Tone-dependent vascular responses to astrocyte-derived signals

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Blanco VM, Stern JE, Filosa JA. Tone-dependent vascular responses to astrocyte-derived signals. Am J Physiol Heart Circ Physiol 294: H2855–H2863, 2008. First published May 2, 2008; doi:10.1152/ajpheart.91451.2007.—A growing number of studies support an important contribution of astrocytes to neurovascular coupling, i.e., the phenomenon by which variations in neuronal activity trigger localized changes in blood flow that serve to match the metabolic demands of neurons. However, since both constriction and dilations have been observed in brain parenchymal arterioles upon astrocyte stimulation, the specific influences of these cells on the vasculature remain unclear. Using acute brain slices, we present evidence showing that the specific degree of constriction of rat cortical arterioles (vascular tone) is a key determinant of the magnitude and polarity of the diameter changes elicited by signals associated with neurovascular coupling. Thus elevation of extracellular K+ concentration, stimulation of metabotropic glutamate receptors (mGluR), or 11,12-epoxyeicosatrienoic acid application all elicited vascular responses that were affected by the particular resting arteriolar tone. Interestingly, the data suggest that the extent and/or polarity of the vascular responses are influenced by a delimited set point centered between 30 and 40% tone. In addition, we report that distinct, tone-dependent effects on arteriolar diameter occur upon stimulation of mGluR during inhibition of enzymes of the arachidonic acid pathway [i.e., phospholipase A2, cytochrome P-450 (CYP) \( \omega \)-hydroxylase, CYP epoxidegenase, and cycloxygenase-1]. Our findings may reconcile previous evidence in which direct astrocytic stimulation elicited either vasoconstrictions or vasodilations and also suggest the novel concept that, in addition to participating in functional hyperemia, astrocyte-derived signals play a role in adjusting vascular tone to a range where dilator responses are optimal.

Although much attention has been given to the characterization of the signals that mediate “functional hyperemia,” the rapid and localized increase in blood flow that occurs after neuronal activation (41, 53), a prominent gap remains in our understanding of the vascular factors that influence the overall neurovascular response. Because vasoactive stimuli typically affect changes in two interdependent physiological parameters of vascular smooth muscle cells (VSMC), namely membrane potential \( V_m \) and intracellular Ca2+ concentration [\( [Ca^{2+}]_i \)], it is conceivable that the responsiveness of the vessels when presented to such stimuli is in turn affected by the resting arteriolar tone, defined by the particular status of \( V_m \) and [\( Ca^{2+} \)] in VSMC. Previous work in pial arterioles showed indeed that vascular tone critically influences the type of response evoked by several neurogenic stimuli (3, 52). In contrast, the influence of vascular tone in the responses of brain intracortical arterioles to signals associated with glial activation has not been evaluated.

Astrocytes establish intimate contacts with both neurons and vessels and are thus uniquely positioned to function as bridges between these cell types, contributing to neurovascular coupling (NVC) and functional hyperemia (24). A turning point in our understanding of the role of astrocytes in mediating NVC came with the observation that the dilation of cortical arterioles upon neuronal stimulation involves activation of metabotropic glutamate receptors (mGluR) leading to an increase in [\( Ca^{2+} \)], in astrocytes (60). More recently, rapid increases in astrocyte [\( Ca^{2+} \)], upon somatosensory stimulation have been shown to be temporally correlated with the onset of hemodynamic responses in the mouse cortex in vivo (59). Rises in astrocytic [\( Ca^{2+} \)], are known to mediate the synthesis and/or release of vasoactive agents, with vasodilator [e.g., K+, epoxyeicosatrienoic acids (EETs), and prostaglandins] or vasoconstrictor [e.g., 20-hydroxyeicosatetraenoic acid (20-HETE)] effects importantly involved in the control of the brain microcirculation (28). In addition, astrocytes may respond to signals released by GABAAergic interneurons, which have also been shown to mediate constrictions (e.g., neuropeptide Y and somatostatin) or dilations (e.g., nitric oxide (NO) and vasoactive intestinal peptide) in cortical arterioles (10, 55). Noteworthy, a recent study showed that spatially defined vasodilation and vasoconstriction characterizes the vascular response to somatosensory stimulation in vivo (13). Although the involvement of astrocytes in these phenomena was not specifically addressed, the ability of glial cells to constrict and dilate blood vessels has been clearly demonstrated by studies in the rodent cortex and retina (39, 42). In the cortex, but not in the retina, evidence would suggest that basal vascular tone influences the type of response evoked by rises in glial [\( Ca^{2+} \)], (42). In line with previous suggestions (49), we hypothesize that, although both constricting and dilating agents can be released upon neuronal activation, the specific degree of constriction of the VSMC (vascular tone) is a key determinant of the direction and extent of the ensuing response.

