

Cannabinoid modulation of electrically evoked pH and oxygen transients in the nucleus accumbens of awake rats

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Abstract

Cannabinoid receptors have been implicated in the regulation of blood flow in the cerebral vasculature. Because the nucleus accumbens (NAc) shows high levels of central cannabinoid receptor 1 (CB₁) expression we examined the effects of cannabinoids on the local transient alkaline shifts and increases in extracellular oxygen induced by electrical stimulation of the medial forebrain bundle (MFB) in conscious animals. These changes result from increases in cerebral blood flow (CBF) and metabolism in the NAc that are evoked by the stimulation. Oxygen and pH changes were monitored using fast-scan cyclic voltammetry at carbon-fiber microelectrodes in the NAc of freely moving rats. Administration of the cannabinoid receptor agonist WIN55,212-2 potently inhibited extracellular oxygen and pH changes, an effect that was reversed and prevented by pre-treatment with the CB₁ receptor antagonists SR141716A and AM251. The effects on

pH following WIN55,212-2 were similar to those following nimodipine, a recognized vasodilator. When AM251 was injected alone, the amplitude of electrically evoked pH shifts was unaffected. Administration of AM404 and VDM11, endocannabinoid transport inhibitors, partially inhibited pH transients in a CB₁ receptor-dependent manner. The present findings suggest that CB₁ receptor activation modulates changes in two well-established indices of local blood flow and metabolism resulting from electrically evoked activation of ascending fibers. Although endogenous cannabinoid tone alone is not sufficient to modify these responses, uptake blockade demonstrates that the system has the potential to exert CB₁-specific effects similar to those of full agonists.

Keywords: blood flow and metabolism, cannabinoids, cyclic voltammetry, dopamine, nucleus accumbens.

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Cannabinoids are some of the oldest known psychoactive and medicinal agents known to man (Snyder 1971). The main psychoactive component of the marijuana plant, Δ^9 -tetrahydrocannabinol, exerts its effects through central cannabinoid receptors (CB₁) (Lupica *et al.* 2004). More recently, both endogenous cannabinoids and synthetic cannabinoid agonists have been shown to elicit hypotensive and bradycardic states establishing a new family of vasoactive substances (Gardiner *et al.* 2001; Randall *et al.* 2002). Such vascular effects are mediated by CB₁ receptors since they are blocked by specific receptor antagonists (Wagner *et al.* 2001). Cannabinoid perfusion increases the diameter of cerebral arterioles (Ellis *et al.* 1995) and cerebral arteries (Hillard 2000) in a CB₁ receptor-dependent fashion, indicating that the main cerebrovascular effect of cannabinoids is vasodilation. This notion is in agreement with findings that marijuana smoking in humans is accompanied by an increase in cerebral blood flow (CBF; Mathew and Wilson 1993).

The energy utilization that accompanies neuronal activity results in increased metabolism that lowers extracellular

oxygen concentration (Zimmerman *et al.* 1992) and extracellular pH (Chesler 2003). At the same time, chemical messengers are released that communicate with the vascular microenvironment promoting vasodilation and an increased supply of nutrients (Raichle 1998). This vasodilation increases blood flow in the intact animal, re-supplying oxygen and clearing carbon dioxide, a component of the sodium bicarbonate system that is the predominant physiological pH buffer. Because vasodilation overcompensates for the metabolic effects, the net result is an increase in oxygen levels (Zimmerman *et al.* 1992; Lowry *et al.* 1997) and an

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Abbreviations used: CB₁, cannabinoid receptor 1; CBF, cerebral blood flow; MFB, medial forebrain bundle; NAc, nucleus accumbens; SR, SR141716A; WIN, WIN55,212-2.

alkaline shift (Venton *et al.* 2003) that occur following increased neuronal activity. Both chemical changes are transient and approximately simultaneous, occurring a few seconds after the electrical activity, and can be assessed with fast-scan cyclic voltammetry at carbon fiber microelectrodes (Venton *et al.* 2003). Oxygen is detected by its electroreduction (Zimmerman and Wightman 1991), and pH is detected because it causes a shift in the voltammetric background (Runnels *et al.* 1999). In freely moving animals, background shifts caused by alkaline pH shifts have been observed in terminal regions of dopaminergic neurons both following evoked neuronal activity and following behaviorally evoked release of dopamine (Kilpatrick *et al.* 2000; Roitman *et al.* 2004).

At present, there is a lack of overall consensus regarding the local vascular effects of cannabinoids in the brain of awake unrestrained rats due to the modification of their actions by general anesthetics (Randall *et al.* 2004) and to the difficulties of directly measuring cerebral blood flow in moving animals. Thus, the aim of the present study was to use fast-scan cyclic voltammetry in the NAc to directly monitor the effects of cannabinoids on local oxygen transients (Zimmerman *et al.* 1991) and background shifts elicited by pH, two indices that are affected by increased blood flow (Lowry *et al.* 1997; Venton *et al.* 2003). Because systemic cannabinoid administration has widespread effects on neuronal activity and CB₁ receptors are particularly dense in the NAc (Julian *et al.* 2003), our studies were restricted only to changes associated with controlled neuronal activation, achieved by electrical stimulation of ascending fibers in the MFB.

