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Positive Modulation of the Glycine Receptor by Means of Glycine Receptor–Binding Aptamers

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Abstract

According to the gate control theory of pain, the glycine receptors (GlyRs) are putative targets for development of therapeutic analgesics. A possible approach for novel analgesics is to develop a positive modulator of the glycine-activated Cl^- channels. Unfortunately, there has been limited success in developing drug-like small molecules to study the impact of agonists or positive modulators on GlyRs. Eight RNA aptamers with low nanomolar affinity to GlyRα1 were generated, and their pharmacological properties analyzed. Cytochemistry using fluorescein-labeled aptamers demonstrated GlyRα1-dependent binding to the plasma membrane but also intracellular binding. Using a fluorescent membrane potential assay, we could identify five aptamers to be positive modulators. The positive modulation of one of the aptamers was confirmed by patch-clamp electrophysiology on L(tk) cells expressing GlyRα1 and/or GlyRα1β. This aptamer potentiated whole-cell Cl^- currents in the presence of low concentrations of glycine. To our knowledge, this is the first demonstration ever of RNA aptamers acting as positive modulators for an ion channel. We believe that these aptamers are unique and valuable tools for further studies of GlyR biology and possibly also as tools for assay development in identifying small-molecule agonists and positive modulators.

Keywords

glycine receptor, aptamer, surface plasmon resonance, fluorescent membrane potential, patch-clamp electrophysiology

Introduction

The glycine receptor (GlyR) belongs to the family of fast-acting cys-loop ligand-gated ion channels that mediate fast inhibitory neurotransmission in the mature central nervous system.[1] GlyRs mediate chloride (Cl^-) influx through the glycine-gated chloride channels and stabilize the resting membrane potential of neurons. In mature neurons, an influx of Cl^- leads to membrane hyperpolarization, which prevents depolarization and neuronal firing. The GlyR consists of five homologous membrane-spanning subunits, symmetrically assembled around a central pore. The native GlyR is a pentameric protein complex composed of α- and β-subunits. The α-subunit contains the ligand-binding domain, and the β-subunit plays important roles in anchoring at synapses and contributing to ligand binding.[2,3] In vitro, the GlyRα1 and α3 can also form α-homopentamers, which have properties similar to those of the native α1/3β-heteromer.[4] However, the α1/3-homopentamers usually cannot be functionally demonstrated in vivo.[5] The GlyRs are potential targets for the treatment of peripheral inflammatory pain.[6]

Both GlyRα1 and GlyRα3 are expressed in lamina II neurons and have the potential to reduce peripheral input

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from Aδ and C-fibers. Harvey et al.[7] have demonstrated that PGE_2 inhibits GlyRα3 and that GlyRα3$^{-/-}$ mice are less sensitive to pain caused by inflammation in the periphery. Therefore, positive modulation of glycine-activated Cl^- channels will potentiate the inward Cl^- flux, which could decrease pain signaling and be a possible approach for the development of analgesics.

RNA aptamers have been introduced as molecules that are prone to bind to functional domains within target proteins, thereby modulating their biological functions.[8] Aptamers are synthetic, typically 50–100-nucleotide-long single-stranded RNA or DNA oligonucleotides that fold into three-dimensional structures and bind to their respective targets by complementary shape interactions. The affinity and selectivity of aptamers are comparable to those of monoclonal antibodies. Aptamers are generated by a process called SELEX (systematic evolution of ligands by exponential enrichment), which is an in vitro selection process comprising iterative rounds of selection and amplification.[9,10] The process is automated that makes the selection of aptamers against almost any soluble target protein fast and therefore more economical.[11] By using the SELEX approach, aptamers have been generated successfully against several membrane proteins.[12–15]

The GlyRα1 subunit is a relatively large protein (48 kD) with an electrical charge of about pI 8.9, which makes it a good target for generation of negatively charged aptamer ligands. In this study, aptamers were generated to the human GlyRα1 subunit (hGlyRα1), either as a purified recombinant homopentamer or by panning using cells expressing the hGlyRα1 homopentamer.

Materials and Methods

Cell Lines

Murine L(tk) cells expressing the hGlyRα1 homomer and hGlyRα1β heteromer were obtained in house according to Wick et al.[16] The cells were grown in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 medium (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA), 100 U/ml penicillin, 100 g/l streptomycin, 0.3 mg/l G-418 geneticin, and 10 mM HEPES or 10% FBS, 0.3 mg/l G-418 geneticin, and 4 mg/l puromycine (Sigma-Aldrich, St. Louis, MO), respectively. Parental L(tk) cells were cultured in F12 media (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 100 g/l streptomycin.

