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Release of taurine and glutamate contributes to cell volume regulation in human retinal Müller cells: differences in modulation by calcium

Vanina Netti,[1,2] Alejandro Pizzoni,[1,2] Martha Pérez-Domínguez,[3] Paula Ford,[1,2] Herminia Pasantes-Morales,[4] Gerardo Ramos-Mandujano,[4] and Claudia Capurro[1,2]

[1]*Universidad de Buenos Aires, Facultad de Medicina. Departamento de Ciencias Fisiológicas, Laboratorio de Biomembranas, Buenos Aires, Argentina;* [2]*CONICET-Universidad de Buenos Aires. Instituto de Fisiología y Biofísica "Bernardo Houssay," Buenos Aires, Argentina;* [3]*Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, México; and* [4]*División de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de México, México*

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Netti V, Pizzoni A, Pérez-Domínguez M, Ford P, Pasantes-Morales H, Ramos-Mandujano G, Capurro C. Release of taurine and glutamate contributes to cell volume regulation in human retinal Müller cells: differences in modulation by calcium. *J Neurophysiol* 120: 973–984, 2018. First published May 23, 2018; doi:10.1152/jn.00725.2017.—Neuronal activity in the retina generates osmotic gradients that lead to Müller cell swelling, followed by a regulatory volume decrease (RVD) response, partially due to the isoosmotic efflux of KCl and water. However, our previous studies in a human Müller cell line (MIO-M1) demonstrated that an important fraction of RVD may also involve the efflux of organic solutes. We also showed that RVD depends on the swelling-induced Ca^{2+} release from intracellular stores. Here we investigate the contribution of taurine (Tau) and glutamate (Glu), the most relevant amino acids in Müller cells, to RVD through the volume-regulated anion channel (VRAC), as well as their Ca^{2+} dependency in MIO-M1 cells. Swelling-induced [^{3}H]Tau/[^{3}H]Glu release was assessed by radiotracer assays and cell volume by fluorescence videomicroscopy. Results showed that cells exhibited an osmosensitive efflux of [^{3}H]Tau and [^{3}H]Glu (Tau > Glu) blunted by VRAC inhibitors 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)-oxybutyric acid and carbenoxolone reducing RVD. Only [^{3}H]Tau efflux was mainly dependent on Ca^{2+} release from intracellular stores. RVD was unaffected in a Ca^{2+}-free medium, probably due to Ca^{2+}-independent Tau and Glu release, but was reduced by chelating intracellular Ca^{2+}. The inhibition of phosphatidylinositol-3-kinase reduced [^{3}H]Glu efflux but also the Ca^{2+}-insensitive [^{3}H]Tau fraction and decreased RVD, providing evidence of the relevance of this Ca^{2+}-independent pathway. We propose that VRAC-mediated Tau and Glu release has a relevant role in RVD in Müller cells. The observed disparities in Ca^{2+} influence on amino acid release suggest the presence of VRAC isoforms that may differ in substrate selectivity and regulatory mechanisms, with important implications for retinal physiology.

NEW & NOTEWORTHY The mechanisms for cell volume regulation in retinal Müller cells are still unknown. We show that swelling-induced taurine and glutamate release mediated by the volume-regulated anion channel (VRAC) largely contributes the to the regulatory volume decrease response in a human Müller cell line. Interestingly, the hypotonic-induced efflux of these amino acids exhibits disparities in Ca^{2+}-dependent and -independent regulatory mechanisms, which strongly suggests that Müller cells may express different VRAC heteromers formed by the recently discovered leucine-rich repeat containing 8 (LRRC8) proteins.

cell volume regulation; glutamate; human Müller cells; taurine; volume-regulated anion channel

INTRODUCTION

Glial Müller cells are mainly involved in controlling osmotic and ionic homeostasis in the retina (Bringmann et al. 2006). During intense neuronal activity, these cells can be surrounded by a hypotonic environment, since light-evoked changes in the ionic composition of the extracellular fluid cause a decrease in osmolarity (Dmitriev et al. 1999). These osmotic gradients lead to Müller cell swelling, which is followed by a regulatory volume decrease (RVD) response. Previous reports have shown that this RVD is due to the isoosmotic efflux of KCl associated with an outflow of water through aquaporin-4 (AQP4) (Pannicke et al. 2004, 2005; Reichenbach and Bringmann 2010). Our studies using a human Müller cell line (MIO-M1) also demonstrated that RVD partially depends on the immediate activation of K^+ as well as Cl^- channels and, consequently, on membrane potential (V_m) (Fernández et al. 2013; Netti et al. 2017a). However, we also proposed that there is an important fraction of RVD (~70%) that depends on the efflux of other molecules, such as organic osmolytes. In fact, early reports in whole retinas indicate that taurine (Tau) and glutamate (Glu), the most relevant amino acids in Müller cells, exhibit an osmosensitive efflux in response to hypotonic shock, which can be reduced in the presence of several Cl^- channel blockers (Pasantes-Morales et al. 1999). Nevertheless, since these experiments were performed in retinal slices, the particular contribution of Müller cells was not determined. To our knowledge, there is only one report in the Müller cell line

Address for reprint requests and other correspondence: C. Capurro, Laboratorio Biomembranas, IFIBIO Houssay, CONICET-UBA, Dept. de Ciencias Fisiológicas, Facultad de Medicina, Univ. de Buenos Aires. Paraguay 2155, Piso 7 (1121), Buenos Aires, Argentina (e-mail: capurro@retina.ar).

TR-MUL5 that has shown a volume-sensitive Tau efflux under hypotonic conditions (Ando et al. 2012). In addition, vesicular Glu release from Müller cells has been reported during hypotonicity, not as a direct RVD effector but as part of an autocrine signaling cascade that involves the activation of Glu metabotropic receptors and ATP-mediated release of KCl (Brückner et al. 2012). Thus the putative contribution of Tau and Glu release to RVD response in Müller cells and the regulation of these effluxes have not been fully studied.

A key player in the RVD response is the volume-regulated anion channel (VRAC), ubiquitously expressed in most cell types, which mediates characteristic swelling-activated Cl^- currents, but it is also permeable to organic osmolytes including Tau and Glu (Mongin 2016; Pasantes-Morales 2016; Pedersen et al. 2016). Despite the fact that the biophysical characteristics and the diverse physiological roles of VRAC have been extensively studied using unspecific inhibitors, its molecular identity was unknown until very recently. It is now proposed that VRAC is formed by the heteromerization of proteins that belong to the family of leucine-rich repeat containing 8 (LRRC8) (Qiu et al. 2014; Voss et al. 2014). Among the five homologous LRRC8 proteins, LRRC8A appears to be essential for channel function but must heteromerize with at least one additional isoform, LRRC8B-E (Lutter et al. 2017; Voss et al. 2014). Even though numerous intracellular signaling enzymes and cascades have been implicated in the regulation or modulation of VRAC, none of them appear to play the dominant and universal role across many cell types; thus the downstream mechanisms that link changes in cell volume to channel activity still remain unsolved (Mongin 2016). One of the most important discrepancies from different cellular models concerns how these channels are regulated by Ca^{2+} (Akita and Okada 2014). Even more, the influence of Ca^{2+} on amino acid efflux has not been fully studied in Müller cells. We previously reported that during Müller cell swelling, there is a slight and transient increase in Ca^{2+} levels, due to both Ca^{2+} influx and Ca^{2+} release from intracellular stores, this being the last process essential for RVD (Netti et al. 2017a). We therefore hypothesized that Tau and Glu osmosensitive efflux could be Ca^{2+} dependent. This issue is of particular interest in the deep layers of the retina, where extracellular Ca^{2+} concentration may vary in physiological conditions, such as in light-dark adaptation (Dmitriev et al. 1999), probably defining the efficiency of cell volume regulation. Therefore, the aim of this study was to evaluate the contribution of Tau and Glu efflux to the RVD response in Müller cells and to identify the signaling pathways that lead to their release.

MATERIALS AND METHODS

Cell culture. The MIO-M1 cell line (kindly provided by Dr. Astrid Limb, University College London, London, UK) is a spontaneously immortalized retinal Müller glial cell line, originated from the human retina (Limb et al. 2002). Cells were grown as monolayers in the presence of DMEM/Glutamax (GIBCO, Thermo Fisher Scientific) supplemented with 10% FBS (Internegocios), containing 5 μg/ml streptomycin and 5 U/ml penicillin (GIBCO, Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were routinely subcultured every week, and those to be studied were grown on coverslips during 3–4 days before the experiments.

