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The Speed of Swelling Kinetics Modulates Cell Volume Regulation and Calcium Signaling in Astrocytes: A Different Point of View on the Role of Aquaporins

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Abstract

Regulatory volume decrease (RVD) is a process by which cells restore their original volume in response to swelling. In this study, we have focused on the role played by two different Aquaporins (AQPs), Aquaporin-4 (AQP4), and Aquaporin-1 (AQP1), in triggering RVD and in mediating calcium signaling in astrocytes under hypotonic stimulus. Using biophysical techniques to measure water flux through the plasma membrane of wild-type (WT) and AQP4 knockout (KO) astrocytes and of an astrocyte cell line (DI TNC1) transfected with AQP4 or AQP1, we here show that AQP-mediated fast swelling kinetics play a key role in triggering and accelerating RVD. Using calcium imaging, we show that AQP-mediated fast swelling kinetics also significantly increases the amplitude of calcium transients inhibited by Gadolinium and Ruthenium Red, two inhibitors of the transient receptor potential vanilloid 4 (TRPV4) channels, and prevented by removing extracellular calcium. Finally, inhibition of TRPV4 or removal of extracellular calcium does not affect RVD. All together our study provides evidence that (1) AQP influenced swelling kinetics is the main trigger for RVD and in mediating calcium signaling after hypotonic stimulus together with TRPV4, and (2) calcium influx from the extracellular space and/or TRPV4 are not essential for RVD to occur in astrocytes. Main Points: (1) The speed of swelling kinetics is the main trigger for Regulatory Volume Decrease (RVD) and for calcium response in astrocytes; (2) Calcium influx from the extracellular space and TRPV4 are not essential for RVD.

Keywords

AQP4; AQP1; RVD; cell swelling; hypotonic stimulus

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Additional Supporting Information may be found in the online version of this article.

Introduction

Under physiological conditions, cells are able to regulate their volume to balance the perturbations due to osmotic pressure (Hoffmann et al., 2009), which constantly challenge intracellular and extracellular osmolarity, causing cell swelling or shrinkage. Upon exposure to anisosmotic extracellular fluids or to altered intracellular osmolarity (Hoffmann and Dunham, 1995; Lang et al., 1998; Macknight et al., 1994; Okada et al., 2001; Russell, 2000) the activity of ion channels and transporters promotes efflux (regulatory volume decrease, RVD) or influx (regulatory volume increase, RVI) of ions and water, resulting in shrinkage or swelling of the cells back to original levels (Lang et al., 1998). Knowledge of the RVD molecular mechanism is particularly important in the CNS since the astrocyte machinery for RVD is partially or completely suppressed under pathological conditions (Kimelberg, 1992; Mongin and Kimelberg, 2005). For instance, after reduced blood flow associated with a stroke, in brain tumors or during trauma, abnormal swelling of perivascular astrocyte processes is associated with the destruction of the blood-brain barrier (BBB) leading to vasogenic brain edema. Abnormal astrocyte swelling is also induced by intracellular acidification, states associated with energy depletion and hypothermia. Under these circumstances, astrocyte swelling itself may cause ischemia due to compression of small vessels (Kimelberg et al., 1995). It has been suggested that astrocyte RVD is controlled by a mechanism dependent on cytosolic calcium elevation, although calcium fluctuations in response to a hypotonic stimulus are not always present in diverse cell types and tissues (McCarty and O'Neil, 1992). Moreover, while astrocytes exhibit a rise in intracellular calcium concentration in response to hypotonic stimulus (Morales-Mulia et al., 1998; O'Connor and Kimelberg, 1993), the role of calcium as a triggering signal of RVD is still a matter of debate (Benfenati et al., 2011; Morales-Mulia et al., 1998; O'Connor and Kimelberg, 1993; Sanchez-Olea et al., 1995).

The speed of cell swelling and shrinkage can be significantly increased by the expression of water channel proteins called Aquaporins (AQPs), allowing bidirectional water flow only depending on the osmotic and hydrostatic gradients across the cell plasma membrane (Agre et al., 2002; Verkman, 2000, 2002a). The major CNS water channel is Aquaporin-4 (AQP4), highly concentrated at the astrocyte foot processes at the BBB and forming the glial-limiting membrane. AQP4 is expressed as two major isoforms, called AQP4-M1 and AQP4-M23, although other minor isoforms have been cloned (De Bellis et al., 2014; Moe et al., 2008) but their function is still unknown. Interestingly, it has been reported that the AQP4e isoform is vesicle associated and that hypotonic stimulation triggers transient changes in AQP4 plasma membrane localization (Potokar et al., 2013) aimed at increasing *ad hoc* the plasma membrane permeability in this situation.

The co-localization of the AQP4 and Kir4.1 potassium channel on astrocyte foot-processes has led to the hypothesis of their functional relationship (Amiry-Moghaddam and Ottersen, 2003; Connors et al., 2004; Manley et al., 2004; Nagelhus et al., 2004; Nicchia et al., 2005; Walz, 1987). However, the role of AQP4 in potassium homeostasis is debated and controversial. While studies performed using AQP4 null mouse brain have showed slowed accumulation of potassium in brain extracellular space (ECS) during neuro-excitation

(Binder et al., 2006; Strohschein et al., 2011), and slowed clearance of potassium from the ECS after neuro-excitation (Padmawar et al., 2005; Strohschein et al., 2011), Zhang and Verkman (2008) have later provided evidence against AQP4-dependent astrocyte potassium permeability. Interestingly, AQP4 also plays unconventional roles in astrocyte migration and changes in morphology (Nicchia et al., 2003, 2008; Saadoun et al., 2005). Under CNS pathological conditions associated with brain edema, such as injury, stroke, tumors, and others, AQP4 facilitates water transport into and out of the brain and therefore plays a crucial role in brain edema formation and resolution (Verkman, 2012). Abnormal swelling of astrocyte processes is reported for mouse models lacking AQP4 (Amiry-Moghaddam et al., 2004; Frigeri et al., 2001), implying a role in the regulation of cell volume at the glial endfeet.

Cell swelling experiments have frequently been used as a surrogate model for membrane stretch (Hammami et al., 2009). In this study we have focused on the role played by two different Aquaporins (AQPs), AQP4 and AQP1, in triggering RVD and in mediating calcium signaling in astrocytes exposed to hypotonic stimulus. We have also evaluated the role played by TRPV4-mediated calcium influx in the activation of RVD.

Materials and Methods

Ethics Statement

All experiments conformed to international guidelines on the ethical use of animals and were designed to minimize the number of animals used and their suffering (Italian Health Department Approved Project n°100/2014-B). The mice used here were bred in the approved facility at the University of Bari. Mice were kept under a 12-h dark to light cycle, at constant room temperature and humidity (22°C±2°C, 75%), with food and water *ad libitum*, and supplied with environmental enrichment materials, such as toys and shelters.

Animals

AQP4 KO pups with a CD1 genetic background (Basco et al., 2013) and age matched controls were used for astrocyte primary cultures prepared as described below.

Astrocytes Primary Cultures

Mouse astrocyte primary cultures were prepared from newborn pups as previously described (Nicchia et al., 2000). Cells were cultured in DMEM-Glutamax medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, and maintained at 37°C in a 5% CO_2 incubator. All the cell culture products were purchased from Thermoscientific (http://www.thermoscientific.com).

Cell Cultures and Transfection

The DI TNC1 cell line, established from primary cultures of type 1 astrocytes from brain diencephalon tissue of 1-day-old rats, was purchased from American Tissue Culture Collection (http://www.lgcstandards-atcc.org). DI TNC1 cells were cultured and maintained as described previously (Mola et al., 2009). To generate DI TNC1 cells stably transfected with AQP4 or AQP1, pmCherry-N1 human AQP4-M23 and pTarget human AQP1 constructs

were used, respectively. Cells were transfected using Lipofectamine 2000 (http://www.thermoscientific.com) following the manufacturer's instructions. pBABE-puro vector (http://www.addgene.org) was used to confer puromycin resistance.

Antibodies

The following primary antibodies were used: goat anti-AQP4 polyclonal (http://www.scbt.com) and mouse anti-GFAP (https://www.sigmaaldrich.com). The following secondary antibodies were used: AlexaFluor488 donkey anti-goat and AlexaFluor647 donkey anti-mouse (http://www.thermoscientific.com) for immunofluorescence, and horseradish peroxidase (HRP) conjugated donkey anti-goat and donkey anti-mouse IgG (http://www.scbt.com) for Western blot.

