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Recruitment of Dynamic Endothelial Ca^{2+} Signals by the TRPA1 Channel Activator AITC in Rat Cerebral Arteries

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Abstract

Objective—Stimulation of endothelial TRP channels, specifically TRPA1, promotes vasodilation of cerebral arteries through activation of Ca^{2+} -dependent effectors along the myoendothelial interface. However, presumed TRPA1-triggered endothelial Ca^{2+} signals have not been described. We investigated whether TRPA1 activation induces specific spatial and temporal changes in Ca^{2+} signals along the intima that correlate with incremental vasodilation.

Methods—Confocal imaging, immunofluorescence staining and custom image analysis were employed.

Results—We found that endothelial cells of rat cerebral arteries exhibit widespread basal Ca^{2+} dynamics (44 ± 6 events/minute from 26 ± 3 distinct sites in a $3.6 \times 10^4 \mu\text{m}^2$ field). The TRPA1 activator AITC increased Ca^{2+} signals in a concentration-dependent manner, soliciting new events at distinct sites. Origination of these new events corresponded spatially with TRPA1 densities in IEL holes, and the events were prevented by the TRPA1 inhibitor HC-030031. Concentration-dependent expansion of Ca^{2+} events in response to AITC correlated precisely with dilation of pressurized cerebral arteries ($p = 0.93$ by F-test). Correspondingly, AITC caused rapid endothelium-dependent suppression of asynchronous Ca^{2+} waves in subintimal smooth muscle.

Conclusions—Our findings indicate that factors that stimulate TRPA1 channels expand Ca^{2+} signal-effector coupling at discrete sites along the endothelium to evoke graded cerebral artery vasodilation.

Keywords

cerebral artery; endothelium; calcium; TRPA1; AITC

INTRODUCTION

In the cerebral circulation, the endothelium plays a crucial role in real-time regulation of vascular tone. Ca^{2+} signaling drives multiple mechanisms of endothelium-dependent vasodilation, but the physiologic engagement and stimulus-specific tuning of these mechanisms remains unclear. Key Ca^{2+} -activated effectors underlying endothelium-dependent vasodilation and preservation of cardiovascular homeostasis [21] include NO-generating eNOS [9, 26] as well as the Ca^{2+} -activated K^+ channels, $\text{K}_{\text{Ca}2.3}$ [8, 45] and $\text{K}_{\text{Ca}3.1}$ [10, 15], that elicit K^+ efflux and membrane potential hyperpolarization. Recent evidence suggests that localized basal Ca^{2+} dynamics [17, 27, 29, 32] underlie the targeted

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recruitment of endothelial vasodilating effectors. In mouse mesenteric arteries, ongoing Ca^{2+} events (Ca^{2+} pulsars) emitting from IP_3 -sensitive internal stores, couple to intermediate conductance $\text{K}_{\text{Ca}3.1}$ channels in the endothelial cell plasma membrane [32]. These channels are concentrated at myoendothelial junction sites where holes in the IEL permit projections of endothelial and smooth muscle cells to form close interactions [38]. This functional architecture allows periodic Ca^{2+} -dependent activation of $\text{K}_{\text{Ca}3.1}$ channels to elicit persistent hyperpolarization [32] and provides an impetus for continuous endothelium derived hyperpolarization of VSM via heterocellular gap junctions [11, 14, 35, 39, 41] or via the direct effect of effluxed K^+ on subintimal smooth muscle cell Na^+/K^+ -ATPases or inward rectifier K^+ channels [20, 47]. The resulting inhibition of influx through voltage-gated Ca^{2+} channels causes dilation of the arterial vasculature [30].

