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Presynaptic inhibition of GABAergic inputs to rat substantia nigra pars reticulata neurones by a cannabinoid agonist

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WHOLE-CELL patch-clamp recordings were made from substantia nigra pars reticulata (SNR) neurones in rat midbrain slices to investigate the electrophysiological effects of cannabinoids. The cannabinoid receptor agonist WIN 55212-2 (10 μM) significantly reduced intranigrally evoked and spontaneous inhibitory post-synaptic currents (IPSCs) which were mediated by $GABA_A$ receptors. The postsynaptic current induced by bath application of GABA was not affected by the presence of WIN 55212-2. The actions of WIN 55212-2 were not mimicked by the inactive enantiomer WIN 55212-3. WIN 55212-2 also hyperpolarized the membrane of SNR neurones in a tetrodotoxin/0-Ca^{2+}-insensitive manner. These data suggest that cannabinoids modulate the activity of SNR neurones by presynaptic inhibition of GABA inputs. They may also exert a direct post-synaptic inhibition on these neurones.

Key words: Cannabinoid; GABA; IPSC; Substantia nigra

Introduction

There is accumulating evidence that the substantia nigra pars reticulata (SNR), an output centre of the basal ganglia, is one of the sites of action for the motor effects induced by cannabinoids. Thus, very high density of binding sites for cannabinoids were found in rat SNR.[1,2] In addition, intranigral injection of cannabinoids modulated circling behaviour in rats induced by the $GABA_A$ receptor agonist muscimol[3] and dopamine D1 agonists.[4] Electrophysiological techniques have also been applied to study the effect of cannabinoids on the excitability of SNR neurones. For example, extracellular recordings showed that firing of SNR neurones was increased by systemic administration[5] or local pressure ejection[6] of cannabinoid agonists. These results, together with the fact that cannabinoid binding sites were largely eliminated by lesioning the caudate nucleus,[2] suggest that cannabinoids act mainly at the presynaptic striatonigral terminals. However, although these results reveal the *in vivo* action of cannabinoids on SNR neurone activity, they are indirect in proving an inhibitory action of cannabinoids on the release of GABA by the striatonigral terminals. So far, there are no data in the literature indicating that cannabinoids inhibit the release of GABA from striatonigral terminals, nor is there evidence that $GABA_A$ receptor mediated synaptic currents in SNR are inhibited by cannabinoids. In addition, it is not known whether cannabinoids have any direct postsynaptic action on SNR neurones, a question crucial for the interpretation of the *in vivo* data. The present study was designed to answer some of these questions. We make use of the patch-clamp technique to study the presynaptic effects of cannabinoids on SNR neurones in the brain slice preparation. The data provide the first direct electrophysiological evidence that a cannabinoid agonist inhibits $GABA_A$ receptor mediated inhibitory post-synaptic currents (IPSCs) in SNR. In addition, a distinct postsynaptic effect was also observed.

Materials and Methods

Sprague–Dawley rats (2–3 weeks old) were deeply anaesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and sacrificed by decapitation. Midbrain slices (200–250 μm) containing the substantia nigra were prepared by a vibrating microtome. They were maintained at 34 ± 1°C in artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 125; KCl, 2.0; $MgSO_4$ 1.2; $CaCl_2$ 2.5; $KH_2PO_4$1.2; glucose 11 and $NaHCO_3$ 26, bubbled with carbogen. Neuronal somata were visualized by the method described by Stuart *et al.*[7] Medium to large sized neurones (> 20 μm in diameter) in SNR were selected since small neurones were considered as local circuit neurones.[8]