[^{3}H]Tau and[^{3}H]Glu release experiments. MIO-M1 cells were grown on Petri dishes to a 90–95% confluence and were preloaded for 1 h at 37°C with [^{3}H]Tau or [^{3}H]Glu (0.075 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO) in isosmotic medium. Cells were then washed and superfused at 1 ml/min for 5 min to reach a stable efflux baseline. Then, the isoosmotic medium was replaced with the experimental medium and samples were collected for 15 min. Preincubation with different inhibitors was performed after radioactive preload for 20 min at 37°C and maintained throughout the experiment. Radioactivity was collected per minute, and the amount of [^{3}H]Tau or [^{3}H]Glu remaining in cells was measured after cell lysis in a liquid scintillation counter. Results are expressed as the efflux rate constant for each amino acid as a function of time. With this aim, the natural logarithm to the fraction of ^{3}H activity remaining in the cells was plotted vs. time, and the rate constant for the efflux of each amino acid (min^{-1}) at each time point was subsequently estimated as the negative slope of the curve between the time point and the proceeding time point as previously reported (Qiu et al. 2014). Finally, the relative total amount of [^{3}H]Tau and [^{3}H]Glu released during the experiments was calculated as the area under the curve of the plot of the radioactivity released per minute as percentage of the total radioactivity incorporated during loading.

Measurement of cell volume changes, water permeability, RVD response, and intracellular calcium levels. MIO-M1 cells were seeded on glass coverslips (12-mm diameter) at 5–10 $\times$ 10^3 cells/mL densities for 48 h and then were subjected to the different experimental conditions. By using the Ca^{2+}-sensitive dye fura-2 AM (Invitrogen, Thermo Fisher Scientific) and recording at the Ca^{2+}-sensitive (380 nm) and -insensitive (358 nm, isosbestic) wavelengths, we simultaneously registered in single cells changes in cell volume (358 nm) and in intracellular Ca^{2+} levels (358/380) (R_t/R_0, fura-2) (Altamirano et al. 1998; Netti et al. 2017a). MIO-M1 cells grown on coverslips were mounted on a chamber, incubated in 14 μM fura-2 AM for 60 min at 37°C and then washed to remove the excess of dye. To prevent dye compartmentalization upon loading, Pluronic F127 (0.2%; Molecular Probes) was used to dissolve the fura-2 AM dye. The coverslips were incubated in the experimental buffer at 20°C for at least 15 min before the experiment. The chamber was placed on the stage of a Nikon TE-200 epifluorescence inverted microscope (Nikon Planfluor $\times$40 oil immersion objective lens) as previously described (Ford et al. 2005). Fluorescence was collected from a small circular region (pinhole) of 1–3% of the total area of the cell, localized in the central region of the cell. Fluorescence data were acquired every 10 s at 20°C using a charge coupled device camera (Hamamatsu C4742–95) connected to a computer with the Metafluor data acquisition software (Universal Imaging). During experiments, bathing solution was exchanged by aspirating the media and adding new media.

Cells were exposed to hypotonic shock and relative fluorescence (F_t/F_0) was recorded. F_0 represents the pinhole signal when placed in equilibrium with an iso-osmotic medium with an osmolality OsM_0 (300 mosmol/kgH_2O) and F_t is the fluorescence from the same region at time t, when placed in equilibrium with a solution with an osmolality of OsM_t. Under isoosmotic conditions, no changes in relative fluorescence were observed after 1 h, indicating that there was no loss of the fluorophore and/or dye bleaching affecting cell volume measurements (F_t/F_0, 0 vs. 60 min: 1.003 $\pm$ 0.005 vs. 1.002 $\pm$ 0.014, n = 25 cells, ns). Changes in cell volume can be calculated as follows:

$$\frac{V}{V_0} = \frac{\left(\frac{F_0}{F_t}\right) - f_b}{1 - f_b}$$

where V is cell volume at time t; V_0 is cell volume at $t = 0$; F_0/F_t represents the relative fluorescence; and f_b is the relative background. This parameter corresponds to the y intercept of a plot of F_0/F_t vs. OsM_0/OsM_t, which represents relative fluorescence distributed in intracellular compartments and not sensitive to osmotic changes.

RVD after cell exposure to a hyposmotic medium was calculated using the following equation:

$$RVD_{15min} = \left[\frac{\left(\frac{V}{V_0}\right)_{max} - \left(\frac{V}{V_0}\right)_t}{\left(\frac{V}{V_0}\right)_{max} - 1} \right] \times 100$$

where $(V/V_0)_{max}$ is the maximum value of V/V_0 attained during hypotonic swelling (peak), and $(V/V_0)_t$ represents the value of V/V_0 observed at time t. $RVD_{15\ min}$ denotes the magnitude of volume regulation at 15 min and $\%RVD_{15\ min}$ represents the percentage of RVD at this time, with a 100% RVD indicating complete volume regulation and a 0% RVD indicating no volume regulation.

Osmotic water permeability of MIO-M1 cells was estimated from the time course of V/V_0 during the first 2 min after the hypotonic shock. Curves for each experiment were fitted with a single exponential function with the software GraphPad Prism 6.0 and the osmotic water permeability coefficient (P_f), was calculated from the exponential time constant (τ) using the equation:

$$P_f = \frac{V_0}{\tau \cdot A \cdot \Delta OsM \cdot V_w}$$

where V_0 is the initial cell water volume; A is the cell surface area; ΔOsM is the osmotic gradient, and V_W is the partial molar volume of water (18 cm^3/mol). Volume-area relation (V_0/A) of MIO-M1 cells was calculated from confocal images using Imaris 7.1.0 software (Bitplane) with a mean value of 20.85 ± 0.70 ($\times 10^{-5}$ cm, $n = 27$).

Western blotting. Confluent MIO-M1 cells were washed three times in cold PBS and were incubated for 30 min at 4°C in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris·HCl pH 7.5, 0.5 g % sodium deoxycholate, 0.1 g/% SDS, 2.5 mM EDTA, 1% Triton 100, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Cells were then collected with a rubber scraper, homogenized, and sonicated. Cell lysates were subjected to electrophoresis in 7.5% SDS-polyacrylamide gel (Bio-Rad), transferred to a nitrocellulose membrane (Bio-Rad), and blocked 1 h with 5% nonfat dried skimmed milk. Membranes were then incubated with the mouse monoclonal LRRC8A antibody (1/500) raised against amino acids 711–810 representing partial length LRRC8A of human origin (sc-517113; Santa Cruz Biotechnology) overnight at 4°C. The blots were then washed and incubated 1 h at room temperature with an anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:7,500; Sigma-Aldrich). Membranes were visualized using the chemiluminescence method (SuperSignal Substrate; Pierce) and captured on a Gbox (Syngene).

Solutions and chemicals. For functional experiments, cells were first set for at least 10 min in an external isoosmotic solution containing the following (in mM): 126 NaCl, 5.5 KCl, 2.5 $CaCl_2$, 1.25 $MgCl_2$, 20 HEPES, and 10 glucose (osmolarity: 299 ± 2 mosmol/kgH_2O). Ca^{2+}-free solutions were made by addition of EGTA (1 mM) and replacing $CaCl_2$ by $MgCl_2$. Hypoosmotic solutions were prepared from isoosmotic solution by the removal of NaCl (osmolarity: 200 ± 2 mosmol/kgH_2O). For experiments where the hypoosmotic solution contained either 15 mM of Tau or Glu, to maintain ionic strength, control isoosmotic solution had 15 mM NaCl replaced by equiosmolar concentration of mannitol (117.5 mM NaCl + 15 mM mannitol) and hypoosmotic solutions were again prepared by NaCl removal but containing 15 mM of mannitol (control: 0 mM Tau/Glu), Tau, or Glu. All solutions were titrated to pH 7.40 using NaOH (Sigma-Aldrich), and osmolalities were routinely measured by using a pressure vapor osmometer (Wescor).

In most experiments, cells were preincubated for ~20 min in the isoosmotic extracellular solution containing drugs or vehicles and the same concentration was used throughout the experiments. We used 20 μM of 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)-oxybutyric acid (DCPIB), 50 μM of carbenoxolone (CBX), 1 μM of thapsigargin (TG), 100 nM of wortmannin, and 1 μM of 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a] pyrrolo[3,4-c] carbazole (Gö6976). All drugs were purchased from Sigma-Aldrich except DCPIB, which was from Tocris Bioscience, and TG, which was purchased from Invitrogen, Thermo Fisher Scientific. For experiments with BAPTA-AM in Ca^{2+}-free medium, cells were preincubated with 25 μM BAPTA-AM (Molecular Probes, Thermo Fisher Scientific) for 1 h at 37°C. One mM fura-2 AM stock solutions were prepared in DMSO and stored at −20°C until used.

Statistical analysis. Data were evaluated with either Student's *t*-test or one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Bonferroni correction for multiple comparisons (significance cut-off at α/n, being α the level of significance, usually 0.05, and n the number of hypotheses to be tested) was applied when appropriate. Values are reported as means ± SE, and n is either the number of cells evaluated in each condition for cell volume measurements or the number of experiments for [^{3}H]release assays. All statistical procedures were performed using GraphPad Prism 6 statistical software package.