We addressed this hypothesis by evaluating the influence of vascular tone in the response of brain intraparenchymal arterioles to three stimuli associated with NVC: 1) moderate elevation of extracellular K+ concentration [\( [K^{+}]_o \)], a known vasodilator stimulus, 2) mGluR activation and subsequent \( Ca^{2+} \)-dependent activation of the arachidonic acid (AA) pathway, and 3) exposure to 11,12-EET, a purported astrocyte-derived vasodilator signal. Some of these results have been presented in preliminary form (6, 7).
**METHODS**

**Brain slice preparation.** Cortical brain slices were prepared from 3- to 10-wks-old Sprague-Dawley rats following protocols approved by the Office of Animal Care Management at the University of Cincinnati. Following anesthesia with pentobarbital, the brain was rapidly removed, and 300-µm-thick coronal slices were cut in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) 3 KCl, 120 NaCl, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 10 glucose, and 0.4 t-aspartic acid, equilibrated with 95% O₂-5% CO₂. Ascorbic acid was added to reduce cell swelling associated with oxidative stress (8). An aCSF with identical composition was used for bath perfusion in all experiments, except for those assessing the effects of high external K⁺ concentration ([K⁺]), in which control aCSF contained 4.2 mM KCl, and KCl replaced NaCl to increase [K⁺] to 10 mM. Osmolality of aCSF was -290 mosm/kgH₂O. Following the slicing procedure, slices were kept at room temperature in aCSF equilibrated with 95% O₂-5% CO₂ (pH 7.45) until used.

**Reagents.** The thromboxane A₂ receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂α (U-46,619), prostaglandin F₃α, methyl arachidonoyl fluorophosphonate (MAFP), N-hydroxy-4'-(4-butyl-2-methylphenyl)-formamidine (HET-0016), N-methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MS-PPOH), sc-560, and ozagrel (all purchased from Cayman Chemical, Ann Arbor, MI) were prepared as stocks in dimethyl sulfoxide (DMSO) and subsequently added to the aCSF. DMSO content in the experimental solutions was ≤0.1%. Trans-1-aminoacyclopetatone-1,3-dicarboxylic acid (t-ACPD; Tocris, Ellisville, MO) was diluted in aCSF from a 50 mM stock in equimolar NaOH. 11,12-EET was obtained from Biomol (Plymouth Meeting, PA) and added to aCSF from a stock in dH₂O. Tetraethylammonium (TEA; Sigma, St. Louis, MO) was dissolved in aCSF.

**Video microscopy.** Diameter changes in cortical arterioles (8–19 µm internal diameter) were recorded using an upright Zeiss Axio- scope 2FS microscope (Carl Zeiss, Thornwood, NY) equipped with infrared Differential Interference Contrast (IR-DIC) optics, a water-immersion objective (Zeiss 63x, numerical aperture 0.9), and an EMCCD camera (iXon+885; Andor Technology, South Windsor, CT). Images were acquired at 1 frame/s, visualized, and stored using IQ software (Andor Tech). The slices were perfused with aCSF (35 ± 2°C) gassed with 95% O₂-5% CO₂ and were allowed to equilibrate for ≥10 min before beginning of recording. Oxygen activity, as measured in the perfusate arriving to the recording chamber, was 54.8±0.3 min (pooled data for 50–250 nM U-46,619) that was main- tained thereafter. In the rest of the experiments presented here, distinct U-46,619 concentrations (50 to 250 nM) were applied to induce different levels of preconstriction.

