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A-Kinase-Anchoring Protein (AKAP150) is expressed in astrocytes and upregulated in response to ischemia.

Aixa F. Rivera-Pagán¹, Miguel P. Méndez-González^{1,6}, David E. Rivera-Aponte¹, Christian J. Malpica-Nieves¹, Katya V. Melnik-Martínez², Astrid Zayas-Santiago³, Gerónimo Maldonado-Martínez⁴, Yaroslav M. Shuba⁵, Serguei N. Skatchkov^{1,2,*}, and Misty J. Eaton^{1,*}

¹Department of Biochemistry, Universidad Central del Caribe, Bayamón, Puerto Rico, United States of America

²Department of Physiology, Universidad Central del Caribe, Bayamón, Puerto Rico, United States of America

³Department of Pathology and Laboratory Medicine and Universidad Central del Caribe, Bayamón, Puerto Rico, United States of America

⁴Retrovirus Research Center, Universidad Central del Caribe, Bayamón, Puerto Rico, United States of America

⁵Bogomoletz Institute of Physiology and International Center of Molecular Physiology of the National Academy of Sciences of Ukraine, Kiev, Ukraine

⁶University of Puerto Rico, Natural Sciences Department, Aguadilla, Puerto Rico, United States of America

Abstract

A-kinase anchoring proteins, AKAPs, are scaffolding proteins that associate with kinases and phosphatases, and direct them to a specific submembrane site to coordinate signaling events. AKAP150, a rodent ortholog of human AKAP79, has been extensively studied in neurons, but very little is known about the localization and function of AKAP150 in astrocytes, the major cell type in brain. Thus, in this study, we assessed the localization of AKAP150 in astrocytes and elucidated its role during physiological and ischemic conditions. Herein, we demonstrate that AKAP150 is localized in astrocytes and is up-regulated during ischemia both in vitro and in vivo. Knock-down of AKAP150 by RNAi depolarizes the astrocytic membrane potential and substantially reduces by 80% the ability of astrocytes to take up extracellular potassium during ischemic conditions. Therefore, upregulation of AKAP150 during ischemia preserves potassium conductance and the associated hyperpolarized membrane potential of astrocytes; properties of

*Corresponding Authors: Misty J. Eaton, PhD, Department of Biochemistry, Universidad Central del Caribe, P.O. Box 60327, Bayamón, PR 00960-6032, USA, Phone: (787) 798-3001 ext. 2034, Fax: (787) 786-6285, mistyeaton1960@yahoo.com, misty.eaton@uccaribe.edu; Serguei Skatchkov, PhD Department of Biochemistry, Universidad Central del Caribe, P.O. Box 60327, Bayamón, PR 00960-6032, USA, Phone: (787) 798-3001 ext. 2035, Fax: (787) 786-6285, sergeis64@gmail.com; sergei.skatchkov@uccaribe.edu.

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astrocytes needed to maintain extracellular brain homeostasis. Taken together, these data suggest that AKAP150 may play a pivotal role in the neuroprotective mechanism of astrocytes during pathological conditions.

Keywords

Potassium uptake; membrane potential; middle cerebral artery occlusion; siRNA; cortical astrocytes

Introduction

A-kinase anchoring proteins (AKAPs) are scaffolding proteins that recruit and direct protein kinases and phosphatases to a specific submembrane site and contribute to the regulation of phosphorylation events (Michel and Scott, 2002, Carnegie et al., 2009). Many studies have focused on the localization and role of AKAPs in brain, specifically the role of AKAP150 in neurons (Rosenmund et al., 1994, Carnegie and Scott, 2003, Snyder et al., 2005, Chen and Kass, 2005, Dell'Acqua et al., 2006, Zhang et al., 2011). In neurons, AKAP150 has been extensively described in in vivo and in vitro model systems (Rosenmund et al., 1994, Carnegie and Scott, 2003, Snyder et al., 2005, Chen and Kass, 2005, Dell'Acqua et al., 2006, Schnizler et al., 2008, Zhang et al., 2011, Jeske et al., 2011). Histologically AKAP150 along with the human homologue AKAP79 have been described in neuronal density fractions, cell bodies and dendrites (Sandoz et al., 2006; Lilly et al., 2005; Glantz et al., 1992; Carr et al., 1992; Klauck et al., 1996; Dell'Acqua et al., 1998; Sfik et al., 2000; Gomez et al., 2002). Accumulating evidence demonstrates that these AKAPs associate with kinases and phosphatases and direct them to a specific subcellular compartment and modulate NMDAR, AMPAR, Kir2.1, TREK, TASK, TRP and other ion channel activity in neurons (Rosenmund et al., 1994; Dart and Layland, 2001; Gomez et al., 2002; Sandoz et al., 2006; Schnizler et al. 2008; Zhang et al., 2011; Jeske et al., 2011; Efendiev et al., 2013).