A framework similar to endothelial Ca^{2+} pulsar-effector coupling might exist in the cerebral circulation. In addition, Ca^{2+} -permeable ion channels belonging to the TRP family of non-selective cation channels [13, 48] have recently been implicated in the endothelial regulation of cerebral artery tone [1, 18, 31, 49]. In particular, the sole member of the ankyrin (A)-associated TRP subfamily, TRPA1 [44], is closely associated with $\text{K}_{\text{Ca}3.1}$ channels at myoendothelial junction sites in the cerebral circulation [18]. TRPA1 channels are activated by various electrophilic compounds including allicin, a component of garlic [5] and AITC, derived from mustard oil [4, 28]. TRPA1 activation with AITC causes concentration-dependent (1 – 100 μM) dilation of rat cerebral arteries through an endothelium-dependent mechanism involving hyperpolarization of cerebral artery smooth muscle [18]. We surmise that stimulus-dependent activation of TRPA1 induces or augments endothelial Ca^{2+} signals along the myoendothelial interface to evoke this K_{Ca} -dependent cerebral artery vasodilation. However, the specific impact of TRPA1 activation on cerebral artery endothelial Ca^{2+} signals has not been assessed and the role of such signaling in graded TRPA1-related vasodilation remains unknown.

Here, we use confocal microscopy and custom detection/analysis software (previously described in [22]) to measure the impact of TRPA1 activation on cytosolic Ca^{2+} dynamics in rat cerebral artery endothelium. We demonstrate that the cerebral artery endothelium exhibits basal Ca^{2+} dynamics even in the absence of exogenous stimulation, and that TRPA1 activation causes a marked concentration-dependent increase in the prevalence and persistence of distinct active Ca^{2+} sites along the intima, correlating with concentration-dependent cerebral artery vasodilation.

MATERIALS AND METHODS

Animals and tissue preparation

Adolescent rats (250–350 g) were euthanized with pentobarbital (80 mg/kg) and decapitated. All animal procedures were approved by the University of South Alabama Institutional Animal Care and Use Committee, and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Brains were quickly removed and chilled ($\sim 4^\circ\text{C}$) in PSS. Artery segments (both middle cerebral and basilar, ~ 5 mm in length) were dissected from the base of the brains, carefully cut open longitudinally, and pinned down on the surface of the small silicone (sylgard) blocks using 14 μm diameter pins with the endothelium facing up [32]. Notably, net lateral stretch placed on the vessels (~ 1.5 times resting width) was adequate to prevent folding of the lamina and is consistent with vascular distention by pressure based on previous experiments; a 300 μm diameter artery, opened and pinned at a width of 1.5 mm, is approximately equivalent to its circumference at 80 mmHg pressure.

Confocal Ca²⁺ imaging and analysis

Artery samples on blocks were incubated at room temperature for 40 minutes in dark with Ca²⁺ indicator loading solution containing PSS, Fluo-4 AM (15 μ M) and 0.06% Pluronic F-127. After washing and 30 minutes equilibration, blocks were placed in a glass-bottom chamber (separated 100 μ m from glass by two parallel supporting pins) containing PSS. The chamber was mounted on an inverted microscope fitted with a PerkinElmer spinning disk RS-3 confocal unit. Ca²⁺-dependent fluorescence (488 nm excitation, 510 nm emission) was measured at 8 frames/sec at 25°C (20X objective) using Ultraview software. Images were saved as 16-bit raw data during recording, and later converted into 8-bit TIFF format for offline processing. Only recordings with > 90% of total viewable area in focus were processed and analyzed. Different AITC concentrations were evaluated in different vessels to avoid potential effects of protracted experimental times. Data were processed using a custom algorithm implemented as a plug-in with ImageJ software [22] specifically designed to: 1) detect sites of dynamic Ca²⁺ change above statistical ($p < 0.01$) noise, 2) define regions of interest (ROI; 5 pixel or 1.7 μ m diameter) at active sites centers, and 3) analyze average fluorescence intensities at ROIs to determine specific event parameters. Fluorescence data are expressed as F/F_0 , where F_0 is determined by a linear regression of base data at each ROI.

Immunofluorescence staining

Artery samples on blocks (described above) were fixed with 4 % formaldehyde for 15 minutes, permeabilized with 0.5 % Triton X-100, blocked with 1 % bovine serum albumin and exposed to primary antibody (Neuromics; Edina, MN) overnight at 4°C. Arteries were subsequently treated with fluorescent secondary anti-rabbit antibodies (Alexafluor 568; Molecular Probes) and Hoechst nuclear stain. Z-sections were acquired at 0.5 μ m increments over ~5 μ m depth and presented as condensed single images. Data were obtained using a Nikon A1 confocal inverted microscope and processed with Nikon Elements and ImageJ software.