RESULTS

Osmosensitive release of [^{3}H]Tau and [^{3}H]Glu from MIO-M1 cells. We first evaluated the hypotonicity-induced release of Tau and Glu in MIO-M1 cells by loading them with the labeled tracers [^{3}H]Tau and [^{3}H]Glu in isoosmotic medium and then exposing them to a reduction in external osmolarity (ΔOsm: 100 mosmol/kgH_2O). As shown in Fig. 1, the efflux rate constants for both amino acids were low under isotonic conditions but increased dramatically and transiently following the hypotonic shock. These effluxes had similar kinetics and reached their peak at ~6 min (time to peak in minutes, Tau vs. Glu: 6.66 ± 0.80 vs. 6.33 ± 0.88, $n = 5–6$, ns). However, maximal Tau release was larger in magnitude (*inset*: maximal change in rate constant for [^{3}H]Tau or [^{3}H]Glu efflux during the experiments). In line with this, the relative total amount of [^{3}H]Tau released during 15 min of experimental time was also larger in comparison to [^{3}H]Glu (%Tau vs. Glu: 31.4 ± 1.8 vs. 14.9 ± 1.7, $n = 5–6$ experiments, $P < 0.001$). Moreover, both [^{3}H]Tau and [^{3}H]Glu effluxes were proportional to the magnitude of the hypotonic stimuli (data not shown).

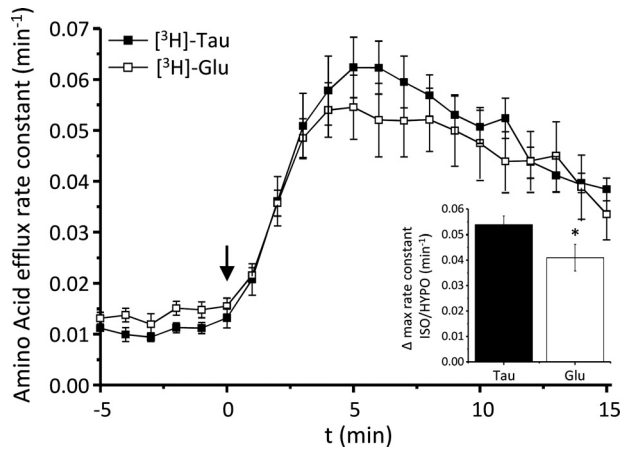

Fig. 1. Osmosensitive release of [^{3}H]taurine ([^{3}H]Tau) and [^{3}H]glutamate ([^{3}H]Glu) from MIO-M1 cells. Time course of efflux rate constants of [^{3}H]Tau and [^{3}H]Glu (min^{-1}) from MIO-M1 cells exposed to a hypoosmotic (HYPO) media ($\Delta OsM = 100$ mosmol/kgH_2O, at the arrow) determined as described in MATERIALS AND METHODS. *Inset*: Δmaximal change in the rate constant from basal isotonic (ISO) conditions to maximal hypotonic release (min^{-1}). Values are expressed as means ± SE from 6 to 7 experiments. *$P < 0.05$, [^{3}H]Tau vs. [^{3}H]Glu.

Since in many cell types VRAC is the main mediator of osmolyte release, we tested the effects of DCPIB and CBX, the most specific and widely used VRAC inhibitors (Benfenati et at. 2009; Decher et al. 2001) on [^{3}H]Tau and [^{3}H]Glu efflux in Müller cells. As shown in Fig. 2, *A* and *B*, DCPIB and CBX significantly reduced the swelling-induced efflux of both amino acids to basal release (Fig. 2, *A* and *B*, *insets*), indicating that VRAC may be the release pathway. In fact, Western blot analysis using a specific antibody against LRRC8A, the obligatory subunit of VRAC, revealed a band of ~90 kDa, confirming the presence of VRAC in MIO-M1 cells (Fig. 2*C*). We next evaluated if the reduction in amino acid release had an impact on RVD by using fura-2 AM to measure cell volume in cells exposed to hypotonic shock. Figure 2*D* shows the kinetics of relative volume changes (V/V_0) and cell volume regulation (insert: $\%RVD_{15\ min}$). It can be observed that while the hypotonic swelling was not modified by the inhibitors, RVD efficiency was markedly reduced by ~80 and 50% in the presence of DCPIB and CBX, respectively.

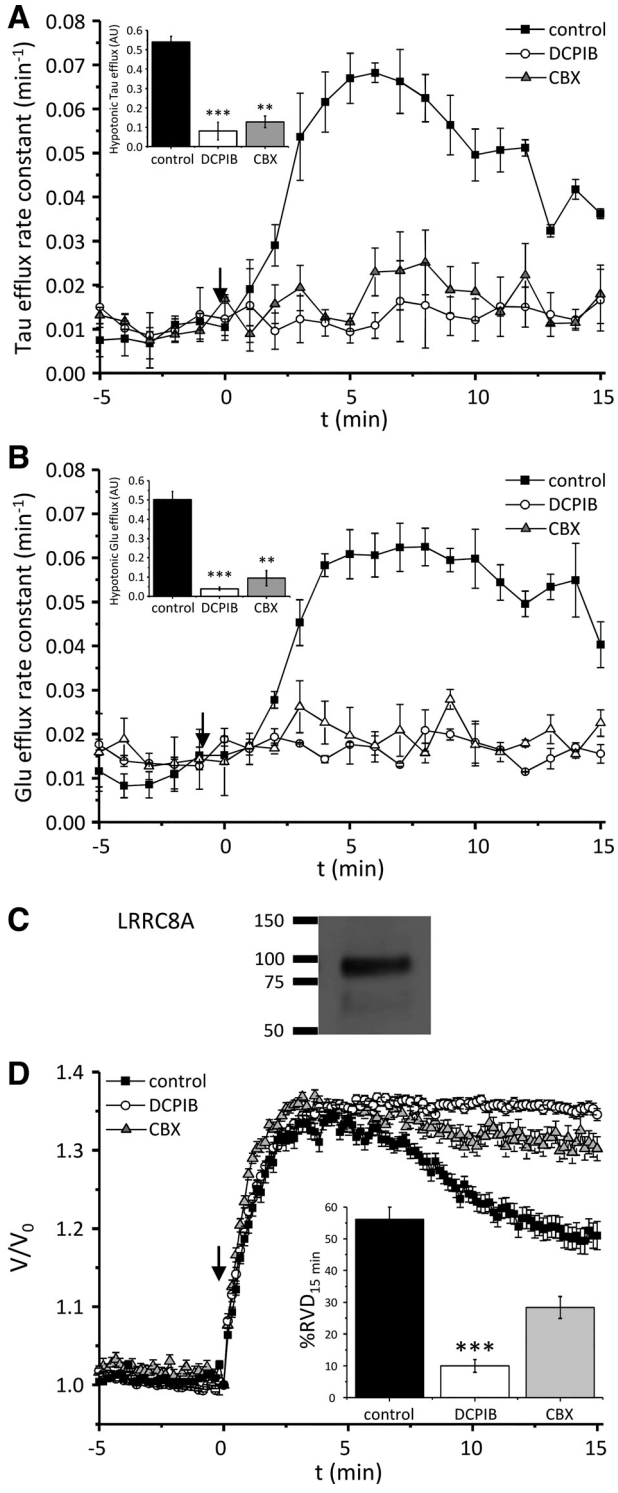

Fig. 2. [^{3}H]taurine ([^{3}H]Tau) and [^{3}H]glutamate ([^{3}H]Glu) efflux and regulatory volume decrease (RVD) response during volume-regulated anion channel (VRAC) inhibition in MIO-M1 cells. Cells were pretreated with the VRAC inhibitors 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)-oxybutyric acid (DCPIB; 20 μM), carbenoxolone (CBX; 50 μM), or vehicle (control), and then exposed to a hypoosmotic gradient (ΔOsM = 100 mosmol/kgH_2O, indicated by arrows). *A* and *B*: time course of efflux rate constants of [^{3}H]Tau (*A*) and [^{3}H]Glu (*B*) from MIO-M1 cells under the above mentioned experimental conditions. *Inset*: Δmaximal change in rate constant from isotonic to hypotonic conditions. Values are expressed as means ± SE from 3 to 4 experiments; $^{**}P < 0.001$, control vs. DCPIB/CBX; $^{***}P < 0.0005$, control vs. DCPIB/CBX. *C*: Western blot showing leucine-rich repeat containing 8 (LRRC8A) expression in MIO-M1 cells. *D*: time course of the relative cell volume changes (V/V_0) in the same experimental conditions. *Inset*: RVD response 15 min after maximum osmotic swelling ($\%RVD_{15\ min}$). Values are expressed as means ± SE from 40 to 58 cells from 4 to 6 experiments. $^{***}P < 0.0005$, control vs. DCPIB/CBX.

