

Vanilloid-Mediated Heterosynaptic Facilitation of Inhibitory Synaptic Input to Neurons of the Rat Dorsal Motor Nucleus of the Vagus

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Vanilloid type-1 receptors (VR1) are abundant in the dorsal vagal complex, where their function is mostly unknown. We examined the role of VR1 in regulating synaptic inputs to neurons of the dorsal motor nucleus of the vagus (DMV). Using patch-clamp recordings from DMV neurons in brainstem slices, capsaicin was found to increase action potential-independent inhibitory input onto DMV neurons. This rapid effect was mimicked by application of the endogenous cannabinoid, anandamide and blocked by VR1 antagonists. The VR1-mediated facilitation of synaptic inhibition was reduced by ionotropic and metabotropic glutamate receptor antagonists, suggesting an indirect, heterosynaptic enhancement of GABA release caused by a VR1-mediated increase in glutamate release from presynaptic terminals of excitatory neurons. Application of L-glutamate also increased GABA release. The paired-pulse ratio was increased for IPSCs evoked after electrical stimulation of the nucleus tractus solitarius, but the effect was slower than for the enhancement of spontaneous and miniature IPSCs. Capsaicin also increased the frequency of glutamatergic postsynaptic currents in a VR1-mediated manner. Results of these studies suggest that VR1-containing glutamatergic terminals contact DMV neurons. Activation of VR1 potently enhances glutamate release onto GABAergic terminals, facilitating GABA release. Endogenous cannabinoids can thereby rapidly enhance inhibitory input to DMV neurons via VR1-mediated presynaptic mechanisms.

Key words: anandamide; cannabinoid; capsaicin; gastric; nucleus tractus solitarius; presynaptic

Introduction

The dorsal vagal complex (DVC) in the dorsomedial medulla consists of area postrema, the nucleus of the solitary tract (NTS), and the dorsal motor nucleus of the vagus (DMV). Neurons in the NTS receive visceral mechanosensory and chemosensory input via primary vagal, glossopharyngeal, and facial nerve afferents, whose central processes form the solitary tract in the hindbrain. Neurons of the DMV constitute the preganglionic motor neurons that project throughout most of the gastrointestinal tract, among other viscera (Laughton and Powley, 1987). Together, the viscerosensory branch, integrative centers within the DVC, and motor fibers from the DMV complete vagal reflex pathways (Raybould et al., 1991; Rogers et al., 1996).

The vanilloid type-1 receptor (VR1) is a ligand-gated nonselective cation channel with high Ca^{2+} permeability that is structurally related to a member of the transient receptor potential (TRP) channel family. These receptors are sensitive to capsaicin and also to temperature and pH changes (Tominaga et al., 1998; Szallasi and Blumberg, 1999), and activation is often associated

with nociception in peripheral nerves. Recently, endocannabinoids, including anandamide (AEA), have also been shown to activate VR1 in the CNS (Szallasi and Di Marzo, 2000; Szolcsányi, 2000; Jennings et al., 2003; Marinelli et al., 2003). Centrally, VR1 are expressed in the spinal cord dorsal horn, as well as several discrete brain regions (Acs and Blumberg, 1994; Szallasi et al., 1995; Acs et al., 1996; Caterina et al., 1997; Mezey et al., 2000; Li et al., 2004), including the DVC (Patterson et al., 2003).

Little is known about the physiological role of VR1 receptors in the hindbrain. Electrophysiological studies in the NTS indicate that capsaicin activates a subset of solitary tract axons to transiently enhance release of glutamate onto second-order viscerosensory neurons (Doyle et al., 2002; Jin et al., 2004a). Synaptic responses to vagal stimulation in cardiac-related DMV neurons are suppressed after >30 min of continuous capsaicin application, but this is probably because of time-dependent inactivation of vagal afferents (Evans et al., 2003). Recent studies have addressed the roles of central VR1 activation on respiratory rate (Geraghty and Mazzone, 2002), gastric acid secretion (Minowa et al., 2001), and emesis (Shiroshita et al., 1997), with emphasis on sensory modulation. However, the rapid effects of capsaicin on neurons of the DMV have not been studied in detail.

We investigated the role of VR1 in the control of excitatory and inhibitory synaptic inputs to DMV neurons using *in vitro* electrophysiological recordings to test the hypothesis that VR1 receptor activation potentiates synaptic input to DMV neurons.

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Materials and Methods

All procedures were performed on male Sprague Dawley rats (Harlan, Indianapolis, IN) and were approved by the Tulane University Animal Care and Use Committee.

Brainstem slice preparation. Transverse brainstem slices were prepared from male Sprague Dawley rats (Harlan), 4–8 weeks of age, as described previously (Derbenev et al., 2004a). Rats were deeply anesthetized by halothane inhalation and killed by rapid decapitation while anesthetized. Brains were rapidly removed and immersed in ice-cold ($0-4^{\circ}\text{C}$) oxygenated (95% O_2 –5% CO_2) artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 3 KCl, 26 NaHCO_3 , 1.4 NaH_2PO_4 , 11 glucose, 1.3 CaCl_2 , and 1.3 MgCl_2 , pH 7.3–7.4, with an osmolality of 290–310 mOsm/kg. Transverse brainstem slices (350–400 μm) containing the DVC were made using a vibrating microtome (Vibratome Series 1000; Technical Products, St. Louis, MO). The slice experiments were performed at 32°C . Slices were transferred to a recording chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus, Melville, NY).