Arterial diameter measurements

For isolated vessel experiments, arteries were cannulated, pressurized with physiological saline solution, and superfused with PSS at 37°C. Inner diameter was continuously monitored using video microscopy and Ionoptix edge-detection software (see ref [18] for additional details).

Reagents and solutions

Reagents and AITC were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA), HC-030031 was obtained from TOCRIS Bioscience (Westwoods Bus. Park Ellisville, MO), and TRPA1 antibody was procured from Neuromics (Edina, MN). Tungsten wires (for making tiny pins) were purchased from Scientific Instrument Services (Ringo, NJ). HEPES/bicarbonate-buffered physiological saline solution (PSS) contained (mM): NaCl 130.0; NaHCO₃ 14.9; KCl 3.7; KH₂PO₄ 1.2; MgSO₄ 1.2; Glucose 11.0; HEPES 10.0; pH 7.4.

Data Analysis

Data are presented as the mean $\pm$ standard error and statistical analysis is performed with GraphPad Prism software. For parametric multiple-set analysis, one-way ANOVA was performed followed by individual comparisons via Dunnett or Tukey post tests. For multiple nonparametric data sets, Kruskal-Wallis and subsequent Dunn's tests were employed. Nonlinear regression curves were fit to concentration-dependent data sets using GraphPad

Prism and statistical comparisons made by F-test. P values < 0.05 were considered significant.

RESULTS

Basal Ca^{2+} dynamics in rat cerebral artery endothelium

We assessed Ca^{2+} signals in the endothelium of fluo-4-loaded opened rat cerebral arteries using confocal imaging. A custom autodetection and analysis algorithm [22] was employed to discriminately evaluate fluctuations of Ca^{2+} above background in intact fields of arterial intima (~150 endothelial cells). Under resting conditions at room temperature and in the absence of flow, endothelial cells of rat cerebral arteries produced widespread dynamic localized Ca^{2+} events (Fig 1A and B; Movie S1). Each event typically encompassed much if not all of an individual cell. As a result, each site that was identified and tracked typically corresponded with a single cell. On average, 44 ± 6 events occurred from 26 ± 3 distinct sites per minute within a $3.6 \times 10^4 \mu\text{m}^2$ sampled field (base data obtained from 10 animals). Replacement of the Ca^{2+} replete bath solution with Ca^{2+} free buffer for 5 min did not alter the occurrence of the basal Ca^{2+} events per minute (44 ± 4) whereas depletion of internal stores with CPA (25 μM) or blockade of IP_3Rs with the combination of xestospongine C (30 μM) and 2-APB (80 μM) significantly decreased the number of events to 1.3 ± 0.9 and 14 ± 3 , respectively ($p < 0.01$ vs. vehicle control; Fig 1B and C). Vehicle time controls at 30 minutes were not significantly different from basal (Fig S1). Composite distributions of various basal Ca^{2+} event parameters are summarized in Fig 2. Overall, event parameter distributions were non-Gaussian. Median event amplitude, duration, spatial spread and frequency (per site) were $1.48 F/F_0$, 3.2 sec, $21.2 \mu\text{m}^2$, and 0.013 hz, with interquartile ranges of 1.35 – 1.66, 2.1 – 5.1 sec, $10.9 - 35.6 \mu\text{m}^2$, and 0.008–0.017 hz, respectively.

Activation of TRPA1 channels expands Ca^{2+} signaling sites along the intima

We assessed whether AITC, a specific agonist of the Ca^{2+} -permeable cation channel TRPA1 [4, 7], would augment cytosolic Ca^{2+} in cerebral artery endothelium. AITC caused marked concentration-dependent increases in the occurrence of dynamic Ca^{2+} events along sampled endothelial fields (Fig 3A). These increases were primarily due to generation of new events at new sites within the field (Fig 3B, $p < 0.05$ vs. corresponding 0 μM AITC). Overall the number of events and sites increased by 54% and 52%, respectively. Within the sampled fields, the overall range of event amplitudes and durations significantly increased at higher AITC concentrations (Fig 3C; $p < 0.01$ by Kruskal-Wallis analysis and subsequent Dunn's tests for nonparametric data) whereas the distribution of event spatial spread values was unchanged. Notably, AITC did not predictably increase the frequency, amplitude, duration or spatial spread of events occurring at previously active sites (Fig 4A). Indeed, the largest events elicited by AITC occurred at sites exhibiting little or no activity before adding the drug (Fig 4B).