Despite the many advances in understanding the role of AKAP150 in neurons, there is a lack of knowledge about AKAP150 function in astrocytes. While given the anatomical location and abundance of astrocytes in cortex, in the literature, a single microscopy study was found showing AKAP150 immunoreactivity within glia (Lilly et al., 2005). However, it was a descriptive observation and the significance of their finding has yet to be determined. Therefore, the presence of AKAP150 in astrocytes and its importance during physiological and pathological conditions remains unknown. The goal of the present study was to address these questions both in vitro and in vivo by examining the localization of AKAP150 in astrocytes and assessing the role of AKAP150 on one of the key astrocytic functions, potassium uptake, during normal and pathophysiological ischemic conditions. Here we show that astrocytes express AKAP150 which is upregulated during ischemia and maintains potassium conductance and the associated hyperpolarized membrane potential of astrocytes.

Experimental Procedures

Animals:

Experiments were carried out in accordance with a protocol approved by the Universidad Central del Caribe Institutional Animal Care and Use Committee. Adequate measures were taken to minimize pain or discomfort of experimental animals. Postnatal 1-2 day old Sprague-Dawley rats were used for preparing primary astrocytes cultures and adult Sprague-Dawley rats (250 – 300 g) were used for transient middle cerebral artery occlusion (tMCAO) experiments.

Astrocyte primary cultures:

Primary cortical astrocyte cultures were prepared from neocortex of 1-2 day old rats as previously described (Kucheryavykh et al., 2007).

Simulated Ischemia:

Astrocytes were exposed to hypoxia/hypoglycemia conditions for 24 hours to simulate ischemia in vitro as previously described in Rivera-Pagán et al., (2015). Briefly, astrocytes were plated in petri dishes and to achieve hypoxia/hypoglycemic conditions the medium from cultures was removed, cells were gently rinsed and a bicarbonate-buffered balanced salt solution (BBSS) containing: 127mM NaCl, 3mM KCl, 19.5mM NaHCO₃, 1.5 mM NaH₂PO₄, 1.4mM MgSO₄, 1mM CaCl₂ and 2.5mM D-glucose and previously gassed with 5 minutes of 95%N₂ and 5%CO₂ was applied to the cells. Afterwards, cells were placed in a chamber flooded with 95% N₂ and 5%CO₂ and incubated at 37°C for 24 hours. Control cells were incubated in a BBSS solution containing 25mM D-glucose and incubated at 37°C for 24 hours under normoxic conditions (95% air and 5%CO₂). We have previously shown using electrophysiological techniques that astrocytes exposed to these conditions are viable (Kucheryavykh et al., 2009).

RNA interference by small double-stranded RNAs:

As we previously described (Kucheryavykh et al., 2007), the cells were transfected using HiPerfect transfection reagent (Qiagen, Germantown, MD, USA) and the Fast-Forward Protocol for Transfection of Adherent Cells with siRNA recommended by the manufacturer (Qiagen, S102000271). Briefly, 20nM of siRNA targeting AKAP150 and 12μL of HiPerfect were diluted in 100μL of culture medium without serum. The mix was incubated for 10 minutes at room temperature to allow the formation of transfection complexes. The complexes were then added to the cells in a drop-wise fashion giving a final volume of 2mL. The plate was gently swirled to evenly distribute the transfection complexes and cells were incubated at 37°C for four days. Cell were either used for electrophysiological studies or harvested and AKAP 150 protein expression was measured and compared in control (no transfection reagent), mock-transfected (transfection reagent (HiPerfect) without siRNA) and AKAP150 siRNA transfected astrocytes (transfection reagent (HiPerfect) with siRNA).