Materials and methods

Animals and surgery

Male Sprague-Dawley rats implanted with a jugular vein catheter (250–350 g; Charles River, Wilmington, MA, USA) were anesthetized with xylazine hydrochloride (10 mg/kg, i.p.) and ketamine hydrochloride (100 mg/kg, i.p.) for recovery surgery. Briefly, rats were fitted with a guide cannula (BAS, West Lafayette, IN, USA) aimed at the NAc (1.3 mm anterior and 1.3 mm lateral relative to bregma; Paxinos and Watson 1986) and a Ag/AgCl reference electrode in the contralateral hemisphere. The medial forebrain bundle including dopaminergic fibers was activated with a bipolar stimulating electrode (Plastics One, Roanoke, VA, USA; 4.2 mm posterior, 1.4 mm lateral and 8.6 mm ventral from the brain surface). Animals were allowed a 48 h post-operative recovery period before data acquisition was initiated. All animal protocols and care were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Fast-scan cyclic voltammetry

Cylindrical carbon-fiber microelectrodes with an exposed tip of 50–100 µm were prepared by sealing carbon fibers (T650, 6 µm diameter, Amoco Corp., Greenville, SC, USA) in capillary glass

(A-M Systems, Carlsborg, WA, USA). Each day, a fresh electrode was lowered to the core of the NAc (6–8 mm, ventral) using a microdrive that locked into the previously implanted guide cannula. Animals were then tethered by the electrode assembly to a rotating commutator situated in the top of the recording chamber, which allowed for virtually unrestrained movement. The electrode was held at –0.6 V versus Ag/AgCl between scans to detect pH and dopamine simultaneously. Every 100 ms, the electrode potential was scanned to +1.4 V and back in a triangular fashion at a scan rate of 400 V/s and the current was recorded (Heien *et al.* 2003). For oxygen detection, the electrode was held at 0 V, ramped to +0.8 V, then to –1.4 V, and back to 0 V at a scan rate of 450 V/s every 100 ms (Zimmerman *et al.* 1992). Dopamine release was evoked with 24 biphasic pulses (2 ms, 125 µA per phase, 60 Hz) delivered every 1 min to the stimulating electrode. Data acquisition and control employed an interface board (National Instruments, Austin, TX, USA) used with a Pentium IV computer and locally written LABVIEW (National Instruments) software.

Background-subtracted cyclic voltammograms (the electrochemical identifier of the detected substances) and color plots (Michael *et al.* 1998) were constructed by subtracting cyclic voltammograms obtained before the stimulation from those after the stimulation. This step is necessary to remove the non-specific current of the electrode. In this work, they are displayed with oxidation current plotted upward and oxidizing potential plotted to the right of 0.0 V. This differs from our previous work but is consistent with IUPAC conventions. To monitor the time course of background changes caused by pH shifts, the current at +0.2 V from successive voltammograms was used. The time course of oxygen and dopamine changes was monitored by analyzing currents at –1.3 V and +0.6 V, respectively. Because of the subtraction, measures of oxygen and pH are differential ones; the absolute pH or oxygen concentration cannot be obtained. A flow injection system was utilized after some experiments to calibrate the response of the electrode to dopamine and pH (Venton *et al.* 2003).

Drugs

WIN55,212-2 (WIN; Sigma-Aldrich, St Louis, MO, USA), SR141716A (SR; Research Triangle Institute/NIDA, Raleigh, NC, USA), AM404 and AM251 (Tocris-Cookson, Ellisville, MO, USA) were freshly suspended in a 1 : 1 : 18 ratio of ethanol, emulphor (Alkamuls EL-620, Rhodia Inc., Cranbury, NJ, USA) and heparinized saline (0.9%) for injection. VDM11 (Tocris-Cookson) supplied pre-dissolved in anhydrous ethanol (5 mg/mL) was dried and re-suspended in the same vehicle. On the experimental day, rats were given intravenous bolus doses (separated by at least 10 min) of vehicle (1 mL/kg), WIN (125 and 250 µg/kg), SR (300 µg/kg) and WIN (250 µg/kg) in the presence of SR. Nimodipine (Nimotop®) was a generous gift from Bayer Pharmaceuticals (Leverküsen, Germany) and was given as a single intravenous bolus in saline (250 µg/kg). AM404 (1 mg/kg), AM251 (300 µg/kg) and VDM11 (1 mg/kg) were also delivered as single boluses. RO-04-1284 was a generous gift from Roche Pharmaceuticals (San Francisco, CA, USA) and was injected intravenously at 1 mg/kg in saline.

Data analysis

Current versus time plots and cyclic voltammograms were generated using GRAPH PAD PRISM (Graph Pad Software Inc., San Diego, CA, USA). Drug effects were determined by averaging traces prior to the

administration of drugs. Peak current data was then compared and analyzed with respect to pre-drug values using conventional ANOVA followed by Scheffé *post hoc* tests and standard Student's *t*-tests; the criterion of significance was set at $p < 0.05$. Statistical analyses were performed using STATISTICA (StatSoft Inc., Tulsa, OK, USA) and data are expressed as mean \pm SEM.

