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Inhibition of N- and P/Q-type Ca^{2+} channels by cannabinoid receptors in single cerebrocortical nerve terminals

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Abstract Since cannabinoid receptors inhibit excitatory synaptic transmission by reducing glutamate release, we have examined whether this might occur through the direct inhibition of presynaptic Ca^{2+} channels. In cerebrocortical nerve terminals, activation of cannabinoid receptors with WIN55,212-2 reduces the KCl-evoked release of glutamate. However, this inhibition is attenuated when N- and P/Q-type Ca^{2+} channels are blocked. Through Ca^{2+} imaging in single nerve terminals, we found that WIN55,212-2 reduced the influx of Ca^{2+} both in nerve terminals that contain N-type Ca^{2+} channels and those that contain P/Q-type Ca^{2+} channels. Thus, cannabinoid receptors modulate the two major Ca^{2+} channels coupled to glutamate release in the cerebral cortex.

Keywords: Cannabinoid; Glutamate release; Ca^{2+} channel; Ca^{2+} imaging in single nerve terminal

1. Introduction

Endocannabinoids released by postsynaptic cells inhibit neurotransmitter release by activating presynaptic cannabinoid receptors in many synapses, including those in the cerebral cortex [1–6]. It has been suggested that inhibition of presynaptic Ca^{2+} channels is responsible for the reduction of glutamate release by cannabinoid receptors. However, it has not been clearly established which types of Ca^{2+} channel can be modulated by this presynaptic mechanism. In cultured rat hippocampal neurons, cannabinoids inhibit somatic N- and P/Q-type calcium channels [7]. However, studies of synaptic transmission have indicated a selective inhibition of N-type Ca^{2+} channels, since blockage of these Ca^{2+} channels subtype with toxins suppresses the response to cannabinoids [1,8,9].

Direct measurement of the effects of cannabinoid on presynaptic Ca^{2+} channels has been limited to synapses with a large presynaptic bouton like those at the Calyx of Held [10]. However, monitoring the presynaptic Ca^{2+} influx and measuring the postsynaptic current amplitude in brain slices reflect the activation of many synaptic terminals that may or may not express the presynaptic receptor under study [11]. Here, we have used a preparation of cerebrocortical nerve terminals to determine the effect that cannabinoids have on glutamate release. In addition, by imaging Ca^{2+} in individual nerve terminals we determined the impact that the activation of cannabinoid receptors has on the Ca^{2+} response evoked by depolarization, identifying the type of Ca^{2+} channel involved. The results show that cannabinoid receptors reduce the depolarization-induced influx of Ca^{2+} in nerve terminals that contain N- or P/Q-type of Ca^{2+} channels. As a consequence, they reduce the glutamate release component associated with the activation of these two Ca^{2+} channels.

2. Materials and methods

2.1. Synaptosomal preparation

The cerebral cortex was isolated from adult male Wistar rats (2–3 months) and the synaptosomes were purified as described previously [12,13] on discontinuous Percoll gradients (Amersham–Pharmacia Biotech, Uppsala, Sweden). Following the final centrifugation at $22\,000 \times g$ for 10 min, the synaptosomes were resuspended in 8 ml of HEPES buffer medium (HBM): 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4. The protein content was determined by the Biuret method, and 1 mg of the synaptosomal suspension was diluted in 8 ml of HBM and spun at $3000 \times g$ for 10 min. The supernatant was discarded and the pellet containing the synaptosomes was stored on ice. Under these conditions, the synaptosomes remain fully viable for at least 4–6 h, as judged by the extent of KCl evoked glutamate release.

2.2. Glutamate release

Glutamate release was assayed by on-line fluorimetry. Synaptosomal pellets were resuspended in HBM (0.67 mg/ml) and preincubated at 37 °C for 1 h in the presence of 16 μM bovine serum albumin (BSA). The BSA served to bind any free fatty acids released from synaptosomes during the preincubation. A 1 ml aliquot was transferred to a stirred cuvette and the release of glutamate was measured as previously described [14]. The Ca^{2+}-dependent release was calculated by subtracting the release obtained during a 5 min period of depolarization in the presence of 200 nM free [Ca^{2+}] from the release at 1.33 mM $CaCl_2$.

