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Identification of Key Signaling Molecules Involved in the Activation of the Swelling-Activated Chloride Current in Human Glioblastoma Cells

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Abstract The swelling-activated chloride current ($I_{Cl,Vol}$) is abundantly expressed in glioblastoma (GBM) cells, where it controls cell volume and invasive migration. The transduction pathway mediating $I_{Cl,Vol}$ activation in GBM cells is, however, poorly understood. By means of pharmacological and electrophysiological approaches, on GL-15 human GBM cells we found that $I_{Cl,Vol}$ activation by hypotonic swelling required the activity of a U73122-sensitive phospholipase C (PLC). $I_{Cl,Vol}$ activation could also be induced by the membrane-permeable diacylglycerol (DAG) analog OAG. Incontrast, neither calcium (Ca^{2+}) chelation by BAPTA-AM nor changes in PKC activity were able to affect $I_{Cl,Vol}$ activation by hypotonic swelling. We further found that R59022, an inhibitor of diacylglycerol kinase (DGK), reverted $I_{Cl,Vol}$ activation, suggesting the involvement of phosphatidic acid. In addition, $I_{Cl,Vol}$ activation required the activity of a EHT1864-sensitive Rac1 small GTPase and the resulting actin polymerization, as $I_{Cl,Vol}$ activation was prevented by cytochalasin B. We finally show that $I_{Cl,Vol}$ can be activated by the promigratory fetal calf serum in a PLC- and DGK-dependent manner. This observation is potentially relevant because blood serum can likely come in contact with glioblastoma cells in vivo as a result of the tumor-related partial breakdown of the blood–brain barrier. Given the relevance of $I_{Cl,Vol}$ in GBM cell volume regulation and invasiveness, the several key signaling molecules found in this study to be involved in the activation of the $I_{Cl,Vol}$ may represent potential therapeutic targets against this lethal cancer.

Keywords Glioma cells · Swelling-activated chloride current · Signal transduction

Introduction

The swelling-activated chloride currents ($I_{Cl,Vol}$) are ubiquitously expressed in mammalian cells, where they play prominent roles in many vital cellular processes. Activation of $I_{Cl,Vol}$ is experimentally induced by cell swelling resulting from exposure to extracellular hypotonic solution. In response to swelling, cells initiate a complex homeostatic process mainly consisting of the activation of $I_{Cl,Vol}$, together with K conductances, that allow a net efflux of K and Cl ions, which is obligatorily followed by osmotic water loss and reestablishment of the original cell volume, a process known as regulatory volume decrease (RVD). As expected, blockade of $I_{Cl,Vol}$ impairs the RVD process and the restoration of the physiological volume of the cell. $I_{Cl,Vol}$ is thus critically important in all those cellular processes that involve a change of cell volume and shape such as cell proliferation, migration, and death, besides processes and functions dependent on the osmolyte transport through the membrane, such as setting the cell resting potential or intracellular pH. In glioblastomas, $I_{Cl,Vol}$ has been found to be critically important for RVD; it has been proposed to facilitate cell infiltration through the narrow spaces of the brain parenchyma where major changes of cell volume and shape are required (Lui et al. 2010; Habela

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et al. 2009; Ransom et al. 2001; Soroceanu et al. 1999; Tysnes and Mahesparan 2001; Cuddapah and Sontheimer 2011; Catacuzzeno et al. 2011). This occurrence points to the $I_{Cl,Vol}$ as a promising therapeutic target against this highly invasive cancer (Cuddapah and Sontheimer 2011).

When subjected to voltage steps, $I_{Cl,Vol}$ displays an instantaneous outward rectification and a significant time-dependent inactivation at high positive voltages, properties thought to be intrinsic to the channel. $I_{Cl,Vol}$ is sensitive to the blocking action of the conventional chloride channel blockers, such as NPPB and DIDS. Often the inhibition of $I_{Cl,Vol}$ by DIDS, but not by NPPB, is markedly voltage dependent, with the inhibition being much stronger at positive potentials. This suggests an interaction of the blocker with a site within the pore where it can sense the transmembrane electric field (Nilius et al. 1996; Okada 1997, 2009).

$I_{Cl,Vol}$ activates slowly upon cell exposure to hypotonic solution (time constant of 100–200 s). This finding suggests that the volume sensor is not part of the channel, and a second messenger transduction pathway is involved. In accordance, many studies have found the involvement of several signaling molecules in the $I_{Cl,Vol}$ activation, although the data appear to be rather varied and sometimes conflicting when different preparations are considered (Okada et al. 2009; Nilius and Droogmans 2003). It is generally accepted that neither cAMP nor intracellular calcium (Ca^{2+}) are needed for $I_{Cl,Vol}$ activation (but see Akita et al. 2011), while the involvement of phospholipase C (PLC), protein kinase C (PKC), and protein tyrosine kinases are controversial and dependent on the cell preparation used. Other suggested modulators of $I_{Cl,Vol}$ activation by hypotonic swelling are represented by the small G proteins of the Rho family, the polymerization status of actin filaments, and the production of reactive oxygen species (ROS). Very few data are available on the transduction pathway mediating $I_{Cl,Vol}$ activation by hypotonic swelling in glioblastoma cells. In a study characterizing the biophysical and pharmacological properties of $I_{Cl,Vol}$ in D54MG glioblastoma cells it was hinted that intracellular Ca^{2+}, ATP/GTP, tyrosine, and serine–threonine kinases and actin depolimerization were not required for $I_{Cl,Vol}$ activation (Ransom et al. 2001). One study reports a modulatory role for Ca^{2+}-calmodulin kinase II in glioblastoma ClC-3 channel activation (Cuddapah and Sontheimer 2010), although the contribution of these channels to $I_{Cl,Vol}$ and their swelling-induced activation has not been clarified.

In the present study, we set out to understand the signal transduction pathway involved in $I_{Cl,Vol}$ activation by cell swelling in GL-15 glioblastoma cells. As in other preparations, we found the involvement of a PLC-induced DAG production and actin polymerization. We also found that the $I_{Cl,Vol}$ activation required the diacylglycerol kinase (DGK)-mediated production of phosphatidic acid (PA) and the activity of the small G protein Rac1. Notably, these signaling pathways had never been involved in $I_{Cl,Vol}$ activation by hypotonic swelling. Finally, we found that fetal calf serum (FCS), a strong motogen for glioblastoma cells, activates $I_{Cl,Vol}$, and it does so by using several signaling molecules involved in $I_{Cl,Vol}$ activation by hypotonicity. Because unknown serum components can infiltrate into high-grade gliomas as result of the breakdown of the blood–brain barrier (Seitz and Wechsler 1987; Lund et al. 2006), this result may help identify the pathophysiological mediators potentially important in the in vivo activation of glioblastoma $I_{Cl,Vol}$.