Results

Electrical stimulation of the medial forebrain bundle causes an increase in local oxygen that is inhibited by WIN55,212-2 in the accumbens of freely moving rats

Stimulation of the MFB with the parameters employed causes release of several neurotransmitters, including dopamine (Nieuwenhuys *et al.* 1982). This stimulation also causes an increase in the local oxygen concentration in anesthetized rats (Zimmerman and Wightman 1991; Venton *et al.* 2003). To evaluate whether the oxygen increase is also

seen in freely moving animals, local oxygen concentrations were monitored during and after similar stimulations with a cyclic voltammetric waveform optimized for its detection. A representative trace is shown in Fig. 1(a). Similar to that found in anesthetized animals, the current due to oxygen reduction monitored at -1.3 V increased immediately after the stimulation and returned to baseline within 10 s. This is illustrated in the color plot beneath the trace by the dark blue area centered at -1.3 V (Venton *et al.* 2003). The vertical white dashed line at the maximal change on the color plot indicates the time when the cyclic voltammogram (Fig. 1b) was obtained. It is identical to the cyclic voltammogram for oxygen (Zimmerman and Wightman 1991). When the stimulation was repeated after intravenous administration of WIN (125 $\mu\text{g}/\text{kg}$), the change in signal at -1.3 V was significantly decreased, indicating that the change in oxygen due to the stimulation was decreased (Fig. 1a, right panel). For all animals tested in this way, the oxygen signal was

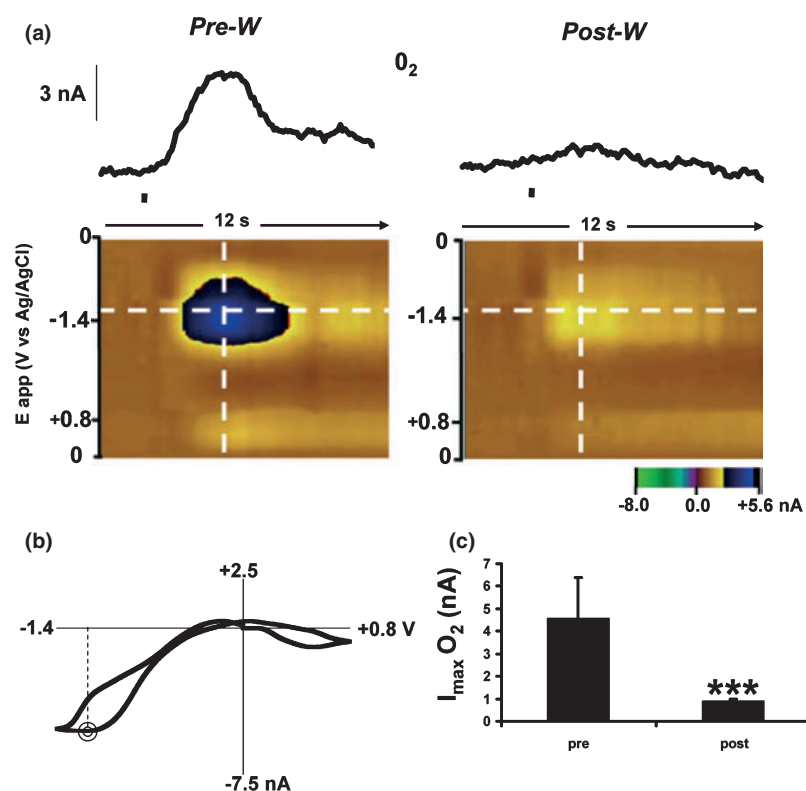


Fig. 1 WIN55,212-2 (WIN) abolishes stimulation-evoked extracellular oxygen transients. (a) Extracellular current monitored at -1.3 V in successive cyclic voltammograms. Left panel: pre-drug (pre-W); right panel: post-WIN (post-W: 125 $\mu\text{g}/\text{kg}$). Electrical stimulation of the medial forebrain bundle (MFB) (biphasic pulses, 0.4 s duration, 60 Hz, \pm 125 μA) occurred at the time indicated by the black bar. Color plot shows the three-dimensional data collected by fast-scan cyclic voltammetry. The x-axis represents time, the y-axis represents the applied potential (E_{app}), and the current is shown in pseudocolor.

The purple circle at 5 s represents the oxygen reduction peak. The dashed white line shows the potential and time at which the cyclic voltammogram and the concentration versus time trace (above) were obtained. (b) The oxygen-specific cyclic voltammogram recorded in the NAc following MFB stimulation reveals that the change is due to oxygen concentration (peak reduction potential shown by the dashed line; $E_p = -1.3$ V). (c) Pooled data ($n = 4$) for the effects of WIN (125 $\mu\text{g}/\text{kg}$) on evoked oxygen levels (***) $p \leq 0.001$ compared to pre-drug, Student's *t*-test.

significantly decreased by $80 \pm 13\%$ ($n = 4$ animals; $p \leq 0.001$, Student's *t*-test, Fig. 1c) following WIN.

Effects of the cannabinoid receptor agonist WIN55,212-2 on electrically evoked alkaline pH shifts

Similar experiments were conducted in another group of rats with a cyclic voltammetric waveform that can detect

dopamine and pH (Venton *et al.* 2003). The time course of the change in current at the potential where background shifts due to pH changes were obtained was similar to the oxygen change, beginning after the stimulation and then returning to pre-stimulation levels within 10 s (Fig. 2a, top left panel). This time course also closely resembles those for pH evoked by similar stimulations in anesthetized animals (Venton *et al.*

