Anandamide modulates carotid sinus nerve afferent activity via TRPV1 receptors increasing responses to heat

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Anandamide modulates carotid sinus nerve afferent activity via TRPV1 receptors increasing responses to heat. J Appl Physiol 112: 212–224, 2012. First published September 8, 2011; doi:10.1152/japplphysiol.01303.2010.—Abnormal respiratory chemosensitivity is implicated in recurrent apnea syndromes, with the peripheral chemoreceptors, the carotid bodies, playing a particularly important role. Previous work suggests that supraphysiological concentrations of the endocannabinoid endovanilloid and TASK channel blocker anandamide (ANA) excite carotid bodies, but the mechanism(s) and physiological significance are unknown. Given that carotid body output is temperature-sensitive, we hypothesized that ANA stimulates carotid body chemosensory afferents via temperature-sensitive vanilloid (TRPV1) receptors. To test this hypothesis, we used the dual-perfused in situ rat preparation to confirm that independent perfusion of carotid arteries with supraphysiological concentrations of ANA strongly excites carotid sinus nerve afferents and that this activity is sufficient to increase phrenic activity. Next, using ex vivo carotid body preparations, we demonstrate that these effects are mediated by TRPV1 receptors, not CB1 receptors or TASK channels: in CB1-null mouse preparations, ANA increased afferent activity across all levels of P0₂, whereas in TRPV1-null mouse preparations, the stimulatory effect of ANA was absent. In rat ex vivo preparations, ANA’s stimulatory effects were mimicked by olvanil, a nonpungent TRPV1 agonist, and suppressed by the TRPV1 antagonist AMG-9810. Using an isolated perfused rabbit carotid body preparation, Kobayashi and Yamamoto (18) report that 3 μM ANA caused a pronounced increase in afferent activity that was not further increased with hypoxia. Therefore, in this preparation, ANA does not appear to augment peripheral chemoreceptor gain. From a control-of-breathing standpoint, if mediated through chemosensory afferents that innervate the carotid body, such an effect might be expected to stave off apnea without further destabilizing the control system. However, the mechanisms by which micromolar concentrations of ANA increase activity in the CSN and the physiological significance, if any, of ANA-mediated carotid body excitation were not resolved.

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they lose their normal response of chemochallenge (10, 17, 38, 46). However, the possibility that the effects in perfused ex vivo preparations are mediated by other mechanisms, namely, involving TRPV1 and/or CB1 receptors on petrosal afferents, has not been tested.

CB1 receptors have been localized in the petrosal ganglia and carotid body (24) but in other systems, activation of CB1 receptors produces outward currents that would be expected to reduce activity. While there are no reports of TRPV1 receptors in the carotid body, several lines of evidence are consistent with TRPV1 receptor involvement in ANA’s effect on CSN discharge. 1) These receptors mediate inward currents. 2) In vivo, intravenous administration of capsaicin (a potent TRPV1 agonist) causes an increase in ventilation only if the CSNs that innervate the carotid body are intact (42). 3) Carotid body afferent activity increases markedly with hyperthermia, and TRPV1 receptors are activated by elevated body temperature (1, 27). 4) TRPV1 receptors are a characteristic of cranial visceral afferent C-fibers, which comprise 85% of petrosal afferents in the CSN (23, 31).

On the basis of these observations, we hypothesize that perfused ANA modulates intact carotid body output by acting on TRPV1 receptors in the CSN. To test this hypothesis, we used novel rodent artificially perfused preparations, immunohistochemistry, and RT-PCR to investigate the effects of ANA on CSN activity. We demonstrate that the excitatory effect of supraphysiological concentrations of ANA on sensory discharge is most likely mediated by TRPV1 receptors located on baro- and chemosensory afferents. However, a concentration of ANA similar to that in blood is not sufficient to activate the carotid body or carotid sinus but may modulate the output of the chemo- and baroreceptors in response to mild hyperthermia.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male 130- to 150-g Sprague-Dawley rats (Charles River, Quebec, PQ, Canada) and CB1−/−, TRPV1−/−, and wild-type (WT) adult mice (all on a C57BL/6 background). CB1−/− and TRPV1−/− colonies were established in our mouse facility from CB1−/− and TRPV1−/− (B6.129X1-Trpv1tm1Jul/J) breeding pairs obtained from Dr. B. Lutz (University of Mainz) (21) and Jackson Laboratories (Bar Harbor, ME), respectively. Genotypes of knockout strains were confirmed by PCR. All experimental procedures were approved by the University of Calgary Animal Care Committee and were carried out in accordance with national guidelines. Drugs were obtained from the following companies: ANA from Calbiochem; casazepine (Cpz) and capsaicin from Sigma-RBI; and olvanil, oleamide, and AMG-9810 from Tocris Bioscience.