SDS-PAGE and Western Blotting Analysis:

Astrocytes were pelleted and resuspended in lysis buffer (pH 7.5) containing: (in mM) Tris-HCl 20, NaCl 150, EDTA 1.0, EGTA 1.0, PMSF 1.0, 1% Triton X-100, and an additional mixture of peptide inhibitors (leupeptin, bestatin, pepstatin, and aprotinin). Total protein of homogenates were determined with the Bradford protein assay (Bio-Rad), followed by addition of an appropriate volume of Urea sample buffer (62mM Tris/HCl pH 6.8, 4% SDS, 8M Urea, 20mM EDTA, 5% β -Mercaptoethanol, 0.015% Bromophenol Blue) to load 15 μ g of protein per lane. Next, samples were boiled in a water bath at 95 °C for 10 minutes, spun to pellet debris, and immediately run on 7% SDS-polyacrylamide gels. Western blotting was performed as previously described (Kucheryavykh et al., 2009) using rabbit polyclonal antibodies against AKAP150 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA; Cat# sc-10765, RRID:AB_2289482). Final detection was performed with enhanced chemiluminescence methodology (SuperSignal® West Dura Extended Duration Substrate; Pierce, Rockford, IL, USA) as described by the manufacturer, and the intensity of the signal was measured in a gel documentation system (Versa Doc Model 1000, Bio Rad, Hercules, CA, USA). In all cases, intensity of the chemiluminescence signal was corrected for minor differences in protein content after densitometry analysis of the India ink stained membrane.

Patch Clamp Electrophysiology: Voltage and K⁺-Steps:

Membrane potentials and currents were measured with the single electrode whole-cell patch-clamp technique. Two Narishige hydraulic micromanipulators (Narishige, MMW-203, Amityville, NY, USA) were used for (1) voltage-clamp recording, and (2) positioning a micropipette with 30 - 50 μ m tip diameter for the application of test solutions. A five valve system for fast drug application (MS Concentration Clamp; VS-2001, Vibraspec, Bear Island, ME, USA), controlled by a second computer, was connected to the outlets. Electrodes from borosilicate glass capillaries (1B150F-4, World Precision Instruments, Inc., Sarasota, Florida, USA) were pulled in four steps using a Sutter P-97 puller (Sutter Instruments Corporation, Novato, CA, USA). Electrodes filled with intracellular solution (ICS) containing (in mM): 120 K-Gluconate, 10 KCl, 20 NaCl, 2 MgCl₂, 0.3 SpermineCl, 1 Ca-EGTA, and 20 HEPES, pH adjusted to 7.2 with NaOH/HCl, had resistances of 4-6 M Ω ; after cell penetration, the access resistance was 10-15 M Ω , compensated by at least 75%. The polyamine spermine was used to keep vital conditions for Kir channel function in glia (Kucheryavykh et al., 2008; Olsen et al., 2015). The extracellular solution (ECS) contained (in mM): 145 NaCl, 3 KCl, 2 MgCl₂, 2.5 CaCl₂ and 10 HEPES. High frequencies (>1 kHz) were cut off, using an Axon Multiclamp-700B amplifier and a CV-7B headstage (Molecular Devices, San Jose, CA, USA), and digitized at 5 kHz through a DigiData 1322A interface (Molecular Devices). The pClamp 9 (Molecular Devices) software package was used for data acquisition and analysis.

The membrane potentials of cultures astrocytes were determined using whole-cell patch clamp recordings from cultured astrocytes. After determining their membrane potential, astrocytes were kept at holding potentials equal to their native resting membrane potentials to hold resting currents at zero and to avoid unaccounted ion redistribution between cell and environment before voltage protocols were applied. To assess the ability to clear potassium, whole cell current measurements in response to potassium steps were performed. Test

solutions were applied without interruption using as a baseline physiological 3mM $[K^+]$. Solutions with decreased extracellular $[K^+]$ 1mM for outward current or increased $[K^+]$ to 10mM for inward current within the physiological range were applied (Skatchkov et al., 1999). In these experiments we used a voltage step protocol as previously described (Skatchkov et al., 2006; Kucheryavikh et al., 2007; 2008; Inyushin et al, 2010).

Transient Middle Cerebral Artery Occlusion (tMCAO) to simulate stroke in vivo:

Adult Sprague-Dawley male rats (250-300 g) were anesthetized with 5% isoflurane for induction and 2% for maintenance and subjected to a tMCAO. Briefly, a ventral midline incision was made in the neck and the left common carotid artery exposed, the external carotid was isolated and permanently ligated as well as the pterygopalatine artery. The occipital and thyroid arteries were electrocoagulated. The blood flow was stopped by using artery clips and the external carotid was transected. A piece of monofilament suture (Doccol) was inserted into the lumen of the stump and advanced into the internal carotid artery until resistance was found, showing that it was in the middle cerebral artery (Ford et al., 2006). Restoration of blood flow was achieved 60 minutes later by withdrawal of the intraluminal monofilament. After 24 hours of reperfusion, animals were anesthetized with isoflurane, decapitated and brains were removed and sectioned by using a rat brain matrix to obtain coronal slices/blocks at 1.0 mm intervals. We corroborated that the tMCAO was effective by using Triphenyltetrazolium chloride (TTC) staining (5% TTC in PBS, pH7.4 for 15-20 minutes).

