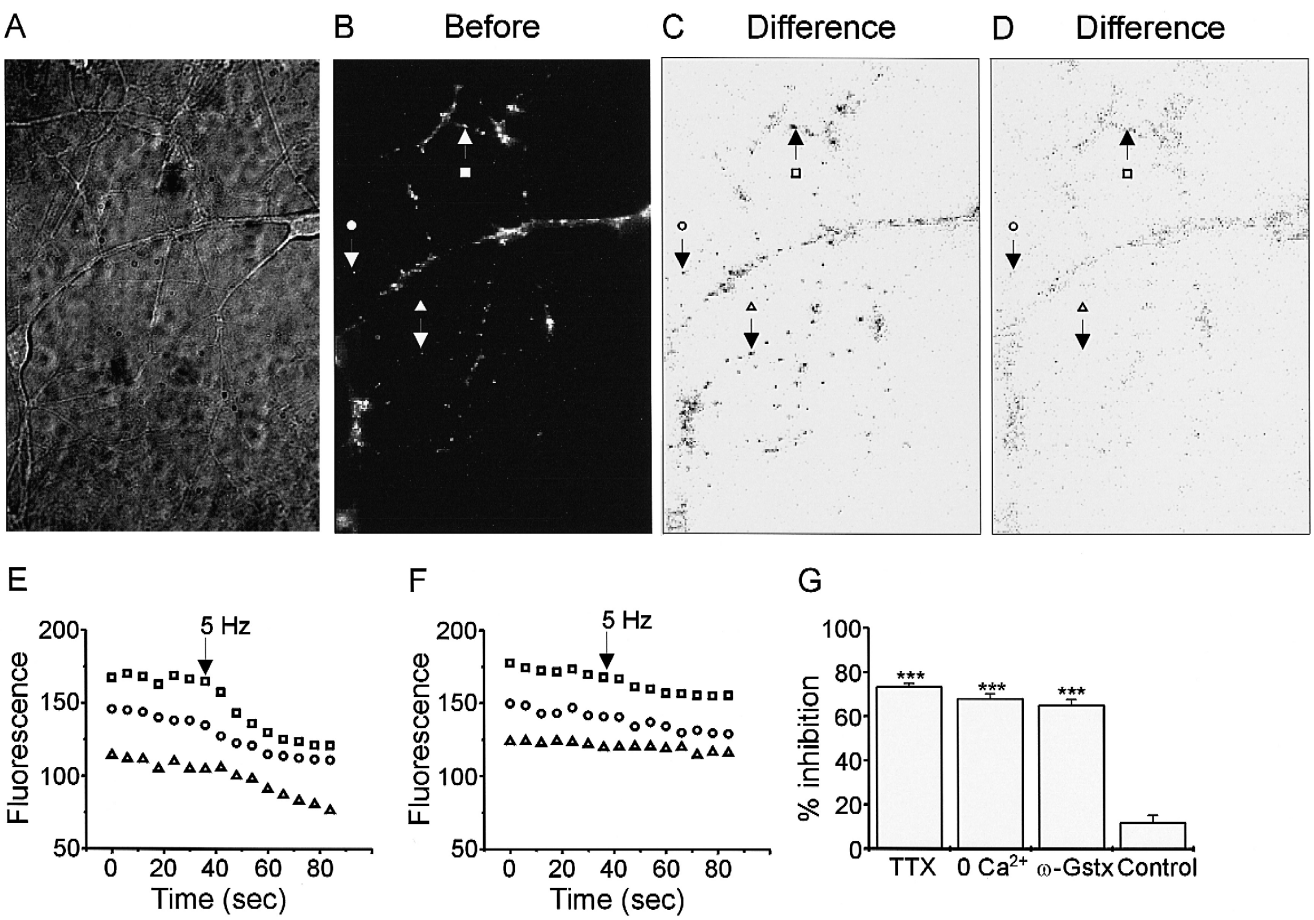

Fig. 2. Pharmacologic manipulation of FM1-43 release. (A) Bright field image of three hippocampal neurons with an extensive network of overlapping processes. (B) Fluorescence image shows punctate staining of synaptic release sites. (C and D) Difference images of the same field shown in B after electrical stimulation (5 Hz for 42 s) in the absence (C) and in the presence (D) of TTX (300 nM). (E and F) Plot of FM1-43 destaining from three representative release sites in the absence (E) and in the presence (F) of TTX (300 nM). (G) Histogram summarizes the inhibition of FM1-43 release by TTX (300 nM), removal of extracellular Ca^{2+} and ω-Gstx (300 nM). Drugs were applied 3 min prior to and during the stimulus. Data were summarized from three separate experiments with 54, 56, and 44 release sites for TTX, Ca^{2+}-free, and ω-GsTx, respectively. $p < 0.001$ relative to control, ANOVA with Bonferoni's post-test.

This approach is also applicable to cultured hippocampal neurons [8,16,19]. The cells shown in Fig. 1A were stimulated with a train of action potentials (15 Hz, 20 s) in the presence of 5 μM FM1-43 to label synaptic vesicles. Washing extracellular dye from the bath revealed fluorescent puncta along neuronal processes (Fig. 1B). Firing another train of action potentials (5 Hz) resulted in a time-dependent decrease in the fluorescent intensity of the puncta as shown in Fig. 1E (42 s, 5 Hz stimulus started at arrow). The destaining was not complete, consistent with the results of Ryan and Smith [20] that showed an action potential early in the stimulus train mobilized about 0.5% of the vesicle pool at hippocampal synapses. Our stimulus protocol provided 210 action potentials and thus, would mobilize approximately 65% of the vesicle pool. Subtracting the fluorescent image recorded after 42 s of stimulation (Fig. 1C) from the initial image (Fig. 1B) produced the difference image shown in Fig. 