Methods

Cell Culture

The GL-15 glioblastoma multiform cell line was grown in minimum essential medium supplemented with 10 % heat-inactivated FCS (Invitrogen, S. Giuliano Milanese, Italy), 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. The flasks were incubated at 37 °C in a 5 % CO_2-humidified atmosphere. The medium was changed twice a week, and the cells were subcultured when confluent. For electrophysiological experiments, cells were seeded in petri dishes at 50,000 cells/ml and used at 3 days after seeding.

Electrophysiology

The whole-cell perforated configuration was used for electrophysiological recordings from GL-15 cells. Currents and voltages were amplified with a HEKA EPC-10 amplifier (List Medical, Darmstadt, Germany), digitized with a 12 bit A/D converter (TL-1, DMA interface; Axon Instruments, Foster City, CA, USA), and analyzed with the Patch Master package (version 2X60, Elektronik) and Microcal Origin 6.0 software. When assessing the reversal potential of the currents activated by hypotonicity, a liquid junction potential of 10 mV, experimentally assessed for our recording solutions, was considered. For online data collection, macroscopic currents were filtered at 3 kHz and sampled at 200 μs/point.

Solutions and Drugs

The external solution contained the following (in mM): NaCl 106.5, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, MOPS 5, glucose 20,

Na-Gluconate 30, (pH 7.25). Octanol (1 mM) was added to the external bathing solution to block gap-junctions (Eskandari et al. 2002). Tetraethylammonium (TEA, 3 mM) and TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole, 3 μM) were added to block Ca^{2+}-activated large- and intermediate-conductance K channels, expressed in these cells (Fioretti et al. 2006). The internal solution contained: K_2SO_4 57.5, KCl 55, $MgCl_2$ 5, MOPS 10 (pH 7.20). Electrical access to the cytoplasm was achieved by adding amphotericin B (200 μM) to the pipette solution. Access resistances ranging between 15 and 25 MΩ were achieved within 10 min after seal formation, and were actively compensated to approximately 50 %. All chemicals used were of analytical grade. Dimethyl sulfoxide (DMSO), tetraethylammonium chloride (TEA), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), 1,2-*Bis*(2-aminophenoxy) ethane-*N,N,N,′N′*-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), U73122, and U73343 were purchased from Sigma (St. Louis, MO, USA). 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), l′1-(cis-9-octadecenoyl)-2-acetyl-*sn*-glycerol, 2-Acetyl-1-oleoyl-*sn*-glycerol (OAG), 6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*thiazolo[3,2-*a*]pyrimidin-5-one (R59022), and 5(5(7(trifluoromethyl)quinolin-4-ylthio)pentyloxy)-2-(morpholinomethyl)-4*H*-pyran-4-one dihydrochloride (EHT1864) were from Tocris Bioscience (Avonmouth, UK). 1-[(2-Chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) was a kind gift of Dr. Heike Wolff. U73122, U73343, NPPB, R59022, EHT1864 TRAM-34 and OAG were prepared in DMSO (to concentrations of 100, 50, 100, 10, 50 mM, respectively). Amphotericin B was similarly dissolved in DMSO to a concentration of 50 mM; the maximal DMSO concentration in the recording solutions was 0.1 %. We verified that application of 0.1 % DMSO did not have significant effects of the membrane currents of GL-15 cells (data not shown). DIDS was prepared in carbonate buffer at 100 mM. To activate the $I_{Cl,Vol}$ current, we used a hypotonic solution prepared by adding 30 % distilled water to the extracellular solution. The 30 % hypertonic solution was prepared by adding to the extracellular solution 100 mM sucrose. The pharmacological agents were dissolved daily in the appropriate solution at the concentrations stated, and bath applied with a gravity perfusion system. Experiments were carried out at room temperature (18–22 °C).

Statistical Analysis

Data are presented as mean ± SE. Statistical differences between experimental groups were verified by the *t* test; significance was considered at a *p* value of 0.05.

Evaluation of the Rac1 Active Form

GL-15 cells after 3 days of subculturing were serum-starved for 24 h. After 20 min in Ringer solution, the cells were treated for 5 min either with 30 % hypotonic or normal Ringer solution. The evaluation of the active form of Rac1 was performed by using a G-LISA Rac1 activation assay (Cytoskeleton) following the instructions of the manufacturer.

Results

Biophysical and Pharmacological Properties of $I_{Cl,Vol}$ in GL-15 Glioblastoma Cell Line

The GL-15 glioblastoma cell line expresses abundant swelling-activated $I_{Cl,Vol}$ (Fioretti et al. 2004). We could activate this current by perfusing the cells with a hypotonic extracellular solution, in the whole-cell perforated configuration of the patch-clamp. To eliminate the contribution of the Ca^{2+}-activated K channels found to be expressed in these cells (Fioretti et al. 2006, 2009; Catacuzzeno et al. 2012), we added to the extracellular solution TRAM-34 (3 μM) and TEA (3 mM), inhibitors of the intermediate- and large-conductance Ca^{2+}-activated K channels, respectively. Under these conditions, we repeatedly stimulated GL-15 cells with voltage ramps from −100 to +100 mV, applied every 5 s from a holding potential of −40 mV, and activated the $I_{Cl,Vol}$ by applying a 30 % hypotonic extracellular solution (Hypo).

