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Serum-Activated K and Cl Currents Underlay U87-MG Glioblastoma Cell Migration

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Glioblastoma cells in vivo are exposed to a variety of promigratory signals, including undefined serum components that infiltrate into high grade gliomas as result of blood–brain barrier breakdown. Glioblastoma cell migration has been further shown to depend heavily on ion channels activity. We have then investigated the modulatory effects of fetal calf serum (FCS) on ion channels, and their involvement in U87-MG cells migration. Using the perforated patch-clamp technique we have found that, in a subpopulation of cells (42%), FCS induced: (1) an oscillatory activity of TRAM-34 sensitive, intermediate-conductance calcium-activated K (IK_{Ca}) channels, mediated by calcium oscillations previously shown to be induced by FCS in this cell line; (2) a stable activation of a DIDS- and NPPB-sensitive Cl current displaying an outward rectifying instantaneous current-voltage relationship and a slow, voltage-dependent inactivation. By contrast, in another subpopulation of cells (32%) FCS induced a single, transient IK_{Ca} current activation, always accompanied by a stable activation of the Cl current. The remaining cells did not respond to FCS. In order to understand whether the FCS-induced ion channel activities are instrumental to promoting cell migration, we tested the effects of TRAM-34 and DIDS on the FCS-induced U87-MG cell migration using transwell migration assays. We found that these inhibitors were able to markedly reduce U87-MG cell migration in the presence of FCS, and that their co-application resulted in an almost complete arrest of migration. It is concluded that the modulation of K and Cl ion fluxes is essential for the FCS-induced glioblastoma cell migration.

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Glioblastoma is the most common and aggressive form of human brain tumors, its poor prognosis largely deriving from its diffuse invasiveness into normal brain that prevents successful surgical resection (Holland, 2001; Maher et al., 2001). In spite of the large body of information available on the biology of glioblastoma cells, the mechanisms underlying their invasiveness are still largely unknown, thus limiting the development of effective therapeutic strategies.

In many cell types, including glioblastoma cell lines, it has been demonstrated that cell migration heavily depends on ion channel activity (Schwab, 2001; McFerrin and Sontheimer, 2006). The large-conductance Ca-activated K (BK_{Ca}) channels and the volume regulated Cl (I_{ClVol}) channels, both largely expressed in glioblastoma cells, have indeed been shown to underlay glioblastoma cell migration, and enhance tumor progression in several experimental tumor models (Ransom et al., 2001; McFerrin and Sontheimer, 2006). These findings led to the hypothesis that ion fluxes during migration cause osmotically driven water movements that result in changes of cell volume and shape, obligatory steps for cell migration (McFerrin and Sontheimer, 2006). We recently demonstrated the expression of the intermediate-conductance Ca-activated K (IK_{Ca}) channel in human GL-15 and U-251 glioblastoma cell lines (Fioretti et al., 2004, 2006). Several studies in normal and tumor cells point to a role of IK_{Ca} channels in cell migration (Sahwab et al., 1999). IK_{Ca} channel inhibition decreases cell migration in a variety of cells, including tumor cells, while its ectopic expression has a promigratory effect (Schwab et al., 2007). Notably, the IK_{Ca} channel is only scantly expressed in the adult central nervous system (Ishii et al., 1997), suggesting that it could represent a target for a pharmacological approach against glioblastomas, provided that its activity is essential for cell migration.

Glioblastoma cells in vivo are exposed to a variety of extracellular matrix components and soluble factors that sensibly affect their migratory ability. Among them, possible candidates are unknown serum components that infiltrate into the tumor area of high grade gliomas as result of the breakdown of the blood–brain barrier (Seitz and Wechsler, 1987; Lund et al., 2006). It has been recently demonstrated that fetal calf serum (FCS) enhances migration of U87-MG glioblastoma cells through the induction of $[Ca]_i$ oscillations (Rondé et al., 2000; Giannone et al., 2002). More specifically, the oscillatory $[Ca]_i$ increases appear to facilitate the detachment of focal adhesions through stimulation of focal adhesion kinase, and the retraction of the cell rear (Giannone et al., 2002). Since the FCS-induced $[Ca]_i$ oscillations reach peaks sufficiently high to potentially activate calcium-activated K channels expressed in these cells, we hypothesized that an oscillatory IK_{Ca} channel and possibly

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BK channel activity would also be present, and that the consequent K efflux could contribute to the volume changes necessary during cell locomotion. We indeed found that FCS triggers an oscillatory activity of IK_{Ca} channels in U87-MG cells, together with a stable activation of a Cl-selective current. Most importantly, both FCS-induced channel activities were found to contribute to cell migration.

Materials and Methods

Cell culture

For the electrophysiological experiments, the U87-MG cell line was grown in DMEM medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10% fetal CALF serum (Invitrogen, S. Giuliano Milanese, Italy). Before electrophysiological recordings U87-MG cells, at 1 day of sub-culturing, were serum starved for 1 day. For migration assays U87-MG cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 1% nonessential amino acids, 1% L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% fetal calf serum (Flow Laboratories) at 37°C in a 5% CO_2 humidified atmosphere in air.

Electrophysiology

The whole-cell perforated patch clamp configuration was used for electrophysiological recordings from U87-MG cells. Currents and voltages were amplified with a HEKA EPC-10 amplifier, and analyzed with the PatchMaster and Origin 4.1 softwares. For on-line data collection, currents were filtered at 3 kHz, and sampled at 100 μsec/point. Membrane capacitance measurements were made by using the transient compensation protocol of PatchMaster.

The external solution contained (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). The internal solution contained: K_2SO_4 57.5, KCl 55, $MgCl_2$ 5, MOPS 10, (pH 7.20).