**Increasing extracellular K⁺ induces tone-dependent vasodilations.** Modest elevations in [K⁺]o result in hyperpolarization and reduction of Ca²⁺ entry in VSMC and constitute a powerful vasodilator stimulus in the cerebral circulation (32, 35,
Recent work provided evidence that K\(^+\) signaling by astrocytes is a key mechanism in NVC (21). As a first step in addressing the influence of resting vascular tone on the response of cortical arterioles to astrocyte-derived signals, the effects of moderately elevated [K\(^+\)]\(_o\) were studied. To this end, we measured changes in diameter from arterioles preconstricted to various degrees using U-46619 (50–250 nM) and exposed to 10 mM K\(^+\), keeping constant the original U-46619 concentration. Raising [K\(^+\)]\(_o\) had a net vasodilator effect. The magnitude of the vasodilation correlated positively with the initial level of preconstriction: as vascular tone increased, proportionally larger vasodilator responses were evoked (r = 0.65). A representative trace of the observed changes is shown in Fig. 2A. In Fig. 2B the changes in diameter in response to 10 mM K\(^+\) are expressed as a function of the initial (preconstricted) diameter of the arterioles (n = 19). The onset of vasodilations occurred 0.9 ± 0.1 min following exposure to 10 mM K\(^+\), and the mean time to peak dilation was 4.5 ± 0.5 min.

It is instructive to examine the tone-dependent effects of elevated [K\(^+\)]\(_o\), when diameters at low (control) and high [K\(^+\)]\(_o\) are plotted as a percent of the initial baseline for individual arterioles ordered by the level of initial preconstriction (Fig. 2C). At low preconstriction levels (>60% of baseline; n = 9), K\(^+\)-induced dilation nearly restored diameter to the initial baseline dilated state, whereas for more preconstricted arterioles the final steady-state diameter, relative to baseline, was lower and more variable.

mGluR activation elicits tone-dependent constrictions and dilations in cortical arterioles. In addition to K\(^+\), a number of constrictor and dilator signals can be produced and released upon increases in astrocyte [Ca\(^{2+}\)]\(_e\), following mGluR activation by glutamate released at the synapse (19). It is not clear, however, how the opposing effects of these vasoactive substances are integrated into a defined vascular response. We hypothesize that the response of brain arterioles to neuronal- and/or glial-derived signals is dictated by both the nature of the signals released and the degree of vascular tone. To assess this hypothesis, we exposed brain cortical slices to t-ACPD, a selective mGluR agonist known to elicit Ca\(^{2+}\) increases in astrocytes (34, 60), and measured the resulting diameter changes in arterioles presenting different levels of preconstriction. As shown in Fig. 3A, exposure to t-ACPD (100 μM; ~10 min duration) consistently elicited constrictions in arterioles having low to moderate preconstriction (up to ~70% of baseline), whereas dilations were observed in vessels initially showing a more pronounced tone (n = 27; see Supplemental Videos 2A and 2B). The onset of constrictions occurred at 1.9 ± 0.6 min, and peak constriction was observed at 3.9 ± 0.7 min (n = 11). Dilatory responses started 2.4 ± 0.5 min after t-ACPD application and reached a plateau at 5.5 ± 0.6 min (n = 16). Comparison of onset and peak times of constrictions vs. dilations revealed no significant differences. To further analyze these responses, data from Fig. 3A were replotted to show the t-ACPD-induced diameter (expressed as percent of maximal, baseline diameter) as a function of the initial diameter (Fig. 3B). Interestingly, the plot shows that, upon t-ACPD exposure, 16 out of 19 vessels initially displaying either low (less than ~30%) or high (greater than ~50%) preconstriction attained a uniform diameter (~36.9 ± 2% constricted), which lies within the range of tone at which vascular responses reversed polarity. Two additional observations suggest that this range of tone (~50–70% of baseline diameter) may represent a set point that defines the polarity and extent of vascular responses. First, the largest vasodilations, in absolute terms (i.e., those that were closer to reaching baseline diameter), were observed in arterioles with initial, resting diameters situated at this putative set point range (Fig. 3B). Second, upon stimulation with t-ACPD, some of the vessels with initial diameters afar from the set point transiently reached the set-point and then showed a dilation (Fig. 3, B and C).

Because the observed responses may be conditioned by the preconstriction treatment, i.e., the specific effects of the thromboxane A\(_2\) receptor agonist U-46619 on vascular tone, we tested the effects of mGluR activation on arterioles preconstricted with a different agonist, i.e., PGE\(_2\). As shown in Fig. 3D, tone-dependent constrictions and dilations were also observed upon exposure to t-ACPD (100 μM; ~10 min duration) in arterioles preconstricted with PGE\(_2\) (5–35 μM). Of a total of nine arterioles recorded, four showing an initial diameter...
arterioles, with resting diameters ≤70% of baseline (namely preconstricted by ≥30%) responded to t-ACPD with vasodilation. The remaining five arterioles, with resting diameters >70% of baseline, showed instead a constriction. As with U-46619, biphasic responses (constriction followed by dilation) were also observed in three out of the five vessels of the latter group. Because comparable responses were observed upon preconstriction with different agonists, these results suggest that constrictions and dilations of brain cortical arterioles in response to mGluR activation are strongly dependent on their resting tone.