Next, we addressed the specific role of TRPA1 channels in the AITC augmented Ca^{2+} signals. To elucidate the spatial relationship between TRPA1 channels and AITC-induced endothelial Ca^{2+} events, we probed for TRPA1 expression immediately following Ca^{2+} measurements in a group of cerebral artery segments. Confocal immunofluorescence images in Fig 5A show distinct densities of TRPA1 mainly along the basolateral surface of the endothelium, and particularly within and around holes in the IEL. Superimposing Ca^{2+} -dependent fluorescence on TRPA1 immunofluorescence reveals the liberation of robust Ca^{2+} signals by 30 μM AITC at several (but not all) distinct TRPA1-dense sites along the intima (Fig 5B). Although these new events consistently initiated at TRPA1 foci, they typically expanded along endothelial cell axes. We found that the TRPA1 specific inhibitor HC-030031 [3] (10 μM for 10 min) had no significant effect on the occurrence of basal

endothelial Ca^{2+} dynamics (Fig 5C) but completely prevented the increase in Ca^{2+} events induced by 30 μM AITC. AITC-induced events were similarly blunted by removal of bath Ca^{2+} for 10 minutes, indicating an essential dependence on extracellular Ca^{2+} influx. Notably, pretreatment with CPA (25 μM) strongly suppressed the AITC-induced events, suggesting a considerable contribution of internal Ca^{2+} stores.

Cerebral artery dilation correlates with concentration-dependent AITC recruitment of spatially distinct endothelial Ca^{2+} events

AITC elicits endothelium-dependent dilation of pressurized cerebral arteries [18]. We evaluated the direct relationship between concentration-dependent AITC-induced increases in discrete Ca^{2+} dynamics along the intima and AITC-induced cerebral artery dilation. Fig 6A shows separate plots of cerebral artery diameter change and relative increases in endothelial Ca^{2+} signals resulting from increasing concentrations of AITC. Notably, global Ca^{2+} change within the entire endothelial field correspond poorly with relative changes in arterial diameter (Fig 6). However, AITC solicitation of new dynamic Ca^{2+} events/sites along the intima corresponded precisely with concentration-dependent arterial diameter changes. Overall, maximal increases in Ca^{2+} events and vessel diameter were 62% and 64%, respectively ($-\log[\text{EC}_{50}]$ values of 4.9 ± 0.2 and 4.8 ± 0.1), and nonlinear regression curves fit to Ca^{2+} events and diameter were not significantly different ($p = 0.93$ by F-test).

AITC causes endothelium-dependent inhibition of smooth muscle Ca^{2+} waves in cerebral arteries

We surmised that if TRPA1 activation expands intima-to-media communication of vasodilatory signals, AITC should elicit endothelium-dependent suppression of Ca^{2+} signals in the smooth muscle cells immediately below the arterial intima. Using open artery preparations, we focused through the IEL to measure Ca^{2+} -dependent fluorescence within the subintimal smooth muscle. In the absence of exogenous stimulus, VSM cells exhibited propagating longitudinal asynchronous waves (Fig 7), previously shown to contribute to cerebral artery vasoconstriction [37]. Addition of 30 μM AITC essentially abolished these VSM Ca^{2+} signals (Fig 7A). However, when the endothelium was removed, the same concentration of AITC failed to inhibit ongoing VSM Ca^{2+} dynamics (Fig 7B).