Arterially Perfused Ex Vivo Carotid Body Preparation

We developed the arterially perfused ex vivo carotid body preparation to assess sensory activity in the CSN originating from the carotid body (6, 34). Animals were heavily anesthetized with halothane and then decapitated (lower cervical level). The carotid bifurcation, including the carotid body, CSN, and superior cervical ganglion, was quickly removed and transferred to a dissection dish containing physiological saline (in mM: 1 MgSO4, 1.25 NaH2PO4·4 KCl, 24 NaHCO3, 115 NaCl, 10 glucose, 12 sucrose, and 2 CaCl2) and equilibrated with carbogen (95% O2-5% CO2). After 10 min, the isolated tissue was transferred to a recording chamber with a built-in water-fed heating circuit. The CSN was desheathed, the carotid sinus region was bisected, and the common carotid artery was cannulated for luminal perfusion with physiological saline. The occipital, internal, and external arteries were ligated, and small incisions were made on the internal and external carotid arteries to allow perfusate to exit. A peristaltic pump was used to set the perfusion rate at ~50 ml/min, which was sufficient to maintain a constant pressure of ~100 mmHg at the tip of the cannula. In experiments to test the involvement of baroreceptors, the carotid sinus region was left intact, and flow rate was transiently increased for 5 min to 60 ml/min. The perfusate was equilibrated with computer-controlled gas mixtures monitored using CO2 and O2 gas analyzers (models CA-2A and PA1B, respectively, Sable Systems, Las Vegas, NV); a gas mixture of 100 Torr Po2 and 36 Torr PCO2 balanced with N2 was used throughout the experiments (yielding pH ~7.4), unless otherwise stated. Before reaching the cannula, the perfusate was passed through a bubble trap and heat exchanger. The temperature of the perfusate, measured continuously as it departed the preparation, was maintained at 37 ± 0.5°C (unless otherwise stated). The effluent from the chamber was recirculated.

Chemosensory discharge was recorded extracellularly from the whole desheathed CSN, which was placed on a platinum electrode and lifted into a thin film of paraffin oil. A reference electrode was placed close to the bifurcation. CSN activity was monitored using a differential AC amplifier (model 1700, AM Systems) and a secondary amplifier (model AM502, Tektronix, Beaverton, OR). The neural activity was amplified, filtered (300-Hz low cutoff, 5-kHz high cutoff), displayed on an oscilloscope, rectified, integrated (200-ms time constant), and stored on a computer using an analog-to-digital board (Digidata 1322A, Axon Instruments) and data acquisition software (Axoscope 9.0). Preparations were exposed to a brief hypoxic challenge to determine viability. Preparations that failed to show a clear-cut increase in activity during this challenge were discarded. After this challenge, preparations were left undisturbed for 60 min to stabilize before the experimental protocol was begun.

Previously, we reported that, after reduction of perfusion pressure, chemosensory afferent responses (i.e., an increase in activity caused by a fall in Po2) dominate over barosensory afferent responses (i.e., a fall in activity caused by baroreceptor unloading) (6). To better understand the relative contribution of chemo- and barosensory afferents to the CSN activity recorded in this preparation, we maximized the effect of increasing flow in three preparations in which we maximized the contribution of barosensory afferents by leaving the carotid sinus region intact (Fig. 1). Hypoxia caused marked increases in CSN activity, whereas increasing flow rate to the common carotid by ~20%, a procedure that would be expected to increase tissue Po2 and stimulate barosensory afferents through the accompanying increase in pressure, decreased activity (P < 0.05, n = 3). Thus, even in preparations with the carotid sinus region intact, barosensory afferents appear to contribute little to activity recorded in the CSN of the ex vivo preparation. For all subsequent experiments, innervation of the carotid sinus was bisected.

Experimental protocol. The following protocol was used for most experiments, unless otherwise stated: 1) the carotid body was perfused for 5 min for determination of baseline CSN activity (used to normalize subsequent neural activity); 2) control neural responses were obtained by challenging the carotid body with hyperoxic (200 Torr Po2 and 36 Torr PCO2 balanced with N2), normoxic (100 Torr Po2 and 36 Torr PCO2 balanced with N2), and hypoxic (60 Torr Po2 and 36 Torr PCO2 balanced with N2) bouts, each 5 min in duration; 3) the same sequence of challenges was repeated in the presence of drug or vehicle; and 4) the drug was washed out in the normoxic condition.

Data analysis. Data were analyzed offline using custom software (written by R. J. A. Wilson). CSN activity was divided into time bins, and activity in each bin was rectified and summed (expressed as integrated neural discharge). The neural responses for different conditions in the protocol were normalized to the baseline (normoxic) condition. Two-way repeated-measures ANOVAs with Student-Newman-Keuls multiple comparisons test were used to determine whether any drug had a significant effect on neural activity compared with vehicle. Differences were considered significant at P < 0.05.
through a heat exchanger, bubble trapper, and 25-ml reservoir and the preparation, central and peripheral perfusate passed once equilibration was achieved in the two reservoirs. Between the custom-built tonometer. This custom-built system was designed to initiate by pulling fresh media from two different reservoirs of a R. J. A. Wilson). A bilateral vagotomy was performed at the midcervical level, and the jugular veins were cut. The common carotid R. J. A. Wilson). Transection and decerebration were performed in 500 ml of cold (4°C) perfusate containing (in mM) 115 NaCl, 24 NaHCO3, 4 KCl, 2 CaCl2, 1 MgSO4, 1.25 NaH2PO4, 10 glucose, and 12 sucrose, equilibrated with 95% O2−5% CO2. After removal of the skin, the preparation was placed in a supine position in a specially designed Plexiglas chamber and secured with ear bars. The descending aorta was cannulated with a double-lumen catheter. One lumen of the catheter was connected to a peristaltic pump (Gilson Minipuls 3) and was cannulated with a peristaltic pump (Gilson Minipuls 3) and used to perfuse the descending aorta in a retrograde direction with perfusate at room temperature (20°C) and equilibrated with 40 Torr PCO2 in O2. By this point, the perfusate was virtually blood-free. Over the next 30 min, the temperature of the preparation and the central perfusion pressure were ramped to 32–33°C and 90 Torr, respectively.