AA metabolites are differentially involved in vascular responses to mGluR activation. Following mGluR activation in astrocytes, the resulting [Ca$^{2+}$]i elevation can trigger AA production, which can be further metabolized to several vasoconstrictor and vasodilator signals (33). To evaluate the participation of AA metabolites in the vasoconstrictions and vasodilations induced by mGluR activation, and the influence of vascular tone on these responses, we performed experiments similar to those described in the previous section, in the presence of selective blockers of AA production or metabolism. Control experiments in which MAFP (100 μM), HET-0016 (100 nM), MS-PPOH (20 μM), or sc-560 (100 nM) were perfused during 30 min showed that baseline diameter (measured during the first 10 min in control aCSF) was not significantly affected by these inhibitors (variation range = −2.6 to 3.5%; P = 0.106, ANOVA followed by Dunnett’s test; n = 4 for each treatment). At the end of these experiments, vessel reactivity was assessed by applying endothelin-3 (100 nM), in the constant presence of the test drug. Endothelin-3 induced marked vasoconstriction (>50%) in all vessels and under all testing conditions, indicating that vascular reactivity was preserved (data not shown).

Results were compared with control data taken from Fig. 3A, and the responses were grouped into three categories encompassing discrete ranges of initial, arteriolar tone: 1) <70% of baseline, a range in which arterioles are presumably dilated beyond resting levels; 2) 70–50% of baseline, a range that corresponds to the active tone developed by pressurized intraparenchymal brain vessels and likely resembles the prevailing physiological myogenic tone (9, 12), and 3) >50% of baseline, a range that may represent constriction beyond physiological levels. First, we tested the effects of selective and irreversible inhibition of phospholipase A2 (PLA2), the enzyme that catalyzes Ca$^{2+}$-dependent AA production from membrane phospholipid. A diminished sensitivity to U-46619 was apparent in vessels preincubated with MAFP (100 μM). In these experiments, the highest dose of U-46619 (250 nM) had to be applied in most cases to achieve clear preconstriction.

Preincubation of brain slices with MAFP (100 μM) significantly inhibited t-ACPD-induced constrictions and dilations (n = 19) (Fig. 4). These results are consistent with previous observations in which the absence of functional PLA2 significantly inhibited astrocyte-induced vasodilation in vivo (56), and vasoconstrictions in vitro (42), and add strong support to the view that AA, or some of its metabolites, are key determinants of the vascular response evoked by mGluR activation.

Previous reports showed that inhibition of cytochrome P-450 (CYP) ω-hydroxylase, the enzyme that mediates the production of the AA-derived metabolite 20-HETE, strongly attenuated vasoconstrictions following glial activation in ro-
dent brain cortex (42) and retina (39). To assess the involvement of 20-HETE in the vascular responses elicited by mGluR activation, brain slices were preincubated with HET-0016 (100 nM), a selective CYP ω-hydroxylase inhibitor (40). After HET-0016 treatment, all constrictions reversed to dilations (n = 9; P < 0.01), while dilations were not significantly affected in vessels with higher resting tone (Fig. 4). Thus these data support 20-HETE as a major vasoconstricting signal following mGluR activation.

A role for astrocyte-derived EETs in functional hyperemia was suggested almost ten years ago (26). Astrocytes in vitro and in situ express a CYP epoxygenase isofrom (2, 48), and its activity is stimulated by glutamate (1). Experimentally, inhibition of CYP epoxygenase reduces hyperemic responses in the brain in vivo (1, 5, 47, 48) and abrogates the late, sustained phase of vasodilation induced by AMPA receptor activation in brain slices (36). To assess the involvement of EETs in t-ACPD-induced vascular responses, brain slices were preincubated with MS-PPOH (20 μM), a selective CYP epoxygenase substrate inhibitor (57). As shown in Fig. 4, MS-PPOH treatment significantly attenuated dilations in vessels with marked preconstriction (>50% of baseline) (n = 6; P < 0.01) but did not affect vascular responses in vessels initially showing intermediate levels of tone. In addition, constrictions were also significantly reduced in the group of vessels initially displaying the lowest preconstriction levels (n = 6; P < 0.05), suggesting that EETs contribute to both constrictor and dilatory responses in a manner strongly dependent on the specific arteriolar tone.