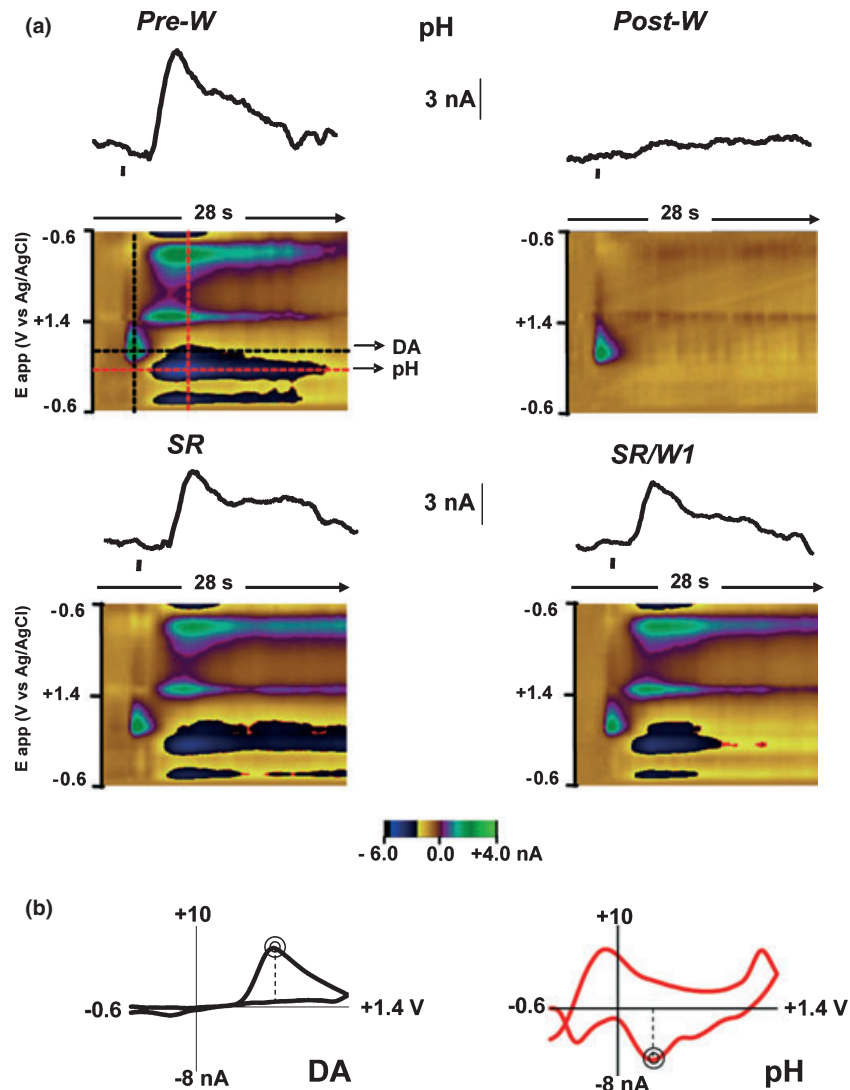
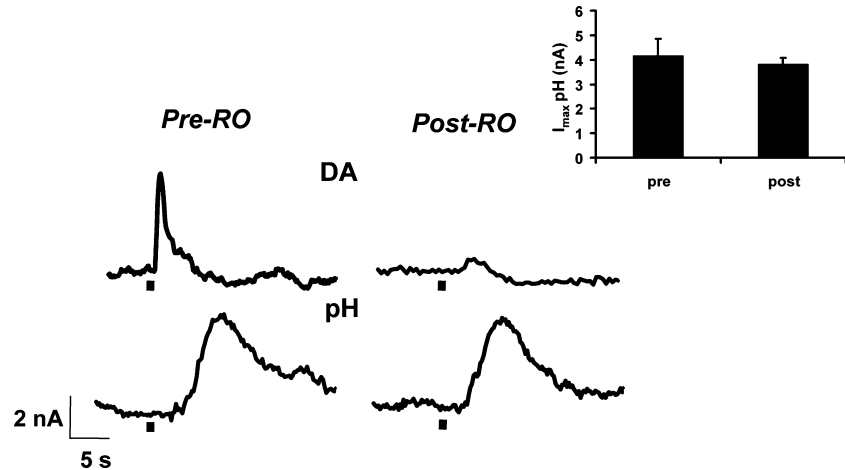


Fig. 2 Electrically evoked pH signals are inhibited by WIN55,212-2 (WIN). (a) The top left panel is a representative trace of the currents measured at 0.21 V in successive voltammograms during electrical stimulation of the medial forebrain bundle (MFB) with the color plot shown below (biphasic pulses, 0.4 s duration, 60 Hz, $\pm 125 \mu\text{A}$). In this case, the purple and green circle at 5 s is the dopamine (DA) oxidation peak and the light colored yellow circle at the same time is due to dopamine reduction. The broad blue and green changes from 7 to 25 s are due to pH changes. The upward deflection following the stimulation is consistent with an alkaline pH shift. The stimulation occurred at the time indicated by the black bar in this and the other panels. Following an intravenous injection of WIN (W: 125 $\mu\text{g}/\text{kg}$), the response

caused by electrical stimulation at this location is abolished (top right panel). Subsequent injection of the cannabinoid receptor antagonist SR141716A (SR, 300 $\mu\text{g}/\text{kg}$) reverses the effect elicited by WIN (bottom left panel) and prevents the inhibition of the electrically evoked pH change following administration of a higher dose of WIN (bottom right panel; W1: 250 $\mu\text{g}/\text{kg}$). (b) Left panel: cyclic voltammogram (obtained at the crossings of the dashed lines above) recorded in the striatum during electrical stimulation of the MFB ($E_P = +0.62$ V, black lines) is identical to that for dopamine. Right panel: cyclic voltammogram recorded at the crossing of the dashed lines 3 s later ($E_P = +0.21$ V, red lines) that is identical to that for an alkaline pH shift.

Fig. 3 Responses induced by administration of RO-04-1284 (RO), a vesicular monoamine transporter inhibitor. Currents from successive voltammograms measured at +0.62 (the oxidative potential of dopamine, upper traces) and at +0.21 V (the alkaline sensitive potential, lower traces) during electrical medial forebrain bundle (MFB) stimulations (indicated by the black bar) before (left) and 90 s after (right) RO administration. Inset: pooled results for the evoked alkaline shifts obtained from three animals.