Whole-cell patch-clamp recordings from SNR neurones were obtained using a patch-clamp amplifier (LM/PCA, List Medical). Whole cell pipettes

typically had a resistance of 4–6 MΩ when filled with an internal solution of the following composition (in mM): K-gluconate 120; KCl 10; EGTA 1.0; $MgCl_2$ 1.0; Na_2-ATP 2.0; HEPES 10. The pH was adjusted to 7.3 with KOH. To study the presynaptic action of cannabinoids on $GABA_A$ receptor-mediated synaptic currents, K-gluconate was replaced with KCl. The inclusion of 130 mM of KCl in the recording pipettes reversed the polarity of $GABA_A$ receptor-mediated currents from outward to inward and enhanced their detection. Synaptic currents were also evoked intranigrally by single shocks (< 0.5 ms, < 99 V, repeated every 3 s) of electrical stimulation delivered via a monopolar pipette filled with ACSF. When the postsynaptic effect of cannabinoids was studied, GTP (0.5–1 mM) was freshly dissolved in the pipette solution. Normally no series resistance compensation was applied. The cell was rejected if the series resistance increased significantly (> 20%) during recording. AP5 (20 μM) and CNQX (20 μM) were included in the ACSF to eliminate glutamate-mediated synaptic currents. Digitization of data was made via the CED 1401plus interface and the Patch and Voltage Clamp software package (Cambridge Electronics Design). Synaptic currents were analysed by a program developed in our laboratory. This program adopts a simple detection algorithm described in detail by Vincent and Marty.[9] Once a synaptic current is detected, information on the time of occurrence and peak amplitude are generated automatically. Histogram and cumulative probability distributions are produced, and statistical comparison of two cumulative probability distributions are possible using the built-in Kolmogorov-Smirnov (K-S) test. All events detected with the computed parameters allowed visual inspection before acceptance.

GTP was purchased from Sigma. All other drugs used were obtained from Research Biochemicals International. A drug was applied by changing the superfusing solution to one added with a known concentration of the drug. The delay of the superfusing system, including the time for heat exchange, was about 30 s. Numerical data are expressed as mean ± s.e.m. K-S test was used to compare two distributions of IPSC inter-event intervals or amplitudes. Otherwise Student's *t*-test was used.

Results

Data were obtained from neurones exhibiting the electrophysiological characteristics of GABA output neurones.[10] These neurones were characterized by a weak hyperpolarization-activated inward rectification, brief action potentials (< 2 ms) and a high firing frequency (8.2 ± 1.0 Hz, $n = 6$). Results were obtained from 41 GABA cells.

As reported previously,[11] in the presence of AP5 (20 μM) and CNQX (20 μM), an IPSC can normally be evoked when a stimulating electrode is placed within the substantia nigra. The IPSC was an inward current when the recording pipette was filled with a high concentration of Cl^-. That these evoked IPSCs were mediated by $GABA_A$ receptor was shown by their sensitivity to 1 μM bicuculline. Bath application of WIN 55212-2, a cannabinoid receptor agonist, significantly reduced the amplitude of the IPSC in a dose-dependent manner while the inactive enantiomer WIN 55212-3 caused only a slight reduction or no effect. Figure 1 shows a typical example in which the effects of 10 μM WIN 55212-3 and two different concentrations (1 and 10 μM) of WIN 55212-2 on the evoked IPSCs of the same neurone were studied. In 18 cells studied, an average inhibition of 49.5% was achieved by 10 μM WIN 55212-2 (control 742 ± 160 pA; WIN 55212-2 344 ± 75 pA, $p < 0.01$) while in six cells, 8.5% inhibition was achieved by 10 μM WIN 55212-3 (control 393 ± 73 pA; WIN 55212-3: 360 ± 114 pA, $p > 0.05$). However, the effect of WIN 55212-2 was typically difficult to remove, even after a period of 30 min. To demonstrate that WIN 55212-2 did not reduce the amplitude of the IPSCs by a direct inhibitory action on the $GABA_A$ receptors, the postsynaptic responses of SNR neurones to GABA were studied. Figure 1B shows that the peak current induced by bath application of 1 mM GABA was not affected in the presence of 10 μM WIN 55212-2. No significant difference was found in the responses obtained from six cells (control 215 ± 53 pA; WIN 55212-2: 203 ± 42 pA, $p > 0.05$). The result indicates that the sensitivity of $GABA_A$ receptors in SNR neurones was not changed by the cannabinoid agonist.