An important role for cyclooxygenase (COX) metabolites in the maintenance of resting cerebral blood flow and as mediators of the neurovascular response has been highlighted by in vivo (44, 45, 56) and in vitro (21, 60) studies. Because attenuation of COX-1, but not COX-2, effectively reduced dilator responses in vivo (56), we assessed whether COX-1 activity could influence arteriolar responses to mGluR activation. COX-1 was selectively inhibited by preincubating slices with sc-560 (100 nM). As shown in Fig. 4, COX-1 inhibition significantly reduced t-ACPD-induced constrictions and dilations. The most prominent effect, however, was observed in the group of vessels with moderate preconstriction levels (70–50% of baseline) in which vasodilations reversed into vasoconstrictions (n = 4; P < 0.01).

To evaluate whether the attenuation of vasoconstrictions observed during COX-1 blockade may represent reduced synthesis of thromboxane, t-ACPD was applied to vessels treated with ozagrel (100 nM), a thromboxane synthase inhibitor (30). As shown in Fig. 4, the extent of vasoconstriction was significantly reduced in vessels with low preconstriction (n = 9; P < 0.05).

11,12-EET induces tone-dependent arteriolar diameter changes and increases intracellular calcium in astrocytes. Glial-derived EETs have been proposed as important mediators of NVC in the brain (26), although the underlying mechanisms are still little understood. In light of this, and to extend our findings, we explored the effects of one of the main glial CYP epoxygenase products, namely 11,12-EET (2), on arteriolar diameter as well as on [Ca2+]i, dynamics in astrocytes. In a first set of experiments, cortical arterioles were preconstricted with U-46619 (50–250 nM) and exposed to 11,12-EET (100–500 nM). Notably, it was found that responses to 11,12-EET were also highly dependent on vascular tone. Thus biphasic responses (i.e., constriction followed by dilation) were observed in four out of six vessels showing relatively low preconstriction (initial diameter more than ~70% of baseline) while, in contrast, only dilations were evoked in arterioles presenting higher resting tones (n = 10; Fig. 5, A–C). There was no relationship between the concentration of 11,12-EET applied and the type of response evoked. Note that, as it was the case for mGluR activation, the tone at which the reversal of constrictrions into dilations occurred corresponded to ~70% of baseline diameter (i.e., ~30% constriction). This observation further supports the notion of a vascular set point that modulates the polarity of the arteriole’s response.

The question arises as to what are the mechanisms that mediate the vascular actions of 11,12-EET? EETs are known to activate large-conductance K+ channels (BK channels) in VSMC (23), leading to vasodilatation. In addition, because some EET regioisomers have been shown to mediate increases in [Ca2+]i in vascular endothelial cells (58) and cultured astrocytes (54), it is possible that other mechanisms may also be at play. To gain

![Fig. 4. Arachidonic acid metabolites are differentially involved in the constrictions and dilations induced by mGluR activation. Summary data showing the diameter changes in cortical arterioles induced by t-ACPD (100 μM) after incubation of brain slices with pharmacological blockers of the AA pathway. Vascular responses were grouped based on the initial degree of preconstriction. Results represent means ± SE. *P < 0.05 and **P < 0.01. Dunnett’s test.](http://ajpheart.physiology.org/Downloadedfrom)
insight into the mechanisms involved in 11,12-EET-induced constrictions and dilations, we investigated the effects of 11,12-EET on astrocytic [Ca\(^{2+}\)]. Using confocal Ca\(^{2+}\) imaging in brain slices loaded with the Ca\(^{2+}\) indicator fluo 4, we found that short application (5–30 s) of 11,12-EET (1 μM) produced rapid and robust increases in [Ca\(^{2+}\)] in astrocytic somata and endfeet (ΔF/Fo = 2.2 ± 0.3; n = 8 slices). A representative example of this response is shown in Fig. 5D (also see Supplemental Video 3). Thus, consistent with previous effects of 5,6-EET in cultured astrocytes (54), our data suggest that, in addition to possible paracrine effects on the vasculature, glial-derived EETs might also act in an autocrine manner, e.g., amplifying and/or sustaining glutamate-induced [Ca\(^{2+}\)], increases, which may in turn affect AA production as well as other Ca\(^{2+}\)-dependent processes. To assess this possibility, brain slices were preincubated with MAFP (100 μM), and vasoconstrictor responses were evaluated in minimally constricted arterioles (<20%). As shown in Fig. 5E, MAFP treatment significantly abolished vasoconstrictor responses to 11,12-EET (300 nM) (n = 4; P < 0.001).