1D. These same sites were reloaded with FM1-43 and stimulated to release comparable amounts of dye (Fig. 1F). Thus, as summarized in Fig. 1G ($n = 61$ sites; three experiments), reproducible, stimulus-dependent decreases in fluorescence were elicited (compare run 1 to run 2). Stimulus-dependent release of FM1-43 was quantified and used as an index of neurotransmitter release.

3.2. Pharmacologic characterization of evoked FM1-43 release

We next characterized stimulus-dependent decreases in FM1-43 fluorescence pharmacologically. Using the same field stimulation protocol described in Fig. 1, we elicited an initial control response, then a second response was elicited in the presence of drug. Drugs were applied 3 min prior to and during the stimulus. As shown in Fig. 2, TTX (300 nM) inhibited the release of FM1-43 by $73 \pm 2\%$ ($n = 54$ sites; three experiments). Thus, electric field stimulation evokes action potentials that depolarize the cell sufficiently to trigger neurotransmitter release. This release relied on extracellular Ca^{2+} as indicated by the $69 \pm 2\%$ ($n = 56$ sites; three experiments) reduction in FM1-43 release observed in nominally Ca^{2+}-free media. The dye loss during stimulation in either TTX or Ca^{2+}-free media resulted from such factors as dye bleaching, spontaneous neurotransmitter release and dye diffusion. High-threshold voltage-gated Ca^{2+} channels mediate action potential-induced Ca^{2+} influx at most central synapses. We confirmed that this was the case for this model system using ω-grammotoxin SIA (ω-GsTx), which blocks N, P and Q-type Ca^{2+} channels in hippocampal neurons [18]. ω-GsTx (300 nM) inhibited $65 \pm 3\%$ ($n = 44$ sites; three experiments) of FM1-43 release evoked by field stimulation (Fig. 2). We conclude that field stimulation elicits action potentials that subsequently activate presynaptic voltage-gated Ca^{2+} channels to trigger the release of FM1-43-labeled synaptic vesicles.