The effect of WIN 55212-2 on spontaneously occurring IPSCs (sIPSCs) was also studied. As reported previously[12] and similar to those of evoked currents, these sIPSCs display characteristics of $GABA_A$ receptor mediated synaptic currents, such as their sensitivity to bicuculline. Bath application of 1–30 μM WIN 55212-2 reduced the amplitudes of the sIPSCs. An example is shown in Fig. 2A in which the sIPSCs were insensitive to WIN 55212-3 but their amplitudes were reduced by WIN 55212-2. Again, no significant recovery was observed within 30 min of washing. The effect of WIN 55212-2 was analysed quantitatively by plotting the cumulative distributions of the amplitudes and inter-event intervals of the sIPSCs. As shown in Fig. 2B, a significant shift of the amplitude towards smaller values is evident, and is supported by the K-S test ($p < 0.01$). In six cells tested, the mean amplitude of the IPSCs was 91.1 ± 19.6 pA, which was reduced to 72.7 ± 12.6 pA by 10 μM WIN 55212-2. Significant

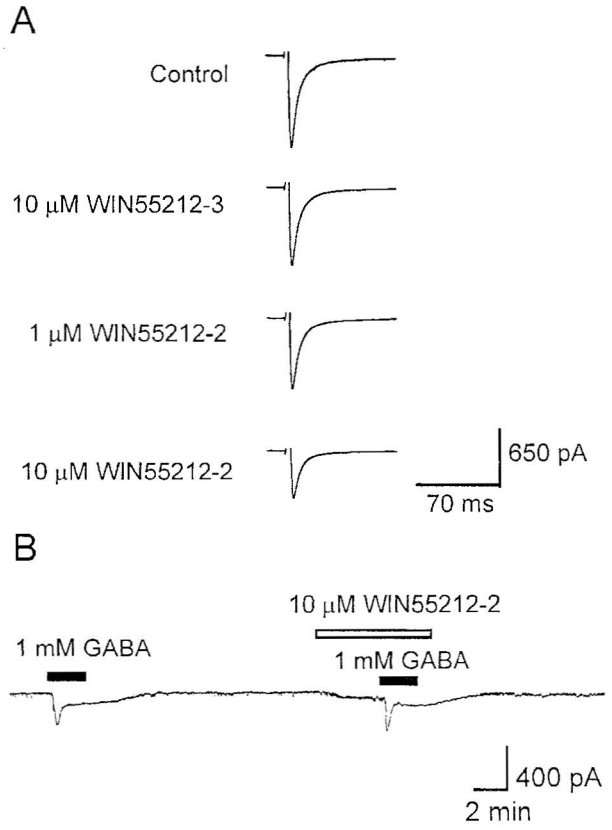

FIG. 1. (**A**) A reversed, inward IPSC was evoked in an SNR neurone by intranigral stimulation and recorded with a high Cl^- electrode. The amplitude of the IPSC was dose-dependently reduced by WIN 55212-2 (1 and 10 μM), while 10 μM WIN 55212-3 caused a small reduction in the IPSC amplitude. The neurone was voltage-clamped at –55 mV and the series resistance remained stable during the experiment. (**B**) The postsynaptic response of an SNR neurone, clamped at –55 mV, to bath application of 1 mM GABA was not affected by the presence of 10 μM WIN 55212-2. A high Cl^- electrode was used resulting in an inward chloride current mediated by $GABA_A$ receptors.