The left phrenic nerve was dissected out, with a small piece of diaphragm attached to its distal end, and placed in a custom-made Plexiglas holder. The nerve was bathed in perfusate and protected with petroleum jelly. Silver bipolar extracellular electrodes were used to record the phrenic neurogram, which was amplified (differential AC amplifier model 1700, A-M Systems), filtered (300-Hz low cutoff, 5-kHz high cutoff), rectified and integrated (amplitude demodulator, Saga Tech), and computer-archived (Digidata 1322A and Axoscope 9.0, Axon Instruments) at a sampling rate of 50 Hz. After a recovery period, the central (brain stem) perfusate was equilibrated with 35 Torr PCO2 in O2, and the peripheral (carotid body) perfusate was equilibrated with 35 Torr PCO2 and 100 Torr PO2 in N2. The temperature of the preparation was maintained at 32–33°C. Phrenic nerve activity (PNA) was used to monitor respiratory motor output.

Experimental protocol. The following protocol was used for all DPP experiments: J) baseline PNA was recorded for 5 min; 2) a 1-min bolus of ANA or vehicle was injected into the central or peripheral perfusate line; and 3) a 10-min period of PNA was recorded postinjection. PO2 and PCO2 of perfusates were unaltered. In preparations with transected CSNs, hypoxic challenges were performed before and after the transection to ensure that transection was complete before the drug was applied.

Data analysis. Data were analyzed offline using custom software (written by R. J. A. Wilson). Only preparations exhibiting a ramping phrenic discharge and inspiratory time ≤1 s under control conditions were included in the study. Preparations with inspiratory time >1 s were considered apneustic. The following respiratory variables were quantified from the integrated phrenic neurogram: respiratory frequency (fR), neural tidal volume (nVT, phrenic amplitude), and neural Ve (nVe, i.e., nVt × nVT). A paired t-test was employed for statistical analysis of each respiratory variable before and after drug application (SigmaStat 2.03). P < 0.05 was considered significant.

TRPV1 Immunohistochemistry

For immunostaining, rats were heavily anesthetized with halothane and perfused with heparinized PBS. The carotid body-CSN complex with bifurcation, glossopharyngeal nerve, and petrosal ganglia were dissected out en bloc. Animals were then euthanized without regaining consciousness with an overdose of anesthetic. En bloc preparations were fixed in 4% paraformaldehyde-PBS for 1 h at 4°C, washed in PBS, and cryoprotected overnight in 20% sucrose-PBS at 4°C. Specimens were frozen in optimal cutting temperature compound (Tissue Tek, VWR Scientific), serially sectioned (10-μm-thick slices), and mounted on Superfrost Plus slides (Fisher Scientific). Slide-
mounted sections were washed three times with PBS + 0.1% Triton X-100 (PBS + TX) for 30 min and then blocked for 1 h in PBS + TX containing 5% goat serum and 5% donkey serum. The slides were incubated overnight at 4°C with a primary antibody diluted in PBS + TX, washed three times with PBS + TX, and incubated for 2 h at room temperature with secondary antibodies. Sections were washed five times with PBS + TX and mounted in Fluoromount Plus mounting medium (Diagnostic BioSystems).

Primary antibodies were rabbit anti-TRPV1 (Ab 31895, Abcam; diluted 1:100), chicken anti-peripherin (Ab 24524, Abcam; diluted 1:1,000), and sheep anti-tyrosine hydroxylase (Ab 1542, Millipore; diluted 1:200). For single labeling of TRPV1 in petrosal ganglia, goat anti-rabbit Alexa 546 (Molecular Probes; diluted 1:1,000) and goat anti-chicken Alexa 488 (Molecular Probes; diluted 1:1,000) were used as secondary antibodies to detect TRPV1 and peripherin, respectively. Digital images were acquired on a Zeiss Apotome confocal microscope. All antibodies were appropriately characterized for their specificity to detect the protein of interest. Briefly, the TRPV1 antibody preincubated (1 h at room temperature) with control peptide (Ab 47034, VRI peptide 825–839; 10 nm) had no effect (P = 0.011 for fR, P = 0.001 for nV˙E, and P = 0.029 for nV˙E, n = 4), followed by a substantive and long-lasting increase in burst amplitude and frequency (P = 0.034 for fR, P = 0.003 for nV˙E, and P = 0.001 for nV˙E, n = 4; Fig. 2, A and E). Vehicle alone had no effect (P = 0.95 for fR, P = 0.99 for nV˙E, and P = 0.98 for nV˙E, n = 4; Fig. 2, B and E). In a separate set of experiments, we resected the CSNs to test whether they were necessary for the phrenic response. We exposed the carotid bodies to hypoxic perfusate (40 Torr PO2 and 40 Torr PCO2 balanced with N2) before and after resection to ensure that resections were complete. In all cases, resection abolished the response to hypoxia (Fig. 2C). We then delivered a bolus of 20 μM ANA via the common carotid arteries under the conditions described above. With CSNs cut, 20 μM ANA had no effect (P = 0.64 for fR, P = 0.25 for nV˙E, and P = 0.33 for nV˙E, n = 5; Fig. 2, D and E).