Immunofluorescence

Primary astrocyte cultures were plated on coverslips and fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), and permeabilized with 0.3% Triton X-100 in PBS. After blocking with 1% BSA in PBS, cells were incubated with primary antibodies for 2 h at RT. After washings in PBS, cells were incubated for 1 h at RT with Alexa conjugated secondary antibodies. Coverslips were mounted on slides, using a mounting medium (PBS, 50% Glycerol, 0.1% N-Propil-Gallate) and examined by using a confocal microscope (TCS SP3, Leica). Once captured, the auto contrast function was applied to all the whole images using Adobe Photoshop CS5 in order to create a more accurate tonal and color correction workflow.

Western Blot Analysis

Astrocyte primary cultures were solubilized in at least ten volumes of RIPA buffer (25 mM Tris–HCl, pH 7,6; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0,1% SDS) added with a cocktail of protease inhibitors (https://lifescience.roche.com). The lysis was performed on ice for 1 h and the samples were then centrifuged at 22,000*g* for 45 min. The protein content of the supernatant was measured with a bicinchoninic acid (BCA) Protein Assay Kit (http://www.thermoscientific.com). Equal amounts of protein samples were separated by 12% Tris-Glycine-SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (http://www.merckmillipore.com/). Membranes with blotted proteins were incubated with primary antibodies, washed, and incubated with peroxidase-conjugated secondary antibodies. Reactive proteins were revealed with an enhanced chemiluminescent detection system (http://www.thermoscientific.com) and visualized on a Versadoc imaging system (http://www.bio-rad.com).

Osmotic Permeability Assays for Cell Swelling and RVD Analysis

TIRF Assay for Cell Swelling Analysis—Cells grown on 20 mm diameter round glass coverslips were washed with DPBS (0.9 mM $CaCl_2$; 0.5 mM $MgCl_2$; 2.7 mM KCl; 1.5 mM KH_2PO_4; 138 mM NaCl; and 8 mM Na_2HPO_4) and incubated with 1 μM of Calcein-AM (http://www.thermoscientific.com) for 45 min at RT. To determine AQP4-dependent water permeability, the osmotic properties of astrocytes were analyzed by total internal reflection fluorescence (TIRF) microscopy, measured in response to the osmotic gradient (Nicchia et

al., 2008). TIRF microfluorimetry allows measuring continuously the volume of adherent cells whose fluorescence is excited by the TIR evanescent field in a thin section (~150 nm) of cytosol at the cell-glass coverslip interface. Because the total number of fluorophores in the cytosol is constant, the fluorophore concentration and therefore the emitted fluorescence is inversely related to the cell volume (Farinas et al., 1995) when 1 μM of Calcein-AM is used. Water permeability was measured using a Nikon Laser TIRF setup equipped with a 488 nm Argon laser mounted on a Nikon TE2000U Microscope that also allows phase-contrast and epifluorescence techniques to be combined with TIRF technology. An incidence angle greater than the critical angle was achieved by the use of a 60× CFI Plan Apo of 1.45 numerical aperture. The coverslips were mounted in a custom perfusion chamber, as described by Solenov et al. (2004), designed for rapid solution exchange without causing cell detachment. Astrocytes in isotonic DPBS were perfused with hypotonic solution (100 mOsm L^{-1}, obtained by adding the appropriate amount of NaCl to NaCl-free DPBS) at high perfusion rate (40 mL min^{-1} with 0.4 s of exchange time between a colorless and dye-containing aqueous solution) at 10°C. The influx of water induced astrocyte swelling and a consequent reduction of Calcein-AM emitted fluorescence. Being WT and KO astrocytes characterized by a comparable morphology, and subjected to the same osmotic gradient, the osmotic properties of their plasma membrane were characterized by comparing the time constant for swelling, obtained from the experimental data, fitted to a exponential function (one phase decay).

Fluorescence-quenching Assay for Cell Swelling and RVD Analysis—Cells were seeded on black, clear bottom 96-well-plates (Corning, NY) at a density of 12,000 cells per well and used 24 h after plating. Nearly 80–85% confluent cells were washed with DPBS and incubated at 37°C for 45 min with 10 μM of Calcein-AM as previously described (Mola et al. 2009). At 10 μM concentration, cytosolic Calcein-AM is quenched by intracellular components (proteins or salts) whose concentrations change upon cell swelling and shrinking. Therefore, differently from the TIRF assay, the measured changes in fluorescence are directly proportional to changes in cell volume (Solenov et al., 2004). Calcein-AM fluorescence was recorded on a Flex Station3 plate reader equipped with an integrated automatic liquid handling module (http://www.moleculardevices.com) able to transfer compounds from a source plate to the assay plate during data acquisition. Cells were rinsed in isotonic DPBS and hypotonicity was applied 15 s after the beginning of each reading by adding an equal volume of NaCl-free DPBS in order to obtain 150 mOsm L^{-1}. The direct addition of the hypotonic solution to the preexisting isotonic solution caused an immediate hypotonic stimulus. Time course fluorescence data following mixing of cells were recorded at 37°C over the indicated period (100 s or 20 min) in order to record the swelling phase and the RVD phase. Data acquisition was performed using SoftMax Pro software, and the data were analyzed with Prism (Graph Pad) software. Being WT and KO astrocytes, as well as AQP transfected and WT-DI TNC1, characterized by a comparable morphology, and subjected to the same osmotic gradient, the osmotic properties of their plasma membrane were characterized by comparing the time constant (for the swelling-phase and the RVD phase) obtained by fitting the data with an exponential function. The RVD was also expressed as a percentage of volume recovery calculated from the maximum level of

fluorescence after exposure to hypotonic solution and the level of fluorescence reached after the RVD.

Intracellular Calcium Measurements

The kinetic of intracellular calcium concentration was measured using the imaging technique based on the use of a Nikon ECLIPSE TE 2000-S microscope and a FlexStation3 plate reader.

Calcium Imaging—Intracellular calcium measurements were performed as previously described (Scemes, 2008) at RT. Briefly, primary cultured astrocytes grown on confocal dishes were loaded with the ratiometric calcium indicator, Fura-2-AM (8 μM; Molecular Probes) for 40 min and then rinsed in DPBS. The hypotonic stimulus was induced 15 s after the beginning of each reading by adding an equal volume of NaCl-free DPBS in order to obtain 150 mOsm L^{-1}. Measurements of calcium concentration in single cells were performed using an inverted fluorescence microscope (Nikon ECLIPSE TE 2000-S) equipped with a cooled CCD camera controlled by the Metafluor 4.6 software (Universal Imaging, Downingtown, PA). For each experiment around 30 cells per coverlip were analyzed.

Fura-2 ratio values were translated into intracellular calcium concentrations according to an *in vitro* calibration curve of the form: [calcium]In=KD{$(R-R_{min})/(R_{max}-R)$} (F380min/F380max), where [calcium]In is the calculated intracellular calcium concentration, KD is the dissociation constant of free-calcium for Fura-2 [KD=224 nM; (Grynkiewicz et al., 1985)], R is the ratio intensity, R_{min} is the ratio of the intensity obtained at zero calcium, R_{max} is the ratio of the intensity at saturated calcium, F380min is the fluorescence intensity measured with zero calcium at 380 nm, and F380max is the fluorescence intensity measured with saturated calcium at 380 nm.

Microplate Reader—Twenty four hours after plating in 96-well black-walled, clear bottom plates, cells were loaded with 8 μM Fura-2-AM in DMEM growth medium for 30 min at 37°C. Cells were then washed with DPBS and stabilized in the same buffer for 10 min at 37°C. Rapid changes in intracellular calcium levels in response of extracellular stimuli were monitored using a FlexStation3 plate reader thermostatically regulated at 37°C. The hypotonic stimulus was induced 40 s after the beginning of the data acquisition by adding an equal volume of NaCl-free DPBS in order to obtain 150 mOsm L^{-1} and the fluorescence was recorded for 200 s. Compounds were added from a 96-well reservoir plate with pipette heights, fluid transfer volume and rate of addition optimized to minimize disturbance of the cells while ensuring rapid mixing. Fluorescence response was measured using the ratio peaks RFU at 340 and 380 nm (F340/F380) and relative timing. Data analysis was performed using SoftMaxPro and Prism (Graph Pad).