2.3. Plasma membrane potential

The plasma membrane potential was determined with 3,3′-dipropylthiadicarbocyanine iodide, $DiSC_3(5)$. Synaptosomes were resuspended (0.67 mg/ml) in HBM without BSA, and 1 ml aliquots were transferred to a stirred cuvette containing 5 μM $DiSC_3(5)$ and 1.33 mM $CaCl_2$. After allowing the mixture to equilibrate for 1 min, the fluorescence was determined at 651 and 675 nm. Data were collected at 1 s intervals.

2.4. Ca^{2+} imaging of the responses in single synaptosomes

Synaptosomes in HBM (2 mg/ml) with 16 μM BSA were preincubated with 5 μM fura-2 AM and 1.33 mM $CaCl_2$ for 40 min and the synaptosomal suspension was then attached to a polylysine-coated coverslip for another hour. Synaptosomes were illuminated alternately at 340 and 380 nm for 0.8 s through a 100× objective with the aid of a monochromator (Kinetic Imaging, Ltd., UK). The fluorescence emitted from the nerve terminals was collected through a band-pass filter

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centered at 510 nm and images were obtained using a slow-scan CCD camera (Hamamatsu C4880) operating at 12-bit digitalization (4096 levels). The output from the camera was stored by the computerized imaging system Lucida 3.0 (Kinetic Imaging, Ltd.) and Ca^{2+} images were analyzed as described previously [15]. To measure $[Ca^{2+}]_{cyt}$, synaptosomes were stimulated by 10 s pulses of 30 mM KCl (a bar in each graph) in the presence or absence of pharmacological agonists or Ca^{2+} channels toxins.

3. Results

3.1. WIN55,212-2 reduces the glutamate release components associated with N- and P/Q-type Ca^{2+} channels

Depolarization of nerve terminals with KCl opens Ca^{2+} channels and initiates the exocytotic release of glutamate. The Ca^{2+}-dependent release of glutamate after 5-min depolarization with KCl was 3.66 ± 0.16 nmol/mg of protein ($n = 5$; Fig. 1A). The prior addition of the cannabinoid receptor agonist WIN55,212-2 reduced the release by 37.4 ± 4.3% ($n = 4$; Fig. 1A). The inhibition by WIN55,212-2 of the KCl-induced release was almost completely abolished (2.4% ± 3.5, $n = 3$, data not shown) by the cannabinoid receptor antagonist AM281 (1 μM). Treatment of synaptosomes with pertussis toxin abolished (0.56 ± 5.5%, $n = 3$, data not shown) the inhibition of release by WIN55,212-2. The blockade of Na^+ channels with tetrodotoxin (TTx) did not alter (37.1 ± 2.1%, $n = 3$, Fig. 1A) the inhibition of release by cannabinoids excluding the modulation of voltage dependent K^+ channels associated to action potentials. Furthermore, depolarization of the synaptosomal plasma membrane measured with a membrane potential-sensitive cationic cyanide dye was not modified by WIN55,212.2. Thus, the increase in fluorescence induced by 30 mM KCl (57.0 ± 2.4 arbitrary units) was not altered by WIN55,212-2 (55.1 ± 4.3 a.u., $n = 5$, data not shown). In order to know to what extent this inhibition resulted from a reduction in the activity of Ca^{2+} channels coupled to glutamate exocytosis, we performed experiments in the presence of Ca^{2+} channel toxins. In cerebrocortical nerve terminals, glutamate release is primarily coupled to Ca^{2+} entry through both N- and P/Q-type Ca^{2+} channels [15,16], channels that can be selectively blocked by ω-CgTx-GVIA [17] and ω-Aga-IVA [18], respectively. Blocking N-type channels with ω-CgTx-GVIA reduced the KCl-evoked release by 30.8 ± 2.6% ($n = 3$) and partially occluded the inhibition by WIN55,212-2 (17.2 ± 3.8%, $n = 5$, Fig. 1A and B). Blocking P/Q-type Ca^{2+} channels with ω-Aga-IVA reduced glutamate release by 65.5 ± 1.3% ($n = 4$, Fig. 1A and B) and also partially occluded the inhibition of release by WIN55,212-2 in this case by 20.9 ± 2.0% ($n = 4$; Fig. 1B). Thus, these data indicate that cannabinoid receptors inhibit glutamate release coupled to both the P/Q- and the N-type Ca^{2+} channels.