Micromolar Concentrations of ANA Increase CSN Afferent Activity Without Increasing the Chemosensory Response to Hypoxia in Ex Vivo Perfused Rat Carotid Body

To characterize the excitatory effect of ANA on peripheral chemoafferent activity in rats, we recorded CSN discharge directly using a novel ex vivo, perfused carotid body preparation. The stimulatory effects of 5, 10, and 20 μM ANA on baseline CSN activity were tested in 18 preparations (n = 6 for each concentration; Fig. 3, A–C). Excitation increased progressively with concentration and lasted ≥30 min. At 10 and 20 μM ANA, the activity at 5 min was not significantly different from that at 40 min after ANA application. After washout, these preparations were still responsive to hypoxia.

In the absence of ANA, lowering PO2 from hyperoxia to hypoxia increased rat CSN activity, as expected (Fig. 3D). However, as reported previously in rabbits (18), 20 μM ANA increased nerve activity during hyperoxia, normoxia, and hypoxia (Fig. 3D). Thus, absolute sensory responses to supraphysiological concentrations of ANA were significantly higher than controls (from 0.79 ± 0.05 to 1.25 ± 0.12 in hyperoxia, from 1.03 ± 0.07 to 1.8 ± 0.18 in normoxia, and from 2.30 ± 0.17 to 3.0 ± 0.25 in hypoxia, P < 0.05, n = 5; Fig. 3E). The change in CSN discharge (Fig. 3E) between normoxia and hypoxia was significantly higher with 20 μM ANA than without ANA (P < 0.05, n = 5). However, the change in neural discharge between normoxia and hypoxia, with and without ANA, was not significantly different. Thus, supraphysiological concentrations of ANA increase CSN afferent activity during hyperoxia and normoxia without increasing the change in chemoreceptor activity for a given change in PO2 when the carotid body is hypoxic.

TRPV1 Receptor Agonists Capsaicin and Olvanil Increase CSN Discharge in the Rat

To determine whether the stimulatory effects of 20 μM ANA are mediated by the CB1 and/or TRPV1 receptor, we tested the effects of the specific CB1 receptor agonist oleamide (20 μM), which is not known to stimulate TRPV1. While oleamide caused a mild inhibition of CSN activity during the hypoxic phase of the protocol in some preparations (Fig. 4A), overall there was no significant effect (Fig. 4B). These data...
suggest that activation of CB1 receptors is not excitatory to the carotid body.

Next, we tested the effects of the TRPV1 receptor-specific and pungent agonist capsaicin and the nonpungent TRPV1 receptor agonist olvanil. Since capsaicin desensitizes TRPV1 receptors with prolonged or repeated applications, we used brief (2 min each) applications of capsaicin. Capsaicin (1 μM) produced a rapid, transient increase in CSN activity, followed by a rapid decrease and undershoot, in which activity dropped below normoxic levels (Fig. 4, C and D; n = 5). Repeated applications of capsaicin diminished CSN activity, an effect that is a hallmark of TRPV1 desensitization (5).

Olvanil (40 μM) mirrored the effects of ANA (Fig. 4, E and F), increasing CSN activity during hyperoxia (0.77 ± 0.02 and 0.99 ± 0.05 for control and olvanil, respectively, P < 0.05, n = 5), normoxia (1.02 ± 0.03 and 1.50 ± 0.06 for control and olvanil, respectively, P < 0.05, n = 5), and hypoxia (2.20 ± 0.20 and 3.00 ± 0.20 for control and olvanil, respectively, P < 0.05, n = 5; Fig. 4F). As with ANA, the change in response between normoxia and hypoxia with olvanil was similar to that
in controls, indicating that the increase in hypoxic activity with olvanil was also due to a basal increase in CSN activity, not an increase in responsiveness to hypoxia.

**TRPV1 Receptor Antagonist AMG-9810 Suppresses ANA-Induced CSN Excitation in Rat Carotid Body**

If the stimulatory effects of micromolar ANA are mediated by the TRPV1 receptor, as the agonist experiments suggest, then the specific TRPV1 receptor antagonist AMG-9810 should block ANA’s effect (14). AMG-9810 (1 μM) alone had no overall effect on CSN activity during hyperoxia, normoxia, or hypoxia (Fig. 5, A and B; P = 0.70, n = 5), but AMG-9810 completely abolished the excitation produced by 20 μM ANA (P < 0.01, ANA vs. AMG-9810 + ANA, n = 5; Figs. 3D and 5B). The TRPV1 antagonist Cpz (20 μM) also suppressed the effects of ANA (20 μM; P < 0.05, n = 5). Interestingly, unlike AMG-9810, Cpz had an additional effect: it blunted the hypoxic response (Fig. 5, C and D). This is likely caused by the nonspecific effects of Cpz on voltage-gated Ca\(^{2+}\) channels (9).