DISCUSSION

The major findings of the current study are 1) rat cerebral artery endothelium generates robust dynamic Ca^{2+} events under basal conditions, 2) activation of TRPA1 channel clusters along the IEL enhances endothelial Ca^{2+} dynamics primarily through recruitment of new distinct Ca^{2+} event sites along the intima, 3) graded dilation of pressurized cerebral arteries in response to TRPA1 activation corresponds precisely with the recruitment of new endothelial Ca^{2+} events, and 4) TRPA1 activation causes endothelium-dependent inhibition of VSM Ca^{2+} waves. Together, these results indicate that increased stimulation of TRPA1 channels discretely expands spatially distinct Ca^{2+} signals along the intima, thereby promoting graded engagement of endothelial effectors and vasodilation. The broad implication of the current findings is that TRPA1-activating compounds, including known electrophilic substances derived from diet and the environment, increase cerebral blood flow through acute augmentation of Ca^{2+} -dependent myoendothelial communication.

In the current study, we employed confocal imaging and a custom signal detection and analysis algorithm [22] to provide a statistically rigorous and comprehensive survey of cellular Ca^{2+} signals along broad fields of vascular intima. We reveal for the first time the occurrence of dynamic basal Ca^{2+} events in rat cerebral artery endothelium. These events were prevented by store depletion and greatly suppressed by IP_3R inhibitors. Our results

suggest that, like Ca^{2+} pulsars [32] in mouse mesenteric artery endothelium, rat cerebral artery endothelial Ca^{2+} dynamics are highly dependent on release from IP_3 -regulated internal stores, although definitive dissection of IP_3 Rs and isoform-specific roles will require the development of more potent and selective pharmacologic tools. Basal endothelial events appear to be functional correlates of Ca^{2+} puffs described in *Xenopus* oocytes that emit locally and underlie a host of complex signals including propagating waves [50]. The occurrence of such basal events in different circulations and species suggests they may be a common underlying feature of the vasculature. In mouse mesenteric arteries, Ca^{2+} pulsars couple to nearby $\text{K}_{\text{Ca}3.1}$ channels along myoendothelial junctions [32], sites where holes in the IEL allow direct endothelial-smooth muscle cell interaction and communication of endothelial-derived hyperpolarization. Structured networks of such coupling sites form a functional nexus for continuous physiologic modulation of vascular tone in vivo. Indeed, $\text{K}_{\text{Ca}3.1}$ channels are integral for EDH-mediated vasodilation in various beds, including cerebral arteries [34, 25], and mice lacking $\text{K}_{\text{Ca}3.1}$ channels are hypertensive [42].

Past investigations have identified Ca^{2+} -permeant TRPA1 channels as important environmental sensors. In nociceptive neurons [44] as well as in non-neuronal tissues such as basal urothelial cells [24] and prostate epithelial cells [16], these channels have primarily been linked to pain and inflammation in response to exogenous chemical irritants [3, 6, 23, 36, 46]. The distinct vascular impact of TRPA1 channels has only recently been exposed. In cerebral arteries, densities of TRPA1 channels associate closely with Ca^{2+} -activated K^+ channels, particularly $\text{K}_{\text{Ca}3.1}$, and TRPA1 stimulation elicits vasodilation explicitly through K_{Ca} -dependent smooth muscle hyperpolarization [18]. Our current data suggest that TRPA1 channels exploit these K_{Ca} channels at the myoendothelial interface through very discrete expansion of endothelial Ca^{2+} signals, thereby evoking acute cerebral artery vasorelaxation.

Our findings indicate minimal contribution of TRPA1 to basal cerebral artery Ca^{2+} signals. Although previous functional data suggest a small increase in resting arterial tone upon TRPA1 inhibition [18], we found no significant effect of TRPA1 inhibition on the occurrence of cerebral artery endothelial events in the absence of direct activation. This may indicate very subtle TRPA1 impacts beyond the limits of our current evaluation or modest nonselective or non-endothelial effects of the inhibitor HC030031. We found that exogenous activation of TRPA1 channels induces multiple new sites of Ca^{2+} activity that are dependent on extracellular Ca^{2+} entry and superimpose on ongoing dynamics. The factors controlling accumulation of these sites along the intima are not clear, but we submit that differences in TRPA1 channel density or specific arrangement per site may contribute to graded stimulation thresholds. Similar triggering of new event sites was observed in mouse mesenteric arteries in response to the endothelial agonist acetylcholine [32], suggesting this is a common mode of Ca^{2+} signal proliferation and titration in the vasculature.