3.3. Cannabinoid receptor agonists inhibit FM1-43 release via CB1 receptors

The cannabimimetic drug Win55212-2 acts as a full agonist to inhibit glutamatergic synaptic transmission between hippocampal neurons grown in primary culture [21]. Based on electrophysiological studies, we hypothesized that cannabinoid receptor agonists act presynaptically to inhibit the release of neurotransmitter. We tested this idea directly by determining the effects of the cannabimimetic, Win55212-2, on the evoked release of FM1-43. A field of hippocampal neurons was loaded with FM1-43 in a stimulus-dependent manner (Fig. 3A). Electric field stimulation produced a marked decrease in the intensity of FM1-43 labeled fluorescent puncta (Fig. 3C, solid symbols). A second stimulation of the same synaptic network in the presence of 300 nM Win55212-2 produced an attenuated response (Fig. 3C, open symbols). Win55212-2 inhibited the evoked release of FM1-43 by $51 \pm 3\%$ ($n = 80$ sites; four experiments). We next determined whether the inhibition produced by Win55212-2 was mediated by the CB1 receptor by applying the cannabimimetic in the presence of the antagonist SR141716 (1 μM) (Fig. 3B and D). A field of neurons was loaded with FM1-43 (Fig. 3B) and shown to release dye in response to field stimulation (Fig. 3D, open squares). Stimulation of the same field in the presence of 300 nM Win55212-2 and 1 μM SR141716 (Fig. 3D, solid squares) evoked release of a comparable amount of dye ($n = 67$ sites; three experiments). We did not examine the effects of SR141716 alone in this study because in previous studies with this cell culture model we have not observed an effect of the antagonist alone [23,24]. Thus, as summarized in Fig. 3E, activation of cannabinoid CB1 receptors significantly inhibits the vesicular release of neurotransmitter evoked by action potentials.

We also performed experiments in which the same FM1-43-labeled sites were stimulated in the presence of Win55212-2 in the absence and presence of SR141716. The field of cells shown in Fig. 4A was loaded with FM1-43 in a stimulus-dependent manner resulting in the fluorescent labeled image shown in Fig. 4B. Stimulation in the presence of 300 nM Win55212-2 alone produced little FM1-43 release as shown by the essentially blank difference image (Fig. 4C) and the absence of stimulus-dependent release from the site identified by the arrow (Fig. 4B and C) and plotted in Fig. 4E (open squares). However, when the same field of cells was reloaded with dye and stimulated in the presence of Win55212-2 and the CB1 receptor antagonist SR141716, a large number of release sites could be identified in the difference image (Fig. 4D). The site marked by the arrow in B–D now showed marked release evoked by the stimulus (Fig. 4E, solid squares). Release evoked in the presence of both Win55212-2 and SR141617 was 2.3-fold greater ($n = 37$ sites; two experiments) than that evoked in the presence of Win55212-2 alone (Fig. 4F).