To confirm a direct contribution of the release of these amino acids to RVD, we tested if RVD could be in fact modulated by altering the Tau and Glu gradient across the plasma membrane. With that aim, we added 15 mM of Tau or Glu to the extracellular hypotonic media (ΔOsm: 100 mosmol/kgH_2O) but maintaining the ionic strength (15 mM NaCl was replaced by equiosmolar concentration of mannitol in both control iso and hypotonic media). As shown in Fig. 3, the addition of Tau or Glu to the hypoosmotic extracellular medium did not affect the time course of V/V_0 during the first ~7 min after cell swelling, but $RVD_{15\ min}$ was significantly reduced by ~25%, in comparison with control conditions (0 mM Tau/Glu), at the time when amino acid release reached the maximal peak. This result confirms a relevant contribution of both Tau and Glu efflux to RVD in Müller cells. Altogether, these results suggest that the osmosensitive VRAC pathway is involved in both Tau and Glu efflux, which contributes to RVD in MIO-M1 cells.

Influence of Ca^{2+} on swelling-induced [^{3}H]Tau and [^{3}H]Glu release and RVD in MIO-M1 cells. We next examined the contribution of extracellular and intracellular Ca^{2+} to swelling-induced [^{3}H]Tau and [^{3}H]Glu release and RVD by removing Ca^{2+} from the extracellular solution ($0Ca^{2+}$) and adding to this condition the membrane-permeable Ca^{2+} chelator BAPTA-AM (BAPTA-AM $0Ca^{2+}$). Figure 4*A* shows the time course of relative changes in intracellular Ca^{2+} levels (R_t/R_0 fura-2) under the mentioned conditions. It can be observed that cell swelling led to a 5% increase in Ca^{2+} levels, but this increase was reduced to 2.5% in Ca^{2+}-free media (*inset*: %max increase in R_t/R_0 fura-2). As expected, treatment of cells with BAPTA-AM $0Ca^{2+}$ completely abolished all swelling-induced changes in intracellular Ca^{2+} levels. These results indicate that intracellular Ca^{2+} changes induced by hypotonic shock involve Ca^{2+} release from intracellular stores as well as Ca^{2+} influx, as previously reported (Netti et al. 2017a). Regarding the efflux of

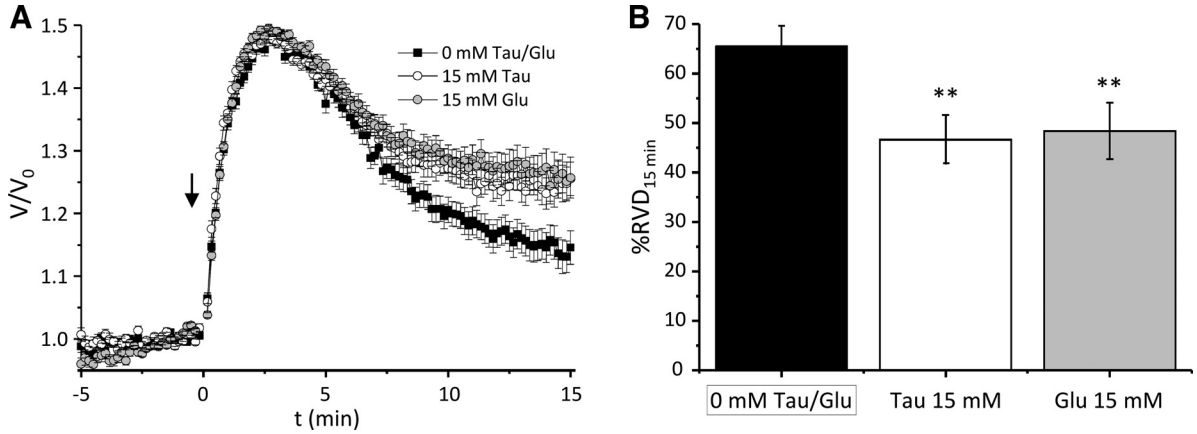

Fig. 3. Effects of extracellular taurine and glutamate in regulatory volume decrease (RVD) response in MIO-M1 cells. Time course of the relative cell volume changes (V/V_0; *A*) and RVD response 15 min after maximum osmotic swelling ($\%RVD_{15\ min}$; *B*) of MIO-M1 cells exposed to control conditions (0 mM of Tau/Glu) or 15 mM of extracellular Tau or Glu. Values are expressed as means ± SE from 38 to 45 cells from 4 to 5 experiments. $^{**}P < 0.001$, control vs. Tau or Glu.

amino acids, the rate constants for Tau or Glu release under isoosmotic conditions were not affected by Ca^{2+} removal (control vs. $0Ca^{2+}$, Tau: 0.011 ± 0.001 vs. 0.014 ± 0.001, n = 4, ns; Glu: 0.012 ± 0.001 vs. 0.011 ± 0.001, n = 4, ns). During the hypotonic shock, [^{3}H]Tau efflux was not modified in Ca^{2+}-free medium, but it was reduced by ~50% in the presence of BAPTA-AM $0Ca^{2+}$, indicating that there was still a Ca^{2+}-insensitive fraction (Fig. 4*B*). However, swelling-induced [^{3}H]Glu release was insensitive to the absence of extracellular and intracellular Ca^{2+} (Fig. 4*C*). Finally, RVD was unaffected in Ca^{2+}-free medium, but it was reduced by 90% when intracellular Ca^{2+} was chelated (Fig. 4*D*), indicating that intracellular stores play a key role in RVD, as we previously showed (Netti et al. 2017a).

Before dismissing the hypothesis that the swelling-induced release of Glu occurred by Ca^{2+}-independent pathways in MIO-M1 cells, we tested if an increase in intracellular Ca^{2+} concentration above basal levels, before the hypotonic shock, may modulate its release. To do so, we activated a store-operated Ca^{2+} entry under isoosmotic conditions by using TG, a noncompetitive inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, which causes in MIO-M1 the depletion of intracellular Ca^{2+} stores and induces a significant and sustained ~25% increase in intracellular Ca^{2+} levels, which remained elevated throughout the hypotonic media exposition (Netti et al. 2017). Then, we investigated the putative contribution of this Ca^{2+} increase to amino acid efflux and RVD. Preincubation of MIO-M1 cells with 1 μM TG did not trigger the release of amino acids under isotonic conditions but significantly increased the rate of [^{3}H]Tau and [^{3}H]Glu efflux when exposed to hypotonic solution (Fig. 5, *A* and *B*), indicating that Ca^{2+} signaling contributes to amino acid efflux. This TG-induced increase in the release of both amino acids was blunted in the presence of DCPIB and CBX. Even more, the increase in Ca^{2+} levels induced by TG was also decreased in the presence of DCPIB and CBX ($\%\Delta R_t/R_0$ fura-2: TG 17.26 ± 1.46; TG + DCPIB 8.05 ± 1.11; TG + CBX 5.04 ± 2.15, n = 48–66 cells, TG vs. TG + DCPIB/CBX; $^{***}P < 0.0005$). Finally, TG pretreatment largely enhanced RVD response and the cell volume was completely recovered at 15 min (Fig. 5*C*). However, in the presence of DCPIB and CBX, the TG-induced RVD acceleration was reduced, being once again this decrease greater for DCPIB in comparison to CBX.

Together, these data indicate that the swelling-induced Tau efflux is dependent on intracellular Ca^{2+} stores, while Glu release seems to be Ca^{2+} independent. However, when Ca^{2+} levels overcome the physiological swelling-activated Ca^{2+} increase (~5%), both Tau and Glu release are notably potentiated, providing evidence that there would be a Ca^{2+} threshold to activate both Tau and Glu efflux under this experimental condition.

Involvement of Ca^{2+}-dependent PKC isoforms on swelling-induced [^{3}H]Tau and [^{3}H]Glu release and RVD in MIO-M1 cells. We further explored whether the observed differences in Ca^{2+} dependence for Tau and Glu efflux were also reproduced when modulating Ca^{2+}-dependent conventional PKC isoforms, which was proposed to regulate Tau efflux, but not Glu, in astrocytes (Estevez et al. 1999). With this aim, we pretreated cells with Gö6976, the selective inhibitor of these PKC isoforms. Results indicated that there were no significant differences in the release rate constants of [^{3}H]Tau and [^{3}H]Glu as compared with control conditions (Fig. 6, *A* and *B*, *insets*). Even more, as shown in Fig. 6, *C* and *D*, hypotonic swelling was faster when cells were pretreated with Gö6976, probably due to the observed increase in osmotic water permeability coefficient (P_f), which is known to be mediated by AQP4 in Müller cells. Probably because of this, the $\%RVD_{15\ min}$ was faster in the presence of inhibitor Gö6976 (Fig. 6*D*). These results indicate that the PKC inhibition-mediated increase in swelling kinetics leads to a faster cell volume regulation in MIO-M1 cells.