An intriguing question is whether TRPA1-triggered Ca^{2+} signals are independent of basally occurring signals or actually interact with them. We found that AITC initiated new events at distinct TRPA1-dense spots, but these events tended to spread considerably through individual cells rather than remaining localized. In fact, spatial spread was not significantly different between events occurring basally and those solicited by AITC. These rather broad cellular Ca^{2+} events may explain why acute AITC-evoked cerebral artery dilation involves not only activation of $\text{K}_{\text{Ca}3.1}$ channels that associate closely with TRPA1 at myoendothelial junctions, but also $\text{K}_{\text{Ca}2.3}$ channels that distribute more peripherally [40].

Overall, our findings suggest that stimulated Ca^{2+} entry at TRPA1-dense sites promotes mobilization of internal stores (i.e. via enhanced Ca^{2+} -induced Ca^{2+} release). Indeed, TRPA1-motivated events were highly suppressed by internal Ca^{2+} store depletion via SERCA inhibition. Recent data from Sonkusare et. al., [43] suggest a similar impact of

activated TRPV4 channels in mesenteric artery endothelium. In these studies, TRPV4 stimulation initiated isolated endothelial Ca^{2+} sparklets, and these focal transients were converted into broad cellular Ca^{2+} rises when internal stores were intact. Taken with our current findings, the general implication is that endothelial TRP channels act as key ignition points where local Ca^{2+} entry triggers or sensitizes broader Ca^{2+} release events. This instigating role of TRPA1 would be predicted to augment Ca^{2+} dynamics by soliciting new signaling sites (i.e. new active cells) and promoting larger events (i.e. amplitude and duration) across the intima, both of which are consistent with our current findings. Notably, the responses of specific sites/cells to acute TRPA1 stimulation may be quite variable since pre-existing activity could impart a relative refractoriness to TRPA1-potentiated store release. Indeed, we found that TRPA1 activation evoked little or no response at sites exhibiting the most robust basal Ca^{2+} dynamics, whereas it solicited the largest responses at sites with little or no basal activity (Fig 4). Overall, we suggest nonselective cation channels afford distinctive widespread tuning of prevailing Ca^{2+} signaling modes in arterial endothelium, and this function likely underpins their influence in real-time endothelial vasoregulation [31, 33, 49].

Previous epifluorescence measurements of global AITC-induced Ca^{2+} elevation in freshly dispersed cerebral artery endothelial cells [19] have suggested an EC_{50} value of $\sim 400 \mu\text{M}$, well beyond concentrations eliciting effective vasodilation (EC_{50} around $10 \mu\text{M}$). In the context of the current study, this discrepancy likely reflects the fact that TRPA1-dependent effector recruitment is not uniform but rather expands in discrete spatial units as populations of Ca^{2+} -dependent targets (e.g. K_{Ca} channels) that were previously out of range of basal signals become enveloped by new Ca^{2+} events. Indeed, we found that TRPA1-induced dilation of cerebral arteries was tightly correlated with progressive accumulation of dynamic Ca^{2+} events at distinct sites across the endothelium, but did not correspond with the modest global Ca^{2+} elevations over the entire endothelial field. It should be noted that previous functional data were acquired at 37°C whereas the current Ca^{2+} measurements were performed at room temperature to avoid potential transient impacts of temperature change during drug solution additions. Specific direct impacts of temperature cannot be excluded and should be addressed in future study. Overall, the data support a close link between dynamic Ca^{2+} site recruitment and vasodilation. Correspondingly, we found that TRPA1 activation at a level that causes an $\sim 80\%$ increase in both endothelial Ca^{2+} events and arterial diameter, effectively suppressed pro-contractile asynchronous Ca^{2+} waves in subintimal VSM cells, and this effect was lost when the endothelium was disrupted. This finding emphasizes the pivotal role of the endothelium in directing TRPA1 dependent vasoregulation. Overall, our data underscore the critical value of temporal/spatial resolution and high-throughput applications for decoding physiologically relevant Ca^{2+} signaling in intact tissues. Ultimately, the discrete localization of Ca^{2+} -activated endothelial effectors, including not only K_{Ca} channels but also eNOS [23], dictate the capacity of functional responses. Differential effector recruitment by proliferating Ca^{2+} dynamics will be a key focus for future investigation. While the current study focused on rat cerebral arteries to preserve continuity with previous work [18], further scrutiny in genetically altered mice, including $\text{K}_{\text{Ca}}3.1$ [42] and TRPA1 [6] knockout models, is warranted. At present, limitations associated with weak Ca^{2+} indicator dye loading in mouse cerebral artery endothelium complicates rigorous dynamic Ca^{2+} imaging and analysis in situ.

