Original Paper

Glycine Induces Migration of Microglial
BV-2 Cells via SNAT-Mediated Cell Swelling

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Key Words

Swelling-dependent chloride current • Cl^- • $\text{ICl}_{\text{swell}}^-$ • VRAC • VSOR • Cell volume regulation • Glycine • Neutral amino acid transporter • Migration • Microglia

Abstract

Background/Aims: The neutral, non-essential amino acid glycine has manifold functions and effects under physiological and pathophysiological conditions. Besides its function as a neurotransmitter in the central nervous system, glycine also exerts immunomodulatory effects and as an osmolyte it participates in cell volume regulation. During phagocytosis, glycine contributes to (local) cell volume-dependent processes like lamellipodium formation. Similar to the expansion of the lamellipodium we assume that glycine also affects the migration of microglial cells in a cell volume-dependent manner. **Methods:** Mean cell volume (MCV) and cell migration were determined using flow cytometry and trans-well migration assays, respectively. Electrophysiological recordings of the cell membrane potential (V_{mem}) and swelling-dependent chloride (Cl^-) currents ($\text{ICl}_{\text{swell}}^-$, VSOR, VRAC) were performed using the whole-cell patch clamp technique. **Results:** In the murine microglial cell line BV-2, flow cytometry analysis revealed that glycine (5 mM) increases the MCV by ~9%. The glycine-dependent increase in MCV was suppressed by the partial sodium-dependent neutral amino acid transporter (SNAT) antagonist MeAIB and augmented by the Cl^- current blocker DCPIB. Electrophysiological recordings showed that addition of glycine activates a Cl^- current under isotonic conditions resembling features of the swelling-activated Cl^- current ($\text{ICl}_{\text{swell}}^-$). The cell membrane potential (V_{mem}) displayed a distinctive time course after glycine application; initially, glycine evoked a rapid depolarization mediated by Na^+ -coupled glycine uptake via SNAT, followed by a further gradual depolarization, which was fully suppressed by DCPIB. Interestingly, glycine significantly increased migration of BV-2 cells, which was suppressed by MeAIB, suggesting that SNAT is involved in the migration process of microglial cells. **Conclusion:** We conclude

that glycine acts as a chemoattractant for microglial cells presumably by a cell volume-dependent mechanism involving SNAT-mediated cell swelling.

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Introduction

Glycine is a simple, non-essential neutral amino acid found in all mammalian body fluids. As primarily inhibitory neurotransmitter it is amply present in the central nervous system (CNS) with heterogeneous distribution. Low concentrations are found in the cerebellum and mid-thoracic spinal cord and high concentrations in the ventral horn of the spinal cord and medulla oblongata [1]. Strict control of the extracellular glycine concentration in the CNS is essential for normal neuronal function. During neuronal activity peak glycine concentrations of 2.2–3.5 mM have been monitored in glycinergic synapses [2] and uptake of the transmitter from the extracellular space is mainly accomplished by astrocytes, which store glycine at concentrations up to 3–6 mM [3]. Under pathophysiological conditions like stroke, ischemia and reperfusion injury or neurodegenerative diseases, when glycine is discharged from dying cells, concentrations in the restricted interstitial space may therefore rise substantially. Moreover, glycine levels both in blood and cerebrospinal fluid are increased under clinical conditions like non-ketotic hyperglycinemia [4] and in inflammatory disorders like meningitis [5], amyotrophic lateral sclerosis [6] and multiple sclerosis [7], where the amino acid shows anti-inflammatory effects.

Beside its functions in the CNS, the systemic elevation of glycine in the blood alleviates symptoms of endotoxin-mediated shock [8], alcoholic liver injury [9] and some forms of cancer [10, 11]. The involvement of glycine in pathophysiological processes in which macrophages play a major role, suggests that it modulates their functions. Effects of glycine on monocytes [12], Kupffer cells [13] or microglial cells [14, 15] are mediated either by ionotropic glycine receptors (GlyRs) or by the Na⁺-dependent neutral amino acid transporter (SNAT; a member of the Slc38 System A amino acid transporter family). In Kupffer cells activation of GlyRs causes a hyperpolarization of the cell membrane potential (V_{mem}) [13, 16], whereas a GlyR-independent, but SNAT-dependent effect of glycine appears in microglial cells [14, 15]. We have shown in microglial cells that glycine uptake via SNAT depolarizes V_{mem} due to Na⁺ influx, which is unaffected by the GlyR antagonist strychnine, but is inhibited by the specific partial SNAT antagonist α -(methylamino)isobutyric acid (MeAIB). Further proof that glycine acts via SNATs rather than GlyRs in microglial cells comes from data showing that the effect of glycine on V_{mem} is sensitive to changes in the extracellular Na⁺, but not the Cl⁻ concentration. In line with this, reverse-transcriptase PCR did not reveal amplification products of GlyR mRNA/cDNA but revealed SNAT transcripts in primary microglial cells and in the murine cell line BV-2 [14, 15].