Cyclopiazonic Acid Treatment

The content of intracellular calcium stores was assessed by using cyclopiazonic acid (CPA), an inhibitor of SERCA pump. Fura-2-AM loaded cells were perfused with 50 μM CPA in

calcium- and magnesium-free DPBS added with 1 mM EGTA. The cells were perfused with regular DPBS after 300 sec of recording to evaluate calcium influx.

Gadolinium and Ruthenium Red Treatment

Gadolinium and Ruthenium Red were used at 10 and 20 μM to assess the contribution of membrane ion channels to the swelling-induced intracellular calcium response and RVD. Cells were exposed to each drug 5 min prior to the functional analysis described above. The possibility that Gadolinium and Ruthenium Red affect the fluorescence over time of Fura-2-AM or Calcein-AM loaded cells was ruled out by measuring their fluorescence before and after the addition of the pharmacological agents (Supp. Info. Fig. 1).

Statistical Analysis

Data are expressed as mean±SE of the number of experiments (n) indicated in the figure legend. In all the assays “n” is referred to the number of independent experiments performed on different cell preparations. For each experiment at least three to four different coverslips or wells were analyzed. Statistically significant differences were computed using one-way Anova or *t* test, the significance level being set at $P<0.05$.

Results

AQP4 and AQP1 Mediated Cell Swelling Accelerates Regulatory Volume Decrease (RVD)

WT and AQP4 KO primary cultured astrocytes were used to assess the rate of water influx conferred by AQP4 water channels and its contribution to RVD. Figure 1A–C shows representative Western blot, Immunostaining, and functional experiments performed exclusively to characterize astrocytes used in this study in terms of AQP4-dependent water permeability. The endogenous AQP4 expression, its plasma membrane localization in WT astrocytes and its absence in AQP4 KO are shown in Fig 1A,B, respectively. In line with previous reports (Nicchia et al., 2005; Solenov et al., 2004), WT and KO astrocytes exhibited a similar morphology (data not shown). The time constant of astrocyte swelling measured by TIRF analysis showed that, at 10°C, AQP4 KO astrocytes displayed a significantly reduced rate of water transport with a 4-fold increase in the time constant of cell swelling (6.57±1.36 s for WT *vs* 24.39±2.19 s for KO astrocytes, Fig. 1C), a result that is in agreement with previous reports (Nicchia et al., 2005; Solenov et al., 2004).

TIRF is the elective technique used to study the biophysics of AQP mediated water transport, since the experiments are performed at 10°C and at a high perfusion rate (Solenov et al., 2004). However, the low temperature and fast perfusion are not-physiological parameters and this seems to interfere with the cell volume regulation, probably by acting on shear stress activated channels (Kuipers et al., 2012). No RVD is in fact detectable during cell volume measurements performed by TIRF (data not shown). For this reason the RVD analysis was performed using a different water transport assay, already shown to be suitable for cell volume regulation studies (Mola et al., 2009), and additionally performed at 37°C, therefore in physiological conditions. In particular, using this assay, which is based on the use of a fluorescence multiplate reader, it is possible to detect a swelling phase immediately followed by an RVD phase (Fig. 1D,E). Using this assay, the time constant of cell swelling

of WT astrocytes was still significantly smaller than that of AQP4 KO cells (3.42±0.13 s for WT, vs. 5.00±0.19 s for KO astrocytes, Fig. 1F). Differences between the time constant values of the swelling phase obtained by the two approaches are compatible with the effect of the temperature (37°C used for the multiplate reader assay versus 10°C used for the TIRF assay) on the fluidity of the lipid bilayer.

Looking at the RVD phase (Fig. 1D), a difference was detected between WT and AQP4 KO astrocytes. WT astrocytes displayed RVD with faster kinetics (Fig. 1G) and larger amplitude (Fig. 1H) than did AQP4 KO.

To evaluate whether AQPs other than AQP4 could provide astrocytes with faster cell swelling and RVD, we used an astrocyte cell line (DI TNC1) transfected with AQP4 or AQP1. The analysis of the swelling phase (Fig. 1E) showed that AQP transfected DI TNC1 are characterized by a faster cell swelling than WT-DI TNC1, as expected (2.39±0.70 s for AQP4-DI TNC1; 3.31±0.18 s for AQP1-DI TNC1; 8.60±1.05 s for WT-DI TNC1, Fig. 1F). The analysis of the RVD phase (Fig. 1E) showed that while WT-DI TNC1 did not show any RVD in response to hypotonic stimulation, DI TNC1 transfected with both AQPs displayed a clear RVD response quantified in terms of a time constant (Fig. 1G) and percentage of recovery (Fig. 1H). These results demonstrate that AQP water channels play a key role in triggering and accelerating RVD by increasing the rate of cell swelling.

AQP4 Plays a Key Role in Mediating Swelling-induced Calcium Influx in Astrocyte Primary Cultures

Osmotic swelling of cultured astrocytes leads to increased levels of cytosolic calcium (Fischer et al., 1997), however the extent to which calcium signaling controls RVD in brain astrocytes is a matter of debate. We here wanted to study whether the fast cell swelling provided by AQP4 contributes to calcium transients in WT and AQP4 KO astrocytes (Fig. 2). In the presence of external calcium (Fig. 2A,B), a hypotonic stimulus induced intracellular calcium elevation in WT astrocytes with a larger amplitude and faster kinetics than in AQP4 KO astrocytes (Fig. 2E, amplitudes: 369.9±35 nM for WT vs. 215.3±34.85 nM for KO; Fig. 2F, time to peak: 32.40±2.9 s for WT vs. 80±4.6 s for KO). To evaluate the relative contribution of intra- and extracellular calcium stores to swelling-induced calcium transients, we performed similar experiments but in the absence of external calcium (Fig. 2C,D). Compared to calcium containing hypotonic solutions, the amplitudes and kinetics of the calcium signals were vastly reduced in WT and AQP4 KO astrocytes exposed to calcium-free hypotonic solution (Fig. 2E, amplitudes: 58.83±5.86 nM for WT vs. 26.89±6.02 nM for KO; Fig. 2F, time to peak: 11.51±1.8 s for WT vs. 25.23±3.5 s for KO), indicating that calcium elevation is mainly contributed by the influx from the extracellular milieu. Interestingly, however, in the absence of external calcium, the hypotonic stimulus-induced calcium transients recorded from WT and KO astrocytes were significantly different in terms of their amplitudes and time to peak (Fig. 2E,F), indicating that more rapid cell swelling, provided by AQP4-mediated water influx, also contributes to calcium release from intracellular stores.

Calcium Release Activated Channels are not Altered in AQP4 KO Astrocytes

Calcium release activated channels (CRAC) are specialized plasma membrane calcium channels activated by depletion of intracellular calcium stores (McNally and Prakriya, 2012). To verify whether the differences in calcium influx recorded between WT and AQP4 KO astrocytes, shown in Fig. 2, were related to altered CRAC contribution in the two genotypes, calcium transients were measured in astrocytes treated with CPA (cyclopiazonic acid), an inhibitor of calcium-dependent ATPases, used to mobilize intracellular calcium deposits (Fig. 3). The results obtained showed no significant differences in calcium amplitudes or time to peak between WT and AQP4 KO astrocytes when a CPA bath was applied, thus indicating similar calcium content within the stores in these two cell types. Moreover, after intracellular calcium depletion, addition of calcium to the extracellular solution resulted in comparable kinetics of calcium influx in both WT and AQP4 KO astrocytes, in the absence of membrane stretch, thus indicating a comparable expression of CRAC. Taken together, the experiments shown in Figs. 2 and 3 indicate that the differences in swelling-induced calcium influx recorded between WT and AQP4 KO astrocytes are likely related to the degree of membrane stretch provided by the AQP water channels.

Stretch-activated Channels Mediate Intracellular Calcium Increase following Cell Swelling

To study the participation of stretch activated plasma membrane channels in mediating calcium influx induced by the hypotonic stimulus applied, we used two pharmacological agents known to partially block stretch activated TRPV4 channels, Gadolinium and Ruthenium Red (Gunthorpe et al., 2002; Yang and Sachs, 1989). Both Gadolinium and Ruthenium Red, used at 10 μM concentration, significantly reduced the amplitude of intracellular calcium increase in WT and AQP4 KO astrocytes (Fig. 4), while in DI TNC1 (Fig. 5), they only reduced calcium influx in the presence of AQP1 or AQP4 and not in WT. Calcium responses to hypotonic stimulus were not further reduced for either astrocyte primary culture or DI TNC1 by doubling the concentration of Gadolinium and Ruthenium Red at 20 μM (data not shown). These results support previous reports (Benfenati et al., 2011) indicating TRPV4 channels as the main stretch activated channel candidate responsible for the calcium influx registered after hypotonic cell swelling, although the mechanism shown here, differently from that shown by Benfenati et al. (2011), is not specific for AQP4 but common to AQP4 and AQP1. The calcium response detectable in WT DI TNC1, and still present in Gadolinium- and Ruthenium Red-treated astrocyte primary cultures and AQP expressing DI TNC1, can be interpreted either as Gadolinium and Ruthenium Red being unable to act on TRPV4 below a certain level of activation or, as due to a calcium influx occurring through a channel different from TRPV4.

