Astrocytic calcium release mediates peri-infarct depolarizations in a rodent stroke model

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Introduction

Stroke is one of the most common diseases and a leading cause of death and disability. cessation of cerebral blood flow (CBF) leads to cell death in the infarct core, but tissue surrounding the core has the potential to recover if local reductions in CBF are restored. In these areas, detrimental peri-infarct depolarizations (PIDs) contribute to secondary infarct growth and negatively affect stroke outcome. However, the cellular pathways underlying PIDs have remained unclear. Here, we have used in vivo multiphoton microscopy, laser speckle imaging of CBF, and electrophysiological recordings in a mouse model of focal ischemia to demonstrate that PIDs are associated with a strong increase of intracellular calcium in astrocytes and neurons.

We found that astroglial calcium elevations during PIDs are mediated by inositol triphosphate receptor type 2–dependent (IP3R2-dependent) release from internal stores. Importantly, IP3R2-deficient mice displayed a reduction of PID frequency and overall PID burden and showed increased neuronal survival after stroke. These effects were not related to local CBF changes in response to PIDs. However, we showed that the release and extracellular accumulation of glutamate during PIDs is strongly curtailed in IP3R2-deficient mice, resulting in ameliorated calcium overload in neurons and astrocytes. Together, these data implicate astroglial calcium pathways as potential targets for stroke therapy.

Results and Discussion

To identify peri-infarct cortex in mice, we created closed cranial windows in mice and subsequently subjected the animals to permanent middle cerebral artery occlusion (pMCAO). Regional CBF in the cortex under the window was measured before and for 4 hours after pMCAO induction using laser speckle contrast imaging in predefined cortical regions of interest (ROIs) (Supplemental Figure 1A and B; supplemental material available online with this article; doi:10.1172/JCI89354DS1). On the basis of these measurements, we defined the peri-infarct zone in our model as an area 2.5–3 mm lateral and 1.5 mm posterior to the bregma, in which CBF consistently remained between approximately 25% and 35% after pMCAO (Supplemental Figure 1C). Moreover, we detected the typical CBF signatures of PIDs, which consisted of a period of hypoperfusion followed by transient hyperemia (Supplemental Figure 1D).

Next, we performed multiphoton microscopy of this cortical area to study the cellular dynamics of PIDs. To this end, we labeled cortical astrocytes and neurons with the calcium indicator OGB-1

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type 2 (Ip3r2−/−, also known as Itpr2−/−) (14). IP3R2 is the predominant IP3 receptor subtype in astrocytes, but shows negligible expression in neurons (15). To identify astrocytes, we crossed Ip3r2−/− mice with Cx43-ECFP mice. We found that the calcium increase in astrocytes during PIDs was reduced by approximately 50% in Ip3r2−/− mice compared with controls (Figure 1C), while the velocity remained unchanged (Figure 1D). Thus, these data identify IP3-mediated release from internal stores as a main component of astroglial calcium changes during PIDs.

Surprisingly, we found that Ip3r2−/− mice also showed a strong reduction of the neuronal calcium amplitude during PIDs (Figure 1E), while the neuronal calcium velocity remained unchanged (Figure 1F), indicating that a significant part of the potentially deleterious (9) calcium overload in neurons during PIDs is determined by the amount of calcium released in astrocytes. To investigate the relationship between neuronal and astrocytic calcium, we performed high temporal and spatial resolution imaging and AM in mice expressing enhanced cyan fluorescent protein (ECFP) under control of the astrocyte-specific connexin 43 promoter (Cx43-ECFP) (13). Blood vessels were labeled with Texas Red dextran (Supplemental Figure 2, A and B, and Supplemental Video 1). Subsequently, we subjected these mice to pMCAO. To verify that the imaging region corresponded to peri-infarct tissue, we determined erythrocyte flux and velocity from line scans along the longitudinal axis of capillaries before and after pMCAO induction (Supplemental Figure 2C). Erythrocyte velocity dropped to 23.3% ± 7.1% compared with baseline, confirming that our measurements were performed in peri-infarct cortex. Following pMCAO, we detected repetitive PIDs in the imaging region (Figure 1, A and B). As previously reported (6–8), the wave of calcium changes during PIDs involved astrocytes and neurons and propagated through the imaging region (Figure 1, A and B, and Supplemental Video 2).

