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ORIGINAL ARTICLE

Glucose is a positive modulator for the activation of human recombinant glycine receptors

Ulrike Breitinger, Karim M. Raafat[1] and Hans-Georg Breitinger

Department of Biochemistry, The German University in Cairo, New Cairo, Egypt

Abstract

The inhibitory glycine receptor (GlyR), a cys–loop ion channel receptor, mediates rapid synaptic inhibition in spinal cord, brainstem and higher centres of the mammalian central nervous system. Here, modulation of GlyR function by glucose and fructose was examined in recombinant alpha1 and alpha1/beta GlyRs using patch-clamp methods. Glucose was a positive modulator of the receptor, reducing the average EC_{50} for glycine up to 4.5-fold. Glucose reduced cell-to-cell variability of glycine-mediated currents by stabilizing receptors with low EC_{50}. Pre-incubation with sugars for several hours also produced augmentation of current responses that persisted after sugar removal. Potentiation by sugars was most significant in the range between 5 and 20 mM, with EC_{50} values ~ 10 mM, i.e. at physiological levels. Addition of glucose had no significant influence on responses mediated by the other GlyR agonists like taurine, β-alanine or ivermectin, indicating that glucose specifically augmented glycine receptor-mediated responses, and did not act through indirect metabolic effects. Receptor modulation by glucose may account for differences in constants reported in the literature and may be clinically relevant for disorders with elevated blood glucose levels.

Keywords: allosteric modulation, glycine receptors, Ligand-gated ion channels, sugars as channel modulators.

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The inhibitory glycine receptor (GlyR), a member of the cysteine loop superfamily of ligand-gated ion channels (Breitinger and Becker 2002), is one of the principal mediators of rapid synaptic inhibition in the mammalian CNS. GlyR's are involved in the control of muscle tone and movement as well as retinal signalling and pain processing (Lynch 2009). GlyRs have been found in mammalian spinal cord and brainstem, hippocampus (Xu and Gong 2010), cerebellum (Bagnall *et al.* 2009), retina (Grunert and Ghosh 1999; Jusuf *et al.* 2005), as well as non-neuronal cells such as macroglia, immune cells and endothelial cells (den Eynden *et al.* 2009; Breitinger 2014). To date, five different subunits have been identified in mammals, four ligand-binding alpha subunits (α1–α4) and one β subunit (Breitinger and Becker 2002). GlyR dysfunction underlies the human neurological disorder, hyperekplexia (Stiff Baby Syndrome, Startle Disease STHE, OMIM 149400), which is associated with mutations in genes encoding GlyR subunits or associated proteins (Davies *et al.* 2010).

The study of GlyR function requires patch-clamp electrophysiological techniques. Recombinant GlyRs, expressed in HEK293 cells or *Xenopus laevis* oocytes are a well-established model for such studies. Despite their widespread use, large differences in glycine-mediated currents and EC_{50} values have been reported for both expression systems (Table 1). EC_{50} values of recombinant α1 GlyRs reported from different laboratories vary up to 10-fold (De Saint Jan *et al.* 2001). Also, variation in EC_{50} from cell to cell, or oocyte to oocyte by a factor of 5–10 was observed when this phenomenon was specifically addressed (Fucile *et al.* 1999; De Saint Jan *et al.* 2001). So far, the reason underlying this considerable variability has not been identified. Upon surveying the literature, we noticed that some laboratories include 10–20 mM glucose in the extracellular recording medium, as it is considered to contribute to cell durability during recording, other groups work without glucose in the recording buffer. An effect of glucose on ion channel activity has been reported once in a study of enhancement of pre-synaptic 5-HT_3 serotonin receptors by extracellular glucose (Wan and Browning 2008), modulation of post-synaptic receptors by sugars has not been examined to date. As glucose is a vital nutrient,

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Address correspondence and reprint requests to Hans-Georg Breitinger, Department of Biochemistry, German University in Cairo, New Cairo, Egypt. E-mail: hans.breitinger@guc.edu.eg

[1]Present address: Department of Pharmaceutical Sciences, Faculty of Pharmacy, Beirut Arab University, 115020 Beirut, Lebanon.

Abbreviation used: GlyR, glycine receptor.

Table 1 Variability of recombinant α1 glycine receptor responses

Expression system		Species	EC_{50} (μM)	EC_{50} range (μM)	Glucose in bath	References
Xenopus oocytes		Danio	116 ± 75 (n = 83)	73–360		Fucile *et al.* (1999)
		Human	'153' (n = 44)	25–280		De Saint Jan *et al.* (2001)
Transfected cell lines	HEK293	Human	18 ± 2 (n > 3)	9–92	10 mM	Lynch *et al.* (1997)
	HEK293	Human	23 ± 10 (n = 5)		10 mM	Moorhouse *et al.* (1999)
	HEK293	Human	39 ± 17 (n = 22)		0 mM	Bormann *et al.* (1993)
	HEK293	Human	75		Not given	Pribilla *et al.* (1992)
	L(tk^-)[a]	Human	101 ± 7 (n = 6)		11 mM	Wick *et al.* (1999)
	HEK293	Human	46 ± 3		0 mM	Becker *et al.* (2008)
	BOSC[b]	Danio	33 ± 17 (n = 19)		10 mM	Fucile *et al.* (1999)

[a]Expression of GlyRα1 in mouse L(tk^-) cells.

[b]BOSC 23 human cells, derived from Ad5-transformed HEK293T cell line. Note that the references give no information about the concentration of glucose in the cell culture medium.

cell culture in glucose-free medium is not routinely done. HEK293 cells are commonly grown in minimum essential medium (MEM 1 g/l = 5.5 mM glucose, which we used in this study), use of Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/l = 24.8 mM glucose) or DMEM low glucose (1 g/l = 5.5 mM glucose) has also been reported.

Here, we show that glucose and fructose act as potentiators of GlyR function, generating a significant change in the EC_{50} values of recombinant α1 and α1/β-GlyR's in HEK293 cells. This shift was accompanied by a reduction in cell-to-cell variability, with low EC_{50} values predominating in the presence of sugars. Potentiation was observed on time scales of seconds as well as hours. Thus, glucose was identified as an endogenous modulator of glycinergic function, acting as allosteric positive modulator of GlyR currents. Enhancement after glucose pre-treatment was close to the maximum at concentrations below 10 mM, i.e. at physiological levels. Glucose concentration may be important as an experimental parameter in ion channel studies as well as in clinical situations.