Additional experiments were performed in order to assess whether the same calcium responses to cell swelling were analogously obtained (a) in the presence of a smaller and more physiological osmotic gradient (60 mOsm L^{-1}) rather than 150 mOsm L^{-1} and (b) using an osmotic gradient made with Mannitol, instead of NaCl, in order to ascertain whether the calcium influx could be due to an effect of the reduced extracellular NaCl concentration on the membrane potential. The results obtained (Supp. Info. data, Fig. 2) showed that (a) calcium responses were still present, although proportionally reduced, as expected, when a 60 mOsm L^{-1} osmotic gradient, instead of 150 mOsmL^{-1}, was applied

and (b) no differences were observed when the hypotonic stimulus was obtained using a Mannitol-based, rather than an NaCl-based, osmotic gradient. All together these data indicate that the calcium responses obtained under hypotonic stimulation, reported in Figs. 2, 4, and 5, can also occur under a more physiological osmotic gradient and are most likely due to an effect of the cell swelling to the level of membrane stretch (Fig. 7) and not to an altered plasma membrane potential due to a reduced extracellular NaCl concentration.

Calcium Influx and TRPV4 Activation are not Essential for RVD

To evaluate whether calcium influx from extracellular space participates in the RVD process, astrocytes, and DI TNC1 were exposed to hypotonic calcium-free solutions and the RVD phase compared to that obtained in the presence of external calcium. The results (Fig. 6) demonstrate that RVD was still present, when astrocytes or AQP expressing DI TNC1 cells were exposed to hypotonic solution devoid of calcium and the kinetics (Fig. 6A,B) are comparable to the kinetics obtained in the presence of external calcium (Fig. 1D,E). Statistical analyses revealed that the extent of recovery (Fig. 6C) and time constant (Fig. 6D) of RVD obtained for cells exposed to calcium-free hypotonic solution were not statistically different from those obtained in calcium-containing hypotonic solution. Moreover, inhibition of TRPV4 channels with Gadolinium and Ruthenium Red did not affect the extent of recovery or the time constant of RVD in AQP expressing DI TNC1 (Fig. 6C,D). Interestingly, a longer recording of RVD kinetics, from astrocyte primary cultures for 1,200 s in the presence of external calcium (Fig. 6E), showed that after a fast RVD occurring immediately after the hypotonic stimulus, a slower and more complete recovery of cell volume was registered, most likely sustained by mechanisms different from those analyzed here. All these results indicate that (1) the influx of calcium (and the activation of TRPV4) is not required for the activation and completion of the RVD mechanism, (2) the stretch-activated channels responsible for the RVD in the cells analyzed are not inhibited by Gadolinium or Ruthenium Red, and (3) the fast RVD analyzed in this study is followed by a slower RVD mechanism for a further cell volume recovery.

As for the calcium responses described in the previous paragraph, additional experiments were performed in order to assess whether the swelling and RVD kinetics were analogously obtained (a) in the presence of a smaller and more physiological osmotic gradient (60 mOsm L^{-1}) rather than 150 mOsm L^{-1} and (b) using an osmotic gradient made with Mannitol, instead of NaCl, in order to unveil a potential involvement of the membrane potential on the events described. The results obtained (Supp. Info. data, Fig. 3) showed that both the kinetics of swelling and RVD were unaltered when a 60 mOsm L^{-1} osmotic gradient, instead of 150 mOsm L^{-1}, was applied and when the hypotonic stimulus was obtained using a Mannitol-based, rather than an NaCl-based, osmotic gradient. Analogously to the calcium responses, these experiments support the hypothesis that RVD is the consequence of an effect of the cell swelling to the level of membrane stretch (Fig. 7).

Discussion

In this article, we have attempted to dissect the role of AQPs in triggering two important astrocytic responses to cell swelling, namely changes in intracellular calcium levels and

RVD (McCarty and O'Neil, 1992). We have focused on AQP4 expressing astrocytes, where RVD can reduce the swelling that occurs in a variety of physiological and pathological situations. In addition, we have attempted to evaluate to what extent our findings are specific for AQP4 or to any AQP. AQP1 was chosen since, similarly to AQP4, it is a water selective AQP and it is constitutively expressed in the plasma membrane.

Other studies have previously tried to clarify how AQP water channels can affect hypotonic stimulus-dependent cell swelling and the consequent intracellular calcium increase and cell volume regulation. In particular, studies on AQP2 (Galizia et al., 2012) in renal cells, on AQP4 in astrocytes (Benfenati et al., 2011) and on AQP5 in salivary glands (Liu et al., 2006) have shown that TRPV4 is activated by hypotonic stimulus in an AQP-dependent manner and it is responsible for calcium entry which in turn triggers RVD. Briefly, according to Liu et al. (2006) the activation of TRPV4 by hypotonicity depends on AQP5, not on cell swelling *per se*; similarly, Benfenati et al. (2011) have shown that the functionality of AQP4 and TRPV4 complex depends on a molecular interaction between AQP4 and TRPV4, AQP1 not functionally substituting for AQP4. Galizia et al. (2012) do not specifically ascribe the functional association between TRPV4 and AQP2, to a specific interaction or co-expression of the two proteins at the plasma membrane. Interestingly, all these three studies have shown that calcium entry through TRPV4 is required for RVD, indicating calcium signaling as a key trigger for cell volume regulation.

Although the data in the present study identify TRPV4 as the stretch-activated channel responsible for the intracellular calcium increase upon cell swelling, the mechanisms by which TRPV4 activation occurs, the role of AQPs, and the effect of calcium signaling on cell volume regulation are different from those been shown by the studies of others mentioned above. The hypothesis of the present study is that it is exclusively the faster kinetics of cell swelling, conferred by a water channel protein that plays a key role in activating TRPV4 and in promoting the RVD. The experiments described in the present study suggest the conclusions and mechanisms discussed in the following paragraphs.

AQPs Have a Strong Impact on RVD

We show here that both astrocyte primary cultures and an astrocyte cell line (TNC) have the basic machinery required for RVD. However, the activation of this process requires rapid cell swelling which is obtained by the presence of AQPs. It is well established that swelling in the presence of AQPs is rapid due to a much faster water influx. The present study highlights that the presence of AQPs and the associated rapid water influx induces an important plasma membrane stretch in turn activating RVD. Moreover, AQPs facilitate water efflux, therefore improving RVD efficiency. This findings find physiological support in the study performed by Haj-Yasein et al. (2012) in which it is shown that deletion of AQP4 accentuated the shrinkage of the ECS during synaptic stimulation, therefore suggesting an AQP4 role in blunting the shrinkage of ECS by allowing water efflux from astrocytic processes (Haj-Yasein et al., 2012).

The RVD object of this study is the "fast active" volume decrease following rapid cell swelling, also observed by other authors (Benfenati et al., 2011), which can be considered as a sort of emergency protective mechanism that the cell activates only after a certain insult to

the plasma membrane, measured by the stretching force applied (see Fig. 7). We have also shown that, in line with previous reports, there is a further volume decrease, although without a total recovery (Risher et al., 2009), most likely sustained by mechanisms not explored in this manuscript. Obviously, the detection of such "fast" RVD is strictly dependent on the experimental conditions. For instance, there is a study by Risher et al. (2009) showing that astrocytes *in vivo* respond passively to hypotonicity and that there is no significant RVD. We believe that the different technical approach used in Risher's study is at the basis of this discrepancy. After 15–20 min of osmotic challenge, images collected in the first few minutes would not allow the detection of the RVD here shown where the detection of the volume decrease is at its maximal level of recovery in <1 min. With a solution exchange time of >1 s, the kinetics of water transport is already technically seriously affected (Solenov et al., 2004). Interestingly, there is also another study by Pangrsic et al. (2006) in which, although the experimental conditions are very different from those used in the present study, it is possible to register a cell volume recovery similar to that shown here.