Involvement of phosphatidylinositol-3-kinase in swelling-induced [^{3}H]Tau and [^{3}H]Glu release and RVD in MIO-M1 cells. Based on the data presented here showing that extra and intracellular Ca^{2+} removal did not affect neither Glu release nor a fraction of Tau efflux, we next explored the putative contribution of Ca^{2+}-independent phosphatidylinositol-3-kinase (PI3K) pathway on the release of these amino acids, which was reported to be involved in the volume homeostasis in the chicken retina (de la Paz et al. 2002). Thus we tested the effects of wortmannin, a potent and specific PI3K inhibitor, in MIO-M1 cells. As shown in Fig. 7, *A* and *B*, PI3K inhibition

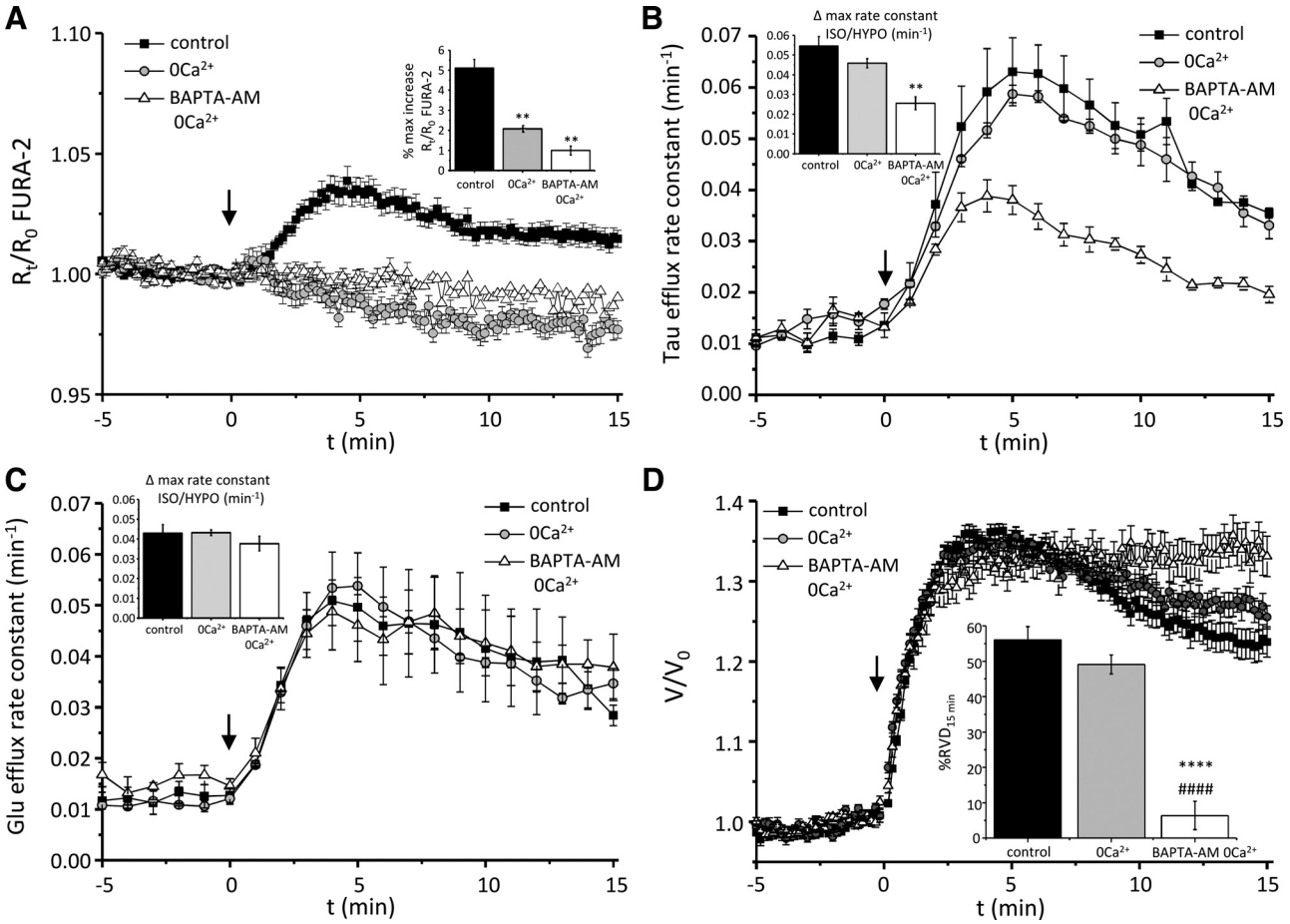

Fig. 4. Contribution of extracellular and intracellular Ca^{2+} to the osmosensitive release of [^{3}H]taurine ([^{3}H]Tau) and [^{3}H]glutamate ([^{3}H]Glu) and regulatory volume decrease (RVD) in MIO-M1 cells. Cells were exposed to *1*) control medium (with 2.5 mM Ca^{2+}), *2*) the absence of external Ca^{2+} ($0Ca^{2+}$), or *3*) the presence of BAPTA-AM during external Ca^{2+} removal (BAPTA-AM $0Ca^{2+}$) and then subjected to a hypoosmotic stress (ΔOsM = 100 mosmol/kgH_2O, indicated by arrows). *A*: kinetics of intracellular Ca^{2+} levels measured as the ratio 358/380 (R_t/R_0 fura-2) of MIO-M1 cells exposed to the above mentioned experimental conditions. *Inset*: %max increase in R_t/R_0 fura-2. Values are expressed as means ± SE for 49–61 cells from 4 to 6 independent experiments. $^{**}P <$ 0.001, control vs. $0Ca^{2+}$/BAPTA-AM $0Ca^{2+}$. *B* and *C*: time course of efflux rate constants of [^{3}H]Tau (*B*) and [^{3}H]Glu (*C*) from MIO-M1 cells under the above mentioned experimental conditions. *Inset*: Δmaximal change in rate constant from isotonic to hypotonic conditions. Values are expressed as means ± SE from 4 to 5 experiments. $^{**}P < 0.005$, control vs. BAPTA-AM $0Ca^{2+}$. *D*: time course of the relative cell volume changes (V/V_0) in the above described experimental conditions. *Inset*: $\%RVD_{15\ min}$ for the different experimental conditions. Values are expressed as means ± SE for 49–61 cells from 4 to 6 independent experiments. $^{****}P < 0.001$, control vs. BAPTA-AM $0Ca^{2;}$ $^{\#\#\#\#}P < 0.001$, $0Ca^{2+}$ vs. BAPTA-AM $0Ca^{2+}$.

reduced the efflux rate constant of both [^{3}H]Tau and [^{3}H]Glu by ~40%, and consequently, RVD was also reduced (Fig. 7*C*). Additionally, when cells were treated with wortmannin in the absence of extra- and intracellular Ca^{2+} (wortmannin + BAPTA-AM $0Ca^{2+}$), an additive effect was observed only for [^{3}H]Tau release (~80% inhibition), indicating that PI3K regulates Tau efflux by a Ca^{2+}-independent mechanism. In line with this, cells continued to swell during the experiment and RVD was essentially impaired under this condition. However, [^{3}H]Glu efflux was not affected by the application of both treatments simultaneously, reinforcing the idea of the Ca^{2+} independency of this mechanism. These results suggest that PI3K activation represents a key Ca^{2+}-independent regulatory mechanism for Tau and Glu osmosensitive efflux in MIO-M1 cells.

DISCUSSION

The present work provides new insights regarding the contribution of Tau and Glu efflux to RVD response in the immortalized retinal Müller cell line MIO-M1, which maintains important functional characteristics of native Müller cells (Limb et al. 2002). Our results of pharmacologic and functional studies showed that Tau and Glu release is mediated by the osmosensitive VRAC pathway and contributes to RVD in Müller cells, the Tau release being higher than the Glu efflux. These data are in line with previous reports, in other cell types, showing that during cell volume regulation, amino acids are predominantly released via VRAC, which is more permeable to Tau than to Glu (Pedersen et al. 2016). The higher loss of Tau as compared with Glu could also be explained by the metabolism of Glu or by the presence of multiple Tau release pathways (Lambert and Hoffmann 1994; Mongin et al. 1999a; Shennan et al. 1994). Nevertheless, both hypothesis should be ruled out if we consider that Schober and Mongin (2015) revealed that intracellular Glu levels are conserved in swollen astrocytes and that it was shown that knockdown of the VRAC component LRRC8A completely blunted the hypotonic loss of Tau in HEK293 cells and astrocytes (Hyzinski-Garcia et al. 2014; Voss et al. 2014). We cannot disregard that differences