Next, to identify the origin and role of PID-related calcium elevations in astrocytes, we used mice lacking the IP3 receptor type 2 (Ip3r2−/−, also known as Itpr2−/−) (14). IP3R2 is the predominant IP3 receptor subtype in astrocytes, but shows negligible expression in neurons (15). To identify astrocytes, we crossed Ip3r2−/− mice with Cx43-ECFP mice. We found that the calcium increase in astrocytes during PIDs was reduced by approximately 50% in Ip3r2−/− mice compared with controls (Figure 1C), while the velocity remained unchanged (Figure 1D). Thus, these data identify IP3-mediated release from internal stores as a main component of astroglial calcium changes during PIDs.
found that the onset of neuronal calcium changes slightly preceded the onset in astrocytes, but that the main signal component in both cell types occurred in synchrony (Figure 1, G–I).

To determine the effects of altered calcium dynamics on overall PID burden, we subjected mice to pMCAO while measuring the intracortical direct current potential and regional CBF using laser Doppler flowmetry (Supplemental Figure 3, A and B). These experiments showed that the total number of PIDs, PID latency, and cumulative PID burden were significantly reduced in Ip3r2-/- mice (n = 14) compared with controls (n = 11 mice). (D–G) Infarct volumetry and cell densitometry 72 hours after MCAD revealed a reduced infarct size (n = 15 mice for each group) and a reduced density of dead neurons (quantified by Fluoro-Jade C [FJC]) (n = 15 mice in each group). Arrows in G indicate cortical neurodegeneration. Scale bar: 1 mm (H) Penetrating arterioles showed strong vasoconstriction during PIDs. Left panel shows the vasculature (labeled with Texas Red dextran) during pMCAO before PID; right panel shows the vasculature during PID. Scale bar: 50 μm. (I) Resliced image (indicated by the dashed line in H) used to determine the arteriolar diameter. Scale bar: 5 μm. Spatial measurements of Texas Red fluorescence (lower trace) demonstrate the diameter decrease during the PID. (J and K) The vascular response to PID, consisting of a vasoconstriction followed by a variable vasodilation, remained unchanged in both groups (n = 4 Cx43-ECFP Ip3r2-/- mice; n = 7 control mice). All P values were determined by Mann-Whitney U test.

Figure 2. IP3R2-mediated astroglial signaling reduces PID burden without affecting PID-related cerebrovascular changes. (A–C) Total number of PIDs, latency to first PID, and cumulative AUC as an indicator of overall PID burden were reduced in Ip3r2-/- mice (n = 14) compared with controls (n = 11 mice). (D–G) Infarct volumetry and cell densitometry 72 hours after MCAD revealed a reduced infarct size (n = 15 mice for each group) and a reduced density of dead neurons (quantified by Fluoro-Jade C [FJC]) (n = 15 mice in each group). Arrows in G indicate cortical neurodegeneration. Scale bar: 1 mm (H) Penetrating arterioles showed strong vasoconstriction during PIDs. Left panel shows the vasculature (labeled with Texas Red dextran) during pMCAO before PID; right panel shows the vasculature during PID. Scale bar: 50 μm. (I) Resliced image (indicated by the dashed line in H) used to determine the arteriolar diameter. Scale bar: 5 μm. Spatial measurements of Texas Red fluorescence (lower trace) demonstrate the diameter decrease during the PID. (J and K) The vascular response to PID, consisting of a vasoconstriction followed by a variable vasodilation, remained unchanged in both groups (n = 4 Cx43-ECFP Ip3r2-/- mice; n = 7 control mice). All P values were determined by Mann-Whitney U test.
PIDs were accompanied by a transient and propagating increase in iGluSnFR fluorescence (Supplemental Figure 6C), indicating glutamate release and diffusion near astrocytic surfaces. Near-astrocyte glutamate kinetics consisted of 2 components: a fast peak followed by a delayed slow rise (Supplemental Figure 6C). We compared PIDs in \( \text{Ip3r2}^{-/-} \) and WT control mice and found that the glutamate signals were significantly shorter in \( \text{Ip3r2}^{-/-} \) mice (Figure 3B). This difference was due to a shortening of the delayed slow-rise component of the glutamate signal, indicated by faster decay times than those in control mice (Figure 3B). To better understand the dynamics between astrocytic calcium elevations and glutamate, we labeled peri-infarct cortex of mice transfected with AAV-GFAP-iGluSnFR with the red-shifted calcium indicator Cal-590 (21), which resulted in colabeling of individual astrocytes with iGluSnFR and Cal-590 (Figure 3C). Our analysis of calcium and glutamate dynamics in individual astrocytes during PIDs revealed that the fast component of the