Finally, to test if BK channels are involved in 11,12-EET-induced vasodilations, we performed experiments in which 11,12-EET (200 nM) was applied in the presence of the BK channel inhibitor TEA (1 mM). In support of BK channel involvement, vasodilation was abrogated in the presence of TEA (Fig. 5, C and E; P < 0.01; n = 3). Further studies are needed to assess whether 11,12-EET-induced dilator effects involved BK channels expressed on VSMC, astrocytic endfeet, or both.

**DISCUSSION**

There is increasing evidence that activation of mGluR in astrocytes, and the subsequent increase in glial [Ca\(^{2+}\)], plays an important role in functional hyperemia (28). However, both in vivo and in vitro studies have associated [Ca\(^{2+}\)] increases in astrocytes not only to vasodilation (20, 36, 39, 55, 60) but also to vasoconstriction (11, 39, 42). Although these responses have in some cases been related to the specific action of constrictor and dilator signals, the factors that determine whether vessels dilate or constrict upon neuronal and/or glial activation are still unclear. In this study, we attempted to shed light on the mechanisms determining the vascular response to astrocyte activation by addressing a fundamental question: do alterations in the properties of the vasculature itself account for the type of response evoked?

A possible influence of vascular tone in the response of brain arterioles to mGluR has been suggested by Mulligan and MacVicar (42). In their work, t-ACPD induced constrictions in vessels that were initially dilated and dilations in vessels preconstricted via inhibition of NO synthesis. However, because NO production was blocked, it is not clear to what extent dilator responses were influenced by alterations in vascular tone per se or rather depended on NO availability. In this sense, a critical role for NO in glial-induced constrictions and dilations remains to be determined.
represents the level of vascular tone at which dilator re-adjust vascular tone around a predefined set point, which may indicate that astrocyte-derived signals act in concert to dilate upon neuronal activation. In summary, our results stabilized around this putative setpoint and hence they readily

intraluminal pressures (20 –100 mmHg) (9, 12), we speculate that, penetrating arterioles in vitro subjected to a broad range of relaxations was demonstrated in the isolated rat retina, albeit here the initial tone of the vessels was reported to have no effect (39).

Using different kinds of stimuli associated with neurovascular signaling mechanisms (modestly elevated [K\(^{+}\)]\(_o\), a mGluR antagonist, and 11,12-EET), we show that the polarity and magnitude of the evoked diameter changes in brain cortical arterioles are in fact dictated by their particular vascular tone. Notably, all stimuli elicited a differential response pattern, defined by the presence of a set point centered at \(\sim 30–40\%\) tone. Thus elevating [K\(^{+}\)]\(_o\) from 4.2 to 10 mM induced consistent, near-maximal dilations in vessels with initial diameters up to \(\sim 60\%\) of baseline, whereas the dilations observed in more constricted vessels, although proportionally larger, did not reach the same final amplitude. This suggests that increases in [K\(^{+}\)]\(_o\) at the gliovascular interface, proposed to be a key mechanism of NVC in the brain (21), may be highly effective in eliciting functional hyperemia when the tone of the arterioles lies at or above the set point. Nevertheless, it must be noted that neuronal influences in the responses to elevated [K\(^{+}\)]\(_o\) cannot be ruled out, inasmuch as 10 mM K\(^{+}\) will decrease \(V_m\) in neurons. Although elevated [K\(^{+}\)]\(_o\) exerted a net dilator effect, both constrictions and dilations were observed upon mGluR activation with t-ACPD. Constrictions were observed in arterioles initially presenting low to moderate levels of tone (constricted up to \(\sim 30\%\)), whereas dilations occurred in vessels preconstricted above \(\sim 30\%).