Figure 1a shows a typical time course of the $I_{Cl,Vol}$ activated by the hypotonic solution, constructed from the current ramps of the type shown in Fig. 1b. Each single point represents the $I_{Cl,Vol}$, which we routinely estimated as the current measured in hypotonic solution minus the basal current, both taken from their appropriate ramp, at +50 mV of applied potential. In the 37 cells (out of 40) that responded to the hypotonic solution the $I_{Cl,Vol}$ density amounted to 9.12 ± 0.73 pA/pF (Fig. 1a, *inset*). The $I_{Cl,Vol}$ could be effectively blocked by both NPPB (100 μM) and DIDS (500 μM) (Fig. 1). Evaluation of the efficacy of $I_{Cl,Vol}$ block by the two inhibitors at different membrane potentials indicated that unlike NPPB, DIDS block was voltage-dependent, being markedly smaller at −80 than at +50 mV (Fig. 1c). The reversal potential of the swelling-activated current, evaluated as the voltage at which the current ramps in control and hypotonic solutions overlap (Fig. 1b, arrow), had a mean of −8.4 ± 1.0 mV, $n = 37$ (corrected for junction potential), a value not significantly different from E_{Cl} under our ionic conditions (E_{Cl}, −6.5 mV; $p > 0.05$). To investigate the basic kinetic features of $I_{Cl,Vol}$, namely the time and voltage dependent

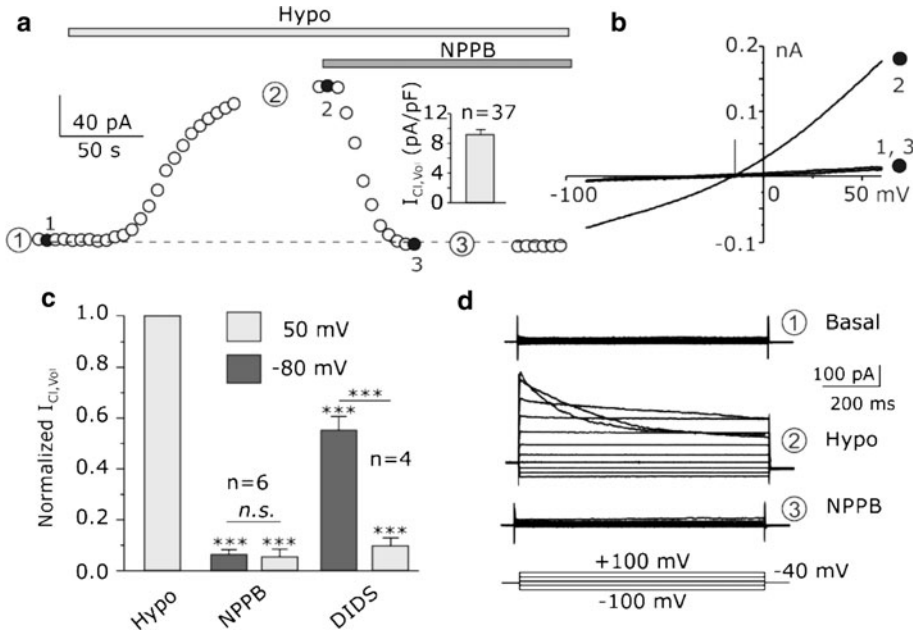

Fig. 1 Biophysical and pharmacological properties of the $I_{Cl,Vol}$ expressed in GL-15 glioblastoma cells. **a** Time course of the current measured at +50 mV showing the effect of 30 % hypotonic solution (Hypo) and subsequent application of NPPB (100 μM), constructed from the current ramps of the type shown in (**b**). Each single point represents the current amplitude taken from the appropriate ramp, at +50 mV, minus the basal current (*dashed line*). *Solid circles* with associated numbers are data taken from the current ramps shown in (**b**), while *circled numbers* indicate the time periods where the traces shown in (**d**), obtained in response to voltage steps, were acquired. *Inset: Bar plot* showing the mean $I_{Cl,Vol}$ density at +50 mV of applied potential in 37 GL-15 cells, assessed as the current recorded after the application of a 30 % hypotonic solution minus the current recorded in basal conditions. **b** Representative current ramps under basal (isotonic) conditions (*1*), in the presence of a 30 % hypotonic solution (*2*), and in the presence of the hypotonic solution containing 100 μM NPPB (*3*). The *arrow* indicates the reversal potential of the hypotonic-activated current, taken as the voltage at which the current traces in hypotonic and in control conditions cross over. **c** *Bar plot* showing the mean fractional residual $I_{Cl,Vol}$ assessed at two different voltages (−80 and +50 mV) in the presence of 100 μM NPPB ($n = 6$) or 500 μM DIDS ($n = 4$); ***$p < 0.001$, *t* test. n.s., $p > 0.05$, *t* test. **d** Families of current traces obtained by applying to the same GL-15 cell shown in (**a**) and (**b**) 1 s voltage steps from −100 to +100 mV, in steps of 20 mV, from a holding potential of −40 mV, under basal conditions (Basal, *1*), in the presence of a 30 % hypotonic solution (Hypo, *2*), and in the presence of a hypotonic solution containing 100 μM NPPB (NPPB, *3*)

activation and inactivation, we applied 1 s voltage steps from −100 to +100 mV, from a holding of −40 mV. Figure 1d shows that the swelling-activated current displayed an instantaneous activation followed by a marked time- and voltage-dependent inactivation, much evident at membrane potentials higher than +40 mV. These results indicate that GL-15 glioblastoma cells express a swelling-activated Cl current with biophysical and pharmacological properties typical of the $I_{Cl,Vol}$ found in many other glioblastoma cell lines, as well as other tissues (Nilius et al. 1996; Olsen et al. 2003; Onesto et al. 2008; Sontheimer 2008).

Involvement of PLC-Mediated Production of DAG in the $I_{Cl,Vol}$ Activation by Hypotonicity

Several studies carried out in various tissues have shown the involvement of PLC in the activation of $I_{Cl,Vol}$ (Ellershaw et al. 2002; Mitchell et al. 1997; Zholos et al. 2005). To verify whether this also applies to glioblastoma GL-15 cells we evaluated the effect of the PLC inhibitor U73122 on the $I_{Cl,Vol}$, estimated by subtraction, from the current ramps at +50 mV, as described above. As shown in Fig. 2a, b, the application of U73122 (5 μM) results in a strong reduction of the $I_{Cl,Vol}$ (residual current 23.5 ± 5.9 % of control, $n = 6$; $p < 0.01$), an effect that was not observed when using the inactive analogue U73343 (5 μM) (residual current 98.7 ± 0.8 % of control, $n = 4$; $p > 0.05$).