Whole-cell recording. Neurons in the DMV were visually targeted for recording under a $40\times$ water-immersion objective using infrared differential interference contrast optics (Olympus). For whole-cell patch-clamp recordings, electrodes (2–4 M Ω) were filled with a solution containing the following (in mM): 130 Cs^+ -gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl_2 , 1 CaCl_2 , 3 CsOH, 2–4 Mg-ATP, and 0.2% biocytin, pH 7.3–7.4, adjusted with 5 M CsOH. IPSCs were examined at a holding potential of -10 mV; EPSCs were recorded at -60 mV. Electrophysiological signals were low-pass filtered at 2–5 kHz, digitized at 88 Hz, recorded using an Axopatch 200B or 700A amplifier (Molecular Devices, Union City, CA), and analyzed with pClamp 8 software (Molecular Devices). Spontaneous and evoked IPSCs and EPSCs were analyzed offline using pClamp 8 or MiniAnalysis (Synaptosoft, Decatur, GA). Synaptic currents were evoked by electrical stimulation using a concentric bipolar platinum-iridium electrode (200 μm outer diameter; FHC, Bowdoinham, ME) placed on the medial portion of the NTS. Evoked EPSCs and IPSCs had short, constant latency (variation in response latency <0.5 ms), and $<20\%$ failure rate. The NTS was stimulated by paired current pulses (300 μs) at pairing frequencies of 10–20 Hz, with 10 s interstimulus interval.

Drug application. Most experiments were performed with tetrodotoxin (TTX; 1–2 μM) in the bath solution. Capsaicin, capsazepine, and 5'-iodoresiniferatoxin (Tocris Bioscience, Ellisville, MO) were dissolved in ethanol and diluted in ACSF (final concentration of ethanol $<0.01\%$ by volume). AEA was purchased predissolved in Tocrisolve (Tocris Bioscience). Vehicle alone (i.e., ethanol or Tocrisolve) had no effect on membrane or synaptic properties ($n = 5$ each). Capsaicin, AEA, or L-glutamate were bath applied for 5–10 min at a final concentration of 1 nM–100 μM . In other experiments, capsaicin (10 μM ; pH 7.3–7.4) or AEA (50 μM ; pH 7.3–7.4) were pressure-applied (5 psi; 100 ms) near the recorded neuron via a glass pipette (~ 2 μm tip diameter) attached to a Picospritzer (Parker-Hannifin, Fairfield, NJ). Antagonists to VR1, capsazepine (20 μM) or 5'-iodoresiniferatoxin (1 μM), or cannabinoid type 1 receptors (CB1R) (AM251, 10 μM ; Tocris Bioscience) were applied with the bath for 10 min before agonist application to isolate effects of endocannabinoid agonists. Ionotropic glutamate receptor antagonists DL-5-aminophosphonovaleric acid (AP-5, 50 μM ; Sigma, St. Louis, MO) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM ; Sigma), or a mixture of type I/II metabotropic [α -methyl-4-carboxyphenylglycine (MCPG), 1 mM and type III [α -methylserine-O-phosphate (MSOP), 250 μM] metabotropic glutamate receptor antagonists (both from Tocris Bioscience), were bath applied for 10 min in some instances before capsaicin application to determine the role of glutamate receptors in effects of VR1 actions on synaptic input. At these concentrations, the antagonists were shown to block all ionotropic and metabotropic glutamate receptors (Derbenev et al., 2004a; Drew and Vaughan, 2004).

Data analysis. The effects of VR1 agonists on spontaneous postsynaptic current (PSC) frequency and amplitude were analyzed within individual neurons using the Kolmogorov–Smirnov test. The effects of agonists on PSC frequency across the neuron population were analyzed

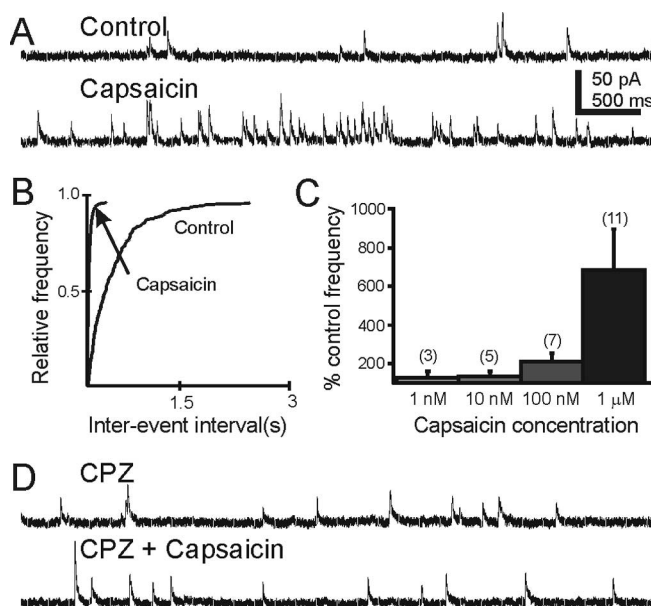


Figure 1. The VR1 agonist capsaicin increased mIPSC frequency in DMV neurons. **A**, Continuous recording of mIPSCs observed at holding potential of -10 mV in the presence of TTX (1 μM ; top) and after application of 1 μM capsaicin (bottom). The recording pipette contained 140 mM Cs^+ . **B**, Cumulative plots of the interevent interval distribution for mIPSCs before and after perfusion of capsaicin ($p < 0.05$, Kolmogorov–Smirnov test). **C**, Concentration-related effect of capsaicin. Numbers of replicates are in parentheses above each bar. **D**, Recordings of mIPSCs at a holding potential of -10 mV in the presence of the VR1 antagonist capsazepine (CPZ; 20 μM) before (top) and after application of capsaicin (1 μM) (bottom). Calibration is the same for **A** and **D**. Error bars indicate SE.