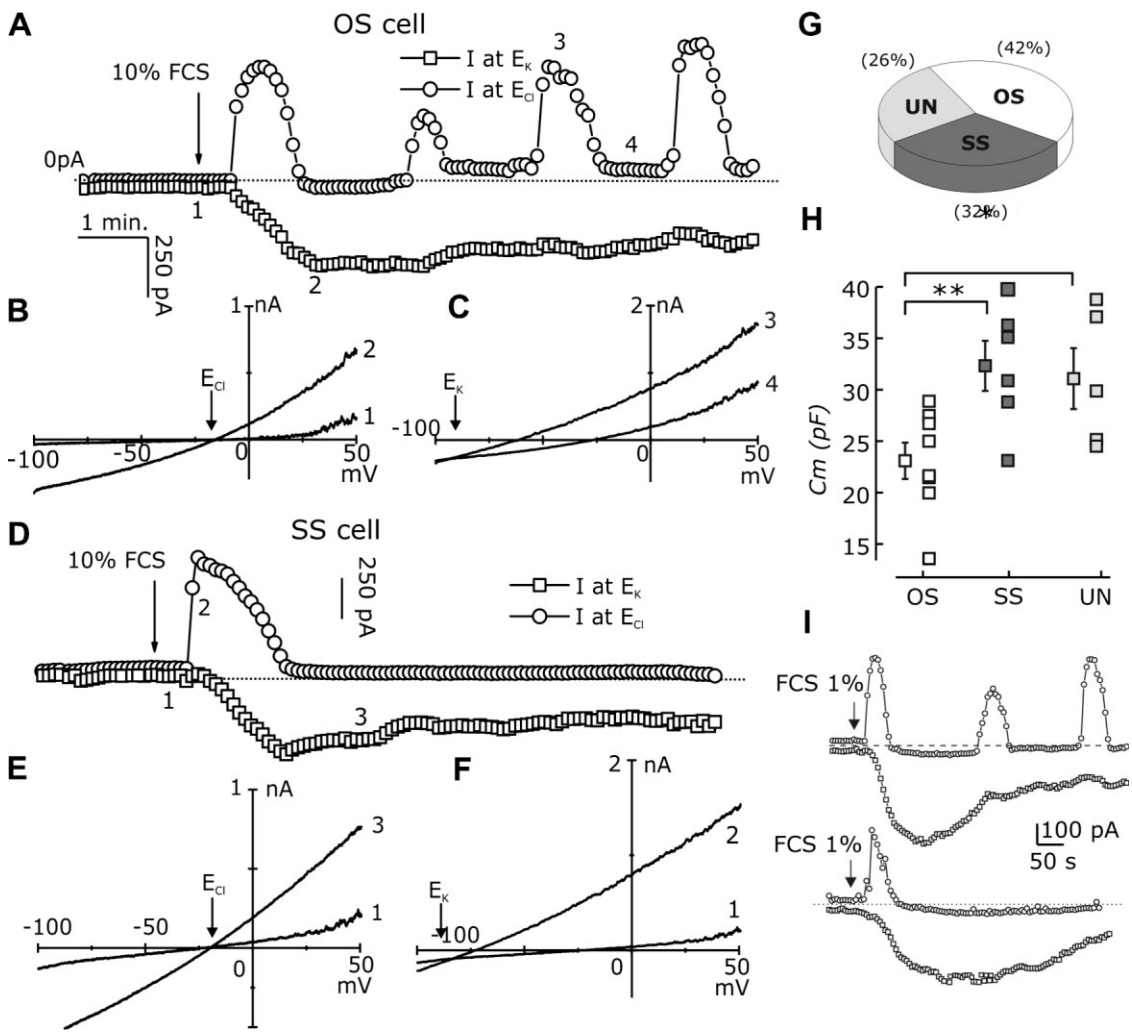

Fig. 1. FCS activates K- and Cl-selective currents in U87-MG cells. A,D: Time courses of the currents measured at the Cl (circles) and K (squares) equilibrium potentials during the application of 10% FCS to an OS cell (A) and an SS cell (D). Each data point represents the current amplitude taken at either E_K or E_{Cl} in response to voltage ramps from −100 mV to +50 mV repeated every 5 sec (cf. parts B, C, E, and F). The reversal potential of the oscillatory current was assessed as the voltage at which the current ramps taken at the minimum and the maximum of the response crossed over. B,C,E,F: Representative current-voltage relationships obtained from voltage ramp protocols applied to the same OS and SS cells of parts A and D. G: Plot showing the percentage of OS, SS, and UN cells. H: Plot comparing the single cell and the mean ± SE electrical capacitance of OS, SS, and UN cells. $^{*}P < 0.05$; $^{**}P < 0.02$. I: Time courses of the currents measured at the Cl (circles) and K (squares) equilibrium potentials during the application of 1% FCS to an OS cell and an SS cell.

Electrical access to the cytoplasm was achieved by adding amphotericin B (200 μM) to the pipette solution. Access resistances ranging between 10 and 20 MOhm were achieved within 10 min following seal formation. All chemicals used were of analytical grade. Dimethyl sulfoxide (DMSO), TEA, clotrimazole, *d*-tubocurarine, NS1619, U73122, U73343, BAPTA-AM, and DIDS were purchased from Sigma (St. Louis, MO), NPPB, DC-EBIO and ionomycin were purchased by Tocris, and Charybdotoxin and Apamin were purchased from Alomone Labs (Jerusalem, Israel). TRAM-34 was a kind gift of Dr. Heike Wolff. TRAM-34, clotrimazole, and NS-1619 were prepared in DMSO (20 mM) as stock solutions. Amphotericin B and BAPTA-AM were similarly dissolved in DMSO to concentrations of 50 mM and 30 mM, respectively; DIDS was prepared in carbonate buffer at 100 mM. Apamin and charibdotoxin were stocked in water at 100 μM concentration. The maximal DMSO concentration in the recording solutions was about 0.1%. Experiments were carried out at room temperature (18–22°C). Data are presented as mean ± SE.