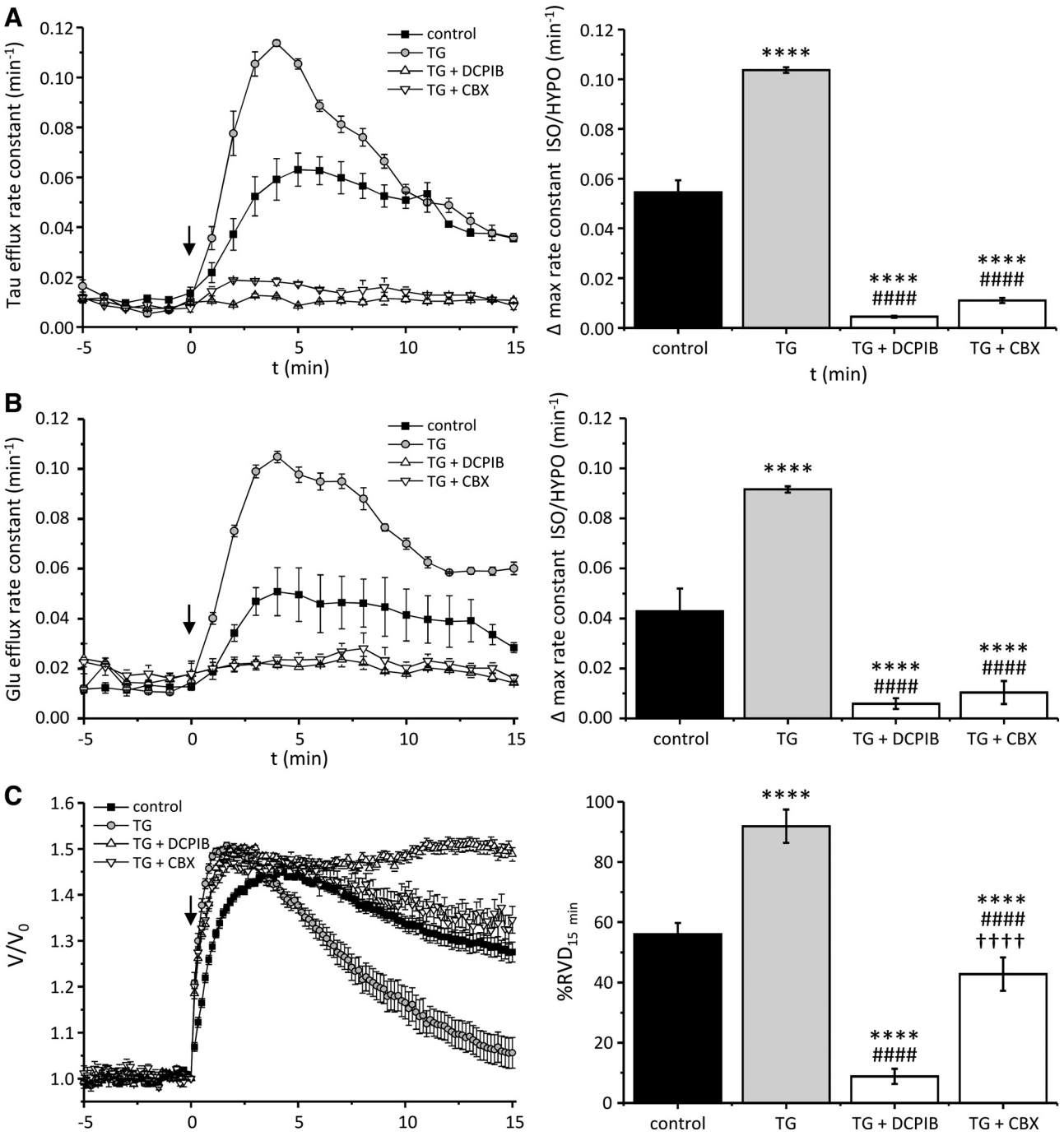

Fig. 5. Osmosensitive release of [^{3}H]taurine ([^{3}H]Tau) and [^{3}H]glutamate ([^{3}H]Glu) and regulatory volume decrease (RVD) in MIO-M1 cells in the presence of thapsigargin (TG). Cells were pretreated with the volume-regulated anion channel (VRAC) inhibitors 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)-oxybutyric acid (DCPIB; 20 μM), carbenoxolone (CBX; 50 μM), or vehicle (control) and then exposed to 1 μM TG in the presence of external Ca^{2+} and then subjected to a hypoosmotic stress (ΔOsM = 100 mosmol/kgH_2O, indicated by arrows). *A* and *B*: time course of efflux rate constants of [^{3}H]Tau (*A*) and [^{3}H]Glu (*B*) from MIO-M1 cells under the above mentioned experimental conditions (*left*) and Δmaximal change in rate constant from isotonic to hypotonic conditions (*right*). Values are expressed as means ± SE from 3 to 5 experiments. ****$P < 0.0001$, control vs. TG/TG + DCPIB/TG +CBX, ####$P < 0.0001$, TG vs. TG + DCPIB/TG +CBX. *C*: time course of the relative cell volume changes (V/V_0) in the same experimental conditions (*left*) and means values of $\%RVD_{15\ min}$ (*right*). Values are means ± SE for 35–65 cells from 4 to 5 independent experiments. ****$P < 0.0001$, control vs. TG/ TG + DCPIB/TG +CBX; ####$P < 0.0001$ TG vs. TG + DCPIB/TG +CBX; ††††$P < 0.0001$, TG + DCPIB vs. TG + CBX.

in Tau and Glu efflux may be due to a larger gradient for Tau release caused by different cytosolic amino acid content. Despite that we did not measure unlabeled Tau/Glu in this study, as previously reported by others; possibly intracellular Tau content is larger than Glu in Müller cells (de Souza et al. 2013). Then, keeping in mind the here observed differences in both the maximal hypotonic increase in the rate constants and the total amount of amino acids released, it is likely that either Tau has a larger gradient for its release and/or that VRAC is indeed more permeable to Tau than to Glu. In addition, we here show

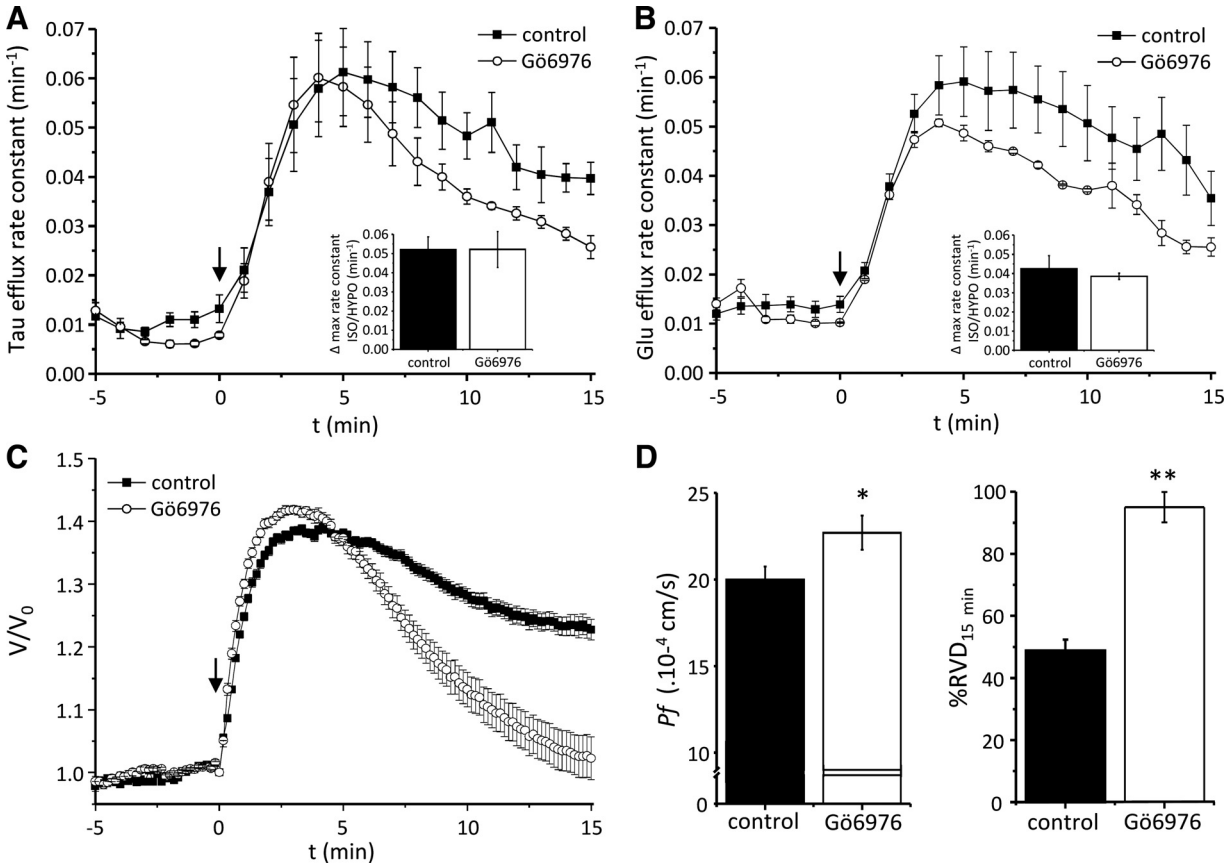

Fig. 6. Effects of PKC inhibitor Gö6976 on the osmosensitive release of [^{3}H]taurine ([^{3}H]Tau) and [^{3}H]glutamate ([^{3}H]Glu) and regulatory volume decrease (RVD) of MIO-M1 cells. Cells were treated with 1 μM of the PKC inhibitor Gö6976 or vehicle (control) and then exposed to a hypoosmotic gradient (ΔOsM = 100 mosmol/kgH_2O, indicated by arrows). *A* and *B*: time course of efflux rate constants of [^{3}H]Tau (*A*) and [^{3}H]Glu (*B*) from MIO-M1 cells. *Inset*: Δmaximal change in rate constant from isotonic to hypotonic conditions. *C*: dynamics of relative cell volume changes (V/V_0) in both experimental conditions. *D*: osmotic water permeability (P_f) and mean values of $\%RVD_{15\ min}$ of MIO-M1 cells in both experimental conditions. Values are means ± SE for 75–90 cells from 5 to 6 independent experiments. *$P < 0.05$ or **$P < 0.001$, control vs. Gö6976.