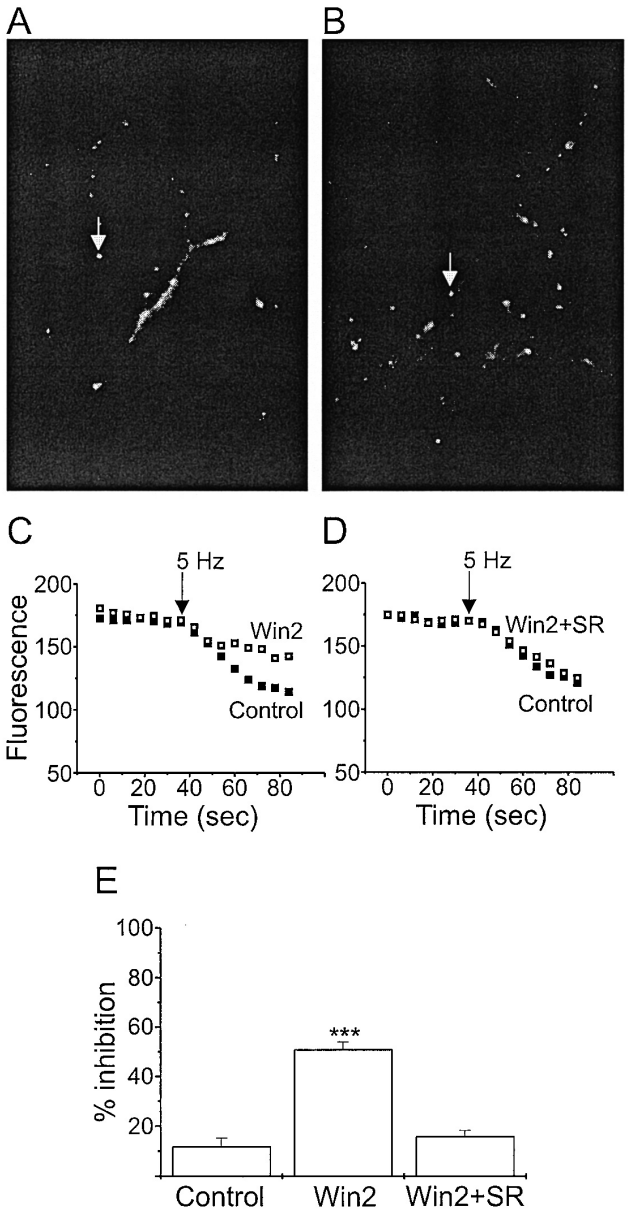

Fig. 3. Inhibition of FM1-43 release by Win55212-2. (A and B) Fluorescence images of FM1-43 labeled synaptic release sites from two separate fields. Drugs were applied 3 min prior to and during the stimulus. (C) A representative plot of FM1-43 destaining from the release site identified by the arrow in A, in the absence (control, solid squares) and presence (Win2, open squares) of 300 nM Win55212-2. (D) A representative plot of FM1-43 release in the absence (control, solid squares) and presence (Win2 + SR, open squares) of both Win55212-2 (300 nM) and SR141716 (1 μM) for the release site identified by the arrow in B. (E) Histogram summarizing the effect of Win55212-2 and SR141716 on the release of FM1-43. *** $p < 0.001$, Win2 vs. control ($n = 80$ sites; four experiments) and Win2 vs. Win2 + SR ($n = 67$ sites; three experiments), ANOVA with Bonferoni's post-test.

3.4. Win55212-2 uniformly inhibits FM1-43 release

We noted that the fluorescent intensity of FM1-43-labelling varied widely even within the same field of cells. Consistent with the stimulus-induced method of dye loading, stimulus dependent release was greater for more intensely labeled puncta (Fig. 5A). The rate of release, when calculated as a percentage of initial fluorescent intensity,