Preparation of Recombinant hGlyRα1

Nucleotides coding for residues 29–449 of hGlyRα1 were added to the *Pichia pastoris* expression vector pPICZαC (Invitrogen). To cleave off unprocessed N-terminus, a TEV site (ENLYFQG) was substituted for the second residue of the mature hGlyR. A point mutation, K383A, was introduced to increase hydrophobicity and thereby protein stability during later purification processes. The expression vector was electroporated into the *P. pastoris* strain SMD1163His+ (Invitrogen), and stable integrants were selected using Zeocin resistance. for the preparation of hGlyRα1.

Fermentation

Fermentation of *P. pastoris* expressing hGlyRα1 was performed using buffered glycerol-complex medium basically as recommended by the supplier of the *Pichia* expression system (Invitrogen). Induction of expression was started at a cell density of 150–200 g/l by switching to a constant level of 0.1% methanol as the sole carbon source and lowering the temperature to 19 °C. After 24 h, the cells were frozen in liquid nitrogen and transferred to −80 °C until further processing.

Purification of hGlyRα1

All procedures were conducted at 4°C unless otherwise stated. Cell pellets were resuspended in 50 mM Tris, 200 mM KCl, 10 mM glycine, 5% glycerol (w/v), 5 mM DTT, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, pH 8.1, supplemented with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Cells were broken by passage once at 35 kpsi in a TS1.1 high-pressure homogenizer (Constant Systems, Daventry, UK) and centrifuged at 5000×g for 10 min. Membranes were subsequently precipitated by adding PEG8000 to the supernatant. After 15 min of stirring on ice, precipitated membranes were collected by centrifugation at 12,000×g for 20 min. Membranes were washed once in 30 mM KPi, 0.3 M KCL, 10 mM glycine, 20% glycerol (w/v), 1 mM PMSF, pH 7.6, then resuspended in the same buffer and transferred to −80 °C until further processing. To solubilize the membrane components, the membranes were diluted to 10 mg/ml membrane protein in 30 mM KPi, 0.8 M KCl, 10 mM glycine, 13% glycerol, 1 mM PMSF, Complete Protease Inhibitor Cocktail, 2 mM β-mercaptoethanol, 1.5% (w/v) dodecylmaltoside (DDM), pH 7.6. Solubilized proteins were separated from nonsolubilized membranes by centrifugation at ~100,000×g for 30 min.

As an initial step in our purification strategy, we used a batch-binding procedure in which the cleared extract from the solubilization was mixed with NiNTA Superflow resin (Qiagen, Venlo, the Netherlands) in the presence of 20 mM imidazole. The capture of his-tagged GlyRα1-protein was done by overnight incubation at 4 °C while stirring at a ratio of 350 mg membrane protein/ml resin. The resin was washed with wash buffer: 25 mM Kpi, 750 mM KCl, 0.1% DDM, 2 mM β-mercaptoethanol, 30 mM imidazole, 0.5 mM PMSF, 10% glycerol, pH 7.6. hGlyRα1 was specifically eluted with wash buffer containing 250 mM imidazole. Fractions containing GlyRα1 as determined by Western blot were pooled and further purified using the GlyRα1-specific affinity resin strychnine agarose (SA). SA was prepared essentially as described in Pfeiffer et al.[17] by coupling 2-aminostrychnine to Affigel 10 (BioRad, Hercules, CA). Approximately 1 ml SA resin/300 mg initial membrane protein was equilibrated with water followed by 25 mM KPi, 750 mM KCl, 0.1 % DDM, pH 7.5 before adding the Ni-NTA eluted hGlyR α1-pool. Nonspecifically bound protein, including misfolded hGlyRα1, was removed from the resin by washing with 25 mM KPi, 750 mM KCl, 2 mM EDTA, 4 mM DTT, 20% glycerol, 0.05% DDM, pH 7.5. GlyRα1 was eluted overnight by incubation in batch with the same buffer containing 200 mM glycine. Fractions containing pure GlyRα1, as determined by Coomassie staining, were pooled. Size exclusion chromatography was used for quality control and/or to remove glycine from the preparation, after which the pure GlyRα1 was applied to a Superdex 200 10/300 and separated using 25 mM KPi, 400 mM KCl, 10 mM glycine, 10% glycerol, 1 mM TCEP, 0.02% DDM, pH 7.4, at a flow rate of 0.4 ml/min. Size standards (HMW-kit; GE, Schenectady, NY) were ran and separated under identical conditions to estimate the size of detergent solubilized GlyRα1. Protein concentration in isolated cellular membranes and of the purified GlyRα1 protein was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA) using bovine serum albumin (BSA) as standard.