Notably, for both slightly and highly preconstricted vessels, mGluR activation tended to bring arteriolar diameter to a consistent level (\(\sim 37\%\) constricted) close to that at which the polarity of the response to t-ACPD reversed. In addition, prominent dilations occurred in vessels in which diameters were already around the set point, as well as in some arterioles that initially constricted and reached the set point. Because the latter resembles the level of myogenic constriction measured in brain arterioles, prominent dilations occurred in vessels in which diameters to a consistent level (\(\sim 30 – 40\%\) tone). Thus elevating [K\(^{+}\)]\(_o\) at the gliovascular interface, proposed to be a key mechanism of NVC in the brain (21), may be highly effective in eliciting functional hyperemia when the tone of the arterioles lies at or above the set point. Nevertheless, it must be noted that neuronal influences in the responses to elevated [K\(^{+}\)]\(_o\) cannot be ruled out, inasmuch as 10 mM K\(^{+}\) will decrease \(V_m\) in neurons. Although elevated [K\(^{+}\)]\(_o\) exerted a net dilator effect, both constrictions and dilations were observed upon mGluR activation with t-ACPD. Constrictions were observed in arterioles initially presenting low to moderate levels of tone (constricted up to \(\sim 30\%\)), whereas dilations occurred in vessels preconstricted above \(\sim 30\%).

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In response to mGluR activation, both dilations and constrictions were dependent on functional PLA\(_2\) activity, confirming the involvement of AA metabolites in these responses. It can be argued that, after pharmacological blockade of AA metabolism, the rise in astrocytic [Ca\(^{2+}\)], resulting from mGluR activation would still have led to activation of BK channels in glial endfeet and subsequent K\(^{+}\)-induced vasodilation. We speculate that, in the absence of neuronal stimulation, K\(^{+}\) gradients are absent or reduced (29), and endfoot [K\(^{+}\)] does not attain substantial levels for this mechanism to operate. We also showed that the effects of blocking specific AA metabolic pathways were highly dependent on vascular tone. Thus, and in agreement with previous reports (39, 42), selective inhibition of CYP \(\omega\)-hydroxylase-mediated 20-HETE synthesis converted constrictions into dilations in vessels with low preconstriction. Because a positive correlation between 20-HETE-mediated vasoconstriction and oxygen pressure has been documented in the skeletal muscle microcirculation (27) and in preliminary form also for the brain (25), it is likely that vasoconstrictions were similarly potentiated in our experiments, in which the perfused aCSF’s O\(_2\) activity was \(\sim 55\%\).

In arterioles with intermediate levels of tone, but not in highly constricted arterioles, t-ACPD-induced vasodilation was clearly attenuated (\(\sim 50\%\)) after inhibition of 20-HETE synthesis. Although this reduction did not attain statistical significance, the possibility that 20-HETE plays a permissive, or perhaps a direct role, in the vasodilation induced by mGluR activation in cortical arterioles needs to be further evaluated (16). More studies are also needed to elucidate whether 20-HETE is generated and released by astrocytes (43), produced by VSMC from glial-derived AA (22), or both of these events.

In weakly preconstricted vessels, t-ACPD-induced vasoconstrictions were significantly attenuated upon inhibition of CYP epoxyenease, COX-1, or thromboxane synthase, which suggests that EETs and COX-1-derived prostanoids (e.g., thromboxane) facilitate this response. The effect of CYP epoxyenease inhibition on t-ACPD-induced constrictions is not clear but may involve

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Fig. 6. Working model. We propose that, under physiological conditions, a combination of intrinsic and extrinsic mechanisms maintains vascular tone at an optimal range, defined here as the “set point” (B). Following neuronal stimulation, released glutamate activates mGluR in astrocytes, resulting in an increase in [Ca\(^{2+}\)]. The rise in [Ca\(^{2+}\), facilitates K\(^{+}\) movement in the perivascular space via large-conductance, Ca\(^{2+}\)-dependent K\(^{+}\) channel (BK) activation at the endfoot, and it also initiates the metabolism of arachidonic acid (AA) via activation of phospholipase A\(_2\) (PLA\(_2\)). Rapid vasodilation (C) is thus elicited by both K\(^{+}\) and vasoactive signals derived from AA metabolism, in particular cyclooxygenase (COX)-1 metabolites (e.g., PGE\(_3\)). We propose that, when vascular tone deviates from its set point, as it might occur under pathological or perhaps physiological conditions (A), astrocyte-derived signals will restore vascular tone to its set point. Although a combination of constrictor and dilator signals may be released by astrocytes, the resultant effect on vascular diameter will be determined by the sensitivity of the vessels to such signals, dictated in turn by their specific tone.
altered Ca$^{2+}$ dynamics in astrocytes (see below), leading to lower production of vasoconstrictors by these cells. Of note, because CYP epoxygenase and COX-1 are present not only in astrocytes (2, 56) but also in vascular endothelial cells (18), a contribution of the latter cell type to the observed effects cannot be excluded. Inhibition of COX-1, but not of CYP epoxygenase, was effective in preventing dilations in vessels with intermediate levels of tone (30–50% preconstriction). Because we showed that maximal dilations in response to t-ACPD occurred in this range of resting tone, which likely resembles the prevailing physiological tone in vivo, our results appear in principle to be consistent with those of Takano et al. (56), which showed that COX-1 inhibition, but not CYP epoxygenase inhibition, reduces vasodilation following astrocytic activation in vivo.