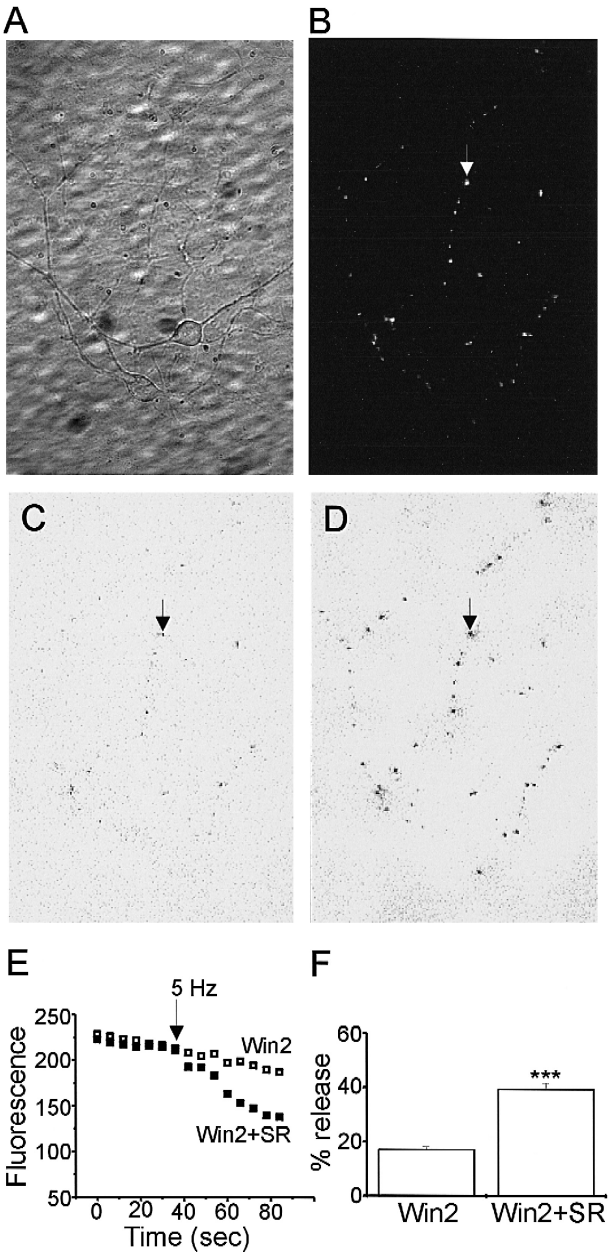

Fig. 4. SR141716 reverses inhibition of FM1-43 release by Win55212-2. (A) A bright field image of two hippocampal neurons making putative synaptic contacts. (B) Fluorescence image of FM1-43 labeled synaptic release sites. Drugs were applied 3 min prior to and during the stimulus. (C and D) Difference images of the same field shown in B after electrical stimulation (5 Hz for 42 s) in the presence of Win55212-2 (300 nM) (C) and in the presence of Win55212-2 (300 nM) plus SR141716 (1 μM) (D), respectively. (E) A representative plot of FM1-43 release in 300 nM Win55212-2 (Win2, open squares) and in the presence of both Win55212-2 (300 nM) and SR141716 (1 μM) (Win2 + SR, solid squares). (F) Histogram summarizes the effects of Win55212-2 on FM1-43 release in the absence and presence of SR141716. *** $p < 0.001$, Student's *t*-test. A total of 37 release sites from two experiments were analyzed.

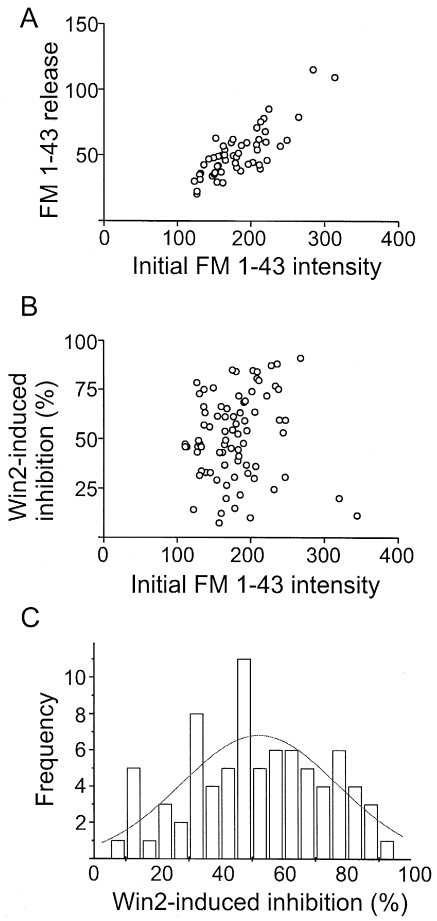

Fig. 5. Distribution of Win55212-2 inhibition of FM1-43 release. (A) Plot of the initial intensity of release sites vs. the amount of FM1-43 released during electrical stimulation shows a good correlation ($r = 0.81$). (B) Plot of initial intensity vs. the percentage inhibition of FM1-43 release by Win55212-2 shows no correlation ($r = -0.08$). (C) Histogram showing frequency distribution of inhibition of FM1-43 release by Win55212-2 and associated Gaussian fit. The distribution of the data was not significantly different from a Gaussian fit ($p > 0.10$, Dallal and Wilkinson's approximation to Lilliefors' method; Kolmogorov–Smirnov distance = 0.054).

was consistent across multiple release sites and was described by a monoexponential equation with a time constant of 40 ± 2 s. In spite of the variation in the amount of FM1-43 released at various synaptic sites, the inhibition of release produced by activation of cannabinoid receptors was not dependent on the intensity of FM1-43 labeling (Fig. 5B). Indeed, the variation in the level of cannabinoid-mediated inhibition observed at individual release sites was normally distributed (Fig. 5C).

4. Discussion

We studied cannabinoid inhibition of neurotransmitter release from a large number of FM1-43 labeled synaptic sites in a network of cultured rat hippocampal neurons. FM1-43 release from labeled synapses was dependent on action potential-induced Ca^{2+} influx through high threshold voltage-gated Ca^{2+} channels. The amount of FM1-43 release at an individual synaptic site correlated with the intensity of FM1-43 labeling. The cannabinoid receptor agonist, Win55212-2, uniformly inhibited vesicular release from these synapses. The response was mediated by CB1 receptors because the CB1 receptor antagonist SR141716 prevented inhibition by Win55212-2.