SELEX S118A and S119B (Automated SELEX against Detergent Solubilized Recombinant hGlyRα1 Pentamer)

SELEX was performed by running six or eight selection cycles on an automated SELEX workstation (Beckman Coulter, Brea, CA) at NascaCell Technologies AG. For S118A, the 2′-F-pyrimidin modified RNA library with a random region of 47 nt was incubated with biotinylated hGlyRα1-pentamer in selection buffer (PBS-Gly, pH 7.5: 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM NaCl, 200 mM KCl, 3 mM $MgCl_2$, 1 µg/µl BSA, 0,016% DDM) for 30 min at 37 °C, and in the S119A library, the biotinylated GlyRα1-pentamer was pre-immobilized to M280-SA Beads (Invitrogen-Dynal). Biotinylated target–aptamer complexes were captured with M280-Streptavidin Beads (Invitrogen-Dynal). Bead target–aptamer complexes were washed with washing buffer (PBS-Gly lacking the BSA: 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM NaCl, 200 mM KCl, 3 mM $MgCl_2$, 0,016% DDM, pH 7.5) to separate away unbound RNA sequences. The washed GlyRα1 bound aptamers were amplified by RT-PCR (RT-PCR-Kit; Qiagen) and subsequently transcribed into the next-round 2′F-pyrimidin modified RNA library.

SELEX S120A (Cellular SELEX against GlyRα1 Expressing L(tk) Cells Using Heat Elution and Counter Selection against L(tk) Parental Cells)

hGlyRα1-addressing aptamers were generated by running 15 SELEX cycles against GlyRα1 expressing L(tk) cells. In brief, a 2′-F-pyrimidine modified RNA library with a random region of 47 nt was incubated with 2×10^7 trypsinated L(tk) parental cells in 1 ml selection buffer (PBS pH 7.6, 3 mM $MgCl_2$, 8 µM tRNA, 0.5 µg/µl BSA) for 20 min at 37 °C to separate 2′-F-RNA irrelevant L(tk)-cell surface proteins. The supernatant with an unbound 2′F-RNA library was transferred to 2×10^7 GlyRα1 expressing L(tk) cells and incubated for an additional 30 min at 37 °C. Cells were washed with 5 ml washing buffer (PBS pH 7.6, 3 mM $MgCl_2$), resuspended in 400 µl H_2O with 0.2% Triton-X and 0.2 mM EDTA, and heated at 60 °C for 5 min to elute bound 2′F-RNA. The 2′F-RNA was phenol-chloroform extracted, precipitated, and pelleted. The pellet was dissolved in 200 µl H_2O and used as a template for reverse transcriptase PCR (RT-PCR) reactions (Qiagen). The PCR reactions were combined, and ~100 pmol of amplicon was transcribed into the next round of the 2′F-pyrimidin modified RNA library (ATP and GTP from Larova, Jena, Germany; 2′F-UTP and 2′F-CTP from Epicentre, Madison, WI; and T7-polymerase from Stratagene, La Jolla, CA). In selection cycles 2–15, the washing volume was stepwise increased: 2×5 ml (2nd round), 3×5 ml (3rd–4th rounds), 4×5 ml (5th–6th rounds), and 5×5 ml (7th–15th rounds). In selection cycles 3–15, the amount of starting library was increased to 1200 pmol (1.2 µM) (**Fig. 1C**).

SELEX S120B (Cellular SELEX against hGlyRα1 Expressing L(tk) Cells, Using Specific Elution with AZ1 and Counterselection against L(tk) Parental Cells)

hGlyRα1 aptamers were generated by 15 rounds of SELEX against GlyRα1 expressing L(tk) cells. Anti-GlyRα1 aptamers were selected by a combination of competitive elution with an anti-GlyR compound, AZ1 (AstraZeneca, London, UK; EC_{50}, 1.2 µM), and counter selection against L(tk) parental cells as described above with the difference that, after washing and pelleting of the GlyRα1 expressing L(tk) cells, the cell pellet was resuspended in 100 µl elution buffer (PBS pH7.6, 3 mM $MgCl_2$, 0.5 µg/µl BSA, 5% DMSO, 1 mM of AZ1) and incubated for an additional 20 min at 37 °C. Cells were centrifuged, and the supernatant bearing the competitive eluted aptamers was phenol-chloroform extracted, precipitated, and pelleted. The RNA pellet was dissolved in 200 µl H_2O and used as a template for RT-PCR