In some physiological processes, glycine acts as an organic osmolyte and participates in cell volume (CV) regulation. In mouse embryo cells, intracellular glycine accumulation leads to cell swelling [17–19]. Furthermore, glycine increases CV in Ehrlich ascites tumor cells (EATCs) [20] as well as in microglial cells [15]. In both cell types, Na⁺ free saline inhibits the glycine-induced cell swelling, thus indicating a contribution of SNAT in cell swelling [15, 20]. The involvement of SNATs in the regulatory volume increase (RVI) process during hypertonic stimulation is well described [21, 22]. However, less evidence exists for an involvement of SNAT in cell volume changes under isotonic conditions. Microglial cells are resident immune cells of the CNS. The need for an expansion of cell volume can be observed during migration and phagocytosis of these cells. Under physiological conditions microglial cells have a ramified morphology, with a small soma and elaborated thin processes. This appearance has been associated with and termed as the microglial “resting” state. Upon any detection of neuronal damage, nervous system dysfunctions or brain lesion, a complex activation process happens, and microglial cells undergo morphological changes by retracting their processes and increasing the size of their cell bodies, i.e. cell swelling. Once activated, they are able to migrate to the site of brain injury and phagocytose affected cells or fragments thereof [23].

Migrating microglial cells undergo cell polarity-dependent CV changes during their movement, which is likely a repetitive cycle of protrusion at the cell front (leading edge or lamellipodium), followed by a retraction of the cell's rear (trailing edge). CV increases during the protrusion of the lamellipodium and decreases during the retraction due to a local water movement across the cell membrane [24-27]. Using the terminology of CV regulation, cell migration can be described as an alternating cycle of regulatory volume increase (RVI) at the leading edge and regulatory volume decrease (RVD) at the trailing edge of the cell. CV is mainly regulated by the ion movement across the cell membrane, accompanied by osmotically obliged water fluxes. CV regulation is therefore critically dependent on the activity of ion channels and transporters [28]. Most of the ion transporters that participate in overall CV regulation are involved also in the migration process [26]. The outward movements of ions like K^+ and Cl^- across ion channels located at the rear part of a migrating cell causes a local RVD process whereas transporters that provoke local RVI are located at the front of the cell and import mainly Na^+ ions via Na^+/H^+ transporters (NHEs) and $Na^+/K^+/2Cl^-$ transporter NKCC1 [29-33]. This suggests that also other Na^+ coupled transporters like Na^+ /amino acid cotransporters are likely to be involved in the migrating machinery, and a role for the cotransport of Na^+ and glycine via SNAT is therefore feasible.

A similar mechanistic explanation has been applied to lamellipodia formation/protrusion during phagocytosis [15]. Accordingly, blockade of a swelling-dependent Cl^- current (ICl_{swell} , ICl_{vol} , VSOR, VRAC) prevents the uptake of Ig-coated microspheres and disrupts the formation of lamellipodia [34, 35]. Moreover, hypertonic conditions decrease particle engulfment [36], suggesting that phagocytosis itself and especially the formation of a phagocytic cup is highly dependent on CV regulation. Importantly, in a previous study we could show that application of glycine rescues the diminished phagocytosis under hypertonic conditions, and that glycine stimulates phagocytosis under isotonic conditions, which is suppressed by MeAIB [15]. These findings strongly suggest that SNAT is involved in phagocytosis. Taken together and based on our previous study [15], we therefore hypothesize that in murine BV-2 microglial cells: (i) glycine increases cell volume via a Na^+ dependent transport (SNAT); (ii) glycine-induced cell swelling triggers isosmotic RVD by activating a swelling dependent Cl^- current (ICl_{swell}); and (iii) glycine acts as a chemoattractant that stimulates microglial cell migration.

Materials and Methods

Cell culture

BV-2 cells were cultured in 25 cm² flask with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2,200 mg/L glucose and 10% (v/v) fetal calf serum (FCS; Biochrom). BV-2 cells were kept at 37° C in a humidified atmosphere of 5% CO₂ (standard culture conditions). Subcultures were established once a week and were used for experiments until passage 30 was reached.