using a paired two-tailed Student's t test. The effects of the glutamate antagonists on capsaicin were compared using one-way ANOVA followed by a Dunn's test. For electrical stimulation analysis, we compared the stimulus amplitudes of the successful responses (i.e., a PSC was evoked by the stimulus) in the presence of capsaicin versus the control amplitudes with an unpaired Student's t test. Paired-pulse ratios were calculated as the average amplitudes of the second PSC divided by the first and compared across neurons using the paired Student's t test. For all analyses, $p < 0.05$ was considered significant. Values are reported as the mean \pm SEM.

Results

Capsaicin effects on mIPSC frequency in DMV neurons

To assess the effects of VR1 activation on GABA release in the DMV, the effects of capsaicin on miniature IPSCs (mIPSCs) were examined in DMV neurons in the presence of TTX (1 μM). Neurons were voltage-clamped at -10 mV using Cs^+ in the recording pipette to block voltage-dependent K^+ channels and consequently improve voltage clamp and facilitate mIPSC analysis (Davis et al., 2003; Derbenev et al., 2004a). Bath application of capsaicin (1 μM) significantly increased the frequency of mIPSCs in each of 11 DMV cells (Fig. 1). The effect of capsaicin was observed within 2 min of the drug reaching the slice and was maximal by 5 min. Under control conditions, the average mIPSC frequency was 1.8 ± 0.3 Hz and ranged from 0.8 to 3.1 Hz. After application of the highest concentration of capsaicin tested (1 μM ; 5–7 min), the mean frequency of mIPSCs was increased to 10.6 ± 3 Hz (range, 2.6–29.2 Hz; $p < 0.05$; $n = 11$) (Fig. 1). Application of different log concentrations of capsaicin (1 nM–1 μM) resulted in a concentration-related increase in the frequency of mIPSCs (Fig. 1C). There was no significant change in mIPSC amplitude after application of 1 μM of capsaicin ($p > 0.05$). The average amplitude before application of capsaicin was 27 ± 2 pA

($n = 11$; range, 17–36 pA) and after bath application of $1 \mu\text{M}$ of capsaicin was 31 ± 3 pA (range, 19–53 pA).

To verify that the effect of capsaicin on mIPSC frequency was mediated by VR1 receptors, capsazepine ($20 \mu\text{M}$) was applied before and during capsaicin application. Capsazepine itself had no effect on the frequency of mIPSCs but completely prevented the effect of capsaicin (Fig. 1D). The frequency of mIPSCs was 2.7 ± 0.5 Hz (range, 1.3–4.2 Hz) in the presence of capsazepine and 2.1 ± 0.4 Hz (range, 1.3–4 Hz) after bath application of capsaicin ($1 \mu\text{M}$) in the presence of capsazepine ($p > 0.05$; $n = 7$).

Dependence of VR1-mediated mIPSC facilitation on glutamate receptors

Under control conditions (i.e., TTX only) in neurons voltage-clamped at -10 mV, the frequency of mIPSCs was 1.8 ± 0.3 Hz ($n = 11$) (Fig. 2A, top trace). After 3–5 min bath application of capsaicin, mIPSC frequency was increased to 10.6 ± 2.5 Hz ($p < 0.05$; $n = 11$) and was 11.6 ± 3.7 Hz ($p < 0.05$; $n = 7$) (Fig. 2A) after 8–10 min capsaicin application. In the presence of ionotropic glutamate receptor antagonists CNQX ($10 \mu\text{M}$) and AP-5 ($50 \mu\text{M}$), mIPSC frequency in another set of neurons was 2.9 ± 0.5 Hz ($n = 10$) (Fig. 2B). The VR1-induced increase in mIPSC frequency in these cells was not significant after 3 min (5.5 ± 1.6 Hz; $n = 10$; $p > 0.05$), but became apparent after 10 min of capsaicin application (9.1 ± 3 Hz; $p < 0.05$; $n = 7$) (Fig. 2B). In another set of neurons, the selective group III metabotropic glutamate receptor antagonist MSOP ($250 \mu\text{M}$) and nonselective group I/group II metabotropic glutamate receptor antagonist MCPG (1 mM) were applied in addition to the ionotropic antagonists. The frequency of mIPSCs in these cells was 2.1 ± 0.7 Hz ($n = 8$). There was no significant effect of capsaicin in these neurons after 3 min (3.7 ± 1.4 Hz; $p > 0.05$; $n = 8$) or 10 min (3.7 ± 1.5 Hz; $p > 0.05$; $n = 8$). Ionotropic and metabotropic glutamate receptor antagonists together significantly reduced the effect of capsaicin on mIPSC frequency (Fig. 2D), even at the later time point (i.e., longer capsaicin application), when ionotropic antagonists alone were ineffective in preventing the effect.