We then tested the involvement of the two PLC products, DAG and inositol-1,4,5-triphosphate (IP_3). As shown in Fig. 2c, isotonic external solutions containing OAG (100 μM), a membrane-permeable analogue of DAG, activated a mean current density of 7.76 ± 0.85 pA/pF ($n = 4$; Fig. 2c, *inset*) that was sensitive to NPPB (100 μM; fractional residual current in NPPB was 0.051 ± 0.027, $n = 3$; $p < 0.01$; Fig. 2e), and had a mean reversal potential of −14.8 ± 5.1 mV, $n = 4$, a value not significantly different from E_{Cl} under our isotonic recording conditions (−15.6 mV; $p > 0.05$; Fig. 2d). Altogether, these data indicate that the OAG-activated current has properties congruent with the $I_{Cl,Vol}$.

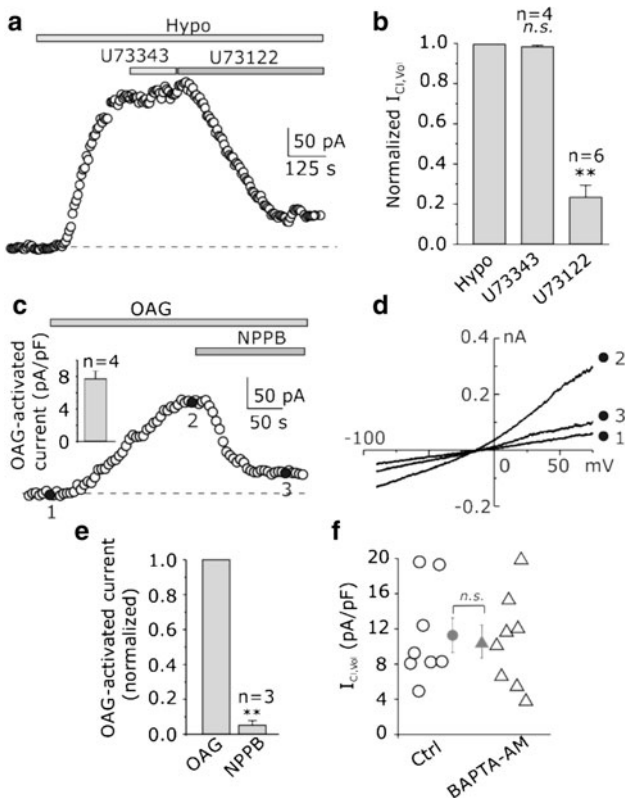

Fig. 2 Involvement of DAG production by PLC in the activation of $I_{Cl,Vol}$. **a** Time course of the current measured from current ramps at +50 mV showing the effects of U73343 (5 μM) and U73122 (5 μM) on the $I_{Cl,Vol}$ activated by a 30 % hypotonic solution. **b** *Bar plot* showing the mean fractional residual current in the presence of 5 μM U73343 ($n = 4$) or 5 μM U73122 ($n = 6$). **c** Time course of the current measured from current ramps at +50 mV showing the effects of OAG (100 μM) and the subsequent application of NPPB (100 μM). *Inset: Bar plot* showing the mean OAG-activated current density recorded at +50 mV in 4 GL-15 cells. **d** Representative current ramps applied under basal conditions (*1*), in the presence of 100 μM OAG (*2*), and 100 μM OAG + 100 μM NPPB (*3*). **e** *Bar plot* showing the mean fractional residual OAG-activated current assessed in the presence of 100 μM NPPB ($n = 3$). **f** Scatter plot showing the swelling-activated $I_{Cl,Vol}$ density in control cells and in cells pretreated for 15 min with 30 μM BAPTA-AM. *Solid symbols* represent means ± SE; ***$p < 0.001$, *t* test; n.s., $p > 0.05$, *t* test

We then tested the involvement of intracellular Ca^{2+} increase that would result from the PLC-mediated IP_3 production. Preincubation of GL-15 glioblastoma cells with the membrane permeable Ca^{2+} chelator BAPTA-AM (30 μM, 15 min) did not prevent the swelling-induced $I_{Cl,Vol}$ activation (the mean swelling-activated current density, obtained as the current measured in hypotonic conditions minus the basal current, was 11.3 ± 2.0 pA/pF, $n = 8$, in control conditions versus 10.6 ± 1.9 pA/pF, $n = 8$, in BAPTA-AM; $p > 0.05$; Fig. 2f). Taken together, these data strongly suggest that a PLC-dependent increase of DAG, but not an IP_3-induced Ca^{2+} increase, is needed for the swelling-induced $I_{Cl,Vol}$ activation.

Involvement of DAG Kinase in the Swelling-Induced $I_{Cl,Vol}$ Activation

Common targets of DAG are the members of the PKC family, already found to be involved in Cl currents modulation in other preparations (Miwa et al. 1997; Vanoye et al. 1999). We then tested whether the activation of PKC by PMA (phorbol 12-myristate 13-acetate), an activator of several DAG-sensitive PKCs, was capable of activating the $I_{Cl,Vol}$. As shown in Fig. 3a, b, an isotonic solution containing 100 nM PMA was not able to activate any current in cells expressing the $I_{Cl,Vol}$. Likewise, preincubation of GL-15 cells with GF109203X, an inhibitor of DAG-sensitive PKCs, did not prevent $I_{Cl,Vol}$ activation by hypotonic swelling (Fig. 3c). These experiments seem to exclude the involvement of DAG-sensitive PKCs in $I_{Cl,Vol}$ activation.

We then turned to investigate whether the DAG metabolite PA was involved in $I_{Cl,Vol}$ activation. Although the bulk of the cellular PA is thought to result from phospholipase D activity, DGKs likely contribute significantly to control the PA level (by catalyzing DAG phosphorylation), especially in specific subcellular districts where PA plays important roles in signal transduction, such as in cytoskeleton dynamics (Zhang and Du 2009; Wang et al. 2006). To verify whether DAG phosphorylation and its product PA were implicated in $I_{Cl,Vol}$ activation, we tested the effect of the DGK inhibitor R59022 (50 μM). As shown in Fig. 3d, e, the application of R59022 markedly reduced the swelling-induced $I_{Cl,Vol}$ activation, and the effect could be fully reversed (data not shown), indicating a major role of DGK/PA in the process. Having shown the insensitivity of the $I_{Cl,Vol}$ activation to the Ca^{2+}-chelator BAPTA-AM (Fig. 2f), our data would indicate the involvement of a Ca^{2+}-independent DGK isoform, such as the DGK_ζ.