in human Müller cells that DCPIB and CBX, the most specific blockers of VRAC, prevented the release of both Tau and Glu, also indicating that VRAC is probably the main mediator of osmolyte efflux and contributes to RVD. Other release pathway for Tau and Glu sensitive to DCPIB and CBX may be connexin-43 hemichannels (Cx43), which are usually activated by Ca^{2+} removal provoking amino acid release in astrocytes (Ye et al. 2009). However, we did not observe differences in Tau or Glu efflux in the absence of Ca^{2+} under isoosmotic conditions in MIO-M1 cells, indicating that Cx43 may not be activated in our experimental conditions, dismissing this possibility. In addition, other osmosensitive pathway for ions and Glu efflux are pannexins (Pnxs), blocked by CBX (Bruzzone et al. 2005) but not by DCPIB. Then, if Pnxs are also involved in the release of solutes, we would expect a greater inhibition of Glu efflux by CBX, and consequently of RVD, in comparison to DCPIB. Since this was not shown, we excluded their contribution. It was recently demonstrated that the intrinsic properties of VRAC, such as conductance and relative permeability to swelling-activated Cl^- currents and to osmolytes, depend on the identity of the associated subunits (Lutter et al. 2017; Syeda et al. 2016). Thus, in MIO-M1 cells, it is tempting to speculate that, in addition to VRACs transporting Tau and Glu, other heteromers that transport exclusively Cl^- or Cl^- plus other organic compounds with different pharmacological properties might also be present, explaining the here observed differences in RVD with DCPIB and CBX. Our results are in contrast with a previous report in the rat Müller cell line TR-MUL5, where DCPIB partially reduced the swelling-induced Tau efflux by ~60%, providing evidence of the contribution of an unknown DCPIB-insensitive release system (Ando et al. 2012). The reason for this apparent discrepancy to our work is unclear, but might be related to differences in experimental models and/or species. The contribution of Tau and Glu release to RVD in MIO-M1 cells was further demonstrated by altering the extracellular levels of these amino acids. Interestingly, increasing Tau and Glu concentration did not affect the %RVD during the first ~7 min after hypotonic shock, indicating that other solutes, such as KCl, may have a more relevant role in the first minutes of RVD. Nevertheless, the time course of cell volume changes became significantly reduced at the time when amino acids release would reach a maximum peak. This agrees with previous studies showing that whereas Cl^- conductance activates rapidly (<1 min) after swelling, Tau efflux activates with a delay of a few minutes (Stutzin et al. 1999), which suggest separate permeability pathways for Cl^- and amino acids, as discussed below.

Despite extensive research efforts, the intracellular mechanisms involved in VRAC activation are not fully understood, but permissive cytosolic Ca^{2+} concentrations seem to be nec-

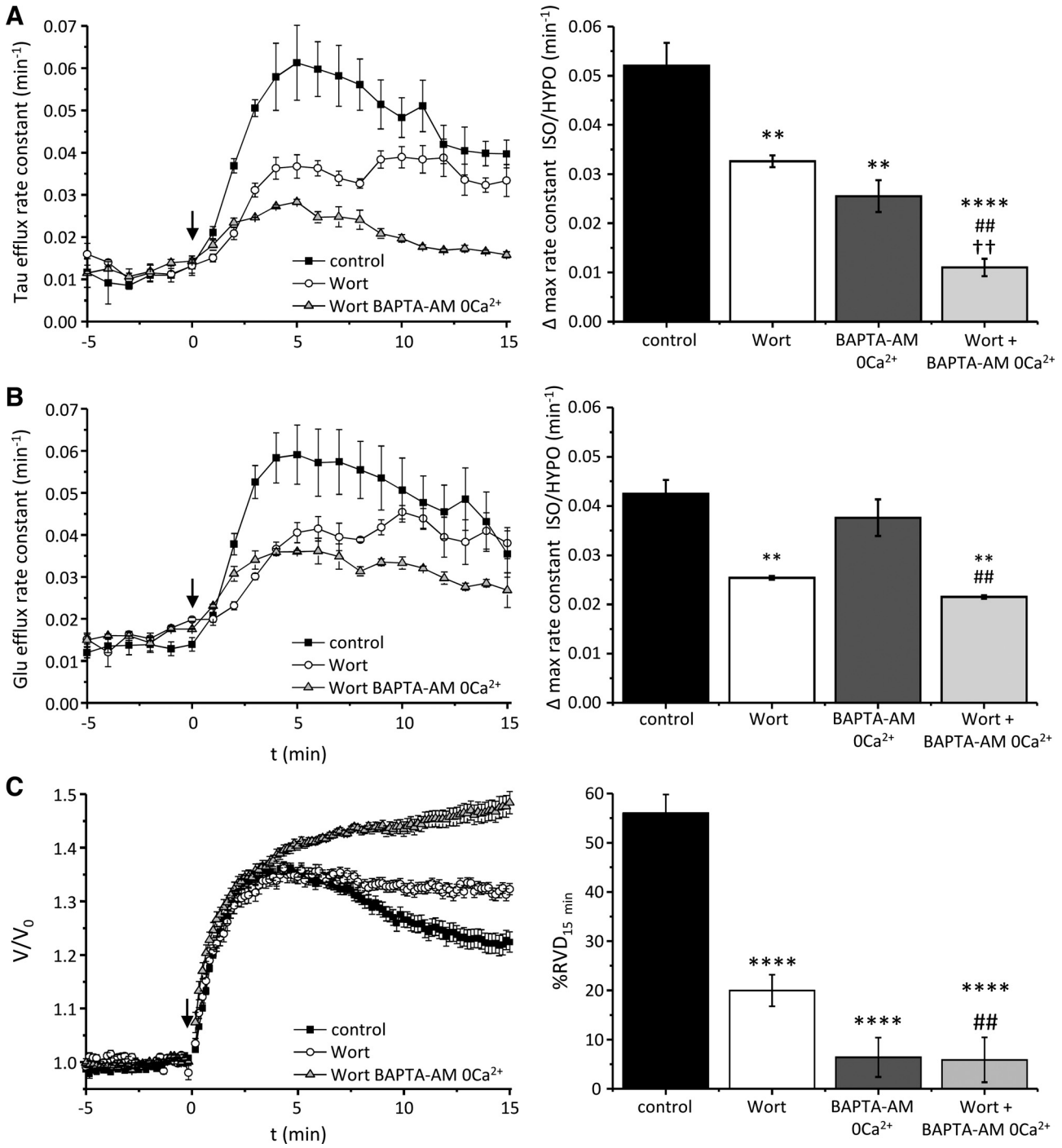

Fig. 7. Effects of the phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin on the osmosensitive release of [^{3}H]taurine and [^{3}H]glutamate and regulatory volume decrease (RVD) of MIO-M1 cells in the presence or absence of Ca^{2+}. MIO-M1 cells were exposed to *1*)control medium (with Ca^{2+}), *2*)100 nM of PI3K inhibitor wortmannin (Wort), *3*) BAPTA-AM during external Ca^{2+} removal (BAPTA-AM $0Ca^{2+}$), or *4*) Wort + BAPTA-AM in the absence of external Ca^{2+} (Wort BAPTA-AM $0Ca^{2+}$) and then subjected to a hypoosmotic stress (ΔOsM = 100 mosmol/kgH_2O, indicated by arrows). *A* and *B*: time course of efflux rate constants of [^{3}H]Tau (*A*) and [^{3}H]Glu (*B*) from MIO-M1 cells under the above mentioned experimental conditions (left) and Δmaximal change in rate constant from isotonic to hypotonic conditions (right). For Tau release (*A*): $^{**}P < 0.005$ control vs. Wort/BAPTA-AM $0Ca^{2+}$; $^{****}P < 0.0001$, control vs. Wort BAPTA-AM $0Ca^{2+}$; $\#\#P < 0.005$, Wort vs. Wort BAPTA-AM $0Ca^{2+}$; $\dagger\dagger P < 0.005$, BAPTA-AM $0Ca^{2+}$ vs. Wort BAPTA-AM $0Ca^{2+}$. For Glu release (*B*): $^{**}P < 0.005$, control vs. Wort/Wort BAPTA-AM $0Ca^{2+}$; $\#\#P < 0.005$, BAPTA-AM $0Ca^{2+}$ vs. Wort BAPTA-AM $0Ca^{2+}$. *C*: dynamics of relative cell volume changes (V/V_0) (*left*) and mean values of $\%RVD_{15\ min}$ for all the mentioned experimental conditions (*right*). Values are means ± SE for 35–45 cells from 4 to 5 independent experiments. $^{****}P < 0.0001$, control vs. Wort/BAPTA-AM $0Ca^{2+}$/Wort BAPTA-AM $0Ca^{2+}$; $\#\#P < 0.005$, Wort vs. Wort BAPTA-AM $0Ca^{2+}$.