PERSPECTIVES

This work provides new mechanistic perspective on dietary and environmental factors that directly influence brain perfusion. We propose a functional basis for TRPA1 channel modulation of cerebral artery tone through definitive spatial and temporal expansion of distinct Ca^{2+} signals along the endothelium. The current work suggests dynamic signaling in

non-excitable endothelial cells of cerebral arteries is analogous to excitable neuronal networks of the brain itself where ongoing patterns are constantly modified by distinctly-positioned and differentially-regulated ion channels. Also, as endothelial targets of mechanical, inflammatory and oxidative stress stimuli [2, 7, 12, 36], TRPA1 channels may be pivotal control points in vascular disease. Future investigations should decipher the tuning of dynamic endothelial Ca^{2+} signals and directly address how their preferential and anomalous effector targeting may contribute to distinctive profiles of vasoregulation and vascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

TRP	transient receptor potential
AITC	allyl isothiocyanate
2-APB	2-Aminoethoxydiphenylborane
CPA	cyclopiazonic acid
PLC	phospholipase C
IP₃R	inositol 1,4,5-trisphosphate receptor
eNOS	endothelial nitric oxide synthase
NO	nitric oxide
ROI	region of interest
IEL	internal elastic lamina
K_{Ca}	Ca^{2+} -activated K^{+} channel
VSM	vascular smooth muscle
PSS	physiological saline solution
AUC	area under curve
SERCA	sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase

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Figure 1.

Basal Ca^{2+} dynamics in rat cerebral artery endothelium. A. 2-minute accumulate image from a continuous time series showing Ca^{2+} -dependent fluorescence measured in endothelial cells of opened Fluo-4 AM-loaded cerebral arteries. Lower panel is a pseudo-color intensity projection of the same basal Ca^{2+} transients. Scale bar is 20 μm . See also Movie S1. B. Recordings obtained from four separate sites corresponding to arrows in (A); 5-pixel diameter regions of interest (ROIs). C. Recordings of basal events in autotransfected ROIs in the absence or presence of Ca^{2+} free buffer (5 minutes), cyclopiazonic acid (CPA; 25 μM for 15 minutes), or the combination of xestospongin C (Xest C; 30 μM) and 2-APB (80 μM) for 40 minutes. Time controls at 5 and 50 min were not significantly different (Fig S1). D. Summary data ($n = 5-7$ per group; * $p < 0.01$).

Figure 2. Properties of basal Ca^{2+} events in rat cerebral artery endothelium. Histograms show distributions of specific event parameters: amplitude, duration, spatial spread and frequency per site. Data were compiled from 10 animals.

Figure 3.

Effect of the TRPA1 channel activator AITC on cerebral artery endothelial Ca^{2+} signals. A. Recordings showing the changes in detected Ca^{2+} dynamics in response to different concentrations of AITC. Inset shows continuous tracings from three distinct sites before and after AITC (15 μM) addition (arrow). B. Panels are two-minute accumulates of Ca^{2+} dynamics (black ellipses depict signals above background threshold) recorded in a representative experiment before and after AITC addition. The merged panel is a composite where red shows events before AITC, green shows events after AITC, and yellow shows sites where events occurred both before and after AITC. The bar graph summarizes the total number of Ca^{2+} events and distinct sites detected at each AITC concentration ($n = 5$; * and ** $p < 0.05$ vs. corresponding 0 μM AITC; each AITC concentration was assessed in a separate vessel). C. Scatter plots show distributions of amplitude, duration and spatial spread of events occurring in sampled endothelial fields ($n = 3-4$) at each AITC concentration (* $p < 0.05$ vs. 0 μM AITC, nonparametric Kruskal-Wallis and Dunn's tests).