2003). As previously reported in freely moving rats (Cheer *et al.* 2004), the electrical stimulation also caused an immediate increase in dopamine (Fig. 2a, top left color plot). In this case, the dopamine oxidation peak is seen as a green circle centered at 0.6 V on the ordinate and at 5 s on the abscissa in the color representation. The reduction peak for dopamine occurs on the reverse scan at -0.2 V and is seen as a lighter yellow circle occurring at the same time. Long-lasting currents shown in dark blue appear following reuptake of dopamine; these correspond to alkaline pH shifts (Venton *et al.* 2003). It is likely that lactate is produced during the stimulation from the glial cells in response to the stimulation (Fray *et al.* 1996). However, our results indicate that the alkalization due to CO₂ removal outweighs possible acidification due to lactate. For all panels in Fig. 2, the change in current shown above the color plots is only for the pH changes. The vertical dashed lines (black for dopamine and red for pH) on the color plot indicate the potentials and times at which the cyclic voltammograms representative of dopamine and pH were taken (Fig. 2b). The shapes of the cyclic voltammograms obtained at the peaks are identical to those obtained for dopamine *in vitro* (Fig. 2b, left panel) and for an alkaline pH shift (Fig. 2b, right panel; compare to data in Heien *et al.* 2003). Like the oxygen signal, the current change attributed to an alkaline pH shift was dramatically suppressed (Fig. 2a, top right panel) following intravenous administration of WIN (125 µg/kg). Intravenous administration of RO-04-1284, a vesicular monoamine transporter inhibitor, obliterated dopamine release (Fig. 3, top panels) in ~120 s (time course not shown) but did not affect the alkaline changes (Fig. 3, bottom panels and inset; $n = 3$).

The time course of the pooled stimulation-evoked alkaline changes during administration of various drugs is shown in the bar graph of Fig. 4(top) ($n = 10$). WIN given at an initial dose of 125 µg/kg (W) significantly reduced alkaline shifts within this epoch ($F_{4,38} = 47.68$; $p \leq 0.05$). The second cumulative bolus dose of 250 µg/kg (W1), a physiologically

relevant dose (Gardiner *et al.* 2001), injected 15 min later, did not modify shifts further. This dose of the cannabinoid agonist also elicited a profound cataleptic state (Cheer *et al.* 2004). Figure 4(bottom) summarizes the changes in the alkaline shift at the different analysis epochs. The time trace

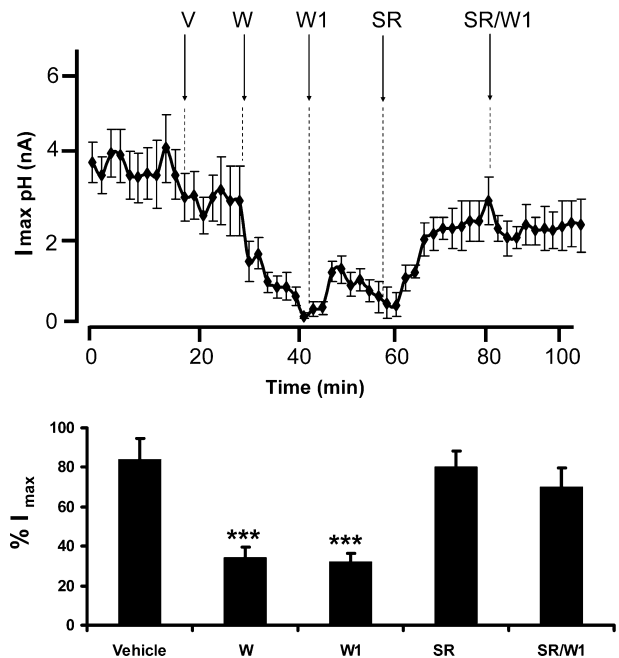


Fig. 4 Time course of the change in amplitude of alkaline shifts following WIN55,212-2 (WIN) and its reversal by SR141716A (SR). Top: Maximal current at the potential where alkaline shifts are apparent (+0.21 V) obtained following medial forebrain bundle (MFB) electrical stimulations delivered at 1 min intervals. Values are the average from responses in 10 rats. Injection times are indicated by the arrows. Bottom: Significant changes in alkaline shifts induced by the different treatments expressed as a percentage change from pre-drug values (*** $p \leq 0.001$ compared to pre-drug, one-way ANOVA followed by Scheffé test). V: vehicle = [1 : 1 : 18, ethanol/emulphor/saline (0.9%) ratio]; W: WIN (125 µg/kg); W1: WIN (250 µg/kg); SR (300 µg/kg); SR/W1: 250 µg/kg WIN in the presence of SR (300 µg/kg).

shown depicts the peak pH current following MFB stimulations at 1 min intervals rather than the effect of the drugs on basal pH levels. Thus, consistent with its assignment as the result of an alkaline pH shift, its amplitude was diminished similarly to the oxygen signal by WIN and, as demonstrated in anesthetized animals (Venton *et al.* 2003), it followed the same time course as oxygen. Thus both signals are responses to transient changes in local blood flow (Lowry *et al.* 1997; Venton *et al.* 2003). In the remaining experiments, only the background shift due to pH was followed.