Transwell cell migration assays

Six transwell filter chambers with 8 μm pores were used for the migration assay (BD Biosciences, Milano, Italy). Recently subcultured, semiconfluent U87-MG cells were harvested and, according to the manufacturer's instructions, resuspended in 2 ml of serum-free medium and seeded at 700,000 cells/well in the Boyden chambers. RPMI containing 10% fetal calf serum was already present into the well underneath the insert. Cells were incubated at 37°C for 21 h. After this time, the inner side of the insert was wiped with a wet swab to remove the cells while the outer side of the insert was gently rinsed with PBS and stained with 0.25% crystal violet (Sigma) for 15 min, rinsed again, and then allowed to dry. Migrated cells were counted under a light microscope counting 8 fields per chamber with 10× objective. For the assay DIDS, TRAM-34 or both, were added to RPMI at different concentrations. The inhibitors were added both in the upper side and in the lower side of each chamber. The complete medium was added to both sides of the chambers. Experiments were repeated three times for each condition of every ion blocker.

MTS assay

MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was performed in 96-well plates using a CellTiter 96 nonradioactive cell proliferation colorimetric assay kit (Promega, Madison, WI). U87MG cell line (2,500 cells in 100 μl medium per well) were plated in 96-well plates and after 24 h were supplemented with various combination of ion channel blockers (500 μM DIDS and 3 μM TRAM-34 used alone or in simultaneous presence). We tested three times: after 30 min, 24 h, and 48 h from the addiction of ion channel blockers by adding 20 μl of MTS solution and after 2 h we measured the samples with at spectrophotometer at wavelength of 490 nm. We related OD absorbance measured with viable cell number with an inverse formula.

Results

To verify whether FCS modulates ion channel activity in U87-MG glioblastoma cells, we made electrophysiological recordings under perforated-patch conditions, and applied voltage ramps from −100 mV to 50 mV every 5 sec from a holding potential of −40 mV. Under control conditions all U87-MG cells studied showed a linear current response to ramp stimulations, with a relatively low amplitude 0.8 ± 0.2 pA/pF at 0 mV of applied potential (n = 19). As shown in Figure 1, following 10% FCS application, in a fraction of cells (42%, 8 out of 19; oscillatory cells, OS) we could record the activation of two distinct types of currents differing for the reversal potential and time course. One, monitored under isolation at the Cl ion equilibrium potential ($E_{Cl} = -20$ mV; Fig. 1A, circles), was oscillatory and had a reversal potential of −81.3 ± 3.7 mV (n = 4), very close, although slightly more depolarized, to the K ion equilibrium potential (E_K) under our recording conditions ($E_K = -90$ mV; cf. Fig. 1B). The oscillating current had a mean density of 10.3 ± 1.8 pA/pF (n = 8). The other FCS-induced current was instead non oscillatory, slightly declined during several minutes recording, was inwardly directed at E_K (Fig. 1A, squares), and had a mean density of −10.8 ± 5.0 pA/pF. The reversal potential of this current measured −26.2 ± 3.0 mV (n = 4; Fig. 1B), a value close to E_{Cl}, suggesting that the current stably activated by FCS is sustained by Cl channels. In about 32% of cells (6 out of 19; single-spiking cells, SS) a concomitant activation of K- and Cl-selective currents in response to FCS was also observed, with the difference that the K-selective current activated transiently in these cells, without giving an oscillatory time course as observed in OS cells (Fig. 1D–F). Finally in the remaining cells (5 out of 19; unresponsive cells, UN) FCS application did not evoke any significant current response. In order to verify whether smaller concentrations of FCS, more likely to enter in contact with glioblastoma cells in vivo, are also able to affect the electrophysiological properties of U87-MG cells, we also performed experiments by looking at the effects of a 10 times lower serum concentrations (1%). We found that also this concentration of FCS is able to affect the electrophysiology of U87-MG cells, by activating both an oscillatory or single-spike K-selective current and a more stable Cl-selective current (Fig. 1I), although the oscillatory response occurred with a substantially smaller frequency than observed with 10% FCS. More specifically, of the 12 cells tested with 1% FCS, 3 responded as oscillatory cells (25%), 5 as single spike cells (42%), and 4 did not present any response.

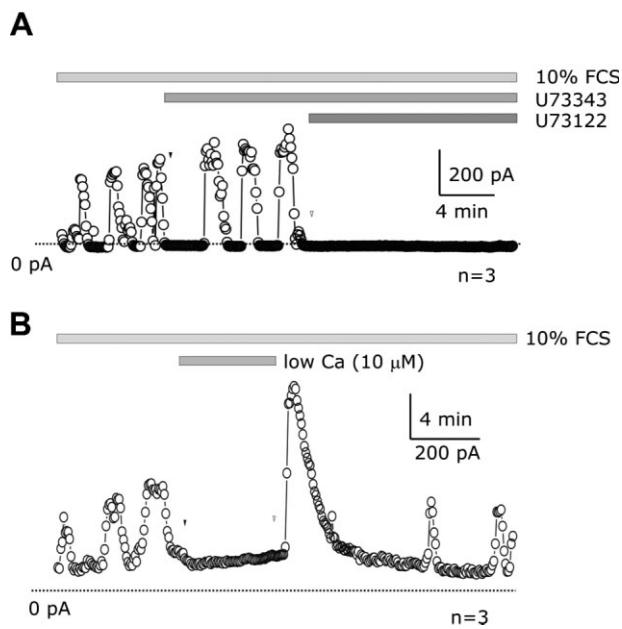

Fig. 2. Dependence of the FCS-induced oscillatory K current activity on the PLC activity and Ca influx. Time plots of the current recorded at E_{Cl} in the presence of 10% FCS, showing the effects of U73343 (5 μM) and U73122 (5 μM) (A), and the effect of lowering the extracellular calcium concentration to 10 μM (B) on the FCS-induced oscillatory current activity.