Electrophysiology

BV-2 cells were seeded on 0.01% poly-D-lysine (PDL)-coated coverslips (Ø 12 mm) and cultured for at least 24 h in DMEM. Coverslips were transferred to a RC-25 recording chamber and mounted on a Nikon Eclipse TE2000-U inverted microscope. All experiments were performed at room temperature and were recorded in the whole cell (ruptured or perforated) patch clamp mode. Ruptured whole cell mode was achieved by applying a slight suction through the pipette whereas the perforated whole cell configuration was reached by adding 130 µM amphotericin B to the pipette solution. Recordings were started as soon as the serial resistance was below 30 MΩ for the perforated and below 10 MΩ for the ruptured configuration. Patch electrode resistances were 3-7 MΩ. After establishing the whole cell configuration, cells were superfused with an extracellular solution and data were recorded using an EPC-10 amplifier controlled by PatchMaster software (HEKA). Cell membrane potential (V_{mem}) recordings were performed in the zero-current clamp mode. The intracellular (pipette) solution contained (in mM): 70 K₂SO₄, 10 NaCl, 10 KCl, 4 MgCl₂, 2 CaCl₂, 5 HEPES free acid, 10 EGTA (243 mOsm/kg, pH 7.2 adjusted with KOH). The extracellular solution contained (in mM): 140 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 HEPES free acid, 4.5 glucose and 5 mannitol (300 mOsm/

kg, pH 7.4 adjusted with NaOH). NaCl was reduced by 40 mM to get a hypotonic (225 mmol/kg) extracellular solution and mannitol was iso-osmotically substituted by 1 mM or 5 mM glycine to assess glycine-induced depolarization of V_{mem} . Cl^- currents ($I_{\text{Cl}_{\text{swell}}}$) were recorded under symmetrical intra- and extracellular Cl^- conditions. The extracellular solution consisted of (in mM): 100 NaCl, 2.5 CaCl_2 , 2.5 MgCl_2 , 10 HEPES free acid and 97 mannitol (306mmol/kg, pH 7.2 adjusted with NaOH). To assess glycine uptake-dependent Cl^- current activation under isotonic conditions, mannitol was substituted iso-osmotically by 1 mM and 5 mM glycine. The pipette solution contained (in mM): 100 CsCl, 5 MgCl_2 , 10 HEPES free acid, 10 EGTA, 65 raffinose, 2 Mg-ATP (306 mmol/kg, pH 7.2 adjusted with CsOH). The currents were monitored every 10 s applying 500 ms voltage ramps from -100 mV to +100 mV. The holding potential between the ramps was kept at 0 mV to avoid voltage-activated currents. Bath solution exchange was performed with a valve-controlled gravity-driven perfusion system (ALA Scientific Instruments) at a flow rate of 3-5 ml/min. Osmolalities of intra- and extracellular solutions were measured using a vapour pressure osmometer (Wescor).

Assessment of the mean cell volume (MCV) by flow cytometry

Subconfluent BV-2 cells were harvested by Trypsin/EDTA after growing for 4-5 days under standard cell culture conditions. To measure the effect of 5 mM extracellular glycine under different conditions, the cell suspension was split into aliquots. Every aliquot was then centrifuged for 5 min at 200×g. Immediately before the first measurement (time point 0) the pellet was re-suspended in 3 ml extracellular solution, which contained (in mM): 140 NaCl, 5.6 KCl, 2.5 CaCl_2 , 1.5 MgCl_2 , 10 HEPES free acid and 4.5 glucose (295 mOsm/kg, pH 7.4 adjusted with NaOH). Under control conditions, the extracellular solution additionally contained 5 mM mannitol. To assess the effect of glycine in combination with the Cl^- blocker DCPIB, we iso-osmotically substituted mannitol with (1) 5 mM glycine and with (2) 5 mM glycine and 10 μM DCPIB. In a second series of experiments cells were exposed to the extracellular solution in presence of (1) 5 mM glycine, (2) 5 mM MeAIB and (3) 5 mM glycine in combination with 5 mM MeAIB. MeAIB and glycine were again iso-osmotically substituted by mannitol (control). The samples of both series were alternately measured every 5 min over a period of 60 min. Mean cell volume (MCV in fl) was directly measured on a CellLab Quanta SC flow cytometer (Beckman Coulter) employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by nonconductive particles suspended in an electrolyte solution. The electronic volume channel was calibrated using 10- μm Flow-Check fluorospheres (Beckman-Coulter) by positioning this size bead in channel 200 on the volume scale. 8,000 single cells with a diameter $\geq 10 \mu\text{m}$ and $\leq 18 \mu\text{m}$ were analyzed per sample. For analysis the data of each condition were normalized to the respective control condition.