Immunohistochemistry:

Brains sections obtained using the rat brain matrix were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 2 hours at 4 °C and subsequently sectioned to 50µm using a vibroslicer (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). The 50µm sections were permeabilized for 30 minutes in PBS containing 0.3% Triton X-100, and 3% normal goat serum. The brain slices were co-incubated in polyclonal rabbit AKAP150 antibody (1/200; Santa Cruz Biotechnology) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody tagged with Cy3 (1/1000; Sigma-Aldrich, St. Louis, MO, USA; cat. # C9205) for 24 hrs at 4°C. After washing, slices were incubated in a mixture of biotinylated goat antirabbit IgG (1:5000) for 24 hours at 4°C. The slices were subsequently incubated in Avidin-FITC in PBS for 2 hours at room temperature. After washing in PBS, the slices were mounted on Superfrost plus microscope slides and coverslipped. As a control, sections were processed as described above with the omission of the primary antibody. Brain sections were visualized using an Olympus Fluoview FV1000 confocal microscope with 40× oil-immersion objectives. Immunostaining for AKAP150 (green labeling) and GFAP (red labeling) were visualized. We compared fluorescent labeling after tMCAO on the ipsilateral (lesion) side with contralateral side (control) in hippocampus and cortex. We also characterized colocalization between GFAP and AKAP150 in astrocytes using Image J.

Results

AKAP150 expression is up-regulated during ischemic-like conditions.

Using Western Blot, we determined that AKAP150 protein was not only present, but that AKAP150 protein expression was up-regulated ($2.0 \text{ fold} \pm 0.2 \text{ SEM}$) after 24 hours of hypoxia/hypoglycemic conditions in cultured astrocytes (Fig. 1, Left). To confirm our findings we knocked-down AKAP150 in cortical astrocytes by using AKAP150 siRNA. To perform this study we first determined the appropriate conditions for AKAP150 RNA interference. We found that transfection of astrocytes with 20nM of AKAP150 siRNA for 4 days effectively knocked-down 68% of AKAP150 protein expression as compared to mock-transfected control astrocytes (Fig. 1, Middle) and 65% of AKAP150 in astrocytes subjected to hypoxia/hypoglycemia (Fig. 1, Right). These results also provide confirmation that the AKAP150 antibody is selectively detecting AKAP150 in astrocytes.

AKAP150 is up-regulated in astrocytes after transient middle cerebral artery occlusion (tMCAO)

We next determined the localization and up-regulation of astrocytic AKAP150 in brain after tMCAO by performing immunohistochemical studies. We examined GFAP positive glial cells and determined qualitatively if AKAP150 co-localizes with GFAP, a marker of normal and particularly reactive glial cells. Double immunofluorescence labeling revealed localization of AKAP150 and GFAP in astrocytes (Fig. 2). Furthermore, we observed an increase in GFAP and AKAP150 expression on the lesioned (ipsilateral) side in hippocampus (Fig. 2A) and in the cortex (Fig. 2B) above the striatum where the lesion is located when compared with the contralateral side.

In this *in vivo* experiment, there are a number of different things going on at one time. 1) The number of GFAP reactive astrocytes is expected to increase in the brain hemisphere ipsilateral to the occlusion relative to the contralateral side. 2) AKAP150 expression may increase in both neurons and astrocytes in the brain hemisphere ipsilateral to the occlusion as well. In an attempt to best quantify the differences in AKAP150 expression in astrocytes in the cortex ($n=3$ rats), we randomly assigned numbers to confocal images obtained from 3 rats receiving tMCAO. An investigator (blinded to the treatment) manually counted the number of astrocytic soma in the confocal image as well as the number of yellow spots (areas of GFAP (red) and AKAP150 (green) overlap). The number of GFAP labeled astrocytes in the contralateral cortical area was 35.7 ± 1.9 (mean $\pm$ SEM; $n=3$), whereas that found in the cortical region ipsilateral to the occlusion was 54.0 ± 9.5 ($n=3$). The number of double labeled yellow spots was significantly increased in the cortical areas ipsilateral to the occlusion (106.7 ± 29.1 ; $n=3$) as compared with the contralateral (non-lesioned) brain hemisphere (24.0 ± 6.0 ; $n=3$). In addition, there was a significant difference in the ratio of double labeled spots to the number of GFAP-positive soma (0.67 ± 0.14 vs. 1.89 ± 0.25 for the contralateral and ipsilateral hemispheres, respectively) with more AKAP150 localizing to astrocytes on the lesioned side of the brain. Taken together, these *in vitro* and *in vivo* studies provide evidence that AKAP150 is expressed in astrocytes and, furthermore, AKAP150 protein expression is up-regulated *in vivo* during ischemia.