Role of the Monomeric G Protein Rac and Actin Polymerization in $I_{Cl,Vol}$ Activation

It has been shown that both DGK_ζ and PA modulate Rac1, a member of the Rho GTPase family of small G proteins that have an important role in the remodeling of the cytoskeleton by controlling the actin polymerization (Hall 1994; Takai et al. 1995; Abramovici et al. 2009; Chianale et al. 2007, 2010). We have thus verified the involvement of Rac1 in the swelling-induced $I_{Cl,Vol}$ activation. More specifically, we first analyzed the effects of short-term exposure to hypotonicity on Rac1 activation, under the same experimental conditions we used for the electrophysiological recordings. As shown in Fig. 4a, the treatment with a 30 % hypotonic solution for 5 min, a time comparable to that needed for $I_{Cl,Vol}$ activation, induced a significant activation of Rac1. In addition, the selective

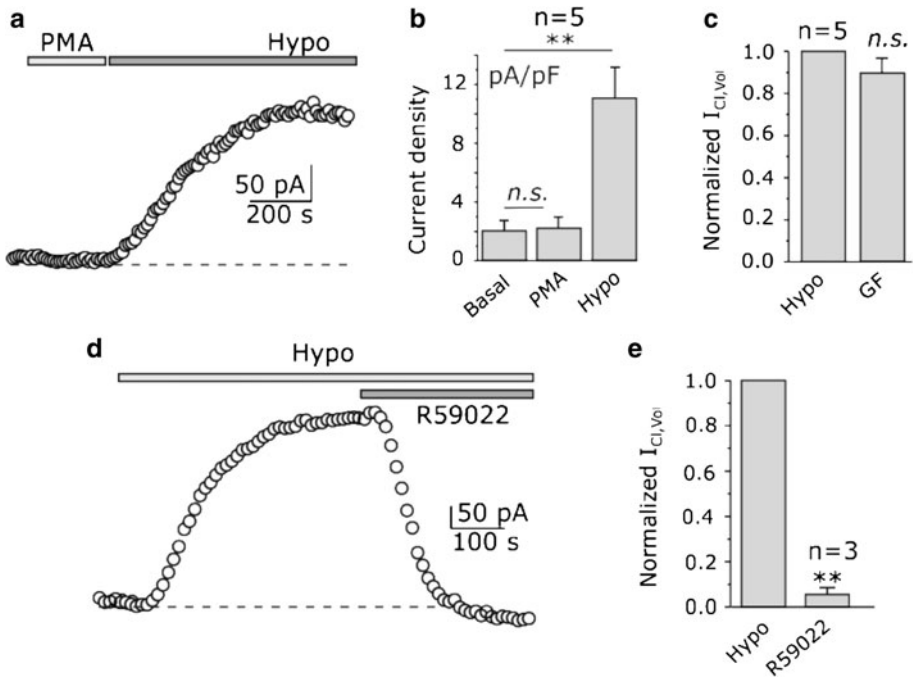

Fig. 3 Involvement of DAG-sensitive PKCs and DGK in the activation of $I_{Cl,Vol}$. **a** Time course of the current measured from current ramps at +50 mV showing the effects of PMA (100 nM) and the subsequent application of a 30 % hypotonic solution. **b** *Bar plot* showing the mean current density recorded from voltage ramps at +50 mV in 5 GL-15 cells in basal conditions (Basal), after the application of 100 nM PMA, and the subsequent application of a 30 % hypotonic solution. **c** *Bar plot* showing the fractional residual $I_{Cl,Vol}$ in the presence of 500 nM GF109203X ($n = 5$). **d** Time course of the current at +50 mV showing the effects of the DAG-kinase inhibitor R59022 (50 μM) on the swelling-induced activation of $I_{Cl,Vol}$. **e** *Bar plot* showing the fractional residual $I_{Cl,Vol}$ in the presence of 50 μM R59022 ($n = 3$); $^{**}p < 0.01$, *t* test; n.s., $p > 0.05$, *t* test

Rac1 inhibitor EHT1864 (50 μM) (Onesto et al. 2008) was able to revert the swelling-induced $I_{Cl,Vol}$ activation (Fig. 4b, c). These results demonstrate the involvement of Rac1 in the hypotonicity-induced $I_{Cl,Vol}$ activation.

Rac1 activation can promote ROS generation through NADPH activation in several cells (Seshiah et al. 2002; Sundaresan et al. 1996; Vignais 2002), and ROS produced by NADPH have been shown to activate $I_{Cl,Vol}$ in several preparations (Browe and Baumgarten 2006; Shimizu et al. 2004; Ren et al. 2008; Deng et al. 2010). We then tested whether ROS, namely H_2O_2, largely used to assess the ability of ROS to activate $I_{Cl,Vol}$ (Browe and Baumgarten 2006; Shimizu et al. 2004; Ren et al. 2008; Deng et al. 2010), was capable to activate the current in glioblastoma cells. As shown in Fig. 4d, left, bath application of H_2O_2 (500 μM) was not able to activate $I_{Cl,Vol}$, suggesting that a Rac1-induced ROS production was not involved in the volume-induced $I_{Cl,Vol}$ activation. In accordance *N*-acetyl-cysteine (NAC, 5 mM), a widely used free radical scavenger, did not affect the hypotonicity-induced activation of $I_{Cl,Vol}$ (Fig. 4d, right). Taken together, these data indicate that $I_{Cl,Vol}$ activation by hypotonicity does not involve Rac1-mediated ROS production.

As one of the most common roles of Rac1 is to promote actin polymerization (Shutes et al. 2007), we also verified whether cytochalasin B, an inhibitor of this process, could prevent the activation of $I_{Cl,Vol}$. As shown in Fig. 4e, incubation of GL-15 cells with cytochalasin B (15 μM for 30 min) markedly reduced the swelling-induced $I_{Cl,Vol}$ density with respect to control (from 8.34 ± 1.67 pA/pF, $n = 6$, in control cells to 1.95 ± 0.27 pA/pF, $n = 8$, in treated cells; $p < 0.05$). These findings suggest that Rac1 plays a key role in the modulation of $I_{Cl,Vol}$, likely through the regulation of actin polymerization.