Materials and methods

Cell culture and transfection

HEK293 cells were grown in 10 cm tissue culture petri dishes in MEM (Sigma, Deisenhofen, Germany) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Karlsruhe, Germany) and Penicillin/Streptomycin at 5% CO_2 and 37°C in a water saturated atmosphere. For experiments, cells were plated on poly-L-lysine treated glass coverslips in 6 cm dishes. Transfection was performed 1 day after cell passage using GenCarrier (Epoch Life Science, Sugar Land, TX, USA): 1.3 μg of receptor DNA, 1.3 μg of green fluorescence protein DNA and 2.6 μL GenCarrier were used, following the manufacturer's instructions. Measurements were performed 2–5 days after transfection. For heteromeric GlyRs, 0.8 μg of α1, 2.4 μg of β cDNA and 4 μL of GenCarrier reagent were used.

Electrophysiological recordings and data analysis

Whole-cell recordings were performed using a HEKA EPC 10 amplifier (HEKA Electronics, Lambrecht, Germany) controlled by Pulse software (HEKA Electronics). Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller (Sutter, Novato, CA, USA). Ligand was applied using a either *U*-tube device (Krishtal and Pidoplichko 1980) that bathed the suspended cell in a laminar flow of solution, giving a time resolution for equilibration of 10–30 ms (Hess 1993), or an Octaflow perfusion system (NPI electronics, Tamm, Germany). The external buffer consisted of 135 mM NaCl, 5.5 mM KCl, 2 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 10 mM Hepes (pH adjusted to 7.4 with NaOH); the internal buffer was 140 mM CsCl, 1.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 5.0 mM EGTA and 10 mM Hepes (pH adjusted to 7.2 with CsOH). Sugars (Sigma-Aldrich, Deisenhofen, Germany) were added to the buffers as indicated. Current responses were measured at room temperature (21–23°C) at a holding potential of −40 mV. Dose–response curves were constructed from the peak current amplitudes obtained with at least seven appropriately spaced glycine concentrations in the range 0.5–2000 μM glycine. Using a non-linear algorithm in Microcal Origin (Additive, Friedrichsdorf, Germany), dose–response data were fitted to the Hill equation

$$\frac{I_{\text{glycine}}}{I_{\text{sat}}} = \frac{[\text{Glycine}]^{n\text{Hill}}}{[\text{Glycine}]^{n\text{Hill}} + \text{EC}_{50}^{n\text{Hill}}}$$

where I_{glycine} is the current amplitude at a given glycine concentration, I_{sat} is the current amplitude at saturating concentrations of glycine, EC_{50} is the glycine concentration at half-maximal current responses and n_{Hill} is the Hill coefficient. Currents from each individual cell were normalized to the maximum response at saturating glycine concentrations. Differences between EC_{50} values recorded in the absence and presence of sugars were compared using one-way ANOVA with $p \leq 0.05$ taken as significant (*), whereas (**) indicates $p \leq 0.01$. For desensitization statistics, total current I_{tot}, the percentage of desensitizing and non-desensitizing current, and the first-order desensitization time constant τ were determined from traces at saturating concentrations of glycine (usually 1 mM).

Two types of whole-cell recording experiments were performed to study the effects of sugars on GlyR-mediated currents: (i) whole-cell recordings in extracellular buffer with or without glucose, (ii) whole-cell recordings in glucose-free extracellular buffer, where GlyR-transfected HEK293 cells had been pre-exposed to glucose in the culture medium for 18–20 h. In all experiments EC_{50} values were determined for each individual cell from a non-linear fit of dose–response data to the logistic equation (above). To assess variation between cells, an unweighed average was calculated from all individual EC_{50} values, without considering the fitting errors. The means and SD from this average are then given for each concentration of glucose.

Results

Upon recombinant expression in HEK293 cells, homomeric GlyR α1 gave stable whole-cell current responses to glycine stimulation. Glycine-evoked currents were investigated in the absence or presence of glucose in the extracellular solution (Fig. 1; Table 2). Dose–response curves showed a distinct, concentration-dependent left-shift when glucose was present (Fig. 1b, Table 2). The average EC_{50} value of GlyR α1 wildtype [EC_{50} (0 mM glucose) = 39 ± 15 μM] shifted by a factor of 2.5 in the presence of 100 mM glucose [EC_{50} (100 mM glucose) = 16 ± 2 μM]. Presence of 10 mM glucose in the intracellular buffer had no effect (Fig. 1c). 20 mM and 50 mM glucose in the extracellular buffer gave half maximum currents at concentrations of 27 ± 5 and 15 ± 3 μM respectively.

EC_{50} values in absence and presence of glucose were inspected for cell-to-cell variability (Fig. 2a). In glucose-free solution, EC_{50} values of GlyRs varied from 15 – 95 μM (> 6-fold), while in presence of 100 mM glucose, the values ranged from 9.2 to 24.6 μM (factor 2.6). Obviously, addition of glucose not only lowered absolute values of EC_{50} (Fig. 2b), but also reduced the variability of EC_{50} values between cells.

Maximum currents (I_{max}), at different glucose concentrations were compared (Fig. 2c). No trend or dependence of I_{max} on glucose concentration was detectable for any of the receptor variants under study. Furthermore, no correlation