Cell migration

BV-2 cells were starved for 12 hours in a serum-free (SF) medium and subsequently seeded in 24-well inserts (24-well inserts with 0.8 μm pores; ThinCert™; Greiner bio-one) at 1×10^6 cells per well. Inserts were transferred in Cellstar® 24-well plates (Greiner) containing 600 μl of SF medium without (control) or with glycine, or glycine plus equimolar MeAIB. As positive control conditions we used medium with 10% FCS, SF medium containing 0.5 $\mu\text{g/ml}$ lipopolysaccharide (LPS from Escherichia coli; Sigma Aldrich) and SF medium containing 1 μM adenosine 5'-triphosphate magnesium salt (Mg-ATP; Sigma Aldrich). Cells were incubated overnight under standard culture conditions. Migrated cells were stained with 8 μM Calcein AM viability dye (Thermo Fisher Scientific) for 45 min and then trypsinized with 500 μl 0.25% Trypsin/EDTA (Sigma Aldrich) for 15 min. 200 μl of the cell suspension were transferred in black, flat bottom 96-well plates (Sarstedt) and fluorescence at 520 nm (485 nm excitation) was measured with a Spark multimode reader® (Tecan).

Statistical Analysis

All data are presented as mean $\pm$ sem (standard error of the means). Statistical analyses were performed using SPSS 18.0 (IBM Corporation) or Prism 7.0 (GraphPad Software). Patch clamp data were analyzed using Fit-Master software (HEKA) and Igor Pro 6 (WaveMetrics). Each experiment was repeated at least three times ($n \geq 3$). Ordinary or repeated measures one-way ANOVA, paired or unpaired double-sided *t*-tests were used as applicable to calculate the levels of significance. Results were regarded as statistically significant at $p < 0.05$.

Results

Glycine-induced cell swelling is SNAT dependent

Fig. 1 shows that iso-osmotic addition of 5 mM glycine caused cell swelling. After 20 and 60 min of glycine application the mean cell volume (MCV) of BV-2 cells was significantly increased by $6.8 \pm 0.6\%$ and $7.5 \pm 0.4\%$, respectively, compared to control conditions in the absence of glycine (Fig. 1A). The time course of glycine-induced cell swelling showed a steep initial increase in MCV reaching a plateau after ~20 min. In presence of the Cl^- channel blocker DCPIB (10 μM) cell swelling was significantly augmented compared to the effect of glycine alone, with a volume gain of $9.6 \pm 1.9\%$ and $14.7 \pm 2.1\%$ after 20 and 60 min, respectively.

To test if glycine-induced cell swelling is mediated by SNAT, MCV was measured in the presence of the partial SNAT antagonist MeAIB (5 mM). As shown in Fig. 1B, glycine led to an increase in MCV by $10.9 \pm 2.5\%$ after 60 min, whereas in the presence of MeAIB cell swelling was almost completely prevented with a volume gain of $0.9 \pm 1.3\%$ after 60 min. MeAIB alone caused only a slight increase in MCV by $2.3 \pm 2.1\%$ within 60 min, which is consistent with partial inhibition of the transporter.

Hypotonicity activates a Cl^- current ($I_{\text{Cl}^-, \text{swell}}$) and leads to depolarization of the cell membrane potential (V_{mem})

In most cells, swelling leads to the activation of distinct volume-regulated Cl^- channels which give rise to a typical swelling-activated Cl^- current $I_{\text{Cl}^-, \text{swell}}$ (VSOR, VRAC). The cellular exit of Cl^- , other anions and organic osmolytes through these channels is accompanied by the outflow of osmotically obliged water and drives the process of regulatory volume decrease (RVD) designated to counteract hypotonic cell swelling [37, 38]. As shown in Fig. 2A, reduction of the extracellular osmolality by 15% led to the gradual activation of a Cl^- current after a delay of ~5-10 min. This current was outwardly rectifying (Fig. 2A-C), inactivated at constant positive potentials (Fig. 2B) and was reversibly inhibited by the Cl^- channel blocker NPPB (150 μM) (Fig. 2B-C).

Fig. 1. Glycine-induced cell volume increase of BV-2 cells is SNAT dependent and augmented by the Cl^- channel blocker DCPIB. Mean cell volume (MCV) was measured by flow cytometry using the Coulter method. Experiments were performed under osmotically balanced (isotonic) conditions. A. Time course of glycine-induced cell swelling in absence and presence of the Cl^- channel blocker DCPIB (10 μM). *, $p < 0.05$ glycine (GLY, 5 mM) vs. GLY+DCPIB (10 μM), $n = 3-4$. B. Inhibition of glycine-induced cell swelling by the partial SNAT antagonist MeAIB (5 mM). * and #, $p < 0.05$ GLY (5 mM) vs. GLY + MeAIB and GLY vs. MeAIB alone, respectively, $n = 4$. CTL, control conditions in absence of GLY, MeAIB and DCPIB.