In contrast, our results suggest that EETs, as well as COX-1 products (e.g., PGE$_2$), contribute to vasodilation in substantially constricted (>50%) vessels. In this respect, a potential target of both PGE$_2$ and EETs is the BK channel in VSMC. Thus, concurrent increases in cAMP [mediated by PGE$_2$ acting through prostaglandins E$_2$ receptor subtypes EP$_2$/EP$_4$ receptors (51)] and [Ca$^{2+}$], [via EETs (14, 17)] may lead to BK channel activation by mechanisms involving phosphorylation (4, 15) and/or by increasing the frequency of Ca$^{2+}$ "sparks" (14, 46, 50). Taken together, these results strongly suggest that, although both constrictor and dilator signals are likely to be released upon mGluR activation in astrocytes, the overall vascular response is determined by the specific resting tone of the arterioles.

The final part of this work focused on the vasoactive effects of a CYP epoxygenase product, i.e., 11,12-EET. Our results showed that, similarly to the responses elicited by t-ACPD, 11,12-EET constricted and dilated cortical arterioles in a tone-dependent manner: biphasic responses (constriction followed by dilation) were predominant in weakly preconstricted vessels, whereas dilations occurred in vessels displaying higher resting tones. Notably, as with t-ACPD-induced constrictions and dilations, the polarity of the primary responses to 11,12-EET also reversed around a similar level of tone (~30% constriction), which reinforces the presence of a putative vascular set point. It is likely that some of the effects of EETs stem from their ability to affect Ca$^{2+}$ changes in astrocytes, as shown previously for 5,6-EET (54) and in this study for 11,12-EET. Because astrocytes are capable of synthesizing EETs, these evidences support a possible autocrine role for EETs in the amplification of Ca$^{2+}$ transients (54), which may be functional for hyperemic mechanisms in vivo. 11,12-EET-induced vasocstriction was prevented by MAPF, which suggests that it depends on AA synthesis and may be mediated by AA-derived vasoconstrictors (e.g., 20-HETE and thromboxane). In line with this, the attenuation of vasocstriction to t-ACPD upon inhibition of EETs synthesis with MS-PPOH (see Fig. 4) could also be due to reduced synthesis of the above AA metabolites. In both cases, however, the cellular sources of AA and its downstream constrictor by-products remain unclear. On the other hand, exogenous 11,12-EET had clear vasodilator effects in vessels preconstricted above ~30%, but vasodilation in response to mGluR activation during CYP epoxygenase inhibition was partially reduced only in highly constricted (>50%) vessels. This may be explained by the relatively large concentration of 11,12-EET used, which may have acted directly on VSMC, or may have stimulated the synthesis of dilator prostanoids secondarily to the Ca$^{2+}$ increase in the astrocyte. Our results also show that, consistent with the role of EETs as activators of BK channels in VSMC (14, 23), 11,12-EET-induced vasodilation was sensitive to 1 mM TEA. Altogether, these data clearly show that the vasoactive effects of CYP epoxygenase products are conditioned by the status of arteriolar tone.

In conclusion, this study provides a comprehensive examination of the influence of vascular tone on the response of brain intracortical arterioles to neuron- and glial-derived signals and supports a novel role for astrocytes in the dynamic control of the cerebral microcirculation. A tentative model of the mechanisms involved is presented in Fig. 6. It must be emphasized that several differences must certainly exist in the mechanisms that operate during pressure-induced myogenic tone and those that determine constriction to U-46619 in vitro. Importantly, endothelial influences are likely to be limited by the lack of intraluminal perfusion characteristic of the brain slice model. Notwithstanding, our results open up the possibility that, in addition to contributing to functional hyperemia, astrocyte-derived signals play an important role in adjusting vascular tone toward a defined “set point” around which the hyperemic response is optimized. It is conceivable that such mechanism is not restricted to circumstances of enhanced neuronal activation but may also be operative during periods of basal brain activity.

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