In the presence of just the metabotropic glutamate receptor antagonists (MCPG, 1 mM , and MSOP, $250 \mu\text{M}$), mIPSC frequency in another set of neurons was 4.0 ± 0.4 Hz ($n = 8$) (Fig. 2C). The VR1-induced increase in mIPSC frequency in these cells was not significant after 3 min (6.2 ± 1.3 Hz; $n = 8$; $p > 0.05$), or 10 min of capsaicin application (5.7 ± 1.6 Hz; $p > 0.05$; $n = 7$) (Fig. 2D). The presence of either ionotropic or metabotropic glutamate receptor antagonists significantly reduced the effect of capsaicin on mIPSC frequency (Fig. 2D). Because the VR1-mediated enhancement of mIPSCs was prevented by glutamate receptor antagonists, we tested the hypothesis that glutamate itself increased mIPSC frequency. Bath application of L-glutamate ($100 \mu\text{M}$) resulted in a small but significant increase in mIPSC frequency (1.4 ± 0.3 Hz; range 0.4–2 Hz in control vs 2.2 ± 0.5 Hz, range 0.6–3 Hz in glutamate; $p < 0.05$, paired t test; $n = 7$) (Fig. 3).

Capsaicin effects on IPSCs evoked after NTS stimulation

Paired electrical stimuli applied to the NTS were used to generate pairs of evoked IPSCs in DMV neurons (Fig. 4). The effects of capsaicin were tested on responses to paired stimuli to further assess whether the VR1 agonist acted at a presynaptic site, with a change in the ratio of amplitudes suggesting a presynaptic site of action (Regehr and Stevens, 2001). For IPSCs evoked at a pairing frequency of 10–20 Hz, paired-pulse depression resulted, with the second evoked IPSC having a smaller amplitude than the first

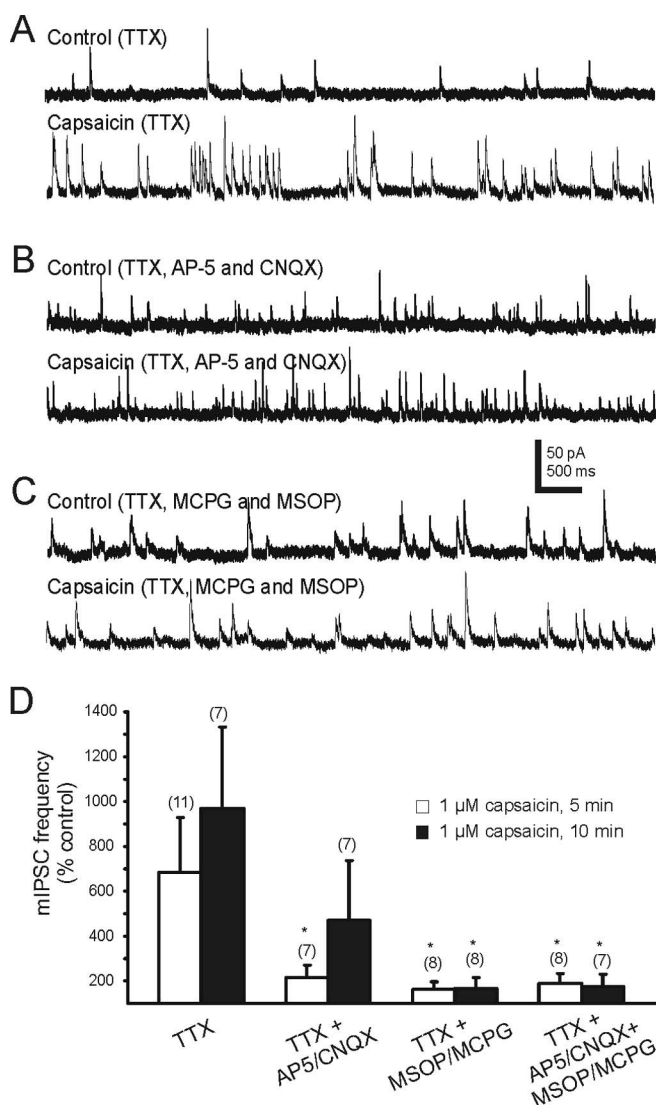


Figure 2. Glutamate receptor antagonists reduced the effect of capsaicin on mIPSCs. **A**, Continuous recordings of mIPSCs at holding potential of -10 mV in control conditions ($1 \mu\text{M}$ TTX; top) and 10 min after addition of $1 \mu\text{M}$ capsaicin (bottom). **B**, Continuous recordings of mIPSCs in the presence of $1 \mu\text{M}$ TTX, $10 \mu\text{M}$ CNQX, and $50 \mu\text{M}$ AP-5 before (top) and 10 min after application of $1 \mu\text{M}$ capsaicin (bottom). **C**, Continuous recordings of mIPSCs at holding potential of -10 mV in control conditions (top) and after 10 min bath application of $1 \mu\text{M}$ capsaicin (bottom). ACSF contained $1 \mu\text{M}$ TTX, 1 mM MCPG, and $250 \mu\text{M}$ MSOP. **D**, Effects of glutamate receptor antagonists on the capsaicin-induced increase in mIPSC frequency, normalized to the frequency before capsaicin application obtained in the presence of drugs indicated, after 3 and 10 min bath application of $1 \mu\text{M}$ capsaicin. Numbers of replicates are in parentheses; the asterisk indicates significance ($p < 0.05$) versus TTX alone. Error bars indicate SE.