To assess whether a correlation existed between cell morphology and the ability to evoke an FCS-induced current response, we compared the mean electrical capacitance of the three subpopulations of U87-MG cells. As shown in Figure 1H oscillatory cells were found to have a significantly lower membrane capacitance as compared to single-spiking or unresponsive cells, indicating that FCS induces an oscillatory response preferentially on cells having a relatively small size.

We next asked whether the oscillatory K channel activity induced by FCS in OS cells was triggered by the $[Ca]_i$ oscillations previously observed in a subpopulation of FCS-treated U87-MG cells, and shown to be relevant in cell migration (Rondé et al., 2000; Giannone et al., 2002). Since the FCS-induced $[Ca]_i$ oscillations have been reported to be inhibited by both low extracellular Ca and the PLC inhibitor U73122, we verified whether these two conditions were also able to suppress the FCS-induced K current oscillations. As shown in Figure 2A, 5 μM U73122, but not its analogue ineffective on PLC activity U73343 (5 μM), was able to inhibit the oscillatory K current monitored at E_{Cl} ($n = 3$). In addition, FCS-induced K currents could also be suppressed by lowering the extracellular [Ca] from 2 mM to 10 μM (Fig. 2B; $n = 3$). These results strongly suggest that the K current activated by FCS in oscillatory cells is sustained by FCS-induced $[Ca]_i$ oscillatory increases, and it is most likely a calcium-activated K current.

As IK_{Ca} channels, expressed in several glioblastoma cell lines (Fioretti et al., 2004, 2006), are sensitive to relatively low intracellular calcium concentrations (Ishii et al., 1997), we first assessed the effect of the specific IK_{Ca} channel inhibitor TRAM-34 to establish the nature of the Ca-activated K channels underlaying the FCS-induced oscillatory K current. As shown in Figure 3, the FCS-induced oscillatory current, recorded at −20 mV (well within the physiological membrane potential range), was mostly blocked by the IK_{Ca} channel inhibitor TRAM-34 (3 μM). The fractional residual current in the presence of 3 μM TRAM-34 is not significantly different from zero ($P > 0.05$). By contrast, the selective BK_{Ca} channel inhibitor TEA (1 mM) and the selective SK_{Ca} channel inhibitor apamin (1 μM) did not show any significant inhibitory effect on the FCS-induced K current oscillations at −20 mV (data not shown). These data indicate that within the physiologically relevant membrane potentials of the cell, the majority of the oscillatory FCS-activated current is sustained by the IK_{Ca} channels.

The expression of the IK_{Ca} channels in these cells was confirmed by recording the IK_{Ca} current evoked by the IK_{Ca}/SK_{Ca} channel agonist DC-EBIO. As shown in Figure 4A, application of DC-EBIO (100 μM) plus the Ca agonist ionomycin (0.5 μM; DC-EBIO/iono) in the continuous presence of 3 mM TEA to block BK_{Ca} channels, activated a voltage-independent current with a reversal potential close to the E_K (Fig. 4A), and having a current density at 0 mV of applied potential of 18.7 ± 3.4 pA/pF ($n = 11$). The DC-EBIO/iono-activated current was present in all U87-MG cells examined and could be mostly blocked by TRAM-34 (3 μM), clotrimazole (10 μM), and charybdotoxin (ChTX; 100 nM), while being only marginally affected by apamin (1 μM; Fig. 4A,B), a