Knock-down of AKAP150 produces a depolarizing shift in the membrane potential of astrocytes subjected to hypoxia/hypoglycemia for 24 hours

Cultured cortical astrocytes display a bimodal distribution of membrane potential (McKhann et al., 1997, Kucheryavykh et al., 2007). At least two types of potassium channels contribute to the resting membrane potential of astrocytes. These include inwardly rectifying K⁺ channels (predominantly Kir4.1) and tandem pore domain K⁺ channels (such as TREK-1 and 2) (Neusch et al., 2006; Sandoz et al., 2006; Kucheryavykh et al., 2007; Djukic et al., 2007; Olsen et al., 2007; Kucheryavykh et al., 2009; Wu et al., 2013). It has been reported that AKAP150 may activate K⁺ channels (Dart and Leyland, 2001; Sandoz et al., 2006), therefore, we investigated whether or not AKAP150 contributes to the hyperpolarized membrane potential of astrocytes. We examined the distribution of membrane potentials in astrocytes subjected to control and hypoxia/hypoglycemic conditions with and without siRNA targeting AKAP150. In control astrocytes subjected to mock transfection (n=117) and control astrocytes transfected with AKAP150 siRNA (n=110), the distribution of membrane potentials was similar to those previously described (Kucheryavykh et al., 2007) with 68% and 65% of cells being in the predominant hyperpolarized population (more hyperpolarized than -55 mV), respectively. When mock transfected astrocytes were subjected to ischemia (n=101), the majority of cells (61%) remained in the hyperpolarized population. This suggests that potassium channels in astrocytes are functioning even after 24 hours of hypoxia/hypoglycemic conditions. In contrast, when astrocytes treated with siRNA targeting AKAP150 were subjected to hypoxia/hypoglycemia (n=89), the distribution of membrane potentials was altered towards depolarization (only 38% of the cells remained in the hyperpolarized population). This depolarization is also apparent when comparing the mean membrane potentials of astrocytes in the different groups (Control/Mock -62.2 mV ± 1.4 SEM, Control/siRNA -61.2 mV ± 2.0 SEM, Ischemia/Mock -57.9 mV ± 2.0 SEM and Ischemia/siRNA -47.1 mV ± 2.1 SEM) with the Ischemia/siRNA group being significantly depolarized as compared with all other groups (ANOVA followed by Tukey's; p<0.05). Taken together these findings suggest that AKAP150 may sustain potassium channel activity in astrocytes during ischemia to maintain the astrocytic membrane potential. Regulation of astrocytic membrane potential plays a fundamental role in the ability of the astrocyte to buffer extracellular potassium channel.

AKAP150 modulates K⁺ uptake by astrocytes

To assess the effect of AKAP150 on the ability of astrocytes to buffer extracellular K⁺, we measured the whole-cell currents reflecting K⁺ uptake/release in response to extracellular [K⁺] steps (Skatchkov et al., 1995; 1999; Zhou and Kimelberg, 2000; Kucheryavykh et al., 2007; Inyushin et al., 2010). The procedure was done during normal and hypoxia/hypoglycemic conditions in presence or absence of siRNA targeting AKAP150. We measured the outward currents induced by decreasing [K⁺]_o from normal 3 mM to 1 mM (hypokalemia) and inward currents induced by increasing [K⁺]_o from 3mM to 10mM (as a model of extracellular K⁺ excess that has been recorded during ischemia in cortex (Nedergaard and Hansen, 1993)). Figure 4 shows a summary of inward and outward currents in response to [K⁺]_o steps. Outward currents evoked in response to 1 mM [K⁺]_o were 27.6 pA ± 5.9 for cells in normal condition and 58.9 pA ± 10.9 for cells subjected to hypoxia/hypoglycemia. This response was significantly (more than three fold) reduced in cells

treated with AKAP150 siRNA (Current evoked was $8.3 \text{ pA} \pm 2.7$) as compared to control cells. In addition, we found a significant increase in inward current in response to $10 \text{ mM [K}^+]_o$ in cells subjected to hypoxia/hypoglycemic conditions for 24 hours. Currents evoked by $10 \text{ mM [K}^+]_o$ were $-68.2 \text{ pA} \pm 23.1$ for cells in normal conditions and $-149.7 \text{ pA} \pm 22.7$, for cells subjected to hypoxia/hypoglycemia. These responses were markedly reduced by 80% in cells treated with AKAP150 siRNA and subjected to hypoxia/hypoglycemic conditions for 24 hours ($-17.0 \text{ pA} \pm 4.0$). These results suggest that AKAP150 mediates the ability of potassium channels to enhance potassium buffering by astrocytes during ischemia.