Fig. 2. Hypotonic stimulation of BV-2 cells leads to ICl_{swell} activation. Ruptured patch clamp recordings. A. ICl_{swell} activation over time. Mean whole cell currents at +100 and -100 mV of 4-5 individual cells evoked by 15% reduction in extracellular osmolality (hypo) recorded in response to voltage ramps (inset). B. Cl^- currents of an individual cell in response to voltage steps (inset) under isotonic conditions, hypotonic conditions (showing typical outward rectification and inactivation over time at constant positive holding potentials), under hypotonic conditions in presence of NPPB (150 μ M) and after washout of the inhibitor. C. Current-voltage relations obtained from the tracings shown in (B).

Fig. 3 shows that hypotonic stimulation by a 30% reduction in extracellular osmolality caused a transient hyperpolarization of V_{mem} due to K^+ current activation [26, 27] from -70.8 ± 1.2 mV to -71.6 ± 1.4 mV, followed by a slower but sustained depolarization to -59.6 ± 2.4 mV resulting from activation of ICl_{swell} (Fig. 3B). This depolarization was fully reversed by the ICl_{swell} inhibitor DCPIB (10 μ M), which caused a drop in V_{mem} back to -71.6 ± 1.7 mV.

Glycine activates a Cl^- current under isotonic conditions ($ICl_{glycine}$) and leads to depolarization of the cell membrane potential (V_{mem})

Since glycine causes cell swelling (Fig. 1), we tested whether it leads to the activation of a Cl^- current similar to ICl_{swell} . As shown in Fig. 4, addition of 1 and 5 mM glycine elicited such a current ($ICl_{glycine}$) under isotonic conditions, which slowly developed over time becoming evident after ~ 10 min and reaching a maximum after ~ 20 min (Fig. 4A, D, F). Currents at -100 and +100 mV increased from -64.8 ± 4.1 pA and 118.3 ± 18.0 pA to -85.8 ± 7.6 pA and 222.3 ± 49.1 pA in the presence of 1 mM glycine, and -123.2 ± 9.6 pA and 342.5 ± 43.3 pA under

Fig. 3. Hypotonic stimulation of BV-2 cells by a 30% reduction in extracellular osmolality (hypo) causes an initial hyperpolarization followed by a sustained depolarization of V_{mem} . Perforated patch clamp recordings. A. Time course of V_{mem} of a single experiment. Each dot is the mean V_{mem} over 15 s. a. Close-up of the time course. The arrow indicates the initial hyperpolarization. B. Mean V_{mem} under isotonic (iso) conditions (n=11) and under hypotonic (hypo) conditions in absence and presence of DCPIB. 1, initial hyperpolarization (n=11); 2, slow ICl_{swell} -mediated depolarization (n=11); 3 (hatched bar), repolarization under DCPIB (10 μ M; n=8). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant.

5 mM glycine (Fig. 4E). $ICl_{glycine}$ showed characteristics of ICl_{swell} such as outward rectification, inactivation at constant positive potentials (Fig. 4B) and sensitivity to the Cl^- channel blocker NPPB (150 μ M) (Fig. 4A, B, C, E), and the current inactivated upon withdrawal of glycine (Fig. 4F).

As shown in Fig. 5A-B, application of 5 mM glycine under isotonic conditions induced a rapid depolarization of V_{mem} from -64.5 ± 5.0 mV to -55.2 ± 5.1 mV resulting from electrogenic cotransport of Na^+ along with glycine via SNAT [15]. Similar to glycine, glutamine (5 mM) had a reversible depolarizing effect (Fig. 5C). Additional hypotonic stimulation in continued presence of glycine caused a significant and sustained further depolarization by ~ 7 mV to -48.0 ± 4.6 mV after a brief initial drop of V_{mem} by ~ 2 mV, similar as during hypotonic stimulation alone (Fig. 3). The hypotonicity-induced depolarization was fully reverted by DCPIB (10 μ M), whereas the SNAT-mediated depolarization was unaffected by the Cl^- channel blocker. After changing to glycine-free isotonic conditions V_{mem} repolarized to -69.4 ± 3.8 mV.