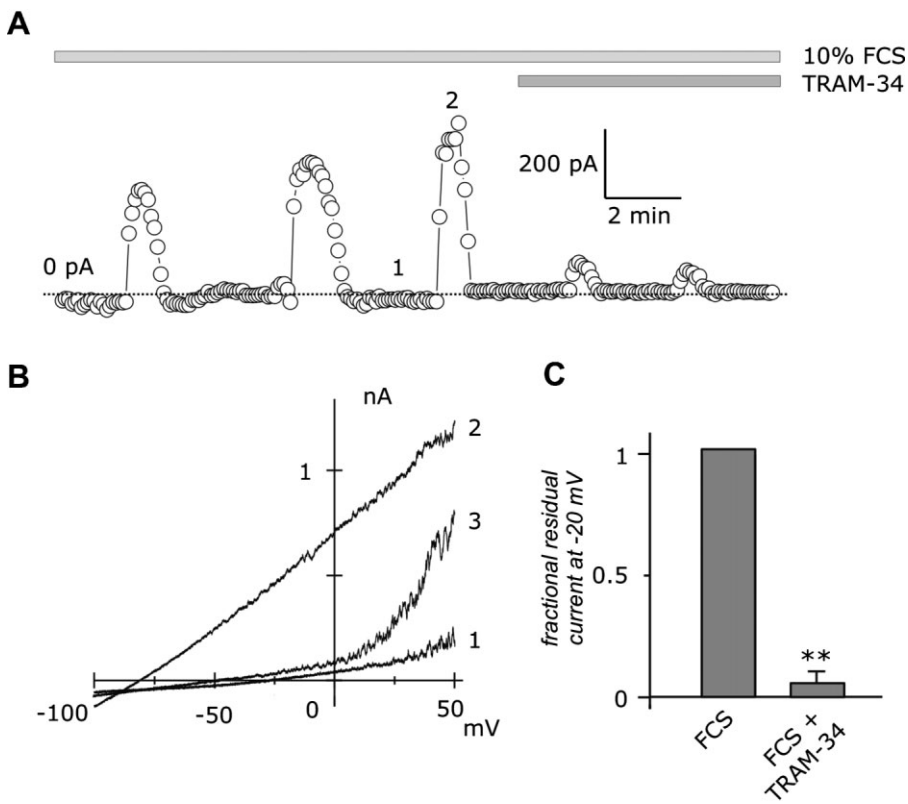

Fig. 3. The FCS-induced oscillatory K current is mainly sustained by the IK_{Ca} channels, at physiological membrane potentials. A: Time course of the current recorded at −20 mV of applied potential in the presence of 10% FCS, showing the effect of bath application of 3 μM TRAM-34 on the FCS-induced oscillatory K current. B: I–V relationships obtained at the indicated time points in the experiment shown in part A. Notice that the I–V record taken at the top of the residual transient current remaining in the presence of TRAM-34 (current trace 3) shows a significant current increase at voltages higher than ca. +20 mV. This current displays in addition the high noise typical of the BK_{Ca} current, suggesting that at high voltages a significant fraction of BK_{Ca} channels is also activated (also see below). C: Bar plot showing quantitatively the mean inhibitory effect of TRAM-34 on the transient K current activated by 10% FCS, assessed on three different OS cells.

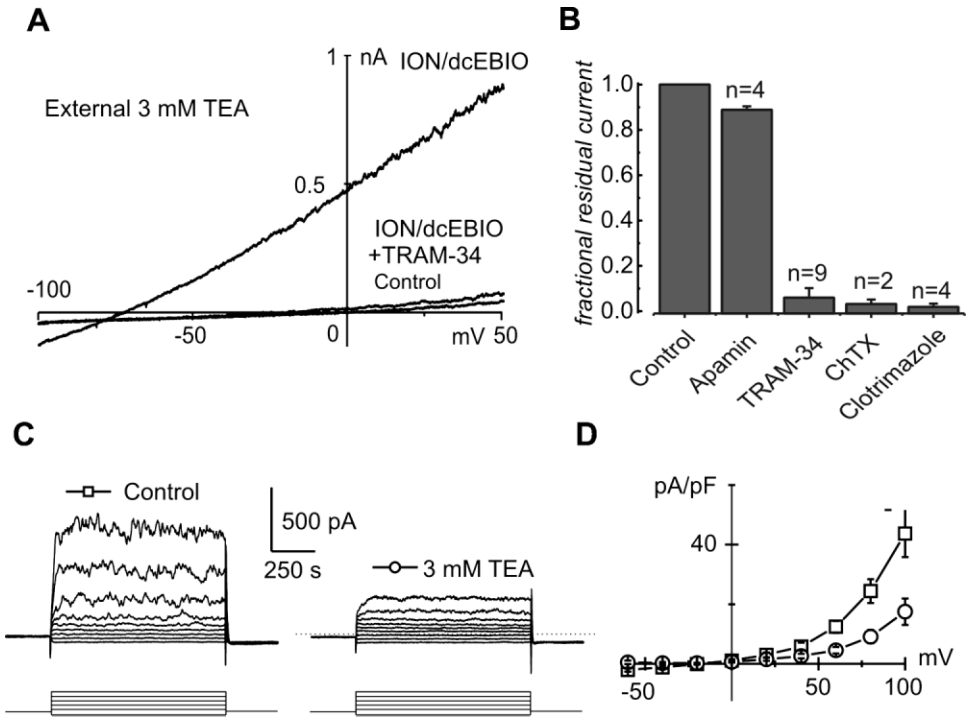

Fig. 4. Expression of Ca-activated K channels in U87-MG cells. A: Current traces obtained in response to voltage ramps from −100 mV to 50 mV under control conditions, following application of 100 μM DC-EBIO plus 0.5 μM ionomycin (DC-EBIO/iono), and following the further addition of 3 μM TRAM-34 in the continuous presence of DC-EBIO/iono. Three millimolars TEA were present throughout the experiment to inhibit BK_{Ca}-mediated currents. B: Bar plot showing the mean residual current measured at 0 mV of applied potential in the presence of 100 μM DC-EBIO plus 0.5 μM ionomycin and following the further addition of 1 μM apamin, 3 μM TRAM-34, 10 μM clotrimazole, and 100 nM ChTX. C: Family of current traces evoked by applying voltage steps from −60 mV to 140 mV in 20-mV increments in a U87-MG cell, under control conditions (left) and following bath application of 3 mM TEA. D: Mean I–V relationships obtained from 14 experiments similar to that shown in panel C.

pharmacological profile consistent with the notion that U87-MG cells express IK_{Ca} channels.

By contrast, within the physiological membrane potentials range, we did not observe any significant BK_{Ca} current component following FCS application, in spite of the abundant BK_{Ca} channel expression we found in U87-MG cells. As already reported by Ducret et al. (2003), at high depolarizing voltages (higher than ca. +30 mV) a current showing the high noise typical of BK_{Ca} channels was activated in all cells examined (n = 14; Fig. 4C). The current could be effectively inhibited by 3 mM TEA (Fig. 4C,D), and enhanced by the BK_{Ca} channel opener NS1619 (100 μM, data not shown), suggesting that it is mainly sustained by BK_{Ca} channels. To this regard, it needs to be added that the only evidence of a minimal BK_{Ca} channel activation at physiological potentials was that the small residual oscillatory currents remaining in the presence of TRAM-34 (cf. Fig. 3A) were sensibly reduced in amplitude following the addition of 3 mM TEA (data not shown). Altogether, these results suggest that, although U87-MG cells coexpress both BK_{Ca} and IK_{Ca} channels, only the IK_{Ca} channels are activated in response to FCS at physiologically relevant membrane potentials. This conclusion well agrees with the relatively high Ca affinity of IK_{Ca} channels as compared to BK_{Ca} channels at physiologically relevant membrane potentials, a property that would allow a selective IK_{Ca} channel activation following moderate $[Ca]_i$ increases (Fioretti et al., 2009).