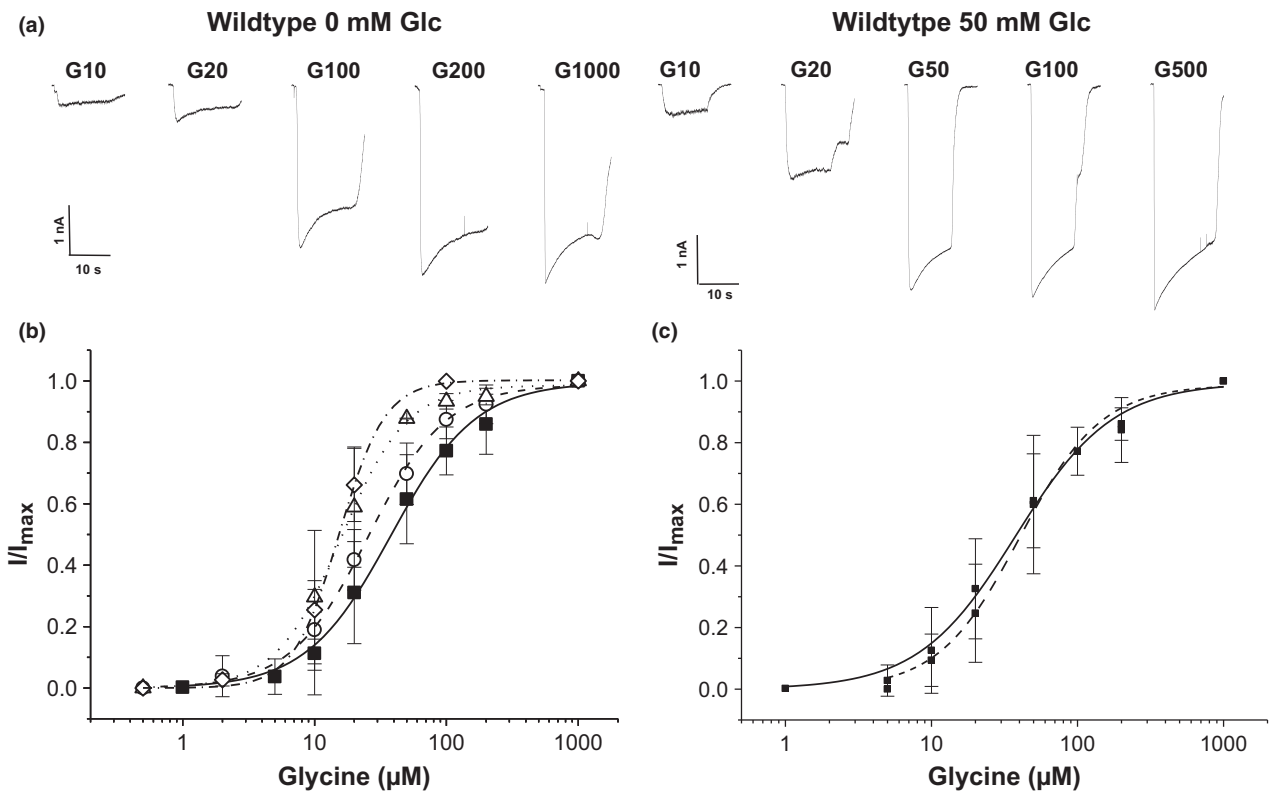

Fig. 1 Patch-clamp characterization of human recombinant α1 glycine receptors (GlyRs) in the absence and presence of glucose. Human α1 glycine receptors were expressed in HEK293 cells as described. (a) Left panel: control current traces of GlyR α1 wildtype (no glucose). Right panel: whole-cell currents of the same receptor recorded in the presence of 50 mM external glucose. (b) Dose–response curves of GlyR wildtype in varying concentrations of glucose: Solid squares, solid line: 0 mM glucose; open circles, dashed lines: 20 mM glucose; open triangle, dotted line: 50 mM glucose; open diamond, dash-dotted line: 100 mM glucose. See Table 2 for EC_{50} and nH values. (c) Dose–response curves of GlyR α1 wildtype in absence and presence of 10 mM glucose in the intracellular buffer. Solid squares, solid line: no internal glucose, EC_{50} = 39 ± 15 μM. Open circles, dashed lines: 10 mM internal glucose, EC_{50} = 40 ± 25 μM.

Table 2 Dose–response characteristics of recombinant glycine receptors: Exp (i): in absence and presence of external glucose; Exp (ii): pre-incubation with glucose or fructose for 18 h, followed by recording in absence of sugars

Exp	Agonist	Sugar application		EC_{50} (μM)	nH	*n*
(i)	Gly	0 mM Glc	hsα1	39 ± 15	1.8 ± 0.7	29
		20 mM Glc	hsα1	27 ± 5	1.5 ± 0.4	10
		50 mM Glc	hsα1	15 ± 3	2,1 ± 0.6	8
		100 mM Glc	hsα1	16 ± 2	2.8 ± 0.8	5
(i)	Gly	0 mM Glc	hsα1–hsβ	48 ± 14	1.8 ± 0.6	6
		50 mM Glc	hsα1–hsβ	11 ± 1	1.4 ± 0.4	4
(i)	Ivm	0 mM Glc	hsα1	1.1 ± 0.3	2.3 ± 0.4	14
		20 mM Glc	hsα1	1.3 ± 0.4	1.8 ± 0.4	6
(ii)	Gly	5.5 mM Glc (ctrl)[a]	hsα1	39 ± 15	1.8 ± 0.7	29
		10 mM Glc PreInc	hsα1	17 ± 4	1.7 ± 0.5	6
		20 mM Glc PreInc	hsα1	11 ± 3	2.0 ± 0.4	6
		50 mM Glc PreInc	hsα1	13 ± 3	2.3 ± 0.4	6
		50 mM Frc PreInc	hsα1	8.6 ± 1	2.3 ± 0.6	5
(ii)	Gly	5.5 mM Glc (ctrl)[a]	hsα1–hsβ	48 ± 14	1.6 ± 0.2	6
		10 mM Glc PreInc	hsα1–hsβ	11 ± 2	2.0 ± 0.5	4
(ii)	Tau	5.5 mM Glc (ctrl)[a]	hsα1	143 ± 23	1.5 ± 0.2	4
		50 mM Glc PreInc	hsα1	119 ± 11	1.9 ± 0.1	5
(ii)	β-Ala	5.5 mM Glc (ctrl)[a]	hsα1	65 ± 9	1.7 ± 0.1	8
		50 mM Glc PreInc	hsα1	74 ± 9	1.5 ± 0.3	7
(ii)	Ivm	5.5 mM Glc (ctrl)[a]	hsα1	1.1 ± 0.3	2.3 ± 0.4	14
		50 mM Glc PreInc	hsα1	1.2 ± 0.2	2.2 ± 0.5	4

Given Errors are standard deviations derived from average values of all cells.
nH = Hill coefficient, *n* = number of cells, PreInc = pre-incubation.
Glc: glucose; Frc: fructose; Gly: glycine; Tau: taurine; β-Ala:β-Alanine; Ivm: ivermectin.
hsa1-hsb: hsa1 and hsb were cotransfected in the ratio 1/3.
[a]Cells were cultured in standard MEM (1 g/L glucose = 5.5 mM).

between EC_{50} and I_{max} was found for homomeric α1 GlyR (Fig. 2d). To test whether glucose had an effect on the desensitization behaviour of recombinant GlyRs, we determined time course and percentage of the desensitizing current fraction at saturating concentrations of glycine. Desensitization differed markedly between cells. However, glucose (0–100 mM) in the bath solution did not produce any significant changes in time constant or fraction of desensitizing currents (Fig. 2e–f), whereas EC_{50} did show significant left-shift under these conditions (Fig. 2b).

Modulation of GlyR responses by sugars was further studied on a time scale of many hours (Fig. 3). HEK293 cells were transfected with GlyR α1 wildtype, and after 1 day of expression, 10 to 50 mM glucose (end concentration) or 50 mM fructose were added to the culture medium for 18 – 20 h. For experiments, the sugar-containing medium was removed, cells were rinsed with sugar-free extracellular buffer and whole-cell recordings were performed in the absence of sugar. Pre-treatment with either monosaccharide gave responses to glycine with very low EC_{50} values of 13 ± 3 μM (n = 6) and 8.6 ± 1 μM (n = 5), for 50 mM glucose (Fig. 3a) and 50 mM fructose (Fig. 3b) respectively. Compared to wildtype control, pre-treatment with sugars reduced EC_{50} by a factor of 3 (glucose), and 4.8 (fructose). Pre-treatment with 10 and 20 mM glucose was also effective, giving EC_{50} values of 17 ± 4 μM (n = 6) and 11 ± 3 μM (n = 6) (Fig. 3a and b, Table 2). Most notably, concentrations as low as 10 mM glucose in the culture medium already lowered the EC_{50} value significantly (to ~ 80% of the maximal reduction), indicating that the EC_{50} for enhancement by glucose is between 5.5 mM (MEM medium, control) and 10 mM (MEM + 4.5 mM glucose), i.e. at physiologically relevant concentrations. The maximum reduction in EC_{50} was already reached at 20 mM glucose (Fig. 3c).