**Effect of ANA on CSN Activity in WT, CB1\(^{-/-}\), and TRPV1\(^{-/-}\) Mice**

To confirm pharmacological data obtained in rats and ensure that the importance of the TRPV1 receptor in mediating the effects of micromolar ANA is not species-specific, we tested the effects of ANA in ex vivo, arterially perfused carotid body preparations from WT, CB1\(^{-/-}\), and TRPV1\(^{-/-}\) transgenic animals (Fig. 6).

**Genotype alone had no effect on carotid body response to PO\(_2\).** Before assessing the effects of genetic deletion of CB1 or TRPV1 receptors on the response to ANA, we tested the effects of genotype on carotid body O\(_2\) sensitivity. Acute reductions in PO\(_2\) produced a graded increase in CSN activity irrespective of genotype (Fig. 6, A–C); at any given PO\(_2\), there was no genotype-dependent difference in discharge (Fig. 6D, −ANA). These data indicate that CB1 or TRPV1 receptor deficiencies do not impair carotid body O\(_2\) sensitivity.

**Excitatory effect of ANA was lost in TRPV1\(^{-/-}\), but not CB1\(^{-/-}\), preparations.** In WT preparations (Fig. 6A), bath application of 10 μM ANA produced marked increases in CSN activity irrespective of genotype (Fig. 6, A–C); at any given PO\(_2\), there was no genotype-dependent difference in discharge (Fig. 6D, −ANA). These data indicate that CB1 or TRPV1 receptor deficiencies do not impair carotid body O\(_2\) sensitivity.

Fig. 3. Effect of micromolar concentrations of ANA on CSN activity of ex vivo arterially perfused rat carotid body preparation. A: integrated CSN activity [arbitrary units (au)] from 3 preparations in response to different concentrations of ANA applied at minute 5. B: summary data showing response to 5 and 40 min after application of ANA (- and ○, respectively, in B). *Significantly different from 0 μM ANA (P < 0.05). D: integrated CSN activity from 1 preparation in response to change in PO\(_2\) with and without ANA. Graded increase in responsiveness to hypoxia (Fig. 5, A and B; P = 0.70, n = 5), but AMG-9810 (1 μM) also suppressed the effects of ANA (20 μM; P < 0.05, n = 5). Interestingly, unlike AMG-9810, Cpz had an additional effect: it blunted the hypoxic response (Fig. 5, C and D). This is likely caused by the nonspecific effects of Cpz on voltage-gated Ca\(^{2+}\) channels (9).

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0.12, \( P < 0.05, n = 6 \), normoxia (from 1.01 ± 0.08 to 2.20 ± 0.21, \( P < 0.05, n = 6 \)), and hypoxia (from 1.70 ± 0.13 to 2.80 ± 0.2, \( P < 0.05, n = 6 \)). Although the response to hypoxia was partially blunted in the presence of 10 \( \mu \)M ANA, CSN activity retained a strong dependence on PO2 (2.20 ± 0.21 vs. 1.45 ± 0.12 for 100 vs. 200 Torr, \( P < 0.009 \); 2.80 ± 0.2 vs. 1.45 ± 0.12 for 60 vs. 200 Torr, \( P < 0.001 \); Fig. 6D, +ANA in CB1−/−). Taken together, these results indicate that, in the absence of CB1 receptors, 10 \( \mu \)M ANA is more excitatory, suggesting that CB1 activation in the mouse has an inhibitory influence on chemosensory activity. Hence, the excitatory effect of 10 \( \mu \)M ANA in WT preparations must be mediated via a different pathway.

In TRPV1−/− preparations (Fig. 6C), the excitatory effect of 10 \( \mu \)M ANA observed in WT preparations (Fig. 6A) during hyperoxia was abolished (1.50 ± 0.12 and 0.79 ± 0.07 for WT and TRPV1−/−, respectively, \( P < 0.05, n = 6 \), and ANA had no effect on the magnitude of the hypoxic response (1.75 ± 0.14 and 1.78 ± 0.17 for WT and TRPV1−/−, respectively, \( P = 0.98, n = 6 \)). Also, lack of TRPV1 receptors significantly reduced the excitatory effect of 10 \( \mu \)M ANA during normoxia (1.65 ± 0.15 and 1.13 ± 0.09 for WT and TRPV1−/−, respectively, \( P < 0.01, n = 6 \)). The slight apparent increase in activity with ANA compared with the preceding within-preparation control during normoxia was not quite significant (from 1.01 ± 0.09 to 1.13 ± 0.10, \( P = 0.06 \)). Importantly, in contrast to the WT preparations treated with 10 \( \mu \)M ANA, CSN activity increased significantly as PO2 decreased (Fig. 6D, TRPV1−/− +ANA; \( P < 0.05, n = 6 \)). Thus these results indicate that TRPV1 receptors are largely responsible for the effects of ANA on CSN activity.