Primary astrocyte cultures lacking AQP4 (AQP4 KO astrocytes) exhibited a faster swelling than the DI TNC1 lacking AQPs (WT-TNC). This is likely to be attributable to a higher water permeability of the astrocyte compared to the DI TNC1 lipid bilayer. Therefore, if the absence of RVD in DI TNC1 might be due to the slow cell swelling not causing a plasma membrane stretch fast enough to activate the RVD machinery, the stretch of AQP4 KO astrocytes seems sufficient to activate RVD, although to a lesser extent compared to WT astrocytes. This is very important to highlight since it gives an example of a situation in which it is the swelling kinetics and not the presence of an AQP that controls the RVD machinery.

On the basis of these observations, and on the results obtained on the kinetics of the volume decrease in the presence or absence of AQPs, we propose a mechanism in which AQPs play a dual function in the swelling-dependent RVD phenomenon: (1) AQPs promote faster swelling, therefore enhancing plasma membrane stretch with more efficient activation of the mechanisms controlling the RVD. (2) AQPs strongly affect and improve the shrinkage kinetics of the water efflux, directly responsible for the volume decrease itself.

AQPs Enhance Calcium Influx through TRPV4 Under Hypotonic Stimulus

The endogenous presence of TRPV4 in DI TNC1 and its role in mediating calcium influx as a consequence of cell swelling has already been reported by Benfenati et al. (2011) using siRNA strategy (Benfenati et al., 2011). Moreover, in line with previous reports (Benfenati et al., 2011; Galizia et al., 2012; Liu et al., 2006) the use of Gadolinium and Ruthenium Red has here identified TRPV4 as the calcium channel responsible for the calcium influx, activated by the membrane stretch. However, the molecular mechanism proposed here is completely different. Similarly to Benfenati et al. (2011), we found that the calcium increase following hypotonic stimulus is mainly due to calcium influx from the extracellular space and, in astrocytes, AQP4 has a major modulatory role. Differently from Benfenati et al. (2011), however, parallel experiments performed with DI TNC1 transfected with AQP4 or AQP1 provided evidence against a role specific for AQP4-TRPV4 interaction in mediating calcium influx. We in fact show that this amplification is mediated by both AQP4 and AQP1

and most likely by any AQP. We here propose that it is the faster cell swelling, characteristic of AQP expressing cells (and not only AQP4), that is responsible for faster distension of the plasma membrane with a consequent higher activation of TRPV4 and a more prominent calcium influx (Fig. 7).

The results here described show that, in the absence of AQP water channels, a different behavior was recorded for the two cell types (astrocyte primary cultures in Fig. 4 and DI TNC1 in Fig. 5) treated with Gadolinium and Ruthenium Red. In AQP4 KO astrocytes, Gadolinium and Ruthenium Red treatment induced a reduction of calcium influx. In contrast, in WT-DI TNC1, the calcium increase was not reduced in the presence of Gadolinium and Ruthenium Red. The calcium response detectable in WT-DI TNC1 and in Gadolinium and Ruthenium Red-treated AQP expressing DI TNC1 can be interpreted as Gadolinium and Ruthenium Red being unable to act on TRPV4 below a certain level of activation, as also reported by Sanchez et al. (2003) or, we cannot exclude, that the calcium influx registered for WT-DI TNC1, as for AQP transfected DI TNC1 in the presence of Gadolinium and Ruthenium Red, occurs through a channel different from TRPV4.

RVD is Independent from Calcium Influx in Astrocytes

It is widely recognized that the hypotonic stimulus causes both an intracellular calcium increase and RVD in astrocytes, implicating calcium as a likely candidate as trigger for RVD (McCarty and O'Neil, 1992). However, the evidence supporting its signal role in RVD is still incomplete and controversial. An older study (O'Connor and Kimelberg, 1993) proposed a mechanism whereby the depolarization caused by the astrocyte swelling is responsible for the calcium influx that triggers the opening of calcium dependent K^+ and Cl^- channels allowing KCl to leave the cell for the RVD to occur. In the more recent studies cited above (Benfenati et al., 2011; Galizia et al., 2012; Liu et al., 2006) it is reported that the stretch activated channel TRPV4 allows the calcium influx, which in turn controls the RVD.

In the present study, experiments performed in the absence of external calcium or in the presence of TRPV4 inhibitors in both primary cultured astrocytes and transfected cells did not prevent RVD, indicating that calcium influx from outside through TRPV4 is not essential for the activation of the RVD machinery. Although we cannot exclude that the smaller calcium increase following hypotonic stimulus registered in the absence of external calcium plays a role in RVD activation, our results support several other reports showing that RVD can occur also as a calcium-independent phenomenon (Grinstein and Smith, 1990; Foskett et al., 1994; Altamirano et al., 1998; Morales-Mulia et al., 1998) and the view that calcium increases elicited by swelling represent a parallel but unrelated phenomenon (Pasantes-Morales and Morales Mulia, 2000).

Conclusions and Speculation

In conclusion, the main finding of the present manuscript is that the fast swelling kinetics, likely associated with AQP plasma membrane expression, plays a key role in modulating two parallel phenomena that we here report to be independent from each other: (1) calcium signaling—together with TRPV4 in astrocytes, and (2) the RVD—while TRPV4 and calcium do not seem to be essential for RVD to occur. AQP function in modulating the

swelling dependent-RVD probably occurs in three different steps: (a) AQPs facilitate rapid initial cell swelling; (b) the rapid AQP-dependent cell swelling activates or increases the activation of calcium-independent stretch-activated ion channels, not inhibited by Gadolinium or Ruthenium Red, responsible for ion extrusion; (c) AQPs promote more efficient water efflux which is directly responsible for the cell shrinkage (Fig. 7).

If RVD and intracellular calcium increase are modulated by the speed of water flux and not by a specific AQP expression, it means that the amount of protein in the plasma membrane also play a key role. For instance, the possibility to regulate the plasma membrane water permeability by controlling the surface expression of AQP4e and AQP4 delta4 may be used by the cells to control these responses.

The patho-physiological role of these effects can only be speculated at present. By facilitating calcium influx AQPs could, for example, control cell migration. It is already known that AQP expression at the leading edge of the cell either facilitates the local swelling necessary for lamellipodium formation (Nicchia et al., 2013; Papadopoulos and Verkman, 2013) or affects the cell cytoskeleton organization (Nicchia et al., 2005, 2008). It is also known that calcium signaling controls cytoskeleton redistribution during cell migration (Chi and Trinkaus-Randall, 2013; Pettit and Fay, 1998). We can here speculate that AQP-dependent local swelling could trigger stretch-activated calcium channels at the leading edge with a resulting enhanced calcium influx and enhanced motility. This mechanism could further explain the reduce glial scar formation in AQP4 KO mice (Saadoun et al., 2005), and the reduced tumor angiogenesis found in AQP1 KO mice (Nicchia et al., 2013).

It is amply documented that in astrocytes calcium signals control the release of chemical mediator (gliotransmitters) which are capable of modulatory actions on other glial, vascular, or neuronal cells (Volterra et al., 2014) and therefore we can speculate that AQP4-depending altered spatiotemporal calcium signaling is at the base of the alteration in neuro-excitability observed in AQP4-null mice (Binder et al., 2004). Interestingly, calcium dynamics in astrocyte processes (highly enriched in AQP4), are characterized by an intense and fast local activity, dissociated from activity in the cell body (where AQP4 is almost absent), that is slower and less frequent (Shigetomi et al., 2013; Volterra et al., 2014).