(Fig. 4). The amplitude of the first response was 212 ± 52 pA and the paired pulse amplitude ratio for these neurons was 0.7 ± 0.1 ($n = 7$). Capsaicin had no effect on the amplitude of the first evoked IPSC (159 ± 27 pA; $p > 0.05$, paired t test) or on the paired-pulse ratio (0.7 ± 0.1 ; $n = 7$; $p > 0.05$) within 5 min of application. However at 10 min, capsaicin reduced the amplitude of the first current (117 ± 32 pA; $p < 0.05$) to a greater degree than the second current, such that the paired-pulse ratio increased to 1.2 ± 0.2 ($n = 7$; $p < 0.05$) (Fig. 4).

Microapplication of capsaicin and endocannabinoids increased frequency of mIPSCs

In the NTS, capsaicin-induced effects desensitized rapidly (Doyle et al., 2002). To examine rapid effects of VR1 activation, agonists

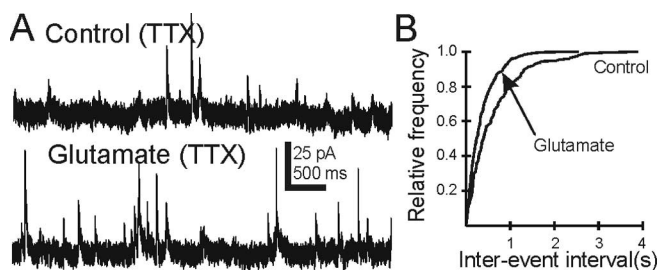


Figure 3. Glutamate increased mIPSC frequency in DMV neurons. *A*, Continuous recording of mIPSCs observed at holding potential of -10 mV in the presence of TTX ($1 \mu\text{M}$; top) and after application of $100 \mu\text{M}$ L-glutamate (bottom). Recording pipette contained 140 mM Cs^+ . *B*, Cumulative plots of the interevent interval distribution for mIPSCs before and after perfusion of capsaicin ($p < 0.05$, Kolmogorov–Smirnov test).

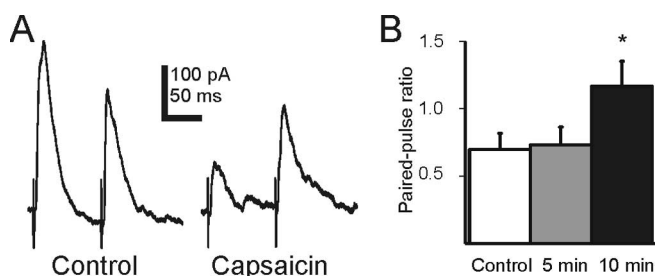


Figure 4. Capsaicin decreased evoked IPSC amplitude via actions at presynaptic receptors. *A*, Average of 15–20 traces of IPSCs evoked by paired current stimuli (10 Hz) applied to the NTS. *B*, Paired-pulse ratio for control and after 5 and 10 min capsaicin ($1 \mu\text{M}$) application. The paired-pulse ratio was unchanged after 5 min, but was increased ($p < 0.05$) after 10 min capsaicin application. * $p < 0.05$ versus control. Error bars indicate SE.

were applied transiently through a glass pipette positioned near the surface of the slice. Capsaicin or AEA was microapplied very near the recorded DMV neuron, which was voltage clamped at -10 mV (Fig. 5). Short-duration (100 ms) pressure application of capsaicin ($10 \mu\text{M}$) resulted in a brief increase in mIPSC frequency from a preapplication frequency of 1.9 ± 0.4 Hz (range, 1–3.2 Hz) to a capsaicin-induced frequency of 17 ± 7 Hz ($p < 0.05$; $n = 5$; range, 4.1–34 Hz) (Fig. 5*A*). A facilitation of mIPSCs was also observed after pressure application of the endogenously produced cannabinoid and vanilloid agonist AEA ($50 \mu\text{M}$) from a control frequency of 2.5 ± 0.5 Hz (range, 1–6.6 Hz) to 16.7 ± 2.5 Hz after AEA application ($p < 0.05$; $n = 10$; range, 5–35.4 Hz) (Fig. 5*B*). Preapplication of the VR1 antagonists capsazepine ($20 \mu\text{M}$; $n = 3$) (Fig. 5*B*) or 5'-iodoresiniferatoxin (500 nM – $1 \mu\text{M}$; $n = 10$), prevented the AEA-induced increase in mIPSC frequency (0.9 ± 0.2 Hz control to 1.3 ± 0.3 Hz in AEA; $p > 0.05$).

The effects of endocannabinoids have been shown to involve a significant suppression of mIPSC frequency over a period of 5–15 min (Derbenev et al., 2004a,b). To determine whether AEA activated CB1R along with VR1, we applied AEA ($1 \mu\text{M}$) in the presence of CB1R antagonist AM251 ($10 \mu\text{M}$) and compared the results with those obtained in the absence of the antagonist. In the presence of AM251, application of AEA increased the frequency of mIPSCs from a control frequency of 3.5 ± 0.8 Hz (range, 0.8–5.4 Hz) to 48 ± 8 Hz after AEA application ($p < 0.05$; $n = 5$; range, 21–73.4 Hz) (Fig. 5). This increase was significantly greater than for the same application in the absence of the antagonist ($p < 0.05$) (Fig. 5*E*), suggesting that both VR1 and CB1R were activated by the endocannabinoid. Endocannabinoids altered mIPSC frequency in DMV neurons by acting at both VR1 and CB1R.