**Nanomolar Concentrations of ANA Affect Temperature Sensitivity of Carotid Body Afferent Activity**

To determine whether the levels of ANA in plasma are likely to be of physiological importance to CSN-mediated chemosensory and barosensory function, we perfused the rat ex vivo carotid body with 20 nM ANA, the upper range likely in blood (8, 16, 37). While 10–20 \( \mu \)M ANA increased basal CSN activity, 20 nM had no effect (Fig. 7A, arrowhead). However, given the fact that TRPV1 receptors and carotid bodies are sensitive to temperature, we hypothesized that nanomolar concentrations of ANA do not affect carotid body afferent activity.

**Fig. 4. Effect of cannabinoid (CB1) and transient receptor potential vanilloid (TRPV1) receptor agonists on rat CSN activity.** A and B: CB1 agonist oleamide (20 \( \mu \)M). C and D: pungent TRPV1 agonist capsaicin (Cap, 1 \( \mu \)M). E and F: nonpungent TRPV1 agonist olvanil (40 \( \mu \)M). A, C, and E: integrated CSN activity from 3 ex vivo arterially perfused rat carotid body preparations. B, D, and E: summary data normalized to normoxic activity before drug perfusion. Values are means ± SE (n = 5). Only olvanil mimicked the stimulatory effects of ANA (see Fig. 3). Hx, 60 Torr PO2. *Significantly different from −olvanil (\( P < 0.05 \)).
ANA may affect temperature sensitivity of CSN afferents. Thus we tested the response of the CSN to short, abrupt bouts of increased temperature (from 37°C to 39°C) during perfusion with 20 nM ANA + vehicle or 20 nM ANA + 1 µM AMG-9810 (with the experimenter blinded as to whether vehicle or AMG-9810 was added; Fig. 7B). ANA + vehicle increased the CSN response to warming twofold (P < 0.01, n = 5), an effect that was blocked by 1 µM AMG-9810 (P = 0.923, n = 6). In some of these preparations, the ANA + AMG-9810 cocktail appeared to blunt the CSN response to warming (Fig. 7B), suggesting that elevated temperature may cause endogenous release of ANA. Therefore, we conducted an additional experiment: we exposed carotid bodies to more severe hyperthermic bouts (from 37°C to 43°C) during perfusion with vehicle or AMG-9810 (with the experimenter blinded as to whether vehicle or AMG-9810 was added; Fig. 8). In the vehicle experiments, the CSN response to the first 43°C (control) bout was greater than the response to all subsequent bouts (bouts 2, 3, and 4; P < 0.001, n = 5; Fig. 8A and Fig. 8C, left). However, there was no significant difference between subsequent bouts. In contrast, in the presence of AMG-9810, the response to bout 2 was not significantly different from the response to the control bout, but the response diminished with each bout thereafter (P < 0.001, n = 5; Fig. 8B and Fig. 8C, right).

**Immunolocalization of TRPV1 in Adult Rat Carotid Body-CSN**

Within the carotid body and on CSN fibers, we found positive immunoreactivity for TRPV1 and peripherin, a marker for C-fibers (Fig. 9). The terminals of some of these fibers appeared to innervate tyrosine hydroxylase-positive glomus cell clusters (Fig. 9, A and B). In addition, immunoreactivity verified that TRPV1 was present in the glossopharyngeal nerve and cell bodies of the petrosal ganglia (Fig. 9C). No immunoreactivity was observed in carotid bodies, petrosal ganglia, or glossopharyngeal nerve in preparations preincubated with blocking peptide or exposed to the secondary antibody without prior exposure to the primary antibody (data not shown).

**RT-PCR Analysis of TRPV1 mRNA in Adult Rat Carotid Body and Petrosal and Superior Cervical Ganglia**

To confirm the immunofluorescence results, expression of TRPV1 mRNA in three tissues was examined by RT-PCR (Fig. 10). cDNAs from petrosal and superior cervical ganglia produced a single band corresponding to the expected size of the PCR product (265 bp). However, no product was observed following amplification of cDNA from the carotid body (Fig. 10). Thus, TRPV1 mRNA is expressed in petrosal and superior cervical ganglia, but not in the carotid body (Fig. 10).

**DISCUSSION**

We have used ex vivo rodent preparations to confirm that perfusion of the common carotid artery with micromolar concentrations of the hybrid endocannabinoid-endovanilloid agonist and TASK channel blocker ANA strongly excites CSN afferent activity. While some of the ANA-induced afferent activity was associated with the baroreflex (see below), our data suggest that chemosensory afferents were also activated. Using selective agonists, antagonists, transgenic mice, immunohistochemistry, and RT-PCR, we found no evidence for a direct effect of perfused ANA on the chemosensing mechanism. Rather, our data demonstrate that perfused ANA acts
predominantly through TRPV1 receptors expressed on petrosal C-fibers, which normally convey sensory information from the carotid body and carotid sinus to the brain stem. Nanomolar concentrations of ANA, approaching physiological levels found in plasma, were without direct effect on CSN activity. However, our data point to physiological levels of ANA modulating CSN responses to elevated temperature and suggest that elevated carotid body temperature may cause endogenous release of ANA.