Discussion

Astrocytes play a critical role in protecting neurons during pathological conditions by helping to maintain extracellular homeostasis and preventing neurotoxicity through glutamate uptake and potassium buffering (Neusch et al., 2006; Kucheryavykh et al., 2007; Djukic et al., 2007; Olsen et al., 2007; Kucheryavykh et al., 2009; Seifert et al., 2009; Olsen et al., 2015). In particular, potassium channels in astrocytes are not only responsible for K^+ uptake, but also have a key role in sustaining glutamate clearance from the synaptic space (Kucheryavykh et al., 2007; Djukic et al., 2007; Kucheryavykh et al., 2009; Inyushin et al., 2010; Wu et al., 2013). In this study, we provide important novel evidence which demonstrates that A-kinase anchoring protein, AKAP150, is up-regulated in astrocytes both *in vitro* and *in vivo*, and may contribute to the sustaining potassium channel activity in astrocytes during ischemia. Several lines of evidence that support this conclusion are presented below.

Histologically, AKAP150 as well as the human homologue AKAP79 have been described in neuronal density fractions, cell bodies and dendrites (Glantz et al. 1992, Carr et al. 1992, Klauck et al. 1996, Dell'Acqua et al. 1998, Sandoz et al., 2006). Accumulating evidence demonstrates that these AKAPs associate with kinases and phosphatases and direct them to a specific subcellular compartment and modulate ion channel activity in neurons (Dart and Leyland, 2001, Sandoz et al., 2006, Schnizler et al., 2008, Zhang et al., 2011). Despite the many advances in our understanding of AKAP150 in neurons, little is known about AKAP150 in astrocytes. In the literature, a single microscopy study noted AKAP150 immunoreactivity within glia (Lilly et al., 2005). However, it was a descriptive observation and the significance of their finding has yet to be determined. The present study confirms the localization of AKAP150 in astrocytes and extends the finding to examine changes in AKAP150 protein expression after ischemia.

Herein, we examined changes in AKAP150 protein expression in cultured astrocytes after exposure to hypoxia/hypoglycemic conditions for 24 hours and observed an increase in AKAP150 protein expression. Before examining the functional consequences of AKAP150 in astrocytes, we first demonstrated the specificity of our antibody by knocking-down AKAP150 in cultured astrocytes using siRNA targeting AKAP150 which, in turn, significantly decreased AKAP150 protein expression. Next, we performed immunohistochemical studies that showed double labeling of GFAP positive astrocytes expressing AKAP150. Moreover, AKAP150 immunolabel was qualitatively increased in astrocytes in the infarction side of the brain 24 hours after tMCAO. Taken together, these

data describe for the first time in both *in vitro* and *in vivo* systems that AKAP150 is located in astrocytes and is up-regulated after ischemia.

We assessed the functional consequences of reducing AKAP150 expression in astrocytes and found that AKAP150 is essential for setting the hyperpolarized membrane potential of cultured cortical astrocytes during ischemic conditions. Our findings demonstrate that control astrocytes in presence and absence of siRNA targeting AKAP150 have a bimodal distribution of their membrane potentials where most of the cells are in the hyperpolarized population in agreement with previous studies (D'Ambrosio et al., 1997, McKhann et al., 1997, Zhou and Kimelberg, 2000, Bolton et al., 2006, Kucheryavykh et al., 2007). This suggests that the presence or absence of AKAP150 in control astrocytes is of little consequence to potassium channel function and astrocytic membrane potential. In contrast, a striking difference was found when comparing mock transfected cells and cells treated with AKAP150 siRNA in astrocytes subjected to hypoxia/hypoglycemia for 24 hours. Down-regulation of AKAP150 significantly depolarized the resting membrane potential of “ischemic” cells suggesting that AKAP150 may sustain potassium channel activity in the astrocytic membrane during ischemic conditions to maintain the hyperpolarized membrane potential, and stimulate key astrocytic functions essential for neuronal viability, such as potassium uptake from the extracellular space.