We next turned to study the properties of the FCS-activated Cl current in U87-MG cells. Experiments were performed in 3 μM TRAM-34 and 3 mM TEA, to inhibit the FCS-induced activity of the Ca-activated K channels. As shown in Figure 5, under these conditions the application of 10% FCS activated in a fraction of cells (75%, 9 out of 12) a current having a reversal potential close to the E_{Cl} (Fig. 5A,B). This current could be effectively blocked by the Cl channel inhibitors DIDS (500 μM) and NPPB (100 μM; Fig. 5B,C). Voltage steps performed in the continuous presence of FCS uncovered for this current an instantaneous outward rectification and a characteristic time- and voltage-dependent inactivation (Fig. 5D). All these features are very similar to those observed for the volume activated Cl-currents expressed in a variety of cells, including glioblastoma cells (Ransom et al., 2001; Fioretti et al., 2004; Kimelberg et al., 2006; Okada et al., 2006, 2009). We also asked whether, similarly to what we observed for the FCS-induced oscillatory IK_{Ca} current, the activation of the Cl current by FCS requires an elevation of intracellular calcium. To this end we performed dedicated experiments where 10% FCS was tested following a 10 min incubation with BAPTA-AM (30 μM), a membrane permeant calcium ion chelator. We obtained a Cl current activation in three of the four cells tested, a percentage similar to that obtained under control conditions, with a mean FCS-induced current measured a +50 mV of applied potential not significantly different from that obtained under control conditions (20.5 ± 8.5 pA/pF, n = 3, in BAPTA-AM and 10.8 ± 5.9 pA/pF, n = 7, in control conditions; $P > 0.05$). This result suggests that the FCS-induced Cl current activation does not require an elevation of the intracellular calcium concentration.

Finally, we looked at the functional relevance of IK_{Ca} and Cl channels activation by FCS, by evaluating the effects of TRAM-

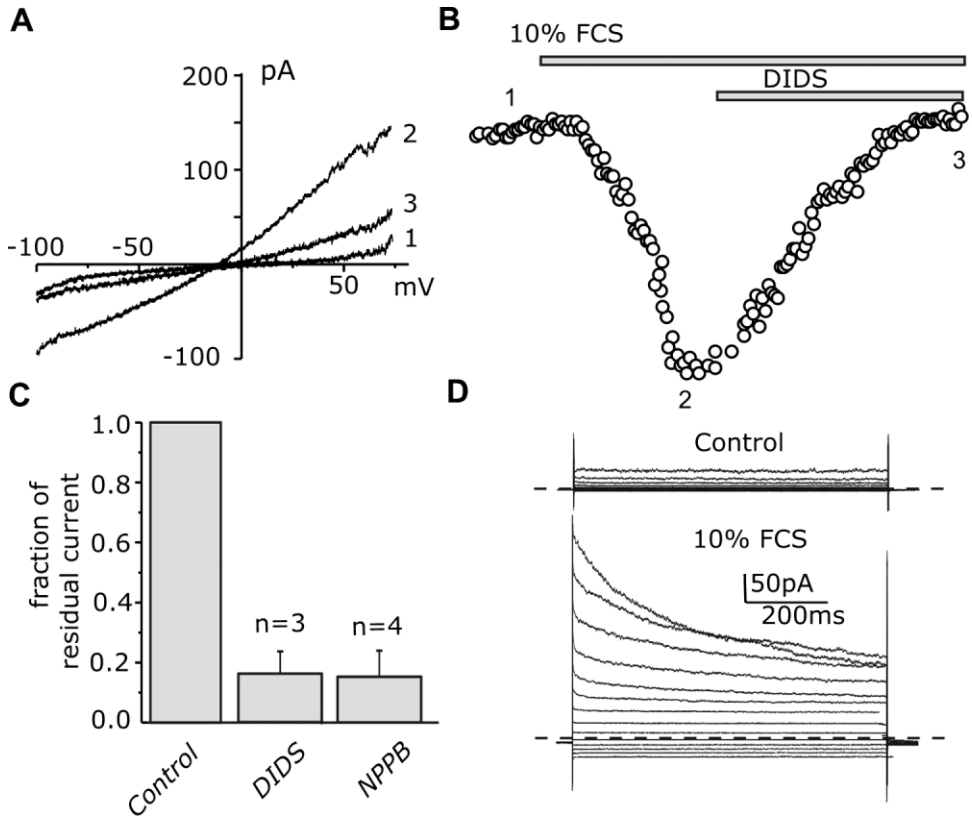

Fig. 5. Properties of the FCS-induced Cl current. A: I–V relationships obtained by applying voltage ramps from −100 to 75 mV in control conditions, in the presence of 10% FCS, and in the presence of 10% FCS + 500 μM DIDS. Three micromolars TRAM-34 and 3 mM TEA were present throughout the experiment to block Ca-activated K channels. B: Time course of the current recorded at −80 mV of applied potential showing the effect of 10% FCS and 10% FCS + 500 μM DIDS. C: Bar plot showing the mean inhibitory effect of 500 μM DIDS (n = 3) and 100 μM NPPB (n = 4) on the FCS-induced Cl current. D: Families of current traces evoked by applying voltage steps from −120 mV to 100 mV (holding potential of −40) in control conditions and in presence of 10% FCS.