RVD is important in a wide variety of cell functions, including epithelial transport, metabolism, excitation, hormone release, migration, cell proliferation, and cell death (Lang et al., 1998). In the CNS, AQP4 control of the RVD could explain some of the effects on ion homeostasis and neuronal signal transmission reported in AQP4-KO mice (Kitaura et al., 2009; Li et al., 2012; Skucas et al., 2011). All together these findings add new molecular insights into the knowledge of the role of water channels in CNS physiology and patho-physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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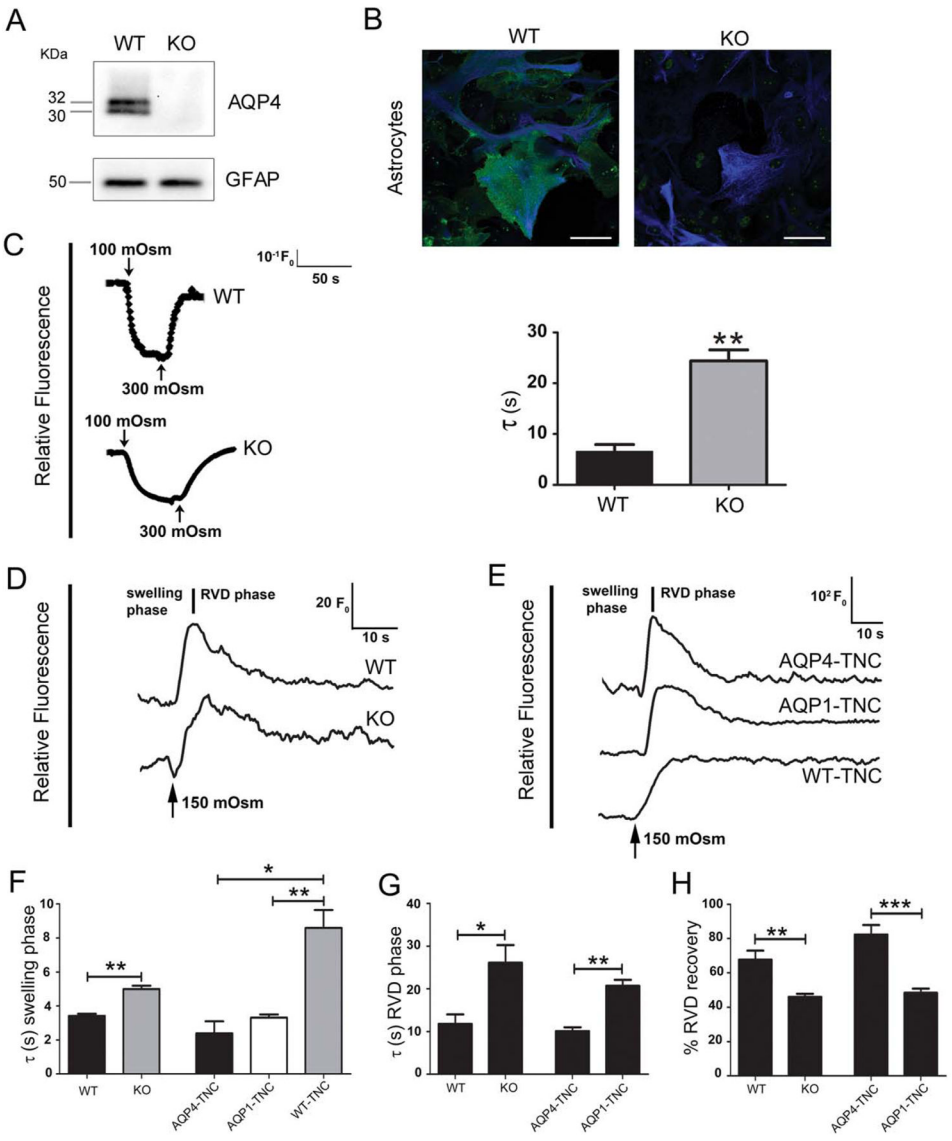

FIGURE 1.

Water transport and RVD in primary cultured astrocytes endogenously expressing AQP4 and in DI TNC1 transfected with AQP4 or AQP1. (**A**) Western blots showing AQP4 and GFAP expression in WT and AQP4 KO (KO) astrocyte primary cultures. Note the expression of both M1 andM23-AQP4 isoforms in WT and their absence in AQP4 KO astrocytes. GFAP was used as the internal standard for protein loading. (**B**) Merged confocal microscopy images of WT and AQP4 KO (KO) astrocytes immunostained for AQP4 (green) and GFAP (blue). Note the absence of AQP4 staining in KO and the typical punctate AQP4 staining in WT (Scale bar=50 μm). (**C**) (Left) Time course of water transport recorded from Calcein-AM loaded WT and AQP4 KO (KO) astrocytes using TIRF microscopy showing changes in fluorescence induced by 200 mOsm L^{-1} hypotonic gradient. The arrows indicate the addition of a hypotonic solution (100 mOsm L^{-1}) and isotonic solution (300 mOsm L^{-1}). (Right) Histogram of the mean±SE values of time constants obtained by fitting an

exponential curve to the cell swelling phase of cells after exposure to 100 mOsm L^{-1} solution (** $P < 0.005$; $n=4$). (**D,E**) Functional analysis for plasma membrane water transport performed by using Flex Station with the Calcein-AM quenching assay, showing the typical swelling kinetics (swelling phase) followed by the RVD kinetics (RVD phase) ofWT and AQP4 KO (KO) astrocytes (D) and DI TNC1 transfected with AQP4 (AQP4-TNC) or AQP1 (AQP1-TNC) and WT (WT-TNC) (E). (**F**) Histogram showing the mean±SE values of the time constants obtained for the "swelling phase" of astrocytes and DI TNC1 cells exposed to 150 mOsm L^{-1} hypotonic solution, obtained using the Calcein-AM quenching assay. (**G**, **H**) Histograms showing the mean±SE values of the time constant (G) and of the extent of recovery (in percent, H) of the RVD phase of the indicated cells. Note that AQP expression promotes faster RVD kinetics and higher efficiency of RVD (F–H: *** $P < 0.0005$; ** $P < 0.005$; * $P < 0.05$; $n=6$ forWT; $n=8$ for KO; $n=5$ for AQP4-TNC; $n=8$ forAQP1-TNC; $n=9$ for WT-TNC).

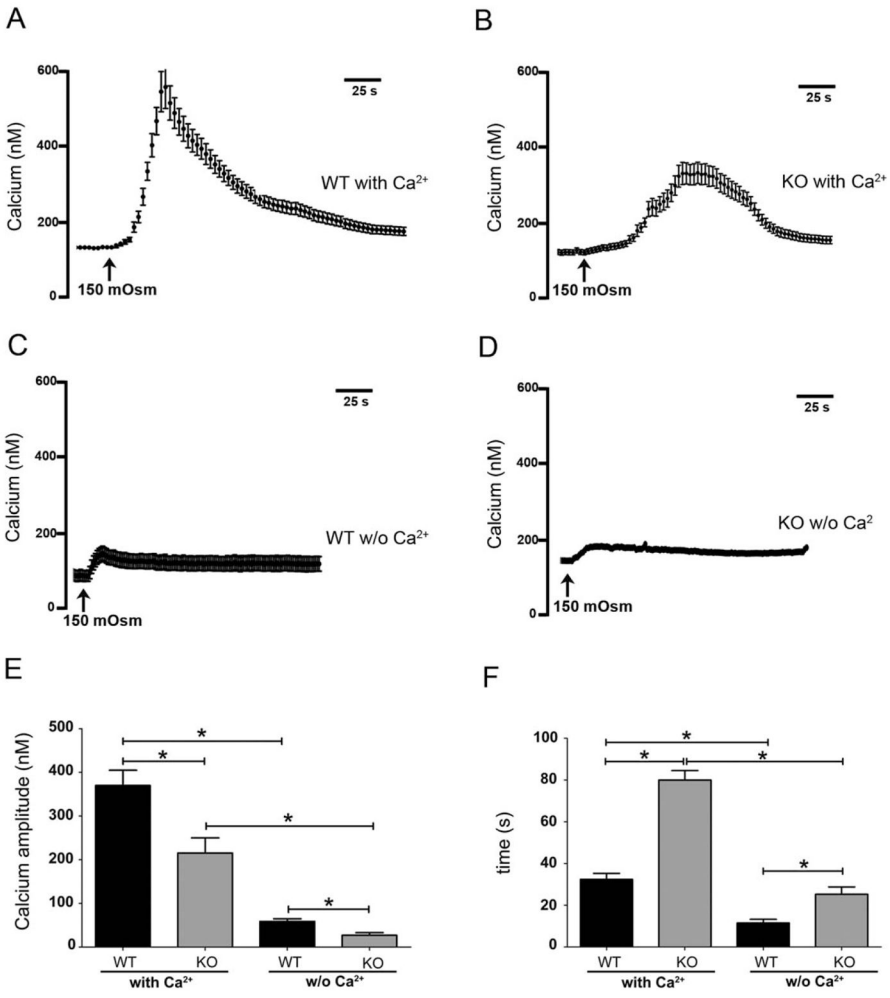

FIGURE 2.