Figure 4.

Analysis of single-site Ca^{2+} dynamics in AITC-stimulated cerebral artery endothelium. Ca^{2+} -dependent fluorescence was measured in open cerebral artery preparations before and after AITC. A. Plots show the effect of AITC (0, 15, 30 and 60 μM) on various Ca^{2+} event parameters only at sites that exhibited basal Ca^{2+} dynamics before AITC addition. Each point is the net change for 1–3 separate experiments at each concentration (3–4 animals per group). No significant change in single-site frequency, amplitude, duration or spatial spread ($p > 0.05$, one-way ANOVA) was indicated at the concentrations tested. B. Effect of pre-existing basal activity on the magnitude of AITC-induced Ca^{2+} increase at a given site. For each site, the total Ca^{2+} signal (AUC; area under curve for all events at that site) after 30 μM AITC is plotted as a function of total Ca^{2+} signal before AITC (quantified 2 minutes before and 2 minutes after AITC). In bar graph, mean Ca^{2+} AUC values are displayed separately for sites that exhibited one or more basal Ca^{2+} events prior to AITC addition and sites exhibiting no basal Ca^{2+} events prior to AITC. Net Ca^{2+} increases were significantly larger at sites that did not discharge prior to AITC exposure ($p < 0.001$, data composite of 4 animals).

Figure 5.

Specific role of TRPA1 in the AITC-stimulated endothelial Ca^{2+} signals. A.

Immunofluorescence images of the intima-media interface in opened rat cerebral arteries showing a merged composite as well as separate images of the internal elastic lamina (IEL; green), TRPA1 (red), and nuclei (blue) of endothelial cells (horizontal) and subintimal smooth muscle cells (vertical). A vessel branch is seen on the right side. Scale bar is 20 μm . Below: Panels show an expanded view of the region circumscribed by the white box in (A); distinct TRPA1 puncta (red) are emphasized by black arrows and Ca^{2+} -dependent fluorescence (white) is shown (30-second accumulate) before and after addition of 30 μM AITC. Ca^{2+} was measured before fixation and immunostaining. Bottom panels show a time series of a single event site, noting the initiation and spread of a Ca^{2+} event from a singular TRPA1 puncta. B. Recordings show AITC-stimulated endothelial Ca^{2+} signals measured in the absence or presence of the TRPA1-selective inhibitor HC-030031 (10 μM for 10 min). C. Data summarized in the bar graph show the relative inhibition of AITC-induced Ca^{2+} events by 10 μM HC-030031, Ca^{2+} -free solution, or 25 μM CPA ($n = 4-7$; * $p < 0.05$).

Figure 6.

Correlation of AITC-induced endothelial Ca²⁺ events and cerebral artery dilation. Left panel shows AITC-induced increases in cerebral artery diameter measured in pressurized isolated arteries [18]. Right panel shows relative AITC-induced increases in endothelial Ca²⁺ signals measured in open cerebral arteries. Nonlinear regression curves were generated for diameter changes (solid line) as well as changes in Ca²⁺; Ca²⁺ sites and total events occurring at these sites (dotted lines). Changes in average Ca²⁺ within the entire endothelial field are plotted as open circles. Points are means of at least three observations from a total of 14 animals.

Figure 7.

Endothelium dependence of AITC-induced inhibition of asynchronous cerebral artery smooth muscle Ca^{2+} waves. Panels show Ca^{2+} -dependent fluorescence measured in open cerebral arteries either in the presence of intact endothelium (A) or following removal of the endothelium (B). In these preparations, distinct subintimal smooth muscle cells (yellow arrows) were monitored before and after addition of 30 μM AITC. Recordings from multiple smooth muscle cells are shown (data representative of six vessels from two animals). For A and B, insets show time-course images of single smooth muscle cells and the corresponding Ca^{2+} recordings from indicated regions of interest (circles) before and after AITC addition (black arrows). ECs; Endothelial cells.