That Cl^- efflux due to activation of ICl_{swell} further depolarized V_{mem} on top of the SNAT (electrogenic glycine/ Na^+ uptake)-mediated depolarization, was not unexpected. The question remained, however, if glycine-induced cell swelling and $ICl_{glycine}$ activation alone, without additional hypotonic stimulation, affects V_{mem} over time. We addressed this question by performing long-time recordings under iso-osmotic conditions in the presence of 5 mM glycine. In these experiments we observed a distinctive biphasic time course of depolarization as shown in Fig. 6: the rapid depolarization by Na^+ -driven glycine uptake via SNAT (from -73.7 ± 3.8 mV to -67.1 ± 4.7 mV) was followed by a slow, gradual further depolarization by ~ 20 mV within 20-25 min to -48.2 ± 5.3 mV that matched the time course of glycine-induced cell swelling (Fig. 1) and $ICl_{glycine}$ activation (Fig. 4), and which was significantly reversed by DCPIB. After washout of DCPIB V_{mem} again strongly depolarized to -39.5 ± 7.1 mV.

Glycine stimulates BV-2 microglial cell migration

Ion transport, depolarization of V_{mem} and (sub)cellular volume regulation are critical events in the regulation of migration of microglial cells [24, 33, 39]. Since glycine modulates these events (a simplified model of these effects on cell volume and electrical properties of microglial cells is depicted in Fig. 7), we tested for the effect of glycine on BV-2 cell migration using trans-well migration system.

Fig. 4. Glycine evokes a Cl^- current in BV-2 cells with characteristics of $\text{ICl}_{\text{swell}}^-$. Experiments were performed under osmotically balanced (isotonic) conditions in the ruptured patch clamp configuration. **A.** Cl^- current activation over time under 5 mM glycine and inhibition of the current by the Cl^- channel blocker NPPB. Whole cell currents at +100 and -100 mV in response to voltage ramps (inset). **B.** Currents elicited by voltage steps (inset) at time points 1-3 indicated in (A) were outwardly rectifying and inactivated over time at constant positive holding potentials (1, 3), and were sensitive to NPPB (2). **C.** Current-voltage relations obtained from the tracings shown in (B). **D.** Current activation in cells under 5 mM glycine (grey circles) versus control cells kept in absence of glycine (empty circles). Mean responses of 3-32 cells. **E.** Mean current amplitudes after 26 min under control conditions (CTL; $n=20$), in presence of 1 mM glycine (1 GLY; $n=10$), 5 mM glycine (5 GLY; $n=32$) and 5 GLY + 150 μM NPPB ($n=5$). **F.** Current inactivation after withdrawal of glycine.

Fig. 5. ICl_{swell} activation by hypotonicity augments the SNAT-mediated depolarization. Perforated patch clamp recordings. A. Time course of V_{mem} of a single experiment (GLY, 5 mM glycine). Each dot is the mean V_{mem} over 15 s. B. Mean V_{mem} (n=9) measured in absence (empty bars) and presence (grey bars) of 5 mM glycine under isotonic (iso)- and hypotonic (hypo) conditions. 1, SNAT-mediated depolarization; 2, transient repolarization; 3, slow ICl_{swell} -mediated depolarization; 4 (hatched bar), repolarization under DCPIB (10 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. C. Depolarization of V_{mem} by 5 mM glutamine (n=5-6).

Fig. 6. Glycine depolarizes V_{mem} in two phases. Experiments were performed under osmotically balanced (isotonic) conditions. Perforated patch clamp recordings. A. The time course of a single recording showed a biphasic depolarization triggered by 5 mM glycine (GLY). The second-phase depolarization was counteracted by the Cl^- channel blocker DCPIB. Each dot is the mean V_{mem} over 15 s. B. Mean V_{mem} measured in absence (empty bars) and presence (grey bars) of 5 mM glycine. 1, rapid SNAT-mediated depolarization (n=15); 2, slow $ICl_{glycine}$ -mediated depolarization (n=15); 3 (hatched bar), repolarization under DCPIB (10 μ M; n=15); 4, depolarization after DCPIB washout (n=9). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

As shown in Fig. 8, migratory activity was significantly increased by 45% under 5 mM glycine compared to control conditions (serum-free medium). The SNAT antagonist MeAIB (5 mM) attenuated the effect of 5 mM glycine, suggesting that the stimulatory effect of glycine is SNAT-dependent. In culture medium containing 10% FCS, 0.5 μ g/ml LPS, or 1 μ M ATP the migratory activity was increased by ~81%, 56% and 74%, respectively.

Fig. 7. Hypothetical (simplified) model of the effects of glycine on cell volume and electrical properties of microglial cells. The electrogenic uptake of glycine by a Na^+ -coupled cotransport via SNAT leads to a rapid depolarization of V_{mem} and increases the intracellular osmolality. The osmotically obliged water influx causes cell swelling and activates swelling dependent Cl^- channels/currents ($\text{ICl}_{\text{glycine}}/\text{ICl}_{\text{swell}}$), which further depolarizes V_{mem} and back-regulates cell volume by regulatory volume decrease (RVD).