The cannabinoid receptor antagonist SR141716A (SR; 300 $\mu\text{g}/\text{kg}$) was given following the highest dose of WIN in order to test whether the inhibition of stimulus-evoked pH transients was mediated by CB₁ receptors (Fig. 2a, bottom left panel and Fig. 4). SR reversed this inhibition so that it returned to $80 \pm 9\%$ of pre-WIN values, an insignificant difference from vehicle (Fig. 4, bottom). Moreover, subsequent administration of WIN (W1: 250 $\mu\text{g}/\text{kg}$) failed to cause the previously elicited suppression of alkaline shifts (Fig. 2a, bottom right panel). Cannabinoid-induced catalepsy, which is known to be mediated by CB₁ receptors, was also reversed by the injection of SR, as indicated by a recovery of the righting reflex (data not shown, Cheer *et al.* 2004). When given alone ($n = 4$), SR did not change the amplitude of alkaline shifts and prevented the effects of a subsequent injection of WIN (125 $\mu\text{g}/\text{kg}$) ($F_{3,124} = 50.17$; $p \leq 0.001$).

Interestingly, SR increased the duration of pH shifts (data not shown). Because SR is known to activate non-CB₁ receptors – particularly vanilloid receptors (Höggestatt and Zygmunt 2002) – we examined the effects of AM251, another CB₁ receptor antagonist devoid of this confound (300 $\mu\text{g}/\text{kg}$; $n = 4$). AM251 did not alter the amplitude of pH shifts or their duration ($p > 0.05$, Student's *t*-test).

Effects of the cerebral vasodilator nimodipine on electrically evoked alkaline signals

To confirm that the alkaline shifts that accompany MFB stimulation can arise from a local change in blood flow in freely moving rats, the dihydropyridine calcium channel blocker nimodipine (250 $\mu\text{g}/\text{kg}$), a known cerebral vasodilator (Yanpallewar *et al.* 2004), was used ($n = 4$). Nimodipine caused a profound (64%) decrease in the amplitude of the stimulation-evoked alkaline transients (Fig. 5a). The inhibition by nimodipine of the amplitude of the alkaline transient failed to return to pre-drug levels 1 h post-injection as shown on the time course plot ($p \leq 0.005$, Student's *t*-test; Fig. 5b, inset for pooled data).

Effects of anandamide transport inhibitors on electrically evoked pH signals

To determine whether an endogenous cannabinoid played a role in the modulation of electrically evoked alkaline shifts, we examined the effects of AM404 ($n = 4$; 1 mg/kg), a blocker of carrier-mediated transport of endocannabinoids.

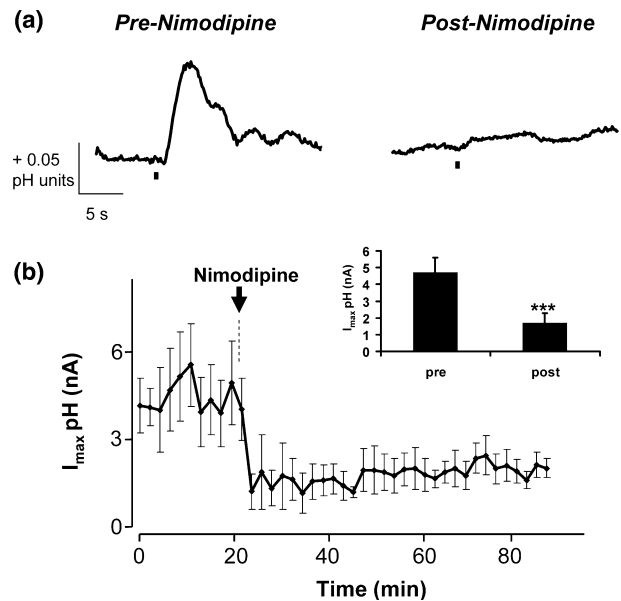


Fig. 5 Nimodipine mimics the inhibitory effects of WIN55,212-2 (WIN) on alkaline shifts. (a) Sample traces of the current at +0.21 V (the pH potential) from successive voltammograms following medial forebrain bundle (MFB) electrical stimulations before (left) and after (right) intravenous administration of nimodipine (250 $\mu\text{g}/\text{kg}$). (b) Maximal currents measured at +0.21 V following MFB electrical stimulation delivered every minute. Values are the average from responses in four rats. Inset: pooled data for the effects of nimodipine on electrically induced alkaline shifts ($***p \leq 0.001$ compared to pre-drug, Student's *t*-test).

Following injection of AM404, a partial suppression of the stimulus-evoked alkaline shift was observed as shown in the example of Fig. 6(a). When data from four animals was pooled, a significant decrease in the amplitude ($37 \pm 8\%$) of alkaline shifts was observed ($p \leq 0.05$, Student's *t*-test, Fig. 6b, inset for pooled data). The amplitude values of alkaline shifts failed to return to pre-injection levels 1 h following AM404 treatment (Fig. 6b). Figure 6(c) displays the inhibitory effects of VDM11 (the *o*-methyl derivative of AM404) on pH shift amplitude, which were similar to those exerted by AM404 ($p \leq 0.05$, Student's *t*-test). Use of VDM11 ($n = 4$) was necessary, because AM404 is also a substrate for fatty acid amidohydrolase and an agonist at vanilloid receptors. By contrast, VDM11 more selectively inhibits anandamide uptake because it is devoid of fatty acid amidohydrolase or vanilloid activity. Both the effects of AM404 and VDM11 were prevented by AM251 pre-treatment [$n = 4$ for each drug; Fig. 6(b) (inset) and Fig. 6(c)].