**Mechanism of Action of ANA**

Several groups have used ANA as a “selective” TASK channel blocker to probe the role of TASK-like channels in carotid body function (3, 17, 18, 36, 46). Similar to our results in mice, Kobayashi and Yamamoto (18) found that 3 μM ANA stimulates the ex vivo perfused carotid body of the rabbit when oxygenated but has no additional effect during hypoxia. Others report that micromolar concentrations of methanandamide ameliorate hypoxia-induced reductions in whole cell outward K+ currents in isolated rat glomus cells, and, in the presence of hypoxia, the inhibitory effects of methanandamide on outward currents are practically abolished (46). These in vivo and in vitro data are interpreted as indicating that ANA-sensitive TASK channels are not necessary for mediating the release of catecholamines in knockouts under normoxic conditions. In some respects, our data are consistent with studies of TASK knockout mice by Mulkey et al. (28), Trapp et al. (39), and Ortega-Saenz et al. (29), in that none of these groups showed an absolute critical role for TASK in O2 sensing. However, in other respects, our data would not be predicted from these studies. Thus, while Ortega-Saenz et al. showed that hypoxia-induced catecholamine secretion from carotid body slices did not differ significantly between TASK1−/−, TASK1/3−/−, and WT mice, their data also demonstrate increased release of catecholamines in knockouts under normoxic conditions. Similarly, in the study of Trapp et al., while ventilatory

**Implications for O2 Sensing?**

If closure of TASK channels is a critical step in the O2-sensing pathway in vivo, then blocking these channels with ANA when the carotid body is well oxygenated might be expected to cause a substantial increase in CSN activity in the absence of functional TRPV1 receptors. Yet we found that the effects of perfused ANA were fully dependent on functional TRPV1 receptors. Therefore, at face value, our results are not consistent with a critical role for ANA-sensitive TASK-like channels in carotid body chemosensing.

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**Fig. 6. Stimulation of mouse CSN by ANA is primarily dependent on TRPV1 receptor.** A, B, and C: CSN activity of ex vivo arterially perfused carotid body preparations from wild-type (WT), CB1−/−, and TRPV1−/− mice. Activity in hyperoxia (200 Torr PO2), normoxia (100 Torr PO2), and hypoxia (60 Torr PO2) was determined before and during perfusion with 10 μM ANA. D: summary data normalized to normoxic activity with and without ANA. Genotype had no effect on chemosensory activity in the absence of ANA (−ANA). However, genotype did affect chemosensitivity in the presence of ANA (+ANA). In preparations retaining the TRPV1 gene, ANA increased normoxic and hyperoxic activity substantially; in TRPV1−/− preparations, CSN activity resembled that of non-ANA-treated preparations. This suggests that TRPV1 is, in large part, necessary for the effects of ANA. Augmented effect of ANA in CB1−/− preparations suggests that, normally, activation of CB1 receptors puts the breaks on chemosensory activity. Values are means ± SE (n = 6). *Significantly different from −ANA (P < 0.05).
and CSN responses to hypoxia persisted in TASK1/−/− and TASK1/3/−/− mice, responses were reduced to 50% of those in WT mice. In attempting to reconcile these observations with our data, we cannot be sure that ANA entered the carotid body of the arterially perfused preparation in sufficient concentration to inhibit glomus cell TASK channels [IC50 = 0.7 μM (20)]. For example, although we consider it unlikely, it is possible that lipid-soluble ANA is barred from entering the carotid body from the circulation while having ready access to petrosal afferents in the CSN. Alternatively, ANA might enter the carotid body and CSN but be more rapidly broken down in the carotid body before it has a chance to bind to TASK channels.

Fig. 7. Nanomolar concentrations of ANA increase temperature sensitivity of CSN afferents. A: CSN response of rat ex vivo arterially perfused carotid body preparation to mild heat challenge (from 37°C to 39°C) was augmented by 20 nM ANA. ANA (20 nM) has no effect on baseline activity at 37°C. B: augmentation of thermal response by ANA was blocked by TRPV1 antagonist AMG-9810. Hx, hypoxic challenge (60 Torr PO2). C: summary data showing response to heat challenge. Values are means ± SE (n = 6). Control, predrug heat challenge; WO, heat challenge after drug washout. *, significantly different from control (P < 0.05).