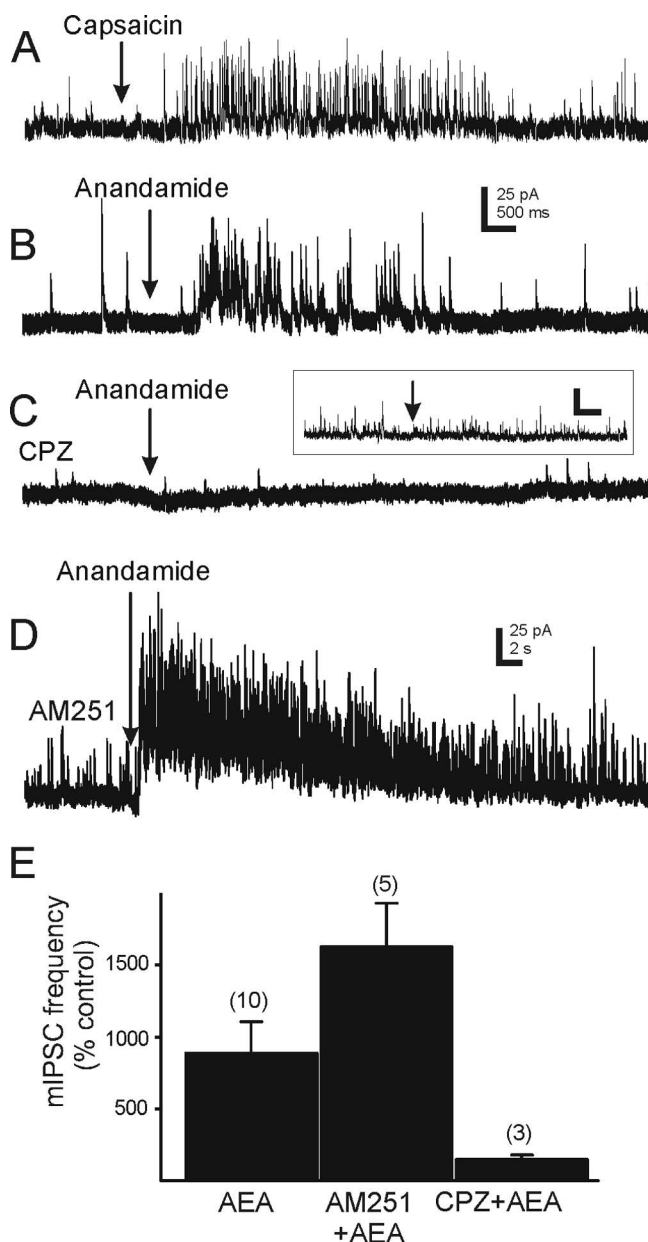


Figure 5. Transient enhancement of mIPSC frequency after microapplication of VR1 agonists. *A*, Rapid (100 ms) application of $100 \mu\text{M}$ capsaicin near the recorded neuron resulted in a barrage of mIPSCs. *B*, Microapplication (100 ms) of AEA ($50 \mu\text{M}$) similarly increased the frequency of mIPSCs. *C*, Preapplication of capsazepine (CPZ; $20 \mu\text{M}$) blocked the effect of microapplied AEA. Calibration is the same for *A*–*C*. Inset, Trace in box between *B* and *C* shows that identical application of vehicle (i.e., Tocrisolve; arrow) had no effect on mIPSC frequency. *D*, Microapplication of AEA in the presence of AM251 ($10 \mu\text{M}$) resulted in a large barrage of mIPSCs. The holding potential was -10 mV for all traces; TTX was present in all traces. *E*, Cumulative data showing the AEA-induced increase in mIPSC frequency in TTX, TTX plus AM251, and TTX plus CPZ ($20 \mu\text{M}$). The AEA-induced increase in mIPSC frequency was significantly greater in the presence of the CB1R antagonist AM251 ($p < 0.05$) than without the antagonist. The effect was prevented by capsazepine. Error bars indicate SE.

Effect of capsaicin on mEPSCs in DMV neurons

In each of seven neurons voltage-clamped at -60 mV, $1 \mu\text{M}$ capsaicin significantly increased frequency of miniature EPSCs (mEPSCs) from 4.6 ± 1.2 Hz before capsaicin application to 16.3 ± 3.8 Hz in the presence of capsaicin ($p < 0.05$) (Fig. 6). There was no change in mEPSC amplitude after application of capsaicin compared with control ($p > 0.05$). The average ampli-