Notably, when glucose was only present in the recording buffer, i.e. for a maximum of 2–3 h, desensitization was not affected (Fig. 2e–f). In contrast we noticed significant changes in time constant and percentage of the desensitizing current fraction after long (18–20 h) pre-incubation (Fig. 3d). The fraction of desensitizing current decreased from 47 to ~ 10 %, and the time constant for desensitization increased from 3 to 11 s. Thus, glucose treatment did not produce fast desensitization that would introduce major

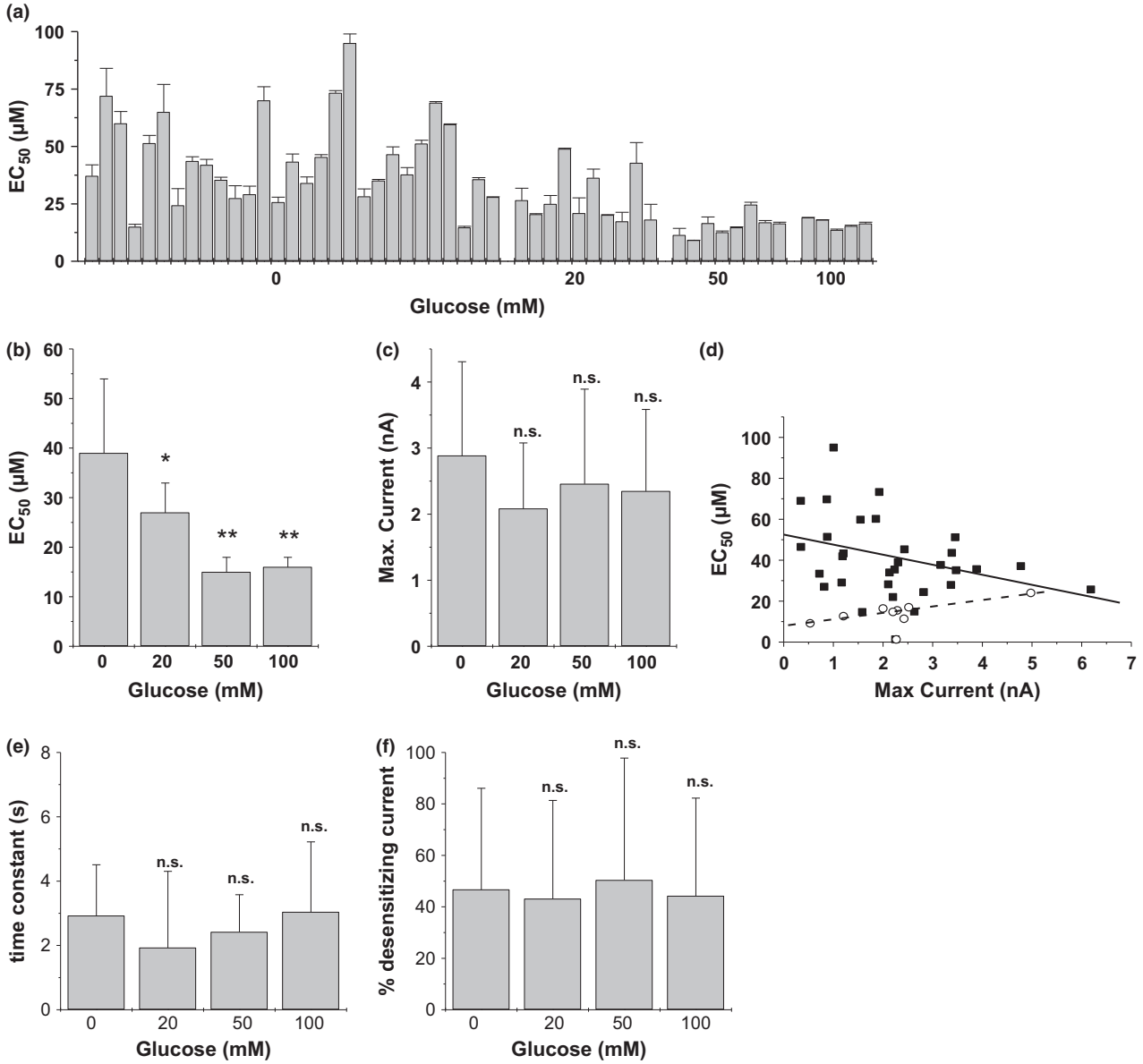

Fig. 2 Dose–response characteristics of recombinant α1 glycine receptor (GlyR) in absence and presence of glucose. Human α1 glycine receptors were expressed in HEK293 cells as described. (a) Individual EC_{50} values of GlyR α1. Error bars are derived from non-linear fitting of individual dose–response curves. (b) Average EC_{50} values of GlyR α1. Error bars indicate SD of the single EC_{50} values. (c) Average maximum currents of GlyR in external buffers of varying glucose concentration. Error bars indicate SD of average maximum currents derived from all cells for each glucose concentration. (d) Correlation plots of EC_{50} values versus maximum current for GlyR wildtype in 0 mM (solid squares, solid line, $R^2 = 0.11$) and 50 mM (open circles, dashed line, $R^2 = 0.37$) glucose. (e) Average time constants of cells in varying glucose concentrations, error bars indicate SD. (f) Average percentage of desensitizing current in varying glucose concentrations, error bars indicate SD. Statistical significance is indicated as follows: $^*p < 0.05$; $^{**}p < 0.01$; n.s.: not significant ($p > 0.05$).

errors in the measurement of I_{max} and EC_{50}. On the contrary, it appears that extended exposure to glucose favoured slow- or non-desensitizing current responses.