essary for channel activation in astrocytes (Mongin et al. 1999b; Mongin and Kimelberg 2005). Our previous (Netti et al. 2017a) and present results show that swelling-induced Ca^{2+} increase in MIO-M1 cells is due to Ca^{2+} influx and release from intracellular stores but only the latter were relevant for RVD. Here we investigate the relevance of Ca^{2+} signaling pathways on swelling-induced amino acid release and RVD in these cells. We observed a differential regulation for Tau and

Glu: Tau efflux was dependent on Ca^{2+} release from intracellular stores, but Glu efflux was essentially Ca^{2+} independent. In line with this, in the absence of external Ca^{2+}, RVD was unaffected because the effluxes of Tau, Glu, and probably also K^+ and Cl^- do not depend on Ca^{2+} entry. However, when intra- and extracellular Ca^{2+} was unavailable, RVD was greatly reduced, probably because Ca^{2+}-activated K^+ channels and CaCCs are not active and this efflux of ions are critical during the first minutes of RVD. Nevertheless, in this condition there are still a Ca^{2+}-independent Tau fraction and the Ca^{2+}-independent Glu efflux, not supporting the fact that Tau and Glu release play a significant role in RVD. However, when these Ca^{2+}-independent effluxes are further reduced by the PI3K inhibitor wortmannin, MIO-M1 cells were not only unable to regulate their volume but continued swelling as well. Then, we hypothesize that Tau and Glu release under Ca^{2+}-free conditions contributes to prevent further swelling, demonstrating that in fact these amino acids are participating in RVD. Differences in Ca^{2+} sensitivity of Tau and Glu effluxes, together with the differences in the time courses of activation between ions and amino acids release, support the fact that MIO-M1 cells express different VRAC isoforms. Interestingly, this may also explain why higher levels of intracellular Ca^{2+} increased Glu release in MIO-M1 cells. This was also observed by Cardin et al. (2003), who reported that a rise in cytosolic Ca^{2+} levels induced by ionomycin potentiated Tau release in cerebellar astrocytes, even though this efflux appeared to be Ca^{2+}-independent in these cells. As previously mentioned, the recently reported molecular identity of VRACs as LRRC8 heteromers leads to the proposal that cells may express VRAC isoforms with different intrinsic properties, as predicted by Stutzin et al. (1999) and Shennan (2008). In fact, it was now reported that the LRRC8A/D heteromer favors permeation of uncharged organic osmolytes (such as Tau) and LRRC8A/E heteromers create a conduit for charged osmolytes (such as Glu), which was shown by disrupting LRRC8 genes by the CRISPR-Cas9 method in HEK293 cells (Lutter et al. 2017; Planells-Cases et al. 2015) and by siRNA experiments in astrocytes (Schober et al. 2017). In MIO-M1 cells, we have provided evidence of the expression of LRRC8A by Western blot and our microarray experiments performed to evaluate changes in gene expression induced by hypotonicity has proven the presence of LRRC8D and E (Netti et al. 2017b). Although our observations do not directly address this point, we hypothesize that the observed disparity in Ca^{2+} dependence for Tau and Glu efflux in Müller cells may provide evidence that the LRRC8 proteins that conform the VRAC isoforms not only determine VRAC substrate selectivity, but also have an influence on how the channel is regulated by Ca^{2+}. The proposed coexistence of at least two functionally different LRRC8-containing heteromeric VRAC channels in rat astrocytes, also supported by our present findings in human Müller cells, may explain why VRAC regulation by signaling events is complex, cell specific, and still not elucidated.

On the other hand, we evaluated if the observed differences for Tau and Glu release in the Ca^{2+}-mediated regulation of VRAC could involve PKC as an effector of Ca^{2+} signaling, since it was recently reported that the predicted topology of VRAC heteromers includes putative phosphorylation sites for PKC (Mongin 2016). Our data demonstrate that in MIO-M1 cells PKC inhibition did not affect the release rates for Tau or Glu effluxes during the hypotonic shock. These results are in line with the reported PKC-independent Tau release in rat supraoptic nucleus and in cultured cerebellar granule neurons (Deleuze et al. 2000; Morales-Mulia et al. 2001) and PKC-independent Glu efflux in astrocytes (Estevez et al. 1999; Rudkouskaya et al. 2008). However, we here show that PKC inhibition significantly accelerated the RVD response, associated, at least in part, to an increase in P_f. In Müller cells, water permeability is determined by AQP4, a key player in cell volume homeostasis (Nagelhus and Ottersen 2013). In other systems, AQP4 phosphorylation in Ser180 by PKC has been shown to decrease P_f (Mc Coy et al. 2010; Zelenina et al. 2002). Thus we suggest that under our experimental conditions, PKC is not directly involved in modulating VRAC-mediated osmolyte efflux but PKC-mediated changes in cell swelling may indirectly accelerate the activation of RVD effectors. Keeping in mind that neural activity generates changes in external osmolarity leading to cell swelling, we speculate that water permeability modulation by PKC could represent a defense mechanism against an abrupt volume change.

Finally, our results showed that even though Tau efflux is largely Ca^{2+} dependent, there is also a Ca^{2+}-insensitive fraction. This, together with the fact that Glu release was insensitive to Ca^{2+} removal, led us to explore the putative contribution of the Ca^{2+}-independent PI3K pathway to the release of both amino acids. In fact, in the retina it was reported that PI3K seems to be an important Ca^{2+}-independent signaling mechanism linking cell swelling to Tau efflux (de la Paz et al. 2002). Moreover, the activation of VRAC currents in cardiac cells also requires the PI3K-mediated increase in phosphatidylinositol (3,4,5)-trisphosphate but only in the presence of osmotic stimulation (Yamamoto et al. 2008). In line with these findings, our results in MIO-M1 cells show that PI3K pathway modulates both Tau and Glu efflux, and consequently, RVD response, indicating that this Ca^{2+}-independent pathway is also relevant in this cell line. Even more, we observed that swelling-induced Tau release has a dual modulation, by Ca^{2+} release from intracellular stores and by the activation of PI3K, mechanisms which seem to operate independently, as previously reported in cultured astrocytes (Cruz Rangel et al. 2008). However, our results are opposed to a previous report showing that the hypotonic release of Glu was unaffected by PI3K inhibition in whole chicken retina (de la Paz et al. 2002). This difference may be due to the studied species and/or to the fact that the retina is a complex tissue in which hypotonic shock may evoke multiple responses from the diverse cell types.

In summary, the present data reveal that VRAC-mediated Tau and Glu release has a relevant role in RVD in human Müller cells. Differences in Ca^{2+}-dependent regulatory mechanisms for Tau and Glu efflux suggest that their transport may be mediated by VRAC isoforms that differ in substrate selectivity and regulatory mechanisms. On the other hand, PI3K activation represents a major Ca^{2+}-independent regulatory mechanism for the efflux of both osmolytes. Since these amino acids play a key role as gliotransmitters in the retina, their release under hypotonic conditions may have an impact on neuronal activity. As Müller cell function in the retina strongly depends on tissue structure and on the presence of other cell types, the extrapolation of our results to the in vivo condition may be limited. Nevertheless, keeping in mind the appropriate

considerations, cell culture is a useful model to study the complex machinery used by Müller cells to regulate their volume. Thus these findings add new insights into knowledge on the role of amino acid efflux in retinal Müller cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.N., G.R.-M., and C.C. conceived and designed research; V.N., A.P., and M.P.-D. performed experiments; V.N., A.P., and C.C. analyzed data; V.N., A.P., H.P.-M., G.R.-M., and C.C. interpreted results of experiments; V.N. and C.C. prepared figures; V.N., P.F., and C.C. drafted manuscript; V.N., A.P., M.P.-D., P.F., H.P.-M., G.R.-M., and C.C. edited and revised manuscript; V.N., A.P., M.P.-D., P.F., H.P.-M., G.R.-M., and C.C. approved final version of manuscript.

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