34 and DIDS on U87-MG cell migration in the presence of FCS. To this end we used transwell migration assays and a cells incubation time of 21 h. As shown in Figure 6, 1 μM TRAM-34 was able to inhibit the U87-MG migration by 33 ± 4.2% (n = 8, $P < 0.0001$), and the inhibitory effect was even more evident at 3 μM concentration (58.5 ± 3.1%; n = 8, $P < 0.0001$). As already demonstrated in other glioma cell lines (Olsen et al., 2003), DIDS decreased U87-MG cell migration in a concentration dependent manner, and the effect was already significant at the minimal concentration tested of 100 μM (Fig. 6C,D). Interestingly, when DIDS (either 100 or 500 μM) and TRAM-34 (3 μM) were applied simultaneously, a strong and more than additive reduction in mobility could be observed (n = 8, $P < 0.0001$; Fig. 6C,D). Similar results were obtained in experiments where the cell incubation time was lowered to 5–6 h (data not shown). Finally, using MST assay, we verified that the maximal concentrations we used for both DIDS and TRAM-34, used alone or in combination, do not significantly affect cell survival ($P > 0.05$ compared to the control; data not shown).

Discussion

In this paper we show that FCS, previously shown to enhance the migratory activity of U87-MG cells by inducing $[Ca]_i$ oscillations, induced in a subpopulation of cells an oscillatory activity of IK_{Ca} channels, mediated by the $[Ca]_i$ oscillations, together with a stable activation of a Cl current. By contrast, in another subpopulation of U87-MG cells FCS induced a single, transient IK_{Ca} current activation, always accompanied by a stable activation of the Cl current. Finally we found that activation of IK_{Ca} and Cl channel activity is a necessary step to promote U87-MG cell migration in the presence of FCS. Unfortunately the serum component/s responsible for the observed electrophysiological effects is for the moment unknown. Even if several growth factors present in serum at relatively high concentration have already been shown to be unable to induce $[Ca]_i$ oscillations similar to those produced by FCS (Rondé et al., 2000), several other substances present in serum have been previously shown to induce $[Ca]_i$ oscillations in glioblastoma cells, such as achetylcoline (Bordey et al., 2000), LPA (Manning et al., 2000), bradykinin (Reetz and Reiser, 1996), ATP and glutamate (Mariggio et al., 2001).

Identity of the ionic currents activated by FCS in U87-MG cells

The identification of the oscillatory K current activated by FCS in OS cells as IK_{Ca} current is supported by the following observations: (a) reducing the extracellular [Ca] or inhibiting PLC activation, maneuvers previously reported to interrupt FCS-induced $[Ca]_i$ oscillations in these cells (Rondé et al., 2000; Giannone et al., 2002), suppressed the oscillatory current; (b) the I–V relationship of the FCS-induced oscillatory current was essentially ohmic, as expected for a voltage independent channel, and with a reversal potential slightly more depolarized than E_K, properties congruent with the IK_{Ca} current; (c) the

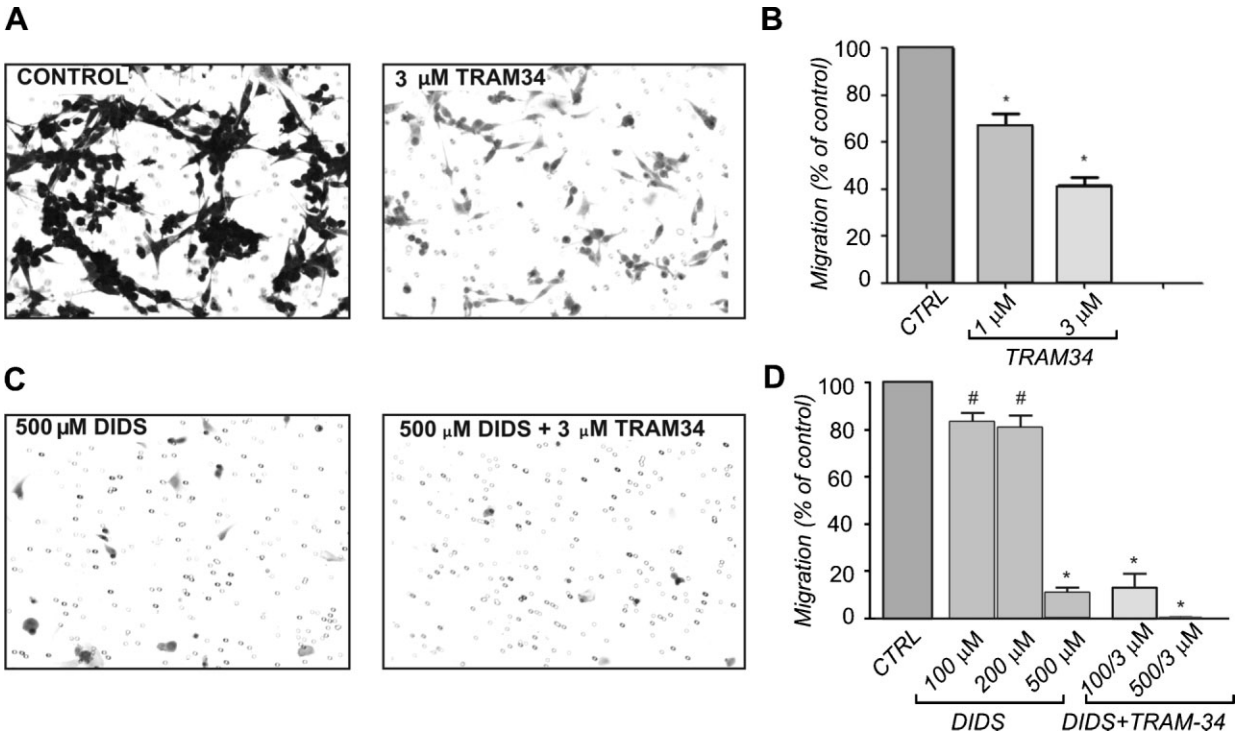

Fig. 6. IK_{Ca} and Cl channels are involved in the U87-MG cell migration. A: Representative microscopic fields of U-87MG human glioblastoma cells that have migrated 21 h across an 8 μm pore size filter in absence and in the presence of 3 μM TRAM-34. B: Bar plot showing the mean number of migrated cells in 10% FCS plus either 1 or 3 μM TRAM-34. C: Representative microscopic fields of U87-MG human glioblastoma cells that have migrated 21 h across an 8 μm pore size filter in presence of 500 μM DIDS or 500 μM DIDS plus 3 μM TRAM-34. D: Bar plot showing the mean number of migrated cells in 10% FCS plus 100 μM, 200 μM DIDS, 500 μM DIDS, 100 μM, or 500 μM DIDS plus 3 μM TRAM-34.