Figure 3. Astroglial IP3R2–mediated signaling contributes to glutamate kinetics during PIDs. (A) An average intensity projection of peri-infarct cortex showed specific and sufficient iGluSnFR expression in cortical astrocytes. The vasculature was visualized with Texas Red (TR) dextran. Scale bar: 50 \( \mu \)m. (B) There was a strong reduction of the duration of glutamate transients that was mostly caused by an attenuation of the second (sustained) phase of glutamate increase, indicated by a significantly shortened peak-to-baseline time and full duration at half-maximum (FDHM) (\( n = 48 \) cells from 9 \( \text{Ip3r2}^{-/-} \) mice; \( n = 70 \) cells from 10 control mice). (C–E) Simultaneous imaging of glutamate (iGluSnFR) and calcium (Cal-590) in individual astrocytes (arrows) showed that the initial glutamate rise preceded the astroglial calcium transient, but that this calcium transient coincided with the secondary glutamate increase (\( n = 25 \) astrocytes from 5 \( \text{Ip3r2}^{-/-} \) mice; \( n = 28 \) astrocytes from 5 control mice). \( \text{Ip3r2}^{-/-} \) mice showed a shortened glutamate signal and an attenuated calcium rise. (F) Spearman’s rank correlation (\( \rho \)) between glutamate and calcium signal durations (FDHM) in overlapping ROIs (yellow lines represent linear regression). (G–I) Inhibition of mGlur groups I and II by MPEP/LY341495 (100 \( \mu \)M and 50 \( \mu \)M, respectively) left the glutamate rise time unchanged, but resulted in significantly shorter FDHM and smaller astroglial calcium amplitudes (\( n = 28 \) cells from 4 mice vs. \( n = 25 \) cells from 4 mice). \( P \) values in B, E, and G–I were determined by Mann-Whitney U test.
iGluSnFR signal preceded astroglial calcium transients (Figure 3, D and E). This finding was consistent with our high-resolution imaging data (Figure 1, G–I) and indicated that the first component of the glutamate signal detected by astrocytic iGluSnFR may represent neuronal glutamate that induces a calcium rise in astrocytes, although iGluSnFR detects a mixture of neuronal and astroglial glutamate (22), and we cannot exclude the possible influence of different indicator kinetics. In turn, this calcium increase coincided with the delayed slower component of the glutamate signal (Figure 3D), and the durations of both signals were strongly correlated (Figure 3F), indicating a secondary glutamate release phase associated with astroglial calcium. Consequently, the attenuated calcium rise in Ip3r2−/− mice was also correlated with a shortened glutamate signal (Figure 3D–F), suggesting that the effects of astroglial IP3-mediated calcium signaling on neuronal calcium, PID threshold, and infarct volume may be mediated by modulation of glutamate release during PIDs. To further test this hypothesis and investigate the potential for pharmacological intervention, we topically applied a combination of drugs that inhibit the effects of neuronal glutamate on astroglial metabotropic glutamate receptors (22). This resulted in shortened glutamate decay times and strongly reduced astroglial calcium amplitudes during PIDs (Figure 3, G–I), supporting the notion that astroglial calcium contributes to secondary glutamate release.

In conclusion, these data suggest that by contributing to extracellular glutamate accumulation, IP3R2-mediated calcium release from astrocytic internal stores is a major determinant of PID burden, neuronal calcium overload, and ischemic neurodegeneration. Interestingly, IP3-gated calcium release from the endoplasmic reticulum is a trigger of glutamate release from astrocytes (19), although the exact mechanisms remain under debate (23). However, our data suggest that astrocytes are an important source of glutamate during PIDs and that this mechanism has previously unrecognized, yet profound effects on the frequency and overall burden of these deleterious depolarization waves. Hence, interference with these pathways may potentially lead to the development of novel treatment strategies to ameliorate PID-induced damage in acute brain disorders.

Methods

Further information can be found in the Supplemental Methods.

Statistics. Comparisons between 2 groups were conducted using a 2-sided Mann-Whitney U test. Correlation was calculated as Spearman’s rank correlation. All data were analyzed using GraphPad Prism 6 (GraphPad Software) and are expressed as the mean ± SEM or as Tukey’s box-and-whisker plots indicating the median, mean, interquartile range (IQR), and 1.5 IQR. A P value of less than 0.05 was considered statistically significant. Six animals were excluded from the analysis (one control mouse died during an imaging experiment; three Ip3r2−/− and two control mice died after MCAO before the infarct volumetry endpoint).

Study approval. All experiments were performed according to animal care guidelines and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz of North Rhine-Westphalia (Germany).

Author contributions

GCP conceptualized and supervised the study. CR performed the experiments. Both authors established the methodology, performed the data analysis, and wrote the manuscript.

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