To assess whether modulation by glucose also occurs on heteromeric α1/β receptors, i.e. the subunit combination prevalent in the human spinal cord, HEK293 cells were transfected with a 1 : 3 ratio of α1 and β cDNA and then tested for glycine-mediated responses and their modulation by glucose (Fig. 4). When α1/β receptors were modulated by glucose, current potentiation was even more pronounced. Both, presence of glucose in the extracellular recording buffer, and pre-incubation with glucose resulted in a left-shift of the dose–response curve (Fig. 4a). Reduction in EC_{50} was statistically significant (Fig. 4b, Table 2, $p < 0.01$). EC_{50}

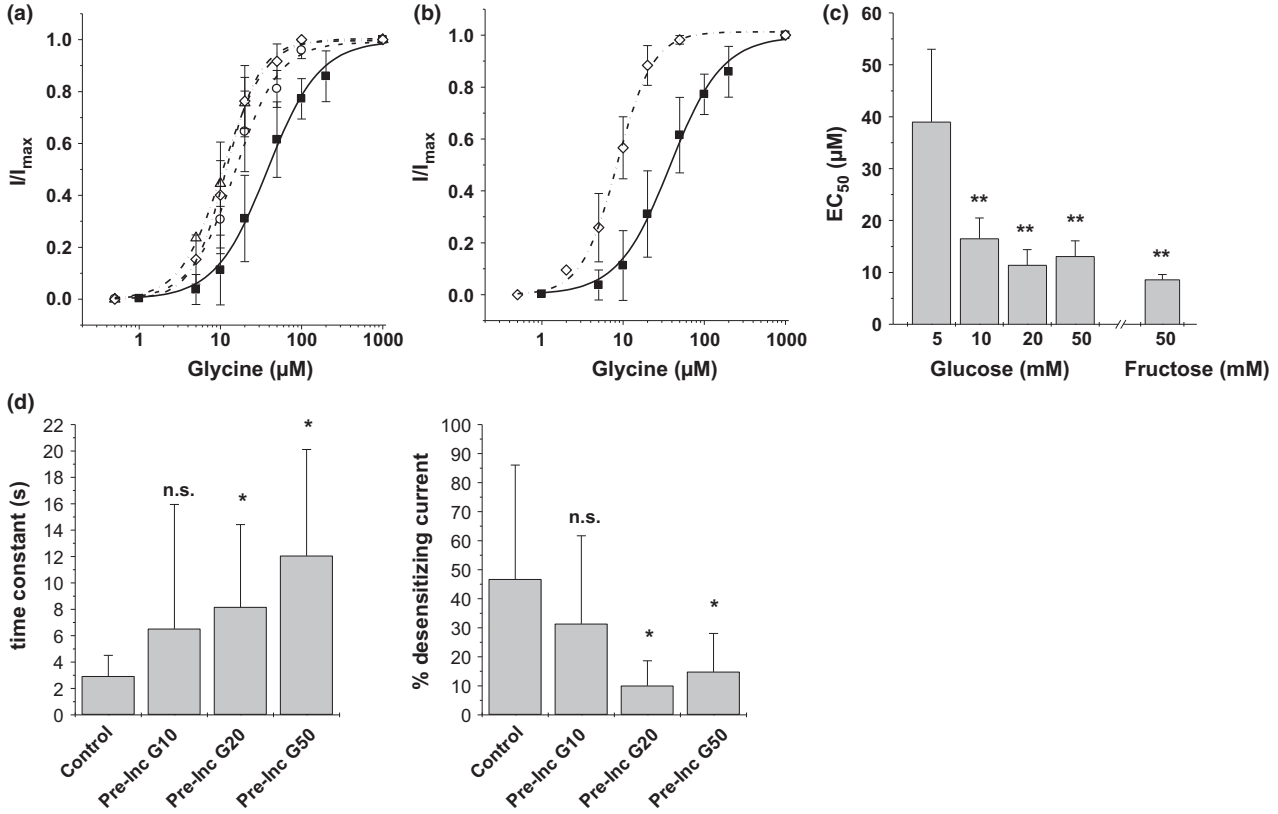

Fig. 3 Modulation of glycine receptor (GlyR) responses by pre-treatment with glucose and fructose. After recombinant expression, receptors were pre-treated with the indicated sugars for 18–20 h, followed by recording in sugar-free external buffer. (a) Glucose pre-treatment: Solid line, solid squares: minimum essential medium (MEM) control (1 g/l = 5.5 mM glucose); dashed line, open circles: 10 mM glucose; dash-dotted line, open triangle: 20 mM glucose; dash-dot-dotted line, open diamond: 50 mM glucose. (b) Fructose pre-treatment: Solid line, solid squares: MEM control (1 g/l = 5.5 mM glucose); dash-dot-dotted line, open diamond: 50 mM fructose. (c) Average EC_{50} values after pre-treatment with 5–50 mM glucose and 50 mM fructose. Errors are SDs of average EC_{50} values derived from single cells. See Table 2 for details. (d) Change in desensitization behaviour after long-term sugar treatment. Time constants as well as percentage of desensitizing current are plotted against glucose concentration in pre-incubation studies. Statistical significance was determined against the control of 5.5 mM glucose present in the cell culture medium. Error significance: $^{*}p < 0.05$; $^{**}p < 0.01$.

values changed from 48 ± 14 μM (0 mM glucose) to 11 ± 1 μM (50 mM glucose) in the extracellular bath or to 11 ± 2 μM after addition of additional glucose in the cell culture medium (10 mM glucose end concentration compared to 5.5 mM glucose for the control). Thus, the maximum reduction in EC_{50} by pre-incubation in 10 mM glucose was 2.5-fold and 4.5-fold for α1 and α1/β receptors respectively (Fig. 4b, Table 2). Glucose was thus able to modulate the synaptic type of GlyRs.

As glucose is a central metabolite and fuel for cellular processes, enhancement of GlyR-mediated currents may be because of general metabolic changes of the HEK293 recombinant host cells. To test this, we performed experiments using ivermectin, an alternative 'unconventional' agonist of the GlyR (Shan *et al.* 2001). Ivermectin-elicited GlyR currents are not sensitive to strychnine, and activation occurs from a binding site and by a mechanism that is different from glycine activation (Shan *et al.* 2001). The effect of glucose on ivermectin-mediated currents was investigated using the same experimental design as for glycine activation, comparing EC_{50} values for ivermectin in the absence (control) and presence of additional glucose (Fig. 5). Notably, glucose had no effect on ivermectin-gated GlyR currents. With glucose added to the recording buffer only, EC_{50} values for ivermectin were 1.1 ± 0.3 μM (0 mM glucose control) and 1.3 ± 0.4 μM (20 mM), i.e. not significantly different (Fig. 5b and c). EC_{50} values of glycine-mediated currents were significantly reduced under these conditions (EC_{50} = 39 ± 15 μM (control) and 27 ± 5 μM (20 mM glucose), see Fig. 2b). In pre-incubation experiments (50 mM glucose, 18–20 h, Fig. 5b and c), EC_{50} values for ivermectin activation were 1.1 ± 0.3 μM (no glucose) and 1.2 ± 0.2 μM (50 mM glucose). Glycine-mediated currents (Fig. 5a and c) under the same conditions showed a shift of EC_{50} values from 39 ± 15 μM (control) to 13 ± 3 μM (50 mM glucose). Thus, glucose augmentation