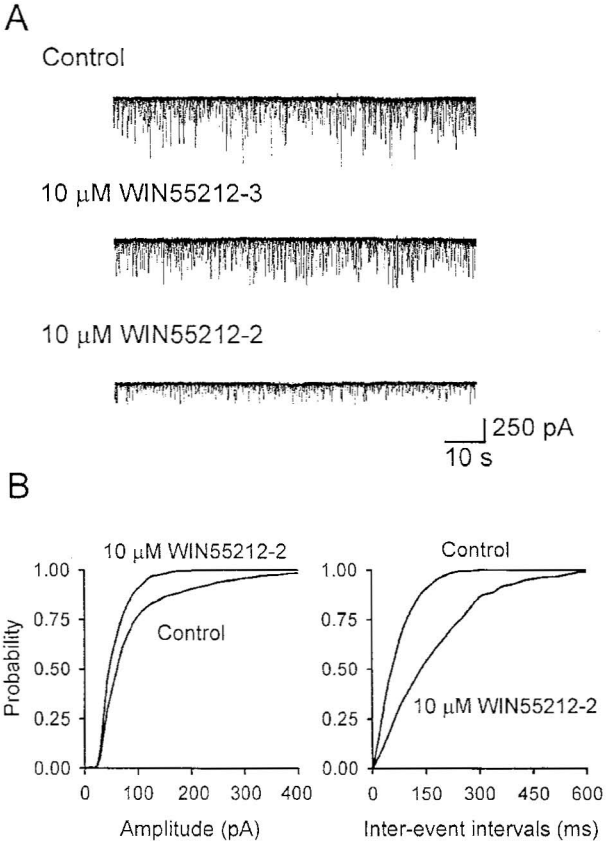

FIG. 2. (**A**) In this SNR neurone with a high frequency of spontaneous IPSCs, bath application of 10 μM of WIN 55212-2 reduced the amplitudes of the IPSCs while the same concentration of WIN 55212-3 was without effect. However, the inhibitory effect of WIN 55212-2 was difficult to wash out. In this experiment, a high Cl^- electrode was used and the neurone was clamped at –55 mV. (**B**) Cumulative distributions of the amplitudes (left) and inter-event intervals (right) of IPSCs reveal that both parameters were significantly changed by WIN 55212-2 ($p < 0.01$, K-S test).

differences ($p < 0.05$) were found in five of the six cells. Figure 2B illustrates that WIN 55212-2 also reduced the frequency of the sIPSCs (control 6.0 ± 1.5 Hz; 10 μM WIN 55212-2 3.0 ± 0.9 Hz, $n = 6$). Significant differences ($p < 0.05$, K-S test) were found in all six cells.

We also observed an inhibitory effect of WIN 55212-2 on the resting firing of SNR GABA neurones in the brain slice. When K-gluconate electrodes with low KCl were used and the cells firing at their resting membrane potential (~ –50 mV), WIN 55212-2 at 10 μM caused a small membrane hyperpolarization (< 8 mV, $n = 4$) and a clear inhibition in firing (Fig. 3). As illustrated, these changes were accompanied by a small reduction in the input resistance of the neurones. This effect of WIN 55212-2 was readily terminated by washing. In addition, these effects were not sensitive to 0.5 μM tetrodotoxin and 0-Ca^{2+}/10 mM Mg^{2+} ($n = 4$, not shown), suggesting that they were postsynaptic responses. However, further characterization of the underlying conductance was not carried out in the present study.

Discussion

The primary objective of the present study is to test whether cannabinoid agonists exert a presynaptic inhibition on GABAergic inputs to SNR neurones. Our data provide a direct electrophysioloigcal evidence supporting this hypothesis. Thus, the amplitudes of the evoked and spontaneous $GABA_A$ receptor-mediated IPSCs were significantly reduced by the application of 10 μM of WIN 55212-2. Cumulative distribution analysis of the spontaneous IPSCs indicated that both the amplitude and frequency were reduced. The reduction in the amplitudes of these IPSCs may result from a presynaptic mechanism, in which the release of GABA was inhibited, or by a direct blockade of $GABA_A$ receptors by WIN 55212-2. The latter possibility is excluded