Fetal Calf Serum Activates $I_{Cl,Vol}$ by Using a PLC- and DKG-dependent Signaling Pathway

In vivo, glioblastoma cells hardly experience hypotonic conditions such as those needed to activate the $I_{Cl,Vol}$. The activation of this current could then occur via other physiopathological mediators. We tested FCS, a physiological stimulus important for cell migration, previously found to activate an outward rectifying Cl current blocked by DIDS and NPPB in another glioblastoma cell line (U87-MG; Catacuzzeno et al. 2011). FCS was able to activate the $I_{Cl,Vol}$, and did it using several transduction molecules involved in the $I_{Cl,Vol}$ activation after exposure to hypotonicity. This is illustrated in Fig. 5, which shows that the application of an isotonic solution containing 10 % FCS

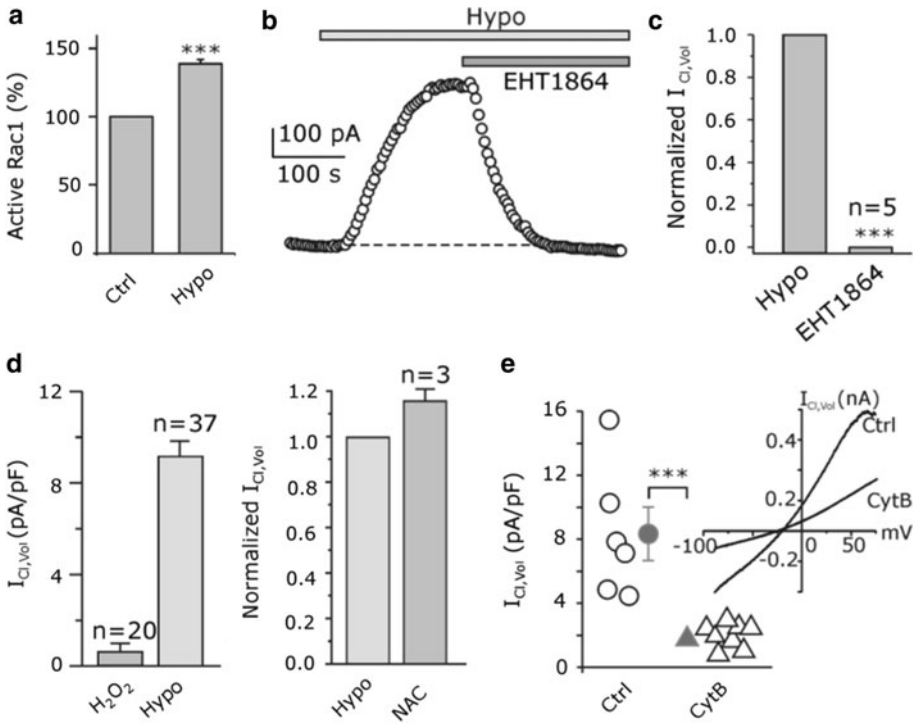

Fig. 4 Involvement of Rac1 and actin polymerization in the activation of $I_{Cl,Vol}$. **a** Plot showing the Rac1 activity assessed by G-Lisa assay after stimulation of 5 min with a 30 % hypotonic solution. Rac1 activity was normalized with respect to that assessed under unstimulated conditions. **b** Time course of the current measured from current ramps at +50 mV showing the effects of the Rac1 inhibitor EHT1864 (50 μM) on the swelling-induced activation of $I_{Cl,Vol}$. **c** *Bar plot* showing the fractional residual $I_{Cl,Vol}$ in the presence of 50 μM EHT1864. **d** *Left*: *Bar plot* showing the mean current density at +50 mV activated by 500 μM H_2O_2 in 20 GL-15 cells. For comparison, the mean hypotonicity-induced current density from Fig. 1 is also reported. *Right*: *Bar plot* showing the fractional residual $I_{Cl,Vol}$ in the presence of 5 mM of *N*-acetylcysteine (NAC). **e** Scatter plot showing the swelling-activated $I_{Cl,Vol}$ density in cells held in control conditions and cells pretreated for 30 min with 15 μM cytochalasin B. *Solid symbols* represent mean ± SE. *Inset*: Representative current ramps from a control cell and a cell pretreated for 30 min with 15 μM cytochalasin B; ***$p < 0.001$, *t* test

induces the activation of a current with a mean density of 5.25 ± 0.9 pA/pF, $n = 17$ (Fig. 5a, *inset*), effectively inhibited by NPPB 100 μM (fractional residual current 0.12 ± 0.06, $n = 3$) and DIDS 500 μM (fractional residual current 0.20 ± 0.05, $n = 3$). As shown in Fig. 5d, the amount of block by NPPB and DIDS under these conditions is comparable to that found in the same glioblastoma cells stimulated by hypotonicity (Hypo; data taken from Fig. 1). The FCS-activated current had a reversal potential of −16.3 ± 1.9 mV ($n = 17$), not significantly different from the E_{Cl} in the ionic condition used ($E_{Cl} = -15.6$ mV, $p > 0.05$), and at potentials above +60 mV showed a marked voltage-dependent inactivation (Fig. 5c). Finally, a 30 % hypertonic solution reverted most of the FCS-activated current (Fig. 5e). All these data strongly indicate that the current activated by FCS is the $I_{Cl,Vol}$.

We then verified whether FCS activates the putative $I_{Cl,Vol}$ using some of the signaling molecules involved in hypotonic activation. The data presented in Fig. 5f show that the serum-activated current is largely inhibited by R59022 (50 μM; fractional residual current 0.25 ± 0.07, $n = 4$, $p < 0.01$) and U73122 (5 μM; fractional residual current 0.28 ± 0.09, $n = 3$, $p < 0.01$), but not by the inactive analogue U73343 (5 μM; fractional residual current 0.97 ± 0.02, $n = 3$, $p > 0.05$). These results indicate that in the activation of $I_{Cl,Vol}$ hypotonicity and serum use several common signaling molecules.