Discussion

The aim of the present study was to assess the local effects of several cannabinoid compounds on previously characterized alkaline and extracellular oxygen signals known to be

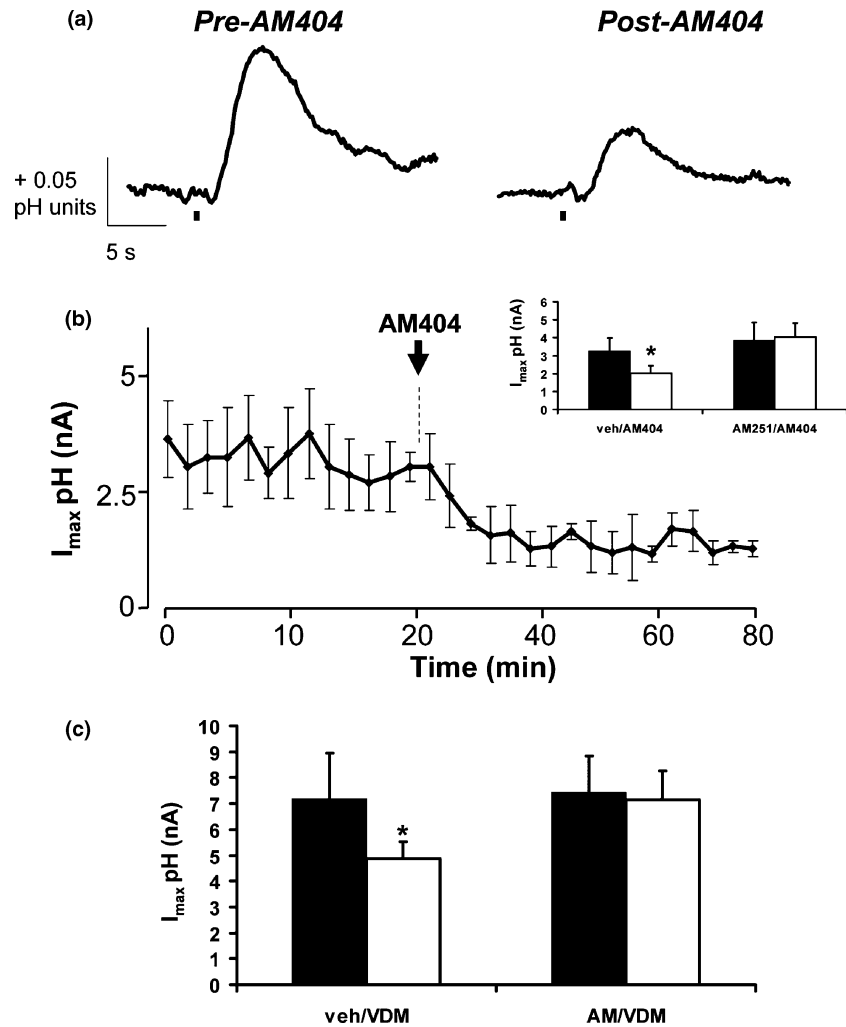


Fig. 6 AM404 and VDM11, anandamide transport inhibitors, partially inhibit pH signals. (a) Sample traces of evoked alkaline shifts before (left) and after (right) intravenous administration of AM404 (1 mg/kg). (b) Current at the pH potential ($E_p = +0.21$ V) during medial forebrain bundle (MFB) stimulation obtained every min recorded for 80 min ($n = 4$). The inset shows pooled data for the effects of AM404 on the detection of pH shifts and its blockade by AM251 ($*p \leq 0.05$ compared to pre-drug, Student's t -test). (c) Bar graph showing pooled data for the effects of VDM11 (VDM) on the detection of pH shifts and its blockade by AM251 (AM) pre-treatment ($*p \leq 0.05$ compared to pre-drug, Student's t -test).

correlated to blood flow (Lowry *et al.* 1997; Venton *et al.* 2003). Since the metabolic and cardiovascular actions of cannabinoids in conscious animals are complicated by differences in experimental approach and data acquisition conditions (Randall *et al.* 2004), we hypothesized that voltammetric detection of local pH and oxygen would optimize our chances of observing cannabinoid-induced *local* changes in blood flow and metabolism. Compared to more conventional measurements of blood flow, electrochemistry provides excellent time resolution for the detection of multiple substances as well as a view of undisturbed oxygen and pH dynamics in subcortical structures of freely moving animals. A CB_1/CB_2 receptor agonist, WIN, blocked the stimulus-evoked changes in oxygen and pH and its actions were reversed by two specific CB_1 receptor antagonists, establishing a role for these receptors in the observed effects.

WIN55,212-2 modulates extracellular oxygen and pH via CB_1 receptors

We studied the effects of WIN in freely moving rats to determine whether this agonist could alter the magnitude of

electrically evoked oxygen changes and background shifts caused by changes in pH. Although not calibrated in all cases in this study, the magnitude of the pH shifts and oxygen transients are ~ 0.06 pH units (Venton *et al.* 2003) and $\sim 50 \mu\text{M}$ (Zimmerman *et al.* 1992), respectively. In the present study, WIN (125 $\mu\text{g}/\text{kg}$) caused a potent inhibition of both pH and oxygen transients of $\sim 80\%$. The voltammetric responses obtained are differential changes that occur during the stimulation, not absolute or baseline measures of the local pH or oxygen level. Thus, substances released as a consequence of stimulating the MFB are unable to affect voltammetric measures of blood flow once the blood vessels are themselves dilated.