The very close location of glial cells to synaptic regions and to ensheath synaptic clefts (Araque et al., 1999; Rusakov and Dityatev, 2014) facilitates their ability to regulate extracellular $[K^+]$ around neurons by potassium spatial buffering (Orkand et al., 1966) and potassium siphoning (Newman et al., 1984). Elevation of extracellular K^+ shifts E_K towards potentials more positive than resting, thereby favoring K^+ influx through Kir4.1 potassium channels under normal conditions (Neusch et al., 2006; Kucheryavykh et al., 2007; Olsen et al., 2007; Seifert et al., 2009; Olsen et al., 2015) and through members of the tandem pore family of K^+ channels (such as TREK channels) particularly during conditions of stress (such as during ischemia) (Kucheryavykh et al., 2009; Rivera-Pagán et al., 2015). AKAP150 has been reported to modulate K^+ channel activity (Henke et al., 2012; Nystoriak et al., 2014; Nieves-Cintrón, et al., 2016). Therefore, in this study we evaluated the ability of cultured astrocytes to take up potassium in presence and absence of AKAP150 by measuring whole-cell currents in response to extracellular $[K^+]_o$ steps in both control and ischemic conditions.

Herein, we provide direct evidence that AKAP150 contributes to astrocytic K^+ buffering capabilities during ischemia. Our study revealed that knock-down of AKAP150 strongly reduces inward current in response to elevated extracellular $[K^+]_o$ in astrocytes subjected to hypoxia/hypoglycemia for 24 hours. Based on these findings, we propose that astrocytic potassium channels are activated by AKAP150 to stimulate potassium uptake during ischemia and may help to maintain the extracellular K^+ concentrations in the penumbra area low during ischemia, therefore, potentially contributing to neuroprotection.

The consequences of ischemia in brain have been well described (for review see Janardhan and Qureshi, 2004). Cessation of local blood flow deprives neurons and glial cells of oxygen and glucose; thus cells fail to generate sufficient ATP resulting in energy failure.

Consequently, ionic gradients are lost and within minutes extracellular homeostasis deteriorates. The extracellular ionic changes include decreased Na^+ , Cl^- and Ca^{2+} as well as increased extracellular K^+ and H^+ . These changes are correlated with depolarization of the neuronal membrane potential. Glutamate is released, reuptake processes are impaired, and glutamate binds to its postsynaptic receptors and promotes excitotoxic processes (Lo et al. 2005). Excitotoxicity due to glutamate receptor overactivation is one of the key mediators of neuronal death after an ischemic insult (Choi and Rothman, 1990). In addition, astrocytes in the brain swell in response to acidification and increased extracellular glutamate levels.

In the present experiment we saw upregulation of AKAP150 in both ischemic brain and in cultured astrocytes that were exposed to anoxia and hypoglycemia for 24 hours. Although the trigger for upregulation of AKAP150 has not been identified, the cultured astrocyte model is simpler than the brain and only some of the changes that occur in brain in response to MCAO are seen in the cultured astrocyte model. The astrocytes are exposed to anoxic conditions for 24 hours. During this period they are placed in a BBSS which initially contains 2.5 mM glucose and it has been previously shown that within 24 hours of anoxic conditions (Kelleher et al., 1993), the concentration of glucose becomes negligible (near zero), therefore the lack of oxygen and glucose seen in ischemic brain are also seen in the cultured astrocytes. Other similarities to the in vivo situation include swelling of astrocytes and liberation of polyunsaturated fatty acids such as arachidonic acid (Yu et al., 1989). Since the consequences of ischemia are complex, future experiments are needed to determine which factor or factors may regulate AKAP150 expression either directly or indirectly.

Although the present study did not seek to identify the potassium channel or channels modulated by AKAP150, Sandoz et al. (2006) have previously demonstrated that AKAP150 binds to and modulates TREK-1 and TREK-2 channel activity. In a heterologous expression system, they found that AKAP150 modulates TREK channel activity from being a low activity outwardly rectifying K^+ channel into a robust open leak channel (Sandoz et al., 2006). Furthermore, studies of TREK-1 and TREK-2 channels in astrocytes indicate that these channels are functionally up-regulated during ischemia (Gnatenco et al., 2002, Kucheryavykh et al., 2009, Zhou et al., 2009, Wang et al., 2012, Wu et al., 2012, Rivera-Pagán et al., 2015) and contribute to neuroprotection by maintaining extracellular homeostasis of glutamate and potassium (Kucheryavykh et al., 2009, Wu et al., 2012). These findings suggest that TREK channels are a possible target of AKAP150 in ischemic conditions. Nevertheless, further studies need to be performed to corroborate this possibility.