Electrophysiological studies have suggested that cannabinoids inhibit neurotransmitter release by a presynaptic mechanism [21]. This study corroborates these observations by providing direct evidence for the inhibition of neurotransmitter release by cannabimimetics at presynaptic terminals. Two lines of evidence support our conclusion that Win55212-2 acted via presynaptic CB1 receptors. First, FM1-43-based recording measures vesicular release directly and thus, does not depend on the postsynaptic cell to report activity, as is the case when recording postsynaptic currents. Second, Win55212-2 does not interfere with action potentials induced by electric field stimulation [21]. Thus, the site of cannabinoid action in these experiments must be downstream from the action potential and independent of the effects of the released neurotransmitter.

Using optical methods similar to those employed here, Reuter [19] found that individual synaptic boutons of cultured hippocampal neurons varied widely in their sensitivity to the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA, indicating a heterogeneous distribution of presynaptic Ca^{2+} channel subtypes. We found that Win55212-2 uniformly inhibited FM1-43 release, suggesting that cannabinoid modulation of neurotransmitter release involves inhibition of calcium influx through multiple calcium channel subtypes. Indeed, cannabinoids inhibit whole-cell currents through both N- and P/Q-type Ca^{2+} channels in hippocampal cultures [26]. The study by Reuter [19] demonstrated that imaging the evoked release of FM1-43 will resolve heterogeneous responses, yet the inhibition produced by cannabinoids was normally distributed across the whole range of FM1-43 labeling intensities. The large range of intensities is consistent with the broad spread of release probabilities previously reported for cultured hippocampal neurons [16]. The uniform response to Win55212-2 was surprising in that cannabimimetic drugs do not affect GABAergic neurotransmission in these cultures and FM1-43 would label both inhibitory and excitatory synapses. It is possible that we did not have a sample of sufficient size to detect a small distinct class of insensitive boutons.

Activation of cannabinoid receptors inhibits less than 17% of the whole-cell Ca^{2+} current [22] whereas inhibition of neurotransmitter release measured with FM1-43 produced greater than 51% inhibition. The presynaptic localization of cannabinoid receptors may make their effects more pronounced at the nerve terminal relative to the soma. Furthermore, neurotransmitter release is related to

$[Ca^{2+}]_i$ by a power function [15] which would allow a small reduction in Ca^{2+} influx to produce a large decrease in FM1-43 release.

The use of the styryl dye FM1-43 provides a direct measure of neurotransmitter release. By using optical imaging to follow loading and unloading of the dye, it was possible to study the effects of cannabimimetics on neurotransmission both at the level of individual synapses and at the level of the larger network. It is possible that CB1-mediated inhibition of FM1-43 destaining resulted from a drug-induced decrease in the duration of fusion pore formation at ''kiss and run'' type synapses [9]. However, our previous data showed that Win55212-2 inhibited excitatory postsynaptic currents indicating that the inhibition of FM1-43 release by cannabimimetic drugs results from a reduction in the release of neurotransmitter rather than a change in the mechanism of endocytosis. FM1-43-based imaging appears generally applicable to study drugs that act presynaptically to inhibit neurotransmitter release as exemplified by the activation of metabotropic glutamate receptors in baroreceptor neurons [6] and GABA(B)-mediated inhibition in hippocampal cultures [8]. Furthermore, the study of individual boutons enables the study of drugs that act at only a subset of active boutons [19]. This optical method has proven useful for studying activity dependent changes in synaptic networks [11] and, as demonstrated here, shows promise for detecting the effects of drugs, including the cannabinoids, on network function. Finally, because optical methods produce little toxicity, FM1-43-based imaging may prove useful for studying the effects of prolonged drug treatment.

In summary, we demonstrate that FM1-43-based recording of neurotransmitter release is a useful tool for studying the actions of drugs that act presynaptically. Specifically, we show that the cannabimimetic drug Win55212-2 acts presynaptically to inhibit neurotransmitter release and that this effect is uniformly distributed across synaptic sites in rat hippocampal cultures. This method may prove useful for studying the effects of prolonged exposure to cannabinoids on the number and activity of individual synaptic boutons.

Acknowledgements

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