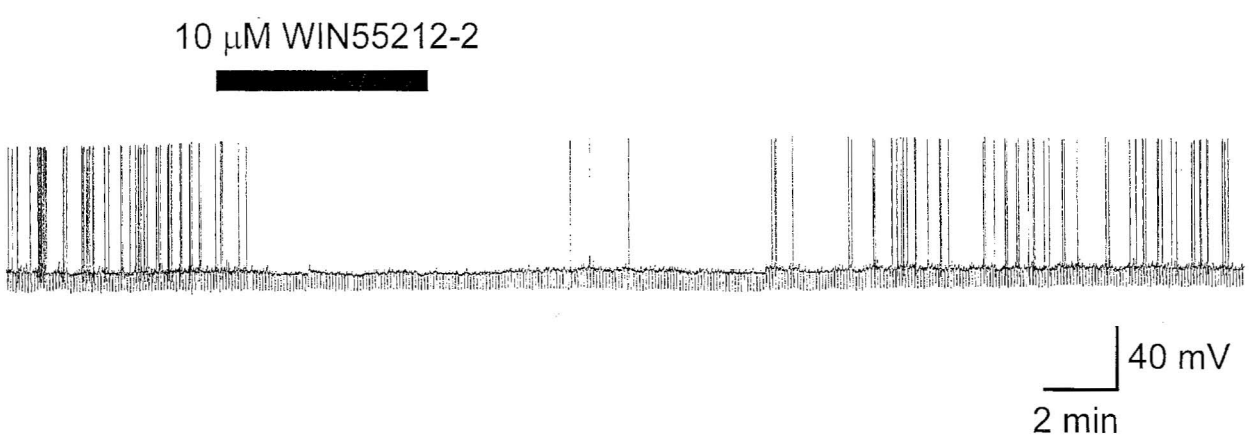

FIG. 3. In current-clamp recording, WIN 55212-2 (10 μM) hyperpolarized an SNR neurone resulting in inhibition of firing. A small reduction in the input resistance was indicated by the decrease in voltage changes in response to periodic current injection. In this experiment, gluconate is the major anion in the internal solution.

by the experiments in which the currents induced by GABA were not affected by the presence of WIN 55212-2. As antagonists for cannabinoid receptor are not yet widely available, the specificity of WIN 55212-2 on cannabinoid receptor is supported by the lack of effect of WIN 55212-3, the inactive enantiomer. However, the mechanism by which WIN 55212-2 inhibits the IPSCs of SNR neurones is not known at present. It is possible that it suppresses the release of GABA by inhibiting voltage-dependent calcium channels,[13] or opening K^+-channels.[14]

Since the striatonigral terminals are the major source of GABA release in SNR,[15] it is reasonable to believe that presynaptic cannabinoid receptors on the striatonigral terminals contribute to the effects of WIN 55212-2 on spontaneous and evoked IPSCs. Such a conclusion therefore supports the hypothesis that cannabinoids excite SNR neurones in anaesthesized rat by removing the inhibition from the striatum.[5,6] However, it should be pointed out that the striatal neurones of anaesthetized rats are likely to be silent and thus the spontaneous firing of SNR neurones are probably not subject to a strong regulation by the striatum. It is therefore also possible that cannabinoids can excite SNR neurones through inhibition of GABA release from other sources including those from globus pallidus[16] and even the axon terminals of neighouring SNR cells.[17]

Another possible mechanism by which cannabinoids excite SNR neurones *in vivo* is by means of a postsynaptic action. Our finding that the firing of SNR neurones were inhibited by WIN 55212-2 in a tetrodotoxin/0-Ca^{2+}-insensitive manner suggests that SNR neurones may express postsynaptic cannabinoid receptors, a hypothesis which may account for the residual cannabinoid receptor binding sites in SNR after ibotenic acid lesion of the caudate nucleus[2] (however, see Ref. 18). It also accounts for the inhibitory effect of WIN 55212-2 on the frequency of sIPSCs, which has a significant contribution from neighbouring SNR neurones via axon collaterals.[12,17] In the present study, we did not elucidate the detailed mechanism of this inhibitory action of cannabinoid. Nevertheless, the fact that WIN 55212-2 is inhibitory means that the excitatory action of cannabinoids on SNR neurones observed in anaesthetized rats cannot be explained by this postsynaptic effect. However, such an action may contribute to the paradoxical observation that intranigral administration of cannabinoid agonists induced contralateral turning in rats,[4] and thus represents an alternative mechanism to the inhibitory effect of cannabinoid agonists on the excitatory inputs from the subthalamic nucleus.[19]

Conclusion

The present experiments indicate that cannabinoids inhibit the release of GABA from inhibitory terminals in SNR neurones by a presynaptic mechanism, an action which can regulate the outflow of the basal ganglia. Therefore, at least some of the motor effects induced by cannabinoids in human and animals are likely to be mediated by the basal ganglia circuit. However, a direct postsynaptic action is also induced by cannabinoids on SNR neurones. To understand the actions of cannabinoids on behaving animals, it is therefore important to know the relative importance of these two mechanisms and conditions in which they are activated.

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