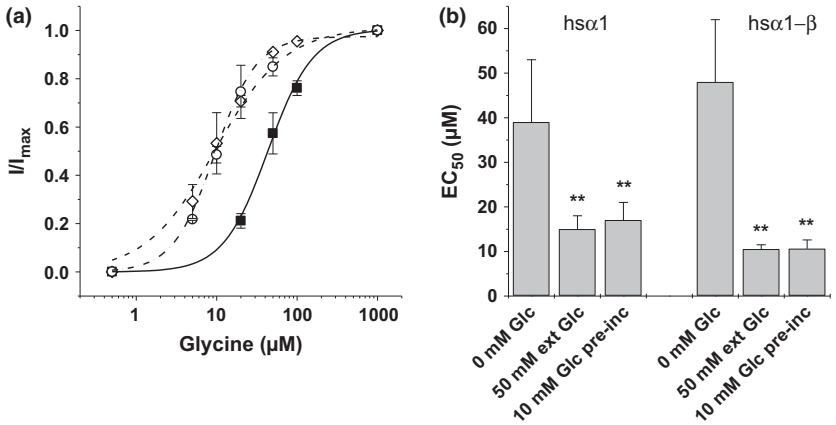

Fig. 4 Modulation of recombinant α1-β receptors by glucose. HEK293 cells were co-transfected with α1 and β cDNA at a ratio of 1 : 3. (a) Changes in EC_{50} after the following glucose additions: Solid squares, solid line: control, no glucose was added; open circles, dashed line: 50 mM glucose was added to the external bathing solution; open diamonds, dash-dotted line: transfected HEK293 cells were pre-incubated with 10 mM glucose for 18 h, electrophysiological measurements were performed in absence of glucose. (b) Summary of EC_{50} shifts of α1 and α1/β receptors by glucose. See Table 2 for details. All changes were statistically significant, $^{**}p < 0.01$. Average EC_{50} values ± SD of all single EC_{50} values are given.

was only observed for glycine-mediated currents, but not for receptor activation by ivermectin.

When analysing the partial agonists taurine and β-alanine in pre-incubation experiments, minor differences in half maximum concentrations were detected (Fig. 5 d–f). A slight decrease in EC_{50} values was measured for taurine, whereas a small increase was noticed for β-alanine when glucose was added to the culture medium between transfection and measurements. Both changes were not statistically significant. For taurine, EC_{50} values decreased from 143 ± 23 to 119 ± 11 μM in the presence of sugar. β-Alanine, on the other hand, showed a lower EC_{50} value in the absence of sugar with 65 ± 9 μM in low glucose MEM compared to 74 ± 9 μM in culture medium with additional glucose (50 mM glucose final concentration).

Discussion

In this study, glucose and fructose were identified as positive modulators of the inhibitory GlyR. Glucose in the extracellular medium produced a robust and statistically significant left-shift of the dose–response curve for recombinant homomeric α1, as well as heteromeric α1/β receptors, i.e. the subunit combination prevalent in spinal cord. Similar allosteric modulation of GlyRs by glutamate (Liu *et al.* 2010a) and cannabinoids (Ahrens *et al.* 2009; Xiong *et al.* 2011) on the GlyR was recently reported, as well as the modulation of retinal GABA receptors by ascorbic acid (Calero *et al.* 2011). To our knowledge, this is the first report of the endogenous metabolite glucose as direct modulator of neuronal GlyRs.

Glucose is an essential metabolite whose presence at elevated concentrations could modify cellular functions, including whole-cell current responses. Here, we observed significant left-shifts of EC_{50} curves in the presence of glucose, while maximum currents were not affected. Currents elicited by 20 μM glycine (~EC_{10}) were directly increased upon addition of 50 mM glucose (data not shown). Such an immediate augmentation of currents would be consistent with a direct action of glucose on the GlyR. No correlation between EC_{50} and I_{max} was observed for homomeric α1 GlyR (Fig. 2d). Desensitization behaviour was heterogeneous throughout all glucose concentrations (0–100 mM glucose in external bath solution), comprising fast as well as slowly desensitizing current fractions. No significant changes in desensitization could be noticed when glucose was applied in the bath solution, i.e. on a time scale of 1–3 h (Fig. 2 e–f). After longer exposure to sugar, there was a notable and statistically significant trend towards slower desensitization and a larger fraction of non-desensitizing current, in agreement with a general increase in glycinergic transmission in the presence of glucose (Fig. 3d). Desensitization can have a major effect on the determination of dose–response relationships, as rapid desensitization may obscure the true maximum current, especially on setups with slow agonist delivery. Our system provides a time course of equilibration over suspended HEK 293 cells within < 100 ms. Desensitization rate constants were on the order of 2–3 s and not affected by glucose concentrations up to 100 mM (Fig. 2e), and current traces did not indicate additional fast desensitization processes. Thus, although it cannot be ruled out from our experiments, it was considered unlikely that desensitization had a major effect on changes in EC_{50} during our time of observation (< 24 h of glucose exposure).

It is noted that both culture medium and recording buffers in patch-clamp experiments may differ in their glucose