current was inhibited by the IK_{Ca} channel selective blocker TRAM-34. An oscillatory IK_{Ca} channel activity induced by $[Ca]_i$ oscillations has also been described in a variety of cell types, including murine C6 glioblastoma cells (Varnai et al., 1993; Verheugen and Vijverberg, 1995; Reetz and Reiser, 1996).

We also found that U87-MG cells abundantly express BK_{Ca} channels, in accordance with a previous report (Ducret et al., 2003), yet they are activated by the FCS-induced $[Ca]_i$ oscillations only at membrane potentials much higher than those relevant physiologically (cf. Figs. 3 and 4). This most likely depends on the relatively low Ca affinity of these channels. The IK_{Ca} channel-induced hyperpolarization during the $[Ca]_i$ peaks would further discourage the voltage dependent BK_{Ca} channels from opening under physiological conditions (Fioretti et al., 2009). It is worth recalling within this context that a recent work on glioblastoma cell lines showed that BK_{Ca} channels are most often strictly colocalized with ryanodine-sensitive intracellular Ca stores, and open in response to chemical stimulations able to activate a calcium-induced calcium release mechanism (Weaver et al., 2007). Our data would then suggest that FCS does not activate this CICR mechanism in U87-MG glioblastoma cells.

FCS was also able to stably activate a Cl current. Although the molecular identity of the underlying channels remains unclear, due to the lack of selective Cl channel antagonists, our data suggest that the FCS-induced Cl current identifies with the volume-sensitive Cl current often described in glioblastoma cells (Ransom et al., 2001; Fioretti et al., 2004; Kimelberg et al., 2006). We indeed found that this current is blocked by the Cl channel inhibitors DIDS and NPPB, has an outward rectifying instantaneous current–voltage relationship, and displays a time- and voltage-dependent inactivation. These properties well agree with those previously found for the volume-sensitive Cl current in several glioblastoma cell lines (Ransom et al., 2001; Fioretti et al., 2004). Moreover, volume-sensitive Cl currents have often been shown to be under the positive control of PLC (Ellershaw et al., 2002; Zholos et al., 2005), and unpublished data from our laboratory indicate that PLC activity mediates the FCS-induced Cl current activation (manuscript in preparation). Volume-sensitive Cl currents are usually assigned a critical role in the regulatory volume decrease occurring upon cell exposure to hypotonic solutions (Hoffmann et al., 2009). The data reported here suggest that this current also activates in response to PLC-stimulating modulators, under isotonic conditions, thus suggesting that it can actively participate in other functional processes involving volume changes, such as cell migration.

Functional significance of FCS modulation of membrane currents in glioblastoma cells

Unlike tumors that form elsewhere in the body or tumors that are metastatic to brain, primary brain tumors are characterized by a relentless drive to invade surrounding healthy tissues. This invasive behavior has long been recognized as a major impediment in the treatment of gliomas (Soroceanu et al., 1999). Most importantly glioma cell movement through the tortuous extracellular space of the brain requires changes in cell volume and shape (Soroceanu et al., 1999), processes that in several cell types are under the control of ion channels (Danker et al., 1996; Lascola et al., 1998; Schwab et al., 1999; Ransom et al., 2000; Schneider et al., 2000).

Our data show that the activation of both IK_{Ca} and Cl currents participate to the FCS-induced migration of U87-MG

glioblastoma cells. This result well agrees with several reports indicating a fundamental role of K and Cl channels in the migration of a number of cell types, including glioblastoma cells (Schwab, 2001; McFerrin and Sontheimer, 2006; Schwab et al., 2007). Cell locomotion is thought to be promoted by the cycling repetition of three fundamental steps: (i) protrusion of the cell front, concomitant with an asymmetric polymerization of an actin-based cytoskeleton; (ii) adhesion of the newly protruded cell front to the substratum, mediated by the binding of focal adhesions to the extracellular polymers in contact with the cell; (iii) unbinding of the focal adhesions localized at the cell rear, and retraction of the main cell body towards the direction of motion (Ananthakrishnan and Ehrlicher, 2007). Two of these steps, namely cell front protrusion and cell rear retraction, involve significant changes in cell volume. The combined K and Cl effluxes are thought to serve this purpose during retraction. As proposed by Schwab (2001), during the protrusion of the cell front the concomitant volume increase is guaranteed by various ion transporters that will promote a KCl influx followed by osmotically driven water. During this phase the $[Ca]_i$ is relatively small (cell protrusion should thus take place between two $[Ca]_i$ transients), the IK_{Ca} channels are closed, and the resting membrane potential is close to the Cl equilibrium potential, thus limiting the loss of KCl through K and Cl channels. By contrast the retraction of the cell rear, with associated cell volume decrease, occurs during the transient $[Ca]_i$ increase, as result of the following cascade of events: the transient $[Ca]_i$ increase translates into the opening of IK_{Ca} channels, and the membrane potential hyperpolarizes to a value between E_{Cl} and E_K, a condition that would allow a significant efflux of KCl with consequent loss of water and cell volume decrease. This hypothesis can explain the slowing of cell locomotion we observed following the inhibition of IK_{Ca} and Cl channels.

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