Hypotonic stimulus induced intracellular calcium rise in WT and AQP4 KO astrocytes. (**A,B**) Time courses of intracellular calcium concentration changes recorded from WT (**A**) and AQP4 KO (B) astrocytes exposed to calcium-containing hypotonic solution. Note the delayed and reduced amplitudes of calcium responses in AQP4 KO astrocytes compared to those of WT cells. (**C,D**) Time course of intracellular calcium concentration changes recorded from WT (C) and AQP4 KO (D) astrocytes exposed to calcium-free hypotonic solution. Note that for both WT and AQP4 KO astrocytes, calcium amplitudes are dramatically reduced compared to those recorded in calcium-containing solution (A,B). (**E**,**F**) Histograms showing the mean±SE values of calcium amplitude (E) and time to peak (F) recorded from WT and AQP4 KO astrocytes exposed to hypotonic calcium containing (with Ca^{2+}) and calcium-free (w/o Ca^{2+}) solution (*$P < 0.05$, n=6).

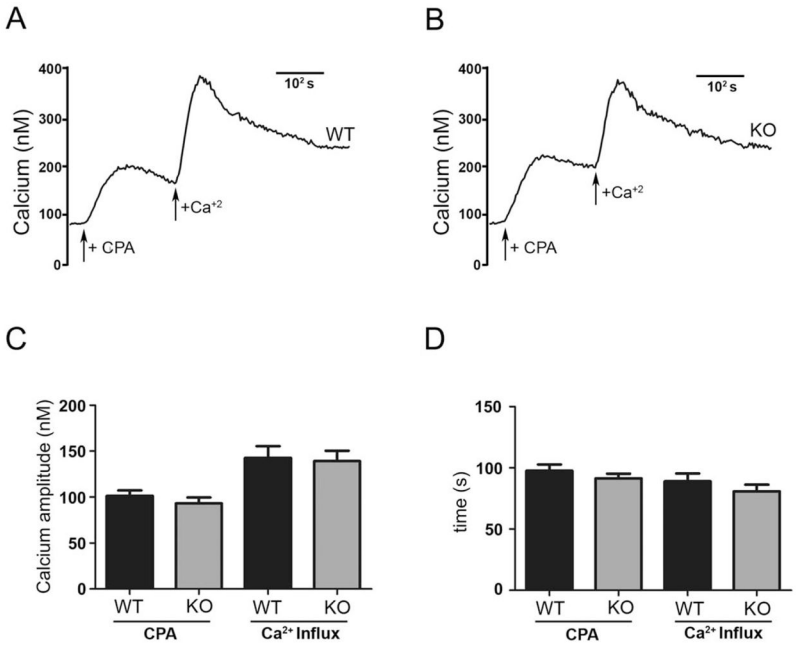

FIGURE 3.

Intracellular calcium content and calcium release activated channels are not altered in AQP4 KO astrocytes. (**A**,**B**) Kinetics of cytosolic calcium changes induced by CPA (+CPA) and following addition of calcium containing external solution (+Ca^{2+}) in WT (**A**) and AQP4 KO (B) astrocytes. (**C**,**D**) Histograms showing the mean±SE values of the amplitudes (C) and time to peak (D) of calcium responses, after store depletion by CPA and addition of extracellular calcium (n=5).

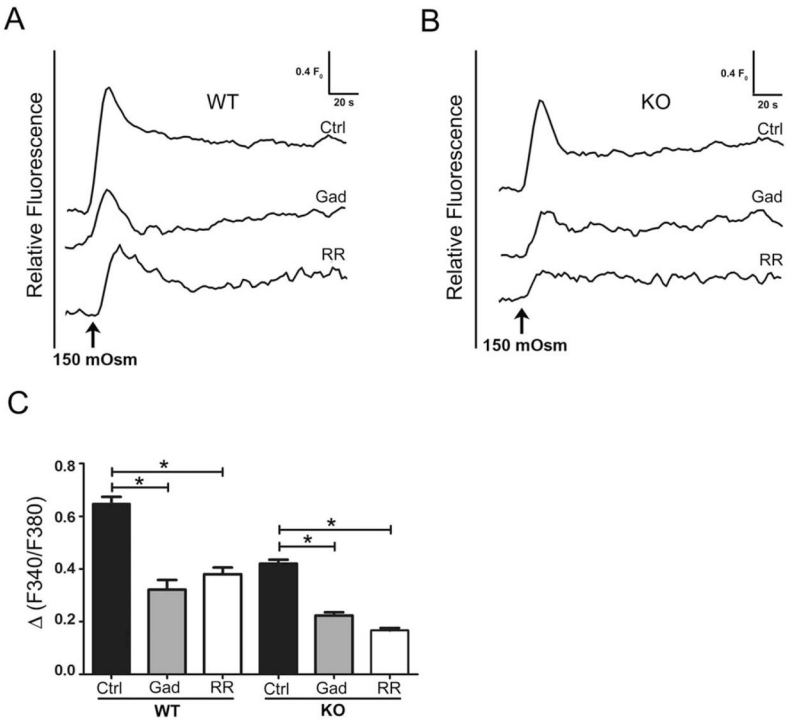

FIGURE 4.

Gadolinium and Ruthenium Red partially block calcium increase induced by hypotonicity-induced astrocyte swelling. Superimposed kinetics of calcium responses recorded from untreated (Ctrl) and Gadolinium (Gad) or Ruthenium Red (RR) treated WT (**A**) and AQP4 KO (**B**) astrocytes exposed to hypotonic stimulus. (**C**) Histograms showing the mean±SE values of the calcium amplitudes measured under conditions shown in A and B. Note that in both WT and AQP4 KO astrocytes Gadolinium and Ruthenium Red significantly reduced the amplitude of intracellular calcium levels induced by hypotonic stimulus (*$P < 0.05$, n=6).

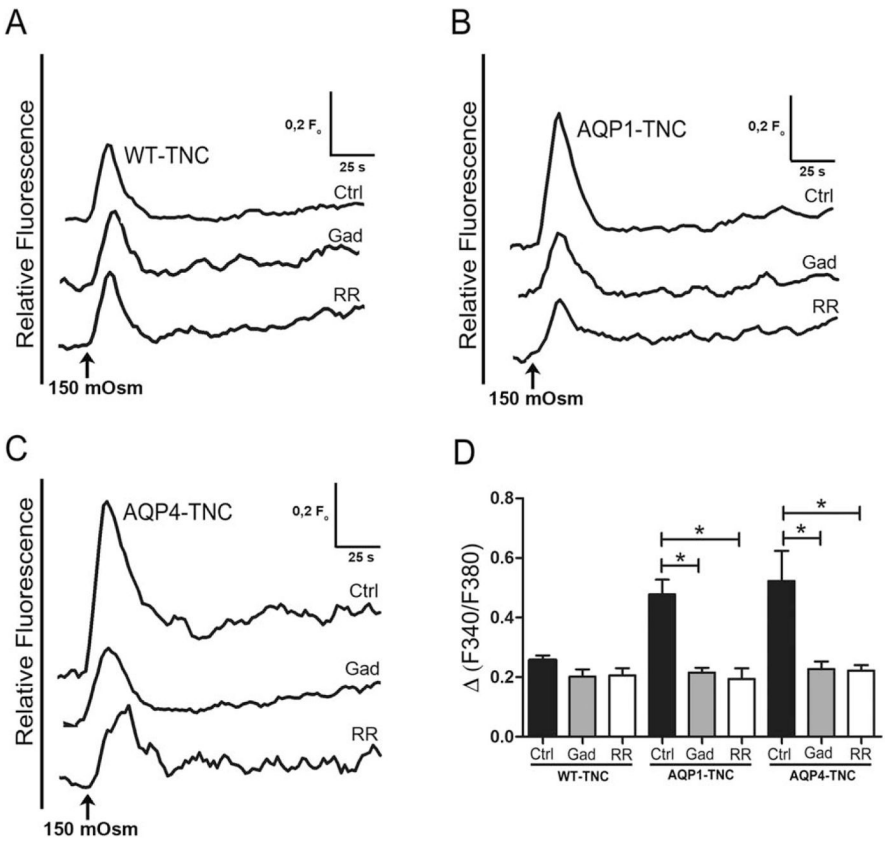

FIGURE 5.