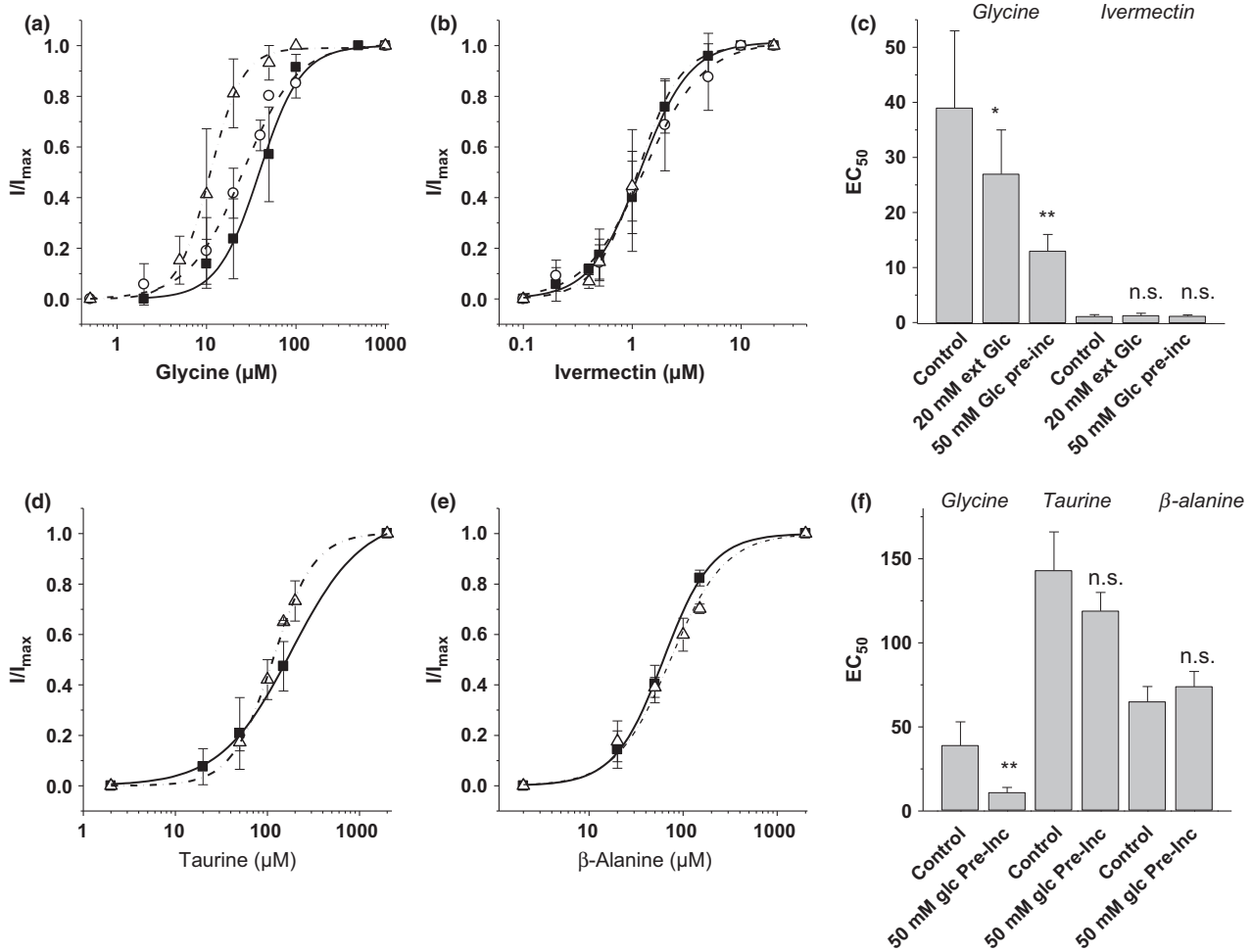

Fig. 5 Recombinant glycine receptor (GlyR) activation by glycine, ivermectin, taurine and β-alanine in presence and absence of glucose. Homomeric a1 glycine recpetors were expressed in HEK293 cells. Solid squares, solid line: control, no glucose. Open circles, dashed line: 20 mM glucose in external bath and during application. Open triangles, dash-dotted line: HEK293 cells were pre-treated with 50 mM glucose 1 day after transfection for 18–20 h, followed by recording in sugar-free buffer. Dose–response curves of glycine receptor agonists (a) glycine, (b) ivermectin, (d) taurine, and (e) β-alanine are shown. (c) Average EC_{50} values of glycine and ivermectin in absence and presence of glucose. (f) Average EC_{50} values for taurine and β-alanine in absence and presence of glucose. Statistical significance was determined against the control. $^{*}p < 0.05$; $^{**}p < 0.01$. Errors are SD of average EC_{50} values of cells used in one experimental condition, see Table 2 for constants and n's.

content. Glucose in the extracellular medium is well tolerated, as are differences in osmolarity of ~ 5–10% between extracellular and intracellular buffer (van Hooft and Vijverberg 1996; Breitinger *et al.* 2000, 2001). At the most relevant concentrations (10 and 20 mM of sugars), osmolarity of the extracellular buffer (300 mosmol) was only changed by 3.3% (+ 10 mM glucose), or 6.7% (+ 20 mM glucose). Even at 100 mM glucose, cells were viable during electrophysiological recordings. Increased viscosity of the extracellular medium because of added sugar was notable only at 50–100 mM concentrations, but even then current rise times and deactivation were not different from those in glucose-free solution, indicating that perfusion of the cells and diffusion of ligands were not greatly affected by changes in viscosity. Likewise, glucose-related changes in desensitization were moderate and did not lead to distortion or loss of signal. As glucose generally improves stability of recordings and longevity of cells, it is added to the recording buffer in patch-clamp experiments in numerous laboratories. Our observations show that current responses of GlyR channels are sensitive to glucose. Modulation of current responses by glucose in the recording buffer may indeed be an important experimental parameter and could help to reconcile some of the variation found in reported EC_{50} values (Table 1). Generally, cell culture in glucose-free medium is difficult if not impossible, as glucose is an essential nutrient. Therefore,

routinely 1 g/l (~ 5.5 mM) of glucose in the medium is used, a concentration similar to the resting value of glucose in human blood serum (Austin *et al.* 1987). While zero sugar concentration was not available, we could examine the concentration range between 5.5 and 50 mM of glucose in pre-incubation studies, where changes in receptor responses were indeed significant.

Left-shifts of dose–response curves upon addition of glucose and fructose were statistically significant at concentrations around 10 mM of sugar, i.e. in the physiological range. The resting plasma glucose level in healthy individuals is around 90–100 mg/dl (0.9–1.0 g/l; 4.5–5.5 mM). In diabetic patients, glucose concentrations exceed 1.3 g/l (~7 mM) reaching up to > 4 g/l (> 22 mM). Over this concentration range, glycation of plasma proteins (i.e. HbA1) was found to increase between 4- and 30- fold (Austin *et al.* 1987). Thus, glycation of plasma proteins under physiological conditions has a very steep dependence on glucose concentration in the range between 5 mM (baseline) and 20 mM (significant glycation). Our data demonstrate that GlyRs show sensitivity to modulation by sugars in precisely this concentration range, with an apparent EC_{50} for receptor potentiation by glucose of ~ 11 mM (Fig. 3a).

We observed a rapid (i.e. immediate) effect of glucose on GlyR currents that may be mediated through non-covalent interactions. However, the magnitude of glucose effects increased with prolonged exposure at a rate that paralleled that of non-enzymatic glycation of proteins. A study of non-enzymatic glycation of haemoglobin (Higgins and Bunn, 1981) suggests a reaction scheme where glucose (G) and haemoglobin (H) form an aldimine intermediate (H = G) which slowly converts to the stable ketoamine (HG).

$$H + G \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} H = G \xrightarrow{k_2} HG$$

Here, $k_1 = 0.3 \times 10^{-3}$/mM/h, $k_{-1} = 0.33$/h, $k_2 = 0.0055$/h (scheme and time constants from Higgins and Bunn, 1981). Fructose and glucose are most efficient glycating agents, with fructose reported to glycate even faster than glucose. Protein glycation is a slow process, with the 'blood glucose memory' (i.e. glycation of haemoglobin) persisting for weeks or even months. On an even longer time scale, advanced glycation end products are formed, with numerous physiological consequences, including protein crosslinking. While on our time scale of observation (< 24 h), formation of advanced glycation end products is not expected, prolonged elevation of blood glucose may favour receptor crosslinking and concomitant increases in receptor density, clustering and associated changes in response behaviour, such as reduced EC_{50} and rapid desensitization (Legendre *et al.* 2002).

As glucose is an essential nutrient, changes in its concentration may be expected to affect cellular metabolism and, possibly, current responses in patch-clamp experiments. To address this question, we investigated the influence of glucose on other known agonists of the GlyR, namely ivermectin, taurine and β-alanine. In contrast to glycine activation, EC_{50} values of ivermectin were not different in the absence or presence of glucose (Fig. 5) in concentrations that had a marked effect on glycine-mediated responses (Figs 2, 3, 5). This clearly indicates that enhancement of GlyR-mediated responses by glucose was not because of general changes in metabolic status, as any metabolic effect would cause ivermectin-mediated currents to be enhanced in the same way. Considering the direct augmentation of subsaturating currents at ~EC_{10} by glucose, we can rule out secondary intracellular processes. Thus, glucose augments current responses of the GlyR by a direct action on the receptor protein. Furthermore, there is evidence that only the glycine-mediated activation of the receptor is susceptible to glucose modulation, and not the alternative activation by ivermectin (Shan *et al.* 2001; Lynagh and Lynch 2012).

When the partial agonists taurine and β-alanine were studied, we did not observe a significant effect of glucose. In the presence of glucose, we found a slight decrease in EC_{50} for taurine, whereas half maximum concentrations of β-alanine in the presence of sugar were slightly higher than those for the control. Both changes were statistically not significant, indicating, that glucose had no or only minor influence on the action of partial agonists. Activation of the GlyR by full (glycine) and partial (taurine, β-alanine) agonists has recently been discussed in light of a mechanism that involves an additional pre-open state, termed the Flip state (Burzomato *et al.* 2004; Lape *et al.* 2008; Sivilotti 2010). Using this model, it was proposed that pre-activation is favourable for full agonists (glycine) and disfavoured for partial agonists (β-alanine, taurine), whereas gating (the open-close transition) is similar for both types of agonists (Lape *et al.* 2008; Sivilotti 2010). A shift of EC_{50} by a factor of ~5 – similar to the magnitude of sugar modulation we observed – was reported for the murine mutation A52S (Plested *et al.* 2007), where changes in agonist affinity of the resting and flipped state were determined to underlie changes of EC_{50} in mutant receptors. A change in EC_{50} by a factor of 5, although physiologically relevant, would only require moderate changes in kinetic constants. In case of partial agonists, it has been suggested that these agonists induce conformational changes in the receptor protein that are different from those caused by a full agonist (Pless and Lynch 2009). While effects of glucose on the pre-activation (flipping) equilibrium could not be excluded from our data, they appear unlikely. If glucose were indeed modulating the flipping equilibrium, one would expect currents elicited by partial agonists to be augmented more than those evoked by the full agonist glycine. This was not observed in our experiments. An alternative explanation might be that glucose affects a fast desensitizing current phase that would

not be resolved in our flow apparatus. Removal of fast desensitization could then account for the observed shifts in EC_{50}. In case of low-affinity (partial) agonists, which do not evoke rapid desensitization, glucose would be of no notable effect, and activation by ivermectin would also be independent of glucose. If such a fast desensitizing current were decreased by glucose, an increase in EC_{50} might also be observed, in contrast to our findings. Thus, our observations could be rationalized under the simple assumption that glucose favours the binding of glycine more than binding of the partial agonists. This would also be consistent with the absence of any effect of glucose on receptor activation by ivermectin.

Although more data are required for a full assignment of an activation mechanism, our observations nevertheless indicate that glucose is an allosteric modulator of the inhibitory GlyR, specifically enhancing currents mediated by the endogenous agonist glycine. Ivermectin, which acts through a different pathway of activation than glycine, is not affected by presence or absence of glucose.

Spinal cord injury often leads to loss of motor or sensory functions and may result in complete or partial paralysis. To date, successful regeneration of cortico-spinal tract axons beyond a lesion has not been achieved, although regeneration of individual axons was observed (Liu *et al.* 2010b). In another approach, spinal networks were activated independent of CNS input (Courtine *et al.* 2009). Indeed, limb movement can be achieved without spinal connection to the brain, by only activating the appropriate electrical circuitry. Thus, training of the injured spinal cord has become a successful therapeutic approach. In rats, both the GABA-ergic and glycinergic inhibitory systems are up-regulated following complete spinal cord transection, and step training results in down-regulation of these transmitter systems to control levels (Talmadge *et al.* 1996; Edgerton *et al.* 2001). In cats with experimental spinal cord lesion, GlyR inhibition with sub-lethal doses of strychnine restored full weight-bearing stepping in cats that had only been trained to stand. The same dose of strychnine given to the step-trained cats has only little effect on their stepping ability (de Leon *et al.* 1999; Edgerton *et al.* 2001). Apparently, spinal cord injury results in an up-regulation of the inhibitory systems in the spinal cord, which appears to hinder the healing process. As reduced GlyR activity seems to be desirable for healing, elevated glucose levels may have the opposite effect, augmenting GlyR-mediated currents. Thus, it may be advantageous to consider glucose levels in neuronal injury, especially in diabetic patients.

Another clinical application of glucose is in the formulation of hyperbaric solutions for slow infusion of local anaesthetics into the human cerebrospinal fluid during surgery. Human cerebrospinal fluid (CSF) contains 2.8–4.4 mM glucose, whereas common hyperbaric injection fluid contains up to 400 mM glucose. Infusion of 3 mL hyperbaric solution into 150 mL cerebrospinal fluid would give a final glucose concentration of 8 mM. However, diffusion in CSF and spinal cord is limited during application of hyperbaric solutions (Flack and Bernards 2010). Hence, glucose is expected to be distributed non-uniformly and local concentration may be considerably higher. Indeed, the concentration range of glucose expected in CSF after application of hyperbaric fluid would be sufficient to induce the high-activity state of spinal GlyRs.

These results show that endogenous metabolites may play important roles in fine-tuning of neuronal signalling. Here, glucose was identified as a positive modulator of the inhibitory GlyR. Glucose concentration should thus be considered as a relevant experimental parameter in ion channel studies. In clinical situations, glucose levels in anaesthesia and treatment of spinal cord injury should be monitored to avoid over-activation of the glycinergic system. Indeed, a high-activity form (P185L; $EC_{50} \sim 5$ μM) of central α3 GlyRs, produced by RNA-editing, was suggested to cause tonic glycinergic inhibition (Meier *et al.* 2005). As elevated glucose produced a similar shift in EC_{50}, GlyR overactivation by long-term elevation of resting blood glucose may be a relevant factor in the pathology and therapy of diabetes and related disorders. Glycation of the receptor protein could be a likely biochemical mechanism for this enhancement.

Thus, our data suggest a direct link between physiologically relevant blood glucose levels and the activity of the inhibitory GlyR, a neuronal ion channel. Given the high structural and functional homology between cys-loop receptors, including acetylcholine, $GABA_A$, and serotonin $5\text{–}HT_3$ receptors, allosteric modulation by glucose may be a general mechanism contributing to the fine-tuning of neuronal ion channel receptors.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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