Discussion

This study confirms previous findings showing that the amino acid glycine – a widely distributed neurotransmitter in the CNS – causes depolarization of the cell membrane potential and cell swelling in microglial BV-2 cells [15]. These effects are mediated by electrogenic uptake of Na^+ and glycine via the sodium-dependent neutral amino acid transporter (SNAT). In a recent study we have shown, that glycine facilitates phagocytosis and that this process is dependent on glycine-induced cell swelling [15]. These findings were fostered by an earlier study showing that in phagocytosing microglial cells a distinctive Cl^- conductance, which is activated to drive regulatory volume decrease (RVD) after cell swelling and which gives rise to the swelling-activated Cl^- current $\text{ICl}_{\text{swell}}$ (ICl_{vol} , VRAC, VSOR), is increased. Accordingly, phagocytosis was suppressed by inhibitors of Cl^- channels and by counteracting glycine-induced cell swelling through MeAIB [15, 35]. It remained unclear, however, whether the activation of $\text{ICl}_{\text{swell}}$ occurs as a consequence of phagocytosis per se or/and as a consequence of SNAT-mediated cellular uptake of Na^+ and glycine and the osmotically driven influx of water.

In our study, glycine increased the mean cell volume (MCV) by ~7% compared to a ~17% volume increase observed in EATCs [20]. In both cell types a plateau of the MCV was slowly reached after 15-30 min and in both microglial cells and EATCs, cell swelling was prevented in Na^+ free extracellular saline [15, 20]. The inhibition of glycine-induced cell swelling by the partial SNAT antagonist MeAIB, as shown here, indicates that the activity of this Na^+ coupled cotransporter underlies cell swelling. In line with this assumption, it has been reported that the transport A system, which includes SNAT, participates in cell volume regulation [40] and that in human fibroblasts SNAT2 silencing lowers the intracellular concentration of amino acids and inhibits the regulatory volume increase (RVI) after hypertonic stress [41].

Na^+ and glycine uptake via SNAT is followed by the activation of a Cl^- current under isotonic conditions ($\text{ICl}_{\text{glycine}}$). This current resembles the one elicited in BV-2 cells by hypotonic cell swelling ($\text{ICl}_{\text{swell}}$, VRAC, VSOR), i.e.; outward rectification, time-dependent inactivation at

Fig. 8. Glycine-stimulated migration in BV-2 cells (n=3-11). Control values (CTL; set to 100%) are indicated by the dashed line. GLY, glycine; LPS, lipopolysaccharide. *, $p<0.05$; **, $p<0.01$; **** $p<0.0001$.

constant positive holding potentials and sensitivity to the Cl^- channel blockers NPPB and DCPIB [15]. In monocytes and Kupffer cells, a glycine-dependent Cl^- current is assigned to the ionotropic glycine receptor (GlyR) [12, 13] and glycine binding increases its intrinsic Cl^- conductance which leads to a hyperpolarization due to Cl^- influx [13, 16, 42]. Based on electrophysiological recordings, Schilling and Eder [14] convincingly argued against GlyR expression in BV-2 cells. In line with our previous results [15], they found that glycine caused a rapid and sustained depolarization, which was insensitive to the GlyR antagonist strychnine. In addition, the glycine-induced depolarization was Na^+ dependent and suppressed by MeAIB [14]. In line with this finding, our earlier results from RT-PCR analysis cells did not reveal expression of GlyR (isoforms Glra1-4) in BV-2 cells and primary microglial cells, but detected those for the Na^+ -coupled neutral amino acid transporter SNAT (solute carrier family 38 isoforms Slc38a1, 2 and 4) [15]. Therefore, the glycine-induced Cl^- current ($\text{ICl}_{\text{glycine}}$) observed here is clearly independent of GlyR. Apart from this, the biophysical properties of $\text{ICl}_{\text{glycine}}$ are different from GlyR-mediated Cl^- currents [43], but they are identical to those of swelling-activated Cl^- currents ($\text{ICl}_{\text{swell}}$, ICl_{vol} , VRAC) described in microglial cells and in a plentitude of other cells [26, 27, 33, 44, 45].