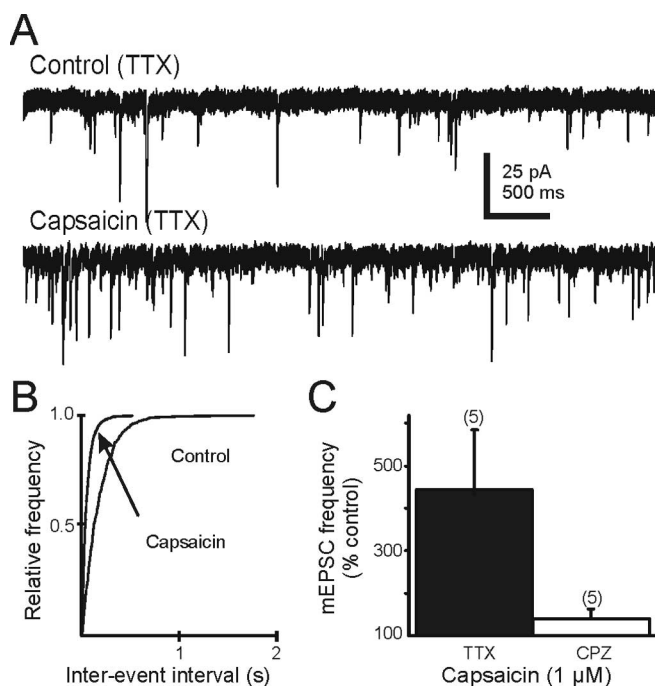


Figure 6. Capsaicin increased mEPSC frequency in DMV neurons. **A**, Continuous recording demonstrating mEPSCs at holding potential -60 mV in control conditions (top) and after bath application of $1 \mu\text{M}$ capsaicin (bottom). ASCF contained TTX ($1 \mu\text{M}$). **B**, Cumulative plots of the interevent interval distribution of mEPSCs before and after perfusion of capsaicin in this recording ($p < 0.05$, Kolmogorov–Smirnov test). **C**, Effects of capsaicin ($1 \mu\text{M}$) on mEPSC frequency at 8 – 10 min after agonist application alone and in the presence of capsazepine, expressed as a percentage of the control frequency in TTX. Error bars indicate SE.

tude before application of capsaicin was 15.1 ± 0.4 pA ($n = 5$; range, 14 – 16 pA) and after bath application of $1 \mu\text{M}$ of capsaicin was 19.8 ± 1.9 pA (range, 13 – 26 pA). The effect of capsaicin on mEPSC frequency was prevented by preapplication of VR1 antagonist capsazepine ($20 \mu\text{M}$; 9.4 ± 2.7 Hz in capsazepine vs 14.2 ± 4.5 Hz in capsaicin; $n = 5$; $p > 0.05$). In 12 additional neurons recorded in current-clamp at rest, the membrane potential changed by <1 mV after capsaicin application (data not shown). These data are consistent with the hypothesis that VR1 increases glutamate release in the DMV.

Discussion

Vanilloid receptors are coupled to ligand-gated ion channels that flux cations, including Ca^{2+} (Montell, 2001). VR1 activation typically increases excitability of neurons and synaptic terminals, and consequently enhances synaptic glutamate release. Activity at VR1 receptors is typically studied at the cellular level in the context of somatic or visceral sensory modulation (Tominaga et al., 1998; Yang et al., 1998; Zygmunt et al., 1999; Hwang et al., 2000; Bailey et al., 2002; Doyle et al., 2002; Evans et al., 2003; Jin et al., 2004a). Activation of VR1 can be achieved by increasing the temperature above 42°C or by lowering the pH below physiological levels (Tominaga et al., 1998; Premkumar and Ahern, 2000), conditions which cannot be met without disturbing normal brain activity. Centrally, several ethanolamide-based endocannabinoids such as AEA bind VR1 as well as CB1R, both of which are located throughout the DVC (Van Sickle et al., 2001; Patterson et al., 2003; Derbenev et al., 2004a). In the DMV, tonically applied AEA and other cannabinoids activate CB1R and suppress synaptic inputs over a period of several minutes (Derbenev et al., 2004a), although AEA often causes a transient increase in synap-

tic frequency. In dorsal horn (Jennings et al., 2003) and substantia nigra (Marinelli et al., 2003), AEA enhances glutamatergic EPSCs, but not GABAergic IPSCs, effects that are suppressed by VR1 antagonists and mimicked by capsaicin. Here, AEA rapidly activated VR1, and capsaicin was used to selectively bind VR1 receptors (i.e., versus CB1R). The most prominent initial response to capsaicin or endocannabinoids was a potent VR1-mediated increase in synaptic GABA release.

In the presence of TTX, capsaicin rapidly increased the frequency of mIPSCs in DMV neurons, supporting a role for VR1 in inhibiting the vagal motor output. There was no capsaicin-induced change in mIPSC amplitude, resting membrane potential, or holding current in the DMV neurons, suggesting a lack of direct postsynaptic VR1 activity. However, these results are consistent with the hypothesis that VR1 activation enhances asynchronous GABA release by acting at presynaptic terminals. Glutamate application increased mIPSC frequency, suggesting that presynaptic glutamate receptors regulate GABA release in the DMV, and glutamate neurotransmission was necessary for the facilitation of mIPSC frequency by VR1 activation. Presynaptic metabotropic glutamate receptors modulate GABA release in the NTS (Jin et al., 2004b) and presynaptic ionotropic glutamate receptors modulate neurotransmitter release in other brain regions (Liu et al., 1999; Cossart et al., 2001), although these have not been examined previously in the DMV. Ionotropic or metabotropic glutamate receptor antagonists suppressed the capsaicin-induced increase in mIPSC frequency in DMV neurons, implicating both receptor subtypes in the response. This may best be explained by activation of VR1 located on glutamatergic terminals that contact GABAergic terminals, which, in turn, synapse on DMV neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In this heterosynaptic arrangement, activation of VR1 increases Ca^{2+} influx in glutamatergic terminals, increasing glutamate release (Li et al., 2004). The glutamate subsequently binds postsynaptically to receptors on DMV neurons, as indicated by an increase in mEPSC frequency. It also binds presynaptically to multiple receptor types on GABAergic terminals that synapse with DMV neurons, increasing terminal excitability. The increased GABA release then inhibits the DMV neurons and decreases their overall excitability. These VR1-containing glutamatergic inputs may originate from several sources, especially neurons of the NTS (Travagli et al., 1991; Davis et al., 2004) or primary vagal afferents (Rinaman et al., 1989). It is also conceivable that VR1-induced glutamate release triggers release of an undefined retrograde messenger from the postsynaptic cell (e.g., occult glutamate, acetylcholine, ATP, etc.), which in turn feeds back and enhances GABA release. Although no direct evidence of activity at postsynaptic receptors was observed after VR1 activation, effects on nearby neural or glial cells cannot be ruled out.