In summary, the present study confirms that AKAP150 is present in astrocytes. Moreover, it demonstrates that AKAP150 in astrocytes is up-regulated during ischemia. Furthermore, our study reveals AKAP150 modulates the astrocytic membrane potential during ischemic conditions. Finally, knock-down of AKAP150 decreases the ability of astrocytes to buffer extracellular potassium. Taken together, our findings suggest that AKAP150 plays a critical role in the mechanism involved in astrocyte regulation of extracellular concentrations of neuroactive substances such as potassium during pathological conditions

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Abbreviations

AKAP	A-Kinase-Anchoring Protein
tMCAO	transient middle cerebral artery occlusion
BSS	bicarbonate-buffered balanced salt solution

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Highlights

- The present study confirms that AKAP150 is present in astrocytes.
- Moreover, AKAP150 in astrocytes is up-regulated during ischemia.
- AKAP150 modulates the astrocytic membrane potential during ischemic conditions.
- Knock-down of AKAP150 decreases the ability of astrocytes to buffer extracellular potassium during ischemic conditions.

Fig. 1. AKAP150 expression in cultured astrocytes during control and ischemia-like conditions. By using Western Blot, levels of AKAP150 protein were determined in control astrocytes and astrocytes exposed to hypoxia/hypoglycemia for 24 hours (Left, n= 4). Quantification of the effect of AKAP150 siRNA on protein levels in astrocytes 4 days after transfection with 20 nM siRNA during normal (Middle, n=3) and hypoxia/ hypoglycemia for 24 hrs (Right, n=3). AKAP150 was detected as a band of around 150KDa which is consistent with the predicted molecular weight. The graph displays the quantification of the relative chemiluminescence intensity $\pm$ standard error of the mean (SEM). The asterisks indicate a significant difference from control (t-test; $p < 0.05$). Representative Western blots are shown above the graph. Data are expressed relative to control.

Fig. 2. Localization of AKAP150 protein *in vivo* in astrocytes after tMCAO.

Immunostaining for AKAP150 (green labeling) and GFAP (red labeling) in hippocampus and cortex after tMCAO. Representative images show a qualitative increase of AKAP150 levels in hippocampus (A) and cortex (B) on the ipsilateral (lesion) side of the brain. White arrows point to astrocytic cell bodies and processes. Insets show higher magnification of the merged image to highlight colocalization between GFAP and AKAP150 in astrocytes. Note: There is an appreciable increase of AKAP150 immunoreactivity in astrocytes on the ipsilateral (lesion) side of the brain.

Fig. 3. Distribution of the Membrane Potentials of Control and AKAP150 siRNA transfected astrocytes with and without exposure to hypoxia/hypoglycemia.
A. Control (mock-transfected) astrocytes in normoxic conditions. B. Astrocytes transfected with AKAP150 siRNA (20 nM) in normoxic conditions. C. Control (mock-transfected) astrocytes exposed to hypoxia/hypoglycemia for 24 hours. D. Astrocytes transfected with AKAP150 siRNA (20 nM) exposed to hypoxia/hypoglycemia for 24 hours. In all cases, astrocytes were transfected 4 days prior to electrophysiology studies. Knock-down of AKAP150 in astrocytes exposed to hypoxia/hypoglycemia for 24 hours alters the distribution of the resting membrane potentials towards depolarization.

Fig.4. Suppression of AKAP150 modulates ability of astrocytes to buffer K⁺.

Mock-transfected astrocytes and astrocytes transfected with AKAP150siRNA were subjected to control (normoxic) or hypoxic/hypoglycemic conditions for 24 hours and subsequently utilized for electrophysiology. Cells were bathed in a solution containing 3 mM [K⁺]_o and this solution was changed to either 1 or 10 mM [K⁺]_o. The currents induced by altering the external concentrations of K⁺ were measured using the whole-cell voltage-clamp technique. (Control/Mock n=14, Control/siRNA n=14, Ischemia/Mock n= 7 and Ischemia/siRNA n=8). Results are expressed as mean current (pA) ± standard error of the mean (SEM). The * indicates a significant difference between Ischemia Control and Ischemia siRNA and ** indicates a significant difference between Control Mock and ischemia Mock (ANOVA followed by Tukey's; p<0.05).