Intracellular calcium response under hypothonic stimulus in WT and AQP1 or AQP4 transfected DI TNC1 treated with Gadolinium or Ruthenium Red. Superimposed kinetics of calcium concentration after hypotonic stimulus of WT (**A**), AQP1 transfected (**B**) and AQP4 transfected (**C**) DI TNC1, untreated (Ctrl) or treated with Gadolinium (Gad) or Ruthenium Red (RR). (**D**) Histograms showing the mean values±SE of calcium amplitude of the conditions shown in A, B and C. Note that when added separately both Gadolinium and Ruthenium Red significantly affect the amplitude of intracellular calcium increase in both AQP4 and AQP1 transfected cells (*$P < 0.05$, n=5).

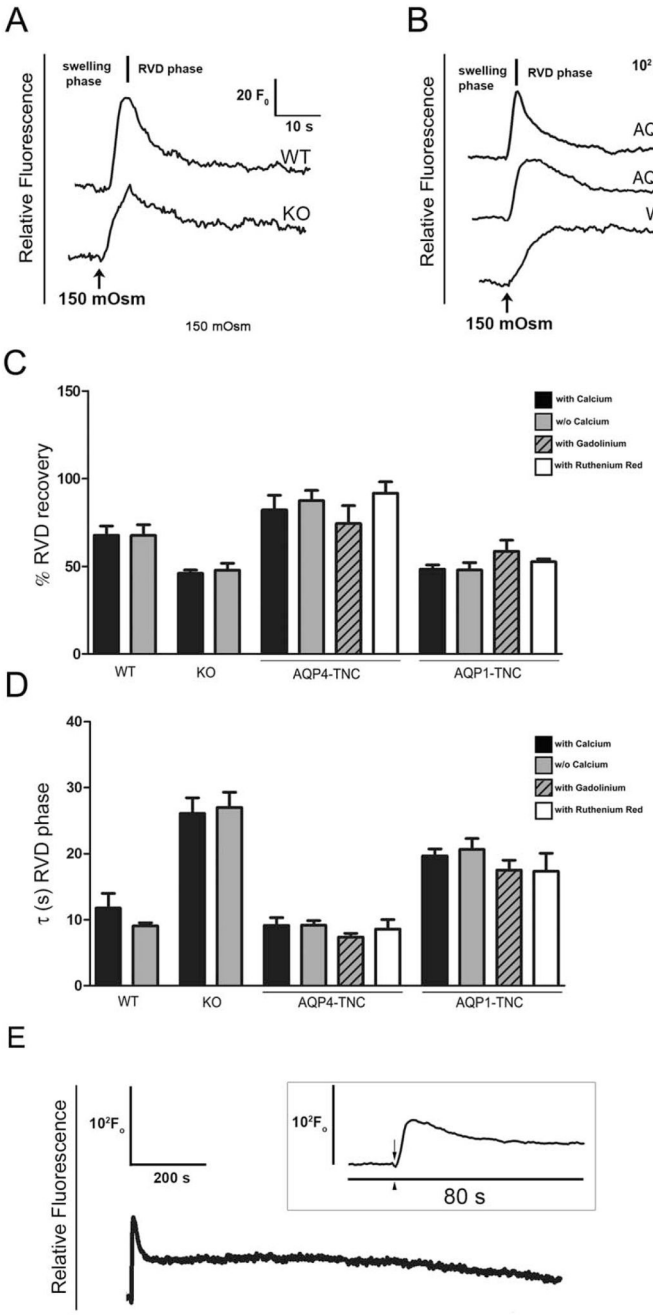

FIGURE 6.

RVD in WT, AQP4 KO astrocytes and AQP expressing DI TNC1 is not dependent on extracellular calcium and TRPV4. (**A**,**B**) Kinetics of RVD recorded from astrocyte primary cultures (A) and DI TNC1 (B) exposed to calcium-free hypotonic solution. (**C**,**D**) Histograms showing the mean±SE values of the extent of recovery (in percent) (C) and time constant (D) of RVD of the indicated cells, in the presence (black bars) and in the absence (grey bars) of external calcium, and in cells treated with Gadolinium (striped bars) or Ruthenium Red (white bars) in the presence of external calcium. In each cell type no significant differences were detected between the conditions analyzed. (**E**) Longer RVD

kinetics recorded from astrocyte primary cultures for 1,200 s. The curve in the inset shows that, immediately after the 150 mOsm L^{-1} hypotonic stimulus (arrow) given at 17 s (arrowhead), the cells undergo a fast RVD which takes <40 s. Recording data for 1,200 s demonstrate that there is a further volume decrease, although without a total recovery.

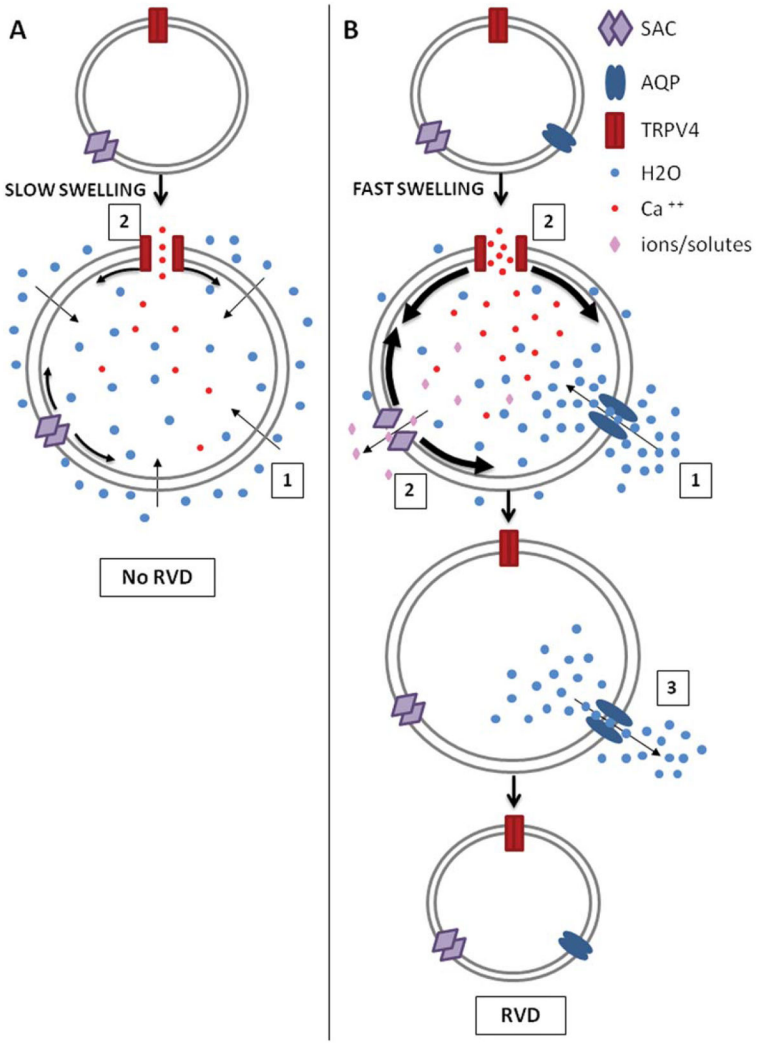

FIGURE 7.

Schematic representation of the mechanism proposed to describe the different effect of slow and fast swelling kinetics on calcium response and RVD. The absence (**A**) or presence (**B**) of AQP water channels in the plasma membrane affects the cell swelling kinetics which is slow (A) and fast (B), respectively. The swelling kinetics modulates the level of plasma membrane stretch, here symbolized by arrows parallel to the plasma membrane. (A) Slow swelling. The swelling in the absence of AQP water channels can be very slow (see WT-TNC), depending on the composition of the lipid bilayer through which the water influx occurs by simple diffusion (1), and produces a weak plasma membrane stretch (thin arrows). This weak stretch is sufficient for the opening of TRPV4 channels (2), with a consequent calcium influx, but not sufficient to open SAC (Stretch Activated Channels), which are responsible for the cell volume regulation, and therefore no RVD occurs. (B) Fast swelling. The swelling in the presence of AQP water channels is always fast (see AQP-TNC or WT astrocyte primary cultures). In this case, AQP mediated water influx (1) is characterized by fast swelling kinetics producing strong plasma membrane stretch (thick arrows). The membrane stretch in this case is strong enough (a) to induce a major opening of TRPV4 (2),

with consequent increased calcium influx, compared to the situation described in A, and (b) to open calcium independent SAC (2) responsible for the cell volume regulation through the efflux of ions/solute. This efflux of ions/solute is followed by a water efflux through AQP water channels (3) itself responsible for the RVD.