Stimulation of the NTS evoked IPSCs in the DMV, which exhibited paired-pulse depression. The evoked IPSCs and the paired-pulse ratio were not altered by capsaicin with the same time course as the increase in mIPSC frequency, consistent with the notion that the VR1 are not primarily located directly on GABA terminals. Interestingly, the eventual decrease in evoked IPSC amplitude occurred concurrently with a VR1-mediated enhancement of mIPSC frequency. Analogous to VR1-mediated effects on EPSCs in substantia nigra or dorsal horn (Yang et al., 1998; Marinelli et al., 2003), the reduction in evoked IPSC amplitude may involve mechanisms resultant from sustained calcium influx or depolarization of afferent neurons or terminals. An inactivating, VR1-mediated inward current is observed in a

subset of NTS neurons (Doyle et al., 2002) and could contribute to the eventual diminution of responses evoked after stimulating the NTS. Thus, VR1 activation could facilitate spontaneous glutamate or GABA release by acting at presynaptic receptors, while reducing action potential-evoked responses via a depolarization block (Katz and Miledi, 1969) or partial depletion of the calcium-dependent, readily releasable vesicle pool in the terminal (Yang et al., 1999; Li et al., 2004). The heterogeneous nature of the axons activated by electrical stimulation of the NTS may also contribute to the change in evoked response over time, which could include VR1-mediated release of a different modulator substance (e.g., a neuropeptide) that secondarily enhances GABA release. Alternatively, a delay in the effect on evoked IPSCs could result from an activity-dependent translocation of VR1 into GABA terminals from the cytosol, as occurs for μ -opioid receptors (Browning et al., 2004). Endogenously released agonists (e.g., AEA) would likely be cleared relatively rapidly by reuptake or degradation mechanisms, which would be expected to minimize the synaptic depletion, depolarization block, or slow activation of other modulatory systems *in vivo*.

Capsaicin also increased the frequency of mEPSCs to a similar extent as mIPSCs. Thus, it is possible that the physiological effects of VR1 agonists in the DMV also depend on the activity state of the nucleus or the predominance of the type of input received by a given set of neurons. Several studies showed that activation of VR1 alters a variety of physiological responses, including increasing gastric acid secretion (Minowa et al., 2001) and decreasing respiratory rate, in rats (Geraghty and Mazzone, 2002), and inducing retching in dogs (Shiroshita et al., 1997). All neurons tested responded to capsaicin with an increase in mIPSC frequency, and the effect of VR1 activation did not appear to differ between neurons in different parts of the DMV. It seems unlikely that the effect is limited to control of a particular organ system. The dual excitatory (i.e., cholinergic) and inhibitory (i.e., non-cholinergic, nonadrenergic) pathways of the vagus peripherally allow for excitation and/or inhibition of DMV neurons to lead to inhibition of smooth muscle (D'Amato et al., 1992; Grundy et al., 1993; Desai et al., 1994; Paterson et al., 2000). Unfortunately, no evidence exists to directly correlate morphological or electrophysiological properties of individual DMV neurons with their final effect on the viscera, so it is currently impossible to determine in a slice preparation which functional vagal pathway is affected by a given DMV neuron. Regardless of the final pathway, during periods of normal neural activity in the DMV, VR1 effects on GABAergic inputs would be prominent. But during rare quiescent periods, enhanced glutamatergic inputs might make neurons more excitable. It has been similarly suggested that the actions of opioids or hypocretins in the DVC may depend on activity state in specific local circuits (Davis et al., 2003; Browning et al., 2004). Understanding mechanisms leading to release of endogenous agonist will be useful for understanding the relationship between VR1 activation and neural activity in the DMV.

The immediate, rapid effect in the DMV of the endocannabinoid, AEA is a VR1-mediated enhancement of synaptic (particularly GABAergic) input, whereas the CB1R-mediated effects include a suppression of that same synaptic input over time (Derbenev et al., 2004a). Notably, we reported previously a suppression of mIPSC frequency by AEA in experiments done in the presence of glutamate receptor antagonists (Derbenev et al., 2004a). Thus, endocannabinoids activate both VR1 and CB1R to alter GABA release in the DMV. As is the case for several other neuroactive substances (e.g., glutamate, serotonin, acetylcholine, etc.), a single ligand (i.e., endocannabinoid) activates a rapid,

ionotropic receptor-mediated response (i.e., VR1) that is replaced by a longer-lasting metabotropic receptor-mediated response (i.e., CB1R). For endocannabinoids in the DMV, understanding the final physiological outcome will require identification of events causing endocannabinoid release in this system. Regardless of the trigger, brief endocannabinoid release will likely affect DMV motor output to the viscera differently than prolonged stimuli. For gastric control, which many of these neurons probably accomplish, this means that a stimulus, such as strong depolarization (Wilson and Nicoll, 2001) or glucocorticoid activity (Di et al., 2003, 2005), might diminish and then increase gastric contractility if the stimulus remains in place. Endocannabinoids released endogenously at specific synaptic sites would likely have selective effects on synaptic input, which could include a rapid enhancement of inhibitory input, but may eventually suppress all input under conditions of prolonged activation. Separating the effects of exogenously applied agonists from activity caused by endogenous release will be required to understand endocannabinoid activity in this and other neural systems that express both CB1R and VR1.

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