Whether WIN induces vasodilation or vasoconstriction cannot be directly discerned from these data because either action would obliterate the chemical changes that occur during the stimulation. This difference would of course be resolved by direct measurements of tissue perfusion or tissue oxygen levels. Nevertheless, we favor vasodilation by WIN because of the known effects on cerebral blood vessels upon CB_1 activation (Gebremedhin *et al.* 1999). Moreover,

nimodipine, an L-type calcium channel blocker known to enhance cerebral blood flow due to its potent vasodilator effects (Mohamed *et al.* 1985; Yanpallewar *et al.* 2004), depressed the stimulus-evoked alkaline changes for up to 1 h post-injection and its actions were similar to the effects induced by WIN (see Hillard 2000). In support of our assignment, human studies show that marijuana smoking produces a dose-related increase in CBF (Mathew and Wilson 1993).

CB₁ receptors are strategically located to simultaneously regulate cerebral blood flow and neuronal activity (Sugiura *et al.* 1998; Gebremedhin *et al.* 1999; Chen *et al.* 2000; Freundt *et al.* 2003). Because our study employed systemic administration to awake unrestrained rats, we cannot determine precisely the contributions of vascular dilation and neuronal activation on the alterations in oxygen and pH transients. Negative feedback regulation of neuronal activity could contribute, because presynaptic CB₁ receptors are activated during periods of intense activity (i.e. that elicited by MFB stimulation), leading to a decreased probability of neurotransmitter release (Wilson and Nicoll 2002; Lupica *et al.* 2004). Peripheral mechanisms, such as lowered vascular tone and heart performance (Jones 2002) coupled to depressed respiration (Pfitzer *et al.* 2004), are less likely contributors to the observed effects because the increase in CBF observed in the basal ganglia following marijuana smoking is independent of sympathetic stimulation, which decreases CBF (Mathew and Wilson 1993).

Because the changes in pH and oxygen follow the phasic release of dopamine in the striatum and dopamine is known to be a vasoconstrictor in both the striatum (Lavyne *et al.* 1977) and the cerebral cortex (Krimer *et al.* 1998), it was important to determine whether evoked dopamine was involved in the observed decrease in pH signals in the NAc. Eliminating vesicular dopamine and its release by blockade of vesicular monoamine transporter did not alter pH transients. Similarly, in anesthetized rats, dopamine synthesis inhibition did not modify stimulated extracellular oxygen transients (Zimmerman *et al.* 1992). Thus, dopamine release does not directly regulate the change in alkaline pH in the NAc that follows electrical stimulation of the MFB.

Role of the endocannabinoid system in the modulation of alkaline shifts

The finding that SR reversed and prevented the reduction of pH signals indicates that CB₁ receptors are involved in this response. Because WIN has higher affinity for CB₂ (peripheral) receptors under certain conditions and CB₂ receptors are expressed in the periphery (Di Marzo *et al.* 2004), the involvement of CB₁ receptors in the effects of WIN needed to be established. The finding that SR affects other signaling systems (i.e. vanilloids), as well as an as yet unidentified non-CB₁ non-CB₂ receptor (Hajos *et al.* 2001), prompted us

to use AM251, a more selective antagonist (White *et al.* 2001). The finding that neither SR nor AM251 modulated the peak amplitude of pH shifts indicates that endocannabinoid signaling during the stimulation is not involved in stimulation-evoked changes in pH. The enhanced dopamine release evoked by MFB stimulation can decrease extracellular anandamide (Giuffrida *et al.* 1999; Patel *et al.* 2003) and 2-AG (Patel *et al.* 2003) concentrations, whereas monoamine depletion with reserpine increases striatal anandamide and 2-AG contents (Di Marzo *et al.* 2000). Thus, our experimental protocol may have masked the effect of endogenous cannabinoids on pH and oxygen signals.

To investigate further the effects of endogenous cannabinoids, endocannabinoid uptake blockers, AM404 and VDM11, were used. Inhibition of anandamide and 2-arachidonoyl glycerol (2AG) uptake elevates extracellular concentrations leading to more occupation of CB₁ receptors. Intravenous injection of AM404 yielded a comparatively small but significant effect to WIN on alkaline shifts. However, AM404 like SR has been reported to target sites other than the endocannabinoid system (Zygmunt *et al.* 2000). In order to circumvent this, we treated rats with VDM11, a more selective uptake inhibitor (Ligresti *et al.* 2004). VDM11 generated a similar indirect agonist profile, indicating that endogenous cannabinoids were responsible for the modulation of pH shift amplitude. These effects are similar to *in vitro* findings showing that anandamide has a lower intrinsic activity compared to WIN but may produce full agonist effects when CB₁ receptor levels are higher than normal (Twitchell *et al.* 1997). The finding that AM251 prevented the effects of both AM404 and VDM11 argues for an involvement of CB₁ receptors in the effects of these agents. Taken together with the results following CB₁ receptor antagonists, these findings suggest that the electrical stimulation used here does not evoke endogenous cannabinoid activity. Nevertheless, the effect of uptake blockers indicates that endogenous cannabinoids have the capacity to be produced following MFB stimulation to target CB₁ receptors.

The present study is the first to show *in situ* CB₁ receptor-mediated changes of local electrochemical indices of CBF and metabolism in freely moving rats. We demonstrate that the administration of an exogenous cannabinoid agonist and two endocannabinoid uptake blockers generates CB₁ receptor-selective reductions in the peak amplitude of pH and extracellular oxygen shifts associated to changes in CBF and metabolism. The results agree with the concept that CB₁ receptor stimulation can affect oxidative metabolism by bridging changes in cerebral blood flow (possibly through vasodilation) with neuronal activity. The present findings further demonstrate the physiological relevance of the endogenous cannabinoid system and might lead to the development of novel therapeutic treatments for patients suffering from cerebrovascular disease.

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