Discussion

In this work we have identified several key steps in the transduction pathway of $I_{Cl,Vol}$ activation after hypotonic stress in GL-15 glioblastoma cells. As found in many cell types, $I_{Cl,Vol}$ activation in response to hypotonic stress develops slowly, suggesting that a cascade of intracellular signaling molecules is involved. To this regard, data from other studies are rather varied and of limited validity beyond the cell type studied. Our results show that in GL-15 glioblastoma cells the activity of $I_{Cl,Vol}$ is modulated by both the PLC/DAG/DGK/PA pathway and by the Rac-mediated cytoskeleton remodeling. Although PLC and actin polymerization have already been implicated in $I_{Cl,Vol}$ activation by hypotonic swelling in other preparations, the

involvement of both DGK-mediated PA production and Rac1 activity has, to our knowledge, never been reported.

The involvement of PLC and its metabolite DAG in the hypotonic activation of $I_{Cl,Vol}$ that we found in our study is in agreement with several previous reports (Ellershaw et al. 2002; Mitchell et al. 1997; Zholos et al. 2005). By contrast we show that PKC, the most common target for DAG, is not involved in the modulation of $I_{Cl,Vol}$, given that neither PKC activators, nor PKC inhibitors affected the hypotonicity-induced $I_{Cl,Vol}$ activation. These data are at variance with previous work showing that in several cell types PKC activity either stimulated (Robson and Hunter 1994; Mitchell et al. 1997), or inhibited the hypotonicity-activated $I_{Cl,Vol}$ (Duan et al. 1995, 1999; Coca-Prados et al. 1995; Hardy et al. 1995), although in some cases it was shown to be uninfluential (Gosling et al. 1995; Miwa et al. 1997; Szücs et al. 1996). Notably, we found that the formation of PA upon DAG phosphorylation by DGK represents a critical step for $I_{Cl,Vol}$ activation, as DGK inhibition by its specific antagonist R59022 fully reverts the hypotonicity-induced $I_{Cl,Vol}$ activation.

In this study we show that a short-term exposure to hypotonicity induces a significant activation of Rac1 (Fig. 4a), and Rac1 is involved in the $I_{Cl,Vol}$ activation by hypotonicity ($I_{Cl,Vol}$ was fully reverted by the selective Rac inhibitor EHT1864; Fig. 4b, c). Rac modulation by changes in the osmolarity of the extracellular solution has already been shown, although the effect was reported to be activatory or inhibitory, depending on the cell preparation under study (Hoffmann et al. 2009; Barfod et al. 2005). As for Rac involvement in $I_{Cl,Vol}$ activation, the observation is consistent with previous studies showing that intracellular addition of the non-hydrolizable GTP analogue GTPγS activates a chloride current with features similar to the hypotonicity-activated $I_{Cl,Vol}$, while the addition of GDPβS, a compound known to block the monomeric G proteins, inhibits or delays the hypotonic activation of $I_{Cl,Vol}$ (Doroshenko et al. 1991; Mitchell et al. 1997; Nilius et al. 1994, 1997; Tilly et al. 1991; Voets et al. 1998). In our GL-15 glioblastoma cells, Rac may activate $I_{Cl,Vol}$ by polymerizing actin filaments, an occurrence already reported to be involved

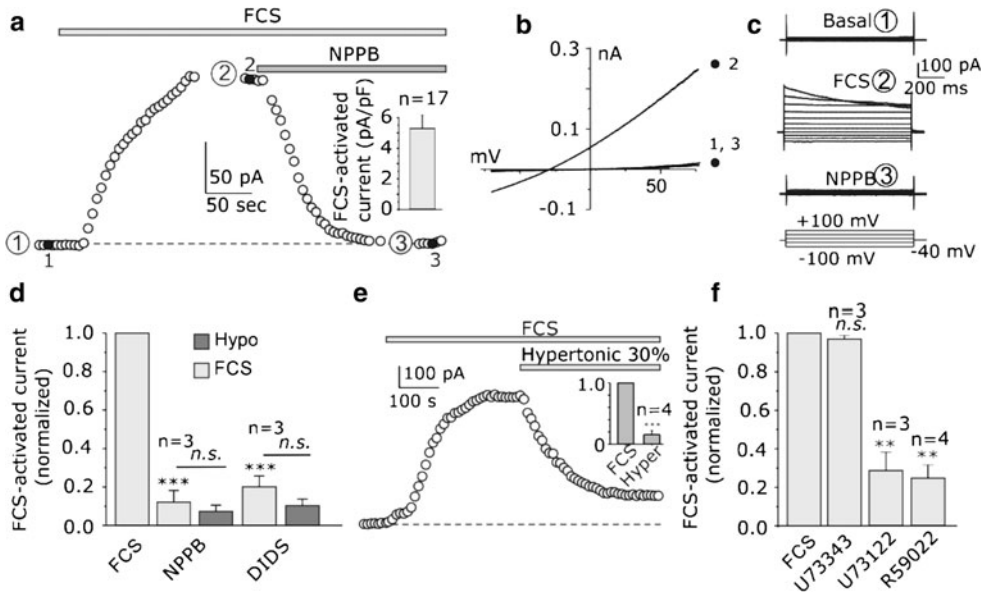

Fig. 5 Fetal calf serum activates $I_{Cl,Vol}$. **a** Time course of the current measured from current ramps at +50 mV showing the effects of 10 % FCS and the subsequent application of 10 % FCS + NPPB (100 μM). *Inset: Bar plot* showing the mean FCS-activated current density, assessed as the current recorded at +50 mV in 17 GL-15 cells after the application of 10 % FCS minus the current recorded under basal conditions. **b** Representative current ramps applied under basal conditions (*1*), in the presence of 10 % FCS (*2*), and in the presence of 10 % FCS + 100 μM NPPB (*3*). **c** Families of current traces obtained by applying to the same GL-15 cell shown in the *inset* of (**a**), 1 s voltage steps from −100 to +100 mV in steps of 20 mV from a holding potential of −40 mV, under basal conditions (Basal), in the presence of 10 % FCS (FCS), and in the presence of 10 % FCS + 100 μM NPPB (NPPB). **d** *Bar plot* showing the mean fractional residual current assessed in the presence of 100 μM NPPB ($n = 3$) and 500 μM DIDS ($n = 3$). For comparison, the mean residual current in NPPB (100 μM) and DIDS (500 μM) of the $I_{Cl,Vol}$ activated by hypotonicity reported in Fig. 1c are also shown. **e** Time course of the current at +50 mV showing the effects of a 30 % hypertonic solution, prepared by adding sucrose, on the current activated by 10 % FCS. *Inset: Bar plot* showing the mean fractional residual current assessed in the presence of the 30 % hypertonic solution ($n = 4$). **f** *Bar plot* showing the mean fractional residual current assessed in the presence of 5 μM U73343 ($n = 3$), 5 μM U73122 ($n = 3$), or 50 μM R59022 ($n = 4$); $^{***}p < 0.001$, *t* test; $^{**}p < 0.01$, *t* test; n.s., $p > 0.05$, *t* test