Glycine-induced cell swelling was enhanced by the Cl^- channel blocker DCPIB. This indicates that the activation of $\text{ICl}_{\text{glycine}}/\text{ICl}_{\text{swell}}$ limits further cell swelling upon amino acid and Na^+ uptake. However, the blocker does not affect the initial kinetics of glycine-induced cell swelling, which is most likely due to the delayed onset of Cl^- current activation. Indeed, $\text{ICl}_{\text{glycine}}$ activation and depolarization of V_{mem} evolve with a considerable delay of several minutes after administration of the amino acid, which is similar to the delayed activation of $\text{ICl}_{\text{swell}}$ by hypotonic stimulation [35, 44]. The changes in V_{mem} caused by glycine were biphasic. Shortly after addition of the amino acid a rapid and stable depolarization occurred. We and others found that this depolarization was reversed by a Na^+ -free extracellular solution [14, 15], showing that this initial depolarization is due to electrogenic glycine uptake along with Na^+ via SNAT. The application of glutamine instead of glycine similarly depolarizes V_{mem} . In the second phase, a further depolarization set in with a delay of 5-10 min, which was antagonized by DCPIB. This supports our assumption that this second-phase depolarization is indeed caused by delayed activation of $\text{ICl}_{\text{glycine}}/\text{ICl}_{\text{swell}}$ over time during glycine-induced cell swelling. That $\text{ICl}_{\text{swell}}$ activation affects V_{mem} in BV-2 cells is evident from depolarizations observed upon hypotonic cell swelling both in absence and presence of glycine, which were reversed by DCPIB.

Cytoskeletal rearrangements as well as ion and water fluxes through ion channels, transporters and aquaporins are basic requirements for cell migration and there is a mutual interaction between these factors. In migrating cells, channels and transporters show a functionally polarized distribution and migrating cells undergo repetitive cycles of local volume gain at the cell front and a volume loss in the rear part. $\text{ICl}_{\text{swell}}$ plays a key role in the retraction of the trailing edge by mediating Cl^- efflux, which drives the efflux of water (reviewed in [24, 33, 39]). The depolarization of the cell membrane potential due to Cl^- efflux generates a driving force for the movement of other ions affecting migration. Blocking these Cl^- channels has been found to inhibit migration in several cell types including human malignant glioma cells, nasopharyngeal glioma cells, HeLa cells and microglial cells [46-49]. Given the effects of glycine on hallmarks of cell migration (cell swelling, Cl^- current activation, depolarization of V_{mem}) we investigated if glycine modulates the migratory activity of microglial cells. *Escherichia coli* bacteria have been shown to be attracted by several amino acids including glycine [50] and glycine in combination with gastrin (glycine-extended gastrin) has been found to stimulate gastrointestinal cell migration [51]. We found that 5 mM glycine significantly increased BV-2 cell migration. To our knowledge, this is the first study showing that glycine stimulates migration in microglial cells. The stimulatory effect of glycine on migration was abrogated by the SNAT inhibitor MeAIB. Similarly, we could previously show that phagocytosis in microglial cells is suppressed by MeAIB [34]. This shows that SNATs play a central role in mediating the effects of glycine on migration and phagocytosis in these cells and suggests that movement of the entire cell during migration and the movement

of the pseudopodium to entangle a cell during phagocytosis are similar processes, which are closely linked to cell volume regulatory mechanisms. In the CNS, the role of glycine as a chemoattractant might be particularly relevant in case of traumata, stroke, ischemia and reperfusion injury or neurodegeneration, when dying neurons and glial cells release glycine and other intracellular compounds. In such a scenario, when released compounds accumulate in the restricted interstitial space, glycine might act as a chemoattractant and guide microglial cells to these regions and concurrently stimulate their phagocytotic activity to remove cell debris and pathogens.

Conclusion

We conclude that the exposure of glycine to microglial BV-2 cells leads to an uptake of glycine by a Na⁺-coupled cotransport system (SNAT), which causes (1) cell membrane depolarization, (2) cell swelling due to osmotically driven water influx, and, (3) activation of a swelling-dependent Cl⁻ current, which further depolarizes V_{mem}. Moreover, we conclude that glycine acts as a chemoattractant and stimulates migration of microglial cells.

Abbreviations

SNAT (sodium neutral amino acid transporter); RVD (regulatory volume decrease); RVI (regulatory volume increase); MeAIB (α-(Methylamino)isobutyric acid); GlyR (glycine receptor); ICl_{swell} (swelling dependent chloride current); VSOR (volume-sensitive outwardly rectifying); VRAC (volume-regulated anion channel); DCPIB (4-[(2-Butyl-6, 7-dichloro-2-cyclopentyl-2, 3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid); DIDS ((4, 4'-Diisothiocyanato-2, 2'-stilbenedisulfonic acid)); NPPB (5-Nitro-2-(3-phenylpropylamino) benzoic acid).

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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