Fig. 8. TRPV1 antagonist affects sensitivity of CSN afferents to severe heat. A: CSN response of rat ex vivo arterially perfused carotid body preparation to severe heat challenge (from 37°C to 43°C) was augmented by 20 nM ANA. ANA (20 nM) has no effect on baseline activity at 37°C. B: augmentation of thermal response by ANA was blocked by TRPV1 antagonist AMG-9810. Hx, hypoxic challenge (60 Torr PO2). C: summary data showing response to heat challenge. Values are means ± SE (n = 6). Control, predrug heat challenge; WO, heat challenge after drug washout. *, significantly different from control (P < 0.05).
on neuronal activity by opening K+ channels and inhibiting Ca2+ channels, leading to membrane hyperpolarization (15). However, a fundamental, cross-species role for CB1 receptor-mediated inhibition in regulating the overall excitatory response of the carotid body to ANA is not fully supported by 1) a previous study that demonstrated only sparse CB1 receptor expression in the rat carotid body/CSN (24) or 2) our ex vivo rat data demonstrating that the CB1 agonist oleamide had no effect on CSN activity (Fig. 4, A and B). With regard to our ex vivo rat data, it is possible that oleamide was restricted from entering the carotid body or was rapidly metabolized therein. While we did not investigate these possibilities further, we note that subcutaneous administration of oleamide has potent central effects (40). Therefore, discordance between enhanced excitation caused by ANA in CB1−/− mice and the lack of effect of oleamide in rats most likely reflect species difference in CB1 levels.

**Localization of TRPV1**

Our RT-PCR and immunolocalization data suggest that TRPV1 receptors are not expressed in carotid body glomus cells but, instead, reside in peripherin-positive CSN afferents. These observations are consistent with reports that C-fibers comprise 85% of afferents in the CSN and are associated with TRPV1 expression in other parts of the peripheral nervous system (11, 23). CSN afferents project from the petrosal ganglia and terminate in the carotid body (chemosensory) or course through the carotid body and terminate in the sinus region of the internal carotid artery (barosensory). Our data from the in situ preparation suggest that both types of fibers are activated by ANA. Specifically, when the common carotid arteries were perfused with 20 μM ANA, we observed a transient decrease in phrenic nerve activity indicative of baroreflex activation (2, 25). However, the transient decrease in activity was followed by a sustained increase above pre-ANA perfusion levels that dominated the response. This excitation is not produced by baroreceptor stimulation and, therefore, must be a consequence of strong chemosensory activation. Both aspects of the biphasic effect of ANA were lost when CSNs were first sectioned, and neither was replicated by addition of 20 μM ANA to the central perfusate [central perfusion with ANA had no significant effect (P = 0.37 for nV˙E, n = 5)]. Furthermore, immunolocalization data demonstrate that the carotid body contains large numbers of peripherin-positive, TRPV1-expressing CSN afferents.

**Role of CB1 Receptors?**

As the excitatory effects of ANA were greatly magnified in preparations from CB1−/− mice, we postulate that CB1 receptor activation in WT mice normally inhibits carotid body output. In other systems, CB1 receptors have inhibitory effects...
TRPV1-expressing fibers that terminate in close proximity to carotid body glomus cell clusters. Together, these data strongly suggest that a sizable proportion of ANA-activated CSN fibers are likely to be chemosensory in nature.

**Physiological Implication of TRPV1 Receptors in Carotid Body Afferents In Vivo?**

Our data suggest that neither endocannabinoid nor endovanillloid signaling is involved in the chemotransduction process per se, but CB1 and TRPV1 receptors may play a role in modulating subsequent neuronal activity, which relays chemosensory information to the brain. Indeed, peripheral chemoreceptor activity and the biophysical properties of TRPV1 share important characteristics, being pH- and temperature-dependent (5).

When expressed in recombinant systems, TRPV1 receptors are stubbornly inactive, exhibiting a temperature sensitivity on the upper limit of the physiological range (≥43°C) (27). Yet endogenous activation of TRPV1 and a role in thermoregulation are strongly indicated in vivo by the hyperthermia produced by administration of specific TRPV1 antagonists (12). A likely explanation for this dichotomy is that modulation of TRPV1 may shift the EC_{50} of the TRPV1 receptor to lower temperatures (4). Consistent with this explanation, we demonstrated that 1) the excitatory effects of 20 μM ANA on CSN activity were entirely dependent on TRPV1, 2) 20 μM ANA increased neuronal ventilation in the in situ preparation, even though this preparation was maintained at 33°C, provided the CSN was intact, and 3) while 20 nM ANA was insufficient to increase CSN activity in the normoxic, normocapnic, and normothermic ex vivo carotid body preparation, it greatly increased the response to mild warming.

These data raise the possibility that a physiological concentration of ANA modulates TRPV1 in carotid body and baroreceptor afferents, contributing to the exquisite sensitivity of chemosensory activity and sympathetic responses to temperature (1). Consequently, TRPV1 in barosensory afferents may contribute to the baroreflex component of body temperature regulation that inhibits sympathoexcitatory pressor responses and increases thermal tolerance (19, 22). Similarly, TRPV1 in chemosensory afferents may provide a mechanism to explain carotid body involvement in exercise/thermal hyperpnea that, paradoxically, is accompanied by respiratory alkalosis or no change in blood gases (32, 45). In this respect, we note that the increase in ventilation during exercise is accompanied by an increase in circulating ANA (8, 26, 37) and that CSN resection or carotid body inactivation in humans blunts the intermediate phase of exercise hyperpnea (i.e., the phase between the initial augmentation at the onset of exercise lasting seconds and the ventilatory plateau that occurs after many minutes) (33, 43, 44).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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