3.2. WIN55,212-2 reduces the entry of Ca^{2+} in nerve terminals containing N- or P/Q-type Ca^{2+} channels

To further examine whether cannabinoid receptors reduce the activity of Ca^{2+} channels, we visualized Ca^{2+} in single nerve terminals. We have previously shown that the two major subpopulations of cerebrocortical nerve terminals exist, containing N- or P/Q-types of Ca^{2+} channels [19]. In these experiments, nerve terminals were first stimulated with KCl in the presence of either ω-CgTx-GVIA to block N-type channels or ω-Aga-IVA to block P/Q-type channels. Subsequently, KCl was added in the presence of the cannabinoid agonist WIN55,212-2 and finally KCl was added alone. In the experiments with ω-CgTx-GVIA and WIN55,212-2 (Fig. 2), a total of 812 nerve terminals from five fields were analyzed. In a subpopulation of nerve terminals (9.3 ± 0.5%), the Ca^{2+} responses were reduced by ω-CgTx-GVIA but not by WIN55,212-2, indicating the presence of N-type channels but the absence of WIN55,212-2-sensitive receptors modulating Ca^{2+} entry. In another subpopulation of nerve terminals (22.5 ± 1.4%), Ca^{2+} responses were insensitive to ω-CgTx-GVIA but they were reduced by exposure to WIN55,212-2, indicating the presence of WIN55,212-sensitive cannabinoid receptors but not of

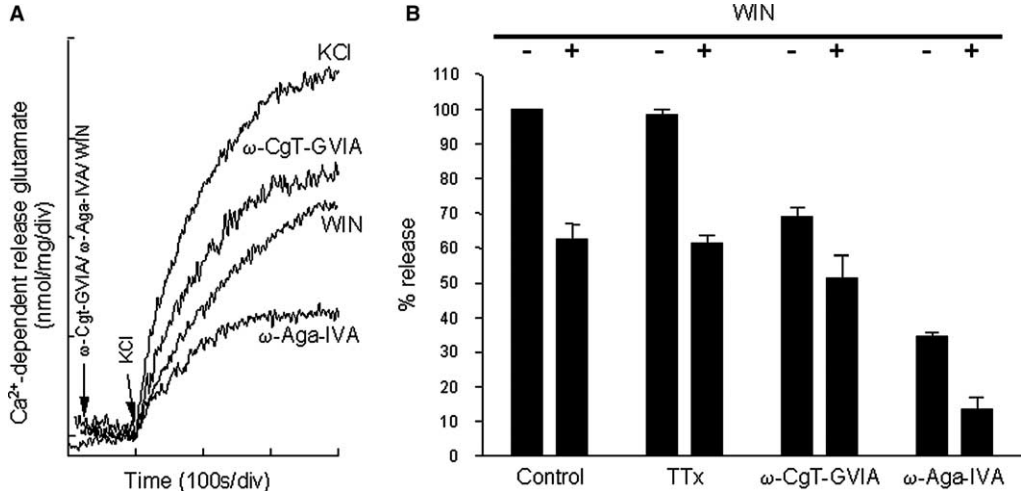

Fig. 1. WIN55,212-2 reduces the release components associated with N- and P/Q-type Ca^{2+} channels. (A) Ca^{2+}-dependent release of glutamate evoked by 30 mM KCl in the presence and absence (control) of 5 μM WIN55,212-2 (WIN), 200 nM ω-Aga-IVA (ω-Aga-IVA) or 2 μM ω-CgT-GVIA (ω-CgT-GVIA). WIN55,212-2 and the Ca^{2+} channel toxins were added 5 and 1 min prior to depolarization with 30 mM KCl. (B) Bar diagrams show the Ca^{2+}-dependent release of glutamate 5 min after depolarization with 30 mM KCl in the presence and absence (Control) of the Ca^{2+}-channels toxins ω-CgT-GVIA and ω-Aga-IVA, both in the presence and absence of WIN55,212-2 (5 μM). In the experiments with tetrodotoxin (TTX), the Na^+ channel blocker (1 μM) was added 1 min prior to depolarization with 30 mM KCl. The results are means ± SEM of 3–5 experiments obtained from the same number of synaptosomal preparations.