in $I_{Cl,Vol}$ activation (Abramovici et al. 2009; Chianale et al. 2007, 2010; Tolias et al. 1998). Several studies have linked actin polymerization to $I_{Cl,Vol}$ activation, although the results are varied: some show that the integrity of actin filaments is needed for the activation of the $I_{Cl,Vol}$ (Cornet et al. 1993), others report that the depolymerization of actin cytoskeleton increases the activity of the channel (Schwiebert et al. 1994), while others still show that the contribution of the actin filaments in the modulation of the $I_{Cl,Vol}$ is null (Oike et al. 1994).

The link between Rac-dependent cytoskeleton remodeling and the activation of $I_{Cl,Vol}$ that we suggest to occur in GL-15 glioblastoma cells is further supported by the observation that the ClC-3 channel, most likely underlying the $I_{Cl,Vol}$, loses its sensitivity to hypotonic stress when the interaction with actin microfilaments is prevented (McCloskey et al. 2007). In this context the issue on the molecular nature of $I_{Cl,Vol}$ is still open, although the ClC-3 remains the strongest candidate. In NIH/3T3 fibroblasts, the expression of ClC-3 generated a Cl^- current activated by hypotonic stress that displayed most physiological and pharmacological properties of the native $I_{Cl,Vol}$ (Duan et al. 1997). Further, the inhibition of the endogenous ClC-3 by siRNA or by an anti-ClC-3 antibody markedly inhibited $I_{Cl,Vol}$ in several cell types (Duan et al. 2001; Jin et al. 2003; Hermoso et al. 2002; Wang et al. 2000). The residual uncertainty with regard to the molecular nature of $I_{Cl,Vol}$ arises from the observation reported by several groups that salivary acinar cells, ventricular myocytes, and hippocampal neurons originating from ClC-$3^{-/-}$ mice displayed a native $I_{Cl,Vol}$ essentially indistinguishable from the wild type ClC-$3^{+/+}$ mice (Stobrawa et al. 2001; Arreola et al. 2002; Gong et al. 2004).

In conclusion, we showed that the activation of $I_{Cl,Vol}$ in GL-15 cells is under the control of both the PLC/DAG/DGK/PA pathway, and the Rac-mediated cytoskeleton remodeling. These two modulatory segments may represent two separate processes that work in parallel, but converge onto the channel to activate the $I_{Cl,Vol}$, or else they form a sequentially coupled linear pathway. In support of the latter view is the observation that PA activates Rac and induces polymerization of actin filaments, an event known to be involved in $I_{Cl,Vol}$ activation (Abramovici et al. 2009; Chianale et al. 2007, 2010; Tolias et al. 1998). In this view, cell swelling leads to the activation of PLC and the production of DAG, the latter being phosphorylated to PA by DGK. PA would then activate Rac, and in turn stimulate the polymerization of actin filaments, and ultimately activate the $I_{Cl,Vol}$. We stress again that this scheme depicts just one of the possible pathways in $I_{Cl,Vol}$ activation, but the issue requires further investigation for a conclusive answer.

Functional Significance

$I_{Cl,Vol}$ has been implicated in a large number of cellular functions that involve changes in cell volume and shape. This current is largely expressed in glioblastoma cells, and is believed to underlay those processes relevant for the tumor growth that depend on the cell shrinkage and shape changes, such as migration and invasion through the healthy brain parenchyma. Glioma cell shrinkage and invasion have in fact been found to be inhibited by Cl channel blockers (Soroceanu et al. 1999; Ransom et al. 2001). We and others showed that $I_{Cl,Vol}$ is activated by rather strong hypotonic stimuli that hardly any cell in vivo experiences. Thus, in addition to hypotonicity, other stimuli are thought to activate the $I_{Cl,Vol}$, and this notion has already been confirmed in some instances (Deng et al. 2010; Zholos et al. 2005). Our study shows that the $I_{Cl,Vol}$ found in GL-15 cells activates when cells are exposed to FCS, an important promigratory stimulus for these cells. This observation is particularly interesting given that glioblastoma cells may come in contact with serum under specific pathological conditions, including brain tumors, that may cause partial degradation of the blood–brain barrier (Lui et al. 2010; Schwiebert et al. 1994). Our results also indicate that the activation of the $I_{Cl,Vol}$ by FCS involves several signaling molecules also activated by hypotonic stress.

Unfortunately the FCS component/s responsible for the observed effects on $I_{Cl,Vol}$ is for the moment unknown. Notably, several growth factors present in FCS at relatively high concentration, such as the fibroblast growth factor, the platelet-derived growth factor and insulin were all able to induce $[Ca^{2+}]_i$ changes in U87-MG cells, although of lower magnitude than those produced by FCS (Rondé et al. 2000). Several other substances, potentially able to activate PLC and present in FCS, have been shown to have their membrane receptors in glioblastoma cells, such as acetylcholine (Bordey et al. 2000), LPA (Manning et al. 2000), bradykinin (Reetz and Reiser 1996), ATP, and glutamate (Mariggio et al. 2001).

Conclusions

This study clarified the transduction pathway mediating $I_{Cl,Vol}$ activation by hypotonicity in GL-15 human glioblastoma cells. More specifically, we found that $I_{Cl,Vol}$ activation by hypotonicity requires DAG production by a U73122-sensitive PLC, and its phosphorylation to PA through the activity of a DGK. In addition, $I_{Cl,Vol}$ activation required the activity of a EHT1864-sensitive Rac1 small GTPase and the resulting actin polymerization, as $I_{Cl,Vol}$ activation was prevented by cytochalasin B. We finally

show that $I_{Cl,Vol}$ can be activated by the promigratory FCS in a PLC- and DGK-dependent manner. The data we report here could represent the starting point for investigating strategies to inhibit the invasiveness of glioblastoma in the healthy brain tissue, centered on the activation of $I_{Cl,Vol}$.

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