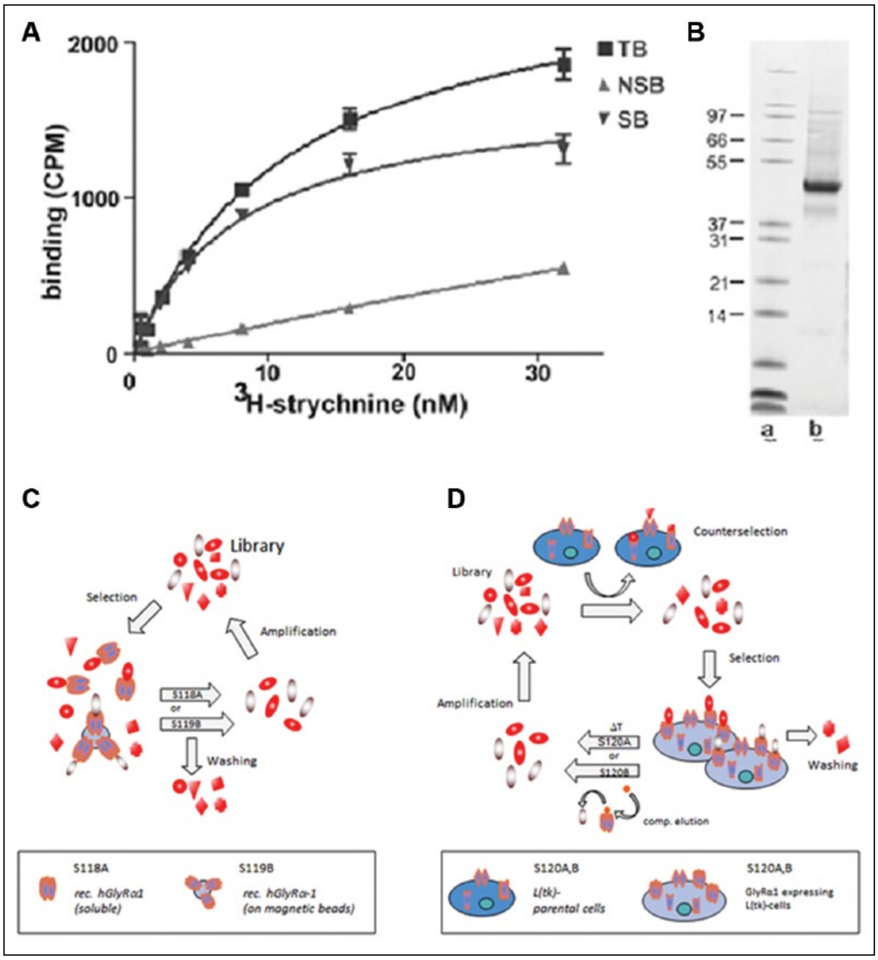

Figure 1. Expression and purification of human GlyRα1 followed by aptamer generation by systematic evolution of ligands by exponential enrichment (SELEX) technology. (**A**) Saturation binding curves from membrane filtration experiment showing specific GlyRα1-dependent 3H-strychnine binding (SB), total binding (TB), and nonspecific binding (NSB) in the presence of 10 mM glycine. (**B**) Coomassie-stained SDS-PAGE showing purity of GlyRα1 after elution from strychnine agarose. (**Lane A**) molecular weight marker; and (**lane B**) purified GlyRα1. Aptamers were generated by NascaCell Technologies AG (Munich, Germany) using two approaches to select and amplify aptamers with 2′-fluoro protective groups: (**C**) selection against detergent solubilized recombinant hGlyRα1-protein (**D**) panning against hGlyRα1 L(tk) cells, followed by counterselection using the parental L(tk) cells and elution with heat or an anti-GlyR compound, AZ1.

reactions (Qiagen), as described above, with the difference that in selection cycles 3–15, the BSA in the selection buffer was increased to 1 µg/µl (**Fig. 1D**).

Binding Analyses of Enriched Libraries and Monoclonal Aptamers

Binding analyses of enriched libraries and of monoclonal sequences derived from the different selection experiments S118A, S119B, S120A, and S120B were performed by radioactive filter retention[17] experiments with recombinant GlyRα1 from *Pichia* membranes. α^{33}P-GTP labeled RNA (~80 nM) was incubated with increasing concentrations of detergent solubilized GlyRα1 target protein (0–1000 nM) and with defined concentrations of control proteins. Incubation was performed in 25 µl PBS, 3 mM $MgCl_2$, 0.016% DDM, 1 mg/ml BSA, and 1 mg/ml heparin for 30 min at 37 °C. The incubation approach was subsequently filtered through a Protran nitrocellulose membrane in a dot blot manifold (Whatman, Little Chalfont, UK) and washed with 5×200 µl washing buffer (PBS, 3 mM $MgCl_2$, 0.016% DDM). The fraction of nucleic acid that was co-retained with the protein on the membrane was quantified with a Phosphoimager (Fuji FLA 5000; Fujifilm, Tokyo, Japan).

Cloning and Sequencing

On the basis of the results from the binding assays with enriched libraries from SELEX experiments S118A, S119B, S120A, and S120B, the following sequences were chosen:

S118A-C3 GGGAGAGGAGGGAGAUAGAUAUCA
ACUCAUGAACUGUCGAGCUAACCCACAGUA
UUAGCGAACCCCCAGCCAGUUUGUCCUCACG
GUGGAUGG
S118A-C13 GGGAGAGGAGGGAGAUAGAUAUCA
AUCGACACCGGGACACACGGCUCAGCACCCAU
AGCUGGGACCCUAUUCAGUUUGUCCUCACGG
UGGAUGG
S118A-C20 GGGAGAGGAGGGAGAUAG
AUAUCAAUAUUGAAACCUACUACAAAACUA
CCUAGAUAACCUUUACUUAGCGUCUAGUUUGU
CCUCACGGUGGAUGG
S119B-C7 GGGAGAGGAGGGAGAUAGAUAUCA

AGGGAUAAGCACCGUCAUGACUACAGGCCA
CAAGUACUUGCAUCACAGUUUGUCCUCACGG
UGGAUGG
S119B-C9 GGGAGAGGAGGGAGAUA
GAUAUCAACCGUGCUUGUGACCAGGUUAAAG
GCGCACGUACUAUUUAGUUCGGACAGUUUGUC
CUCACGGUGGAUGG
S120A-C15
GGGAGAGGAGGGAGAUAGAUAUCAAGCA
CGUCACGUCAGCACUUCAAAGGUAUCCUU
GACCGACAUUUUUACAGUUUGUCCUCACGGUGG
AUGG
S120B-C2 GGGAGAGGAGGGAGAUAGAUAUCA
ACACAACAUGUGAAUAGGAGGACUAACGA
UCACACACCAACAACUUUAGUUUGUCCUCAC
GGUGGAUGG
S120B-C4 GGGAGAGGAGGGAGAUAGAUAUCA
AGCAGAGAGAACUGAUCGCAGGCUAUUGGCU
CUACACCAGCGAUGUUAAGUUUGUCCUCACGG
UGGAUGG

Production of Aptamers

Aptamers were generated by in vitro transcription from DNA templates provided by Nascacell according to the manufacturer's instructions. For some experiments, 2′F-dUTP/dCTP was replaced with unmodified ribonucleotides to produce aptamers without protective groups. Fluorescent aptamers were generated by inclusion of Fluorescein/Bio ApG (IBA BioTagnology, Goettingen, Germany) in the in vitro transcription reaction. The final aptamer preparation was dissolved in RNAse-free H_2O.

Surface Plasmon Resonance

Purified hGlyRα1 subunits were immobilized on a research-grade sensor chip CM5 using amine coupling and inserted into the flow chamber of a Biacore3000 instrument (GE Healthcare, Little Chalfont, UK). Addition of the aptamers results in binding to the immobilized GlyR, producing a small change in the refractive index at the gold surface, which determines the affinity and kinetics.[19] The sensor chip was treated and activated according to the instructions from the manufacturer (GE Healthcare). HBS-EP buffer containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant p20 (GE Healthcare) was used as running buffer, and 3 M guanidine-HCl, pH 1, as regeneration buffer. For stabilization of the protein and to ensure the tertiary structure for the aptamers, an additional 0.5% N-octyl-B-D-glucoside (Anatrace, Maumee, OH) and 2 mM $MgCl_2$ were added to the running and regeneration buffer, respectively. To reduce unspecific binding of the aptamer to the protein, 0.01% Tween 20 was added to the buffer. Flow cells (FCs) 2 and 3 were immobilized with 4000 RU, and FCs 1 and 2 were used as reference surfaces. For kinetic analysis, the aptamers were diluted in running buffer to the following concentrations: 125 nM, 62.5 nM, 31.3 nM, 15.6 nM, and 7.8 nM; then they were injected at a flow rate of 20 µL per min over the FCs, at 25 °C. Between the injections, the surfaces were regenerated by two injections of regeneration buffer for 30 s, at a flow rate of 20 µL/min. To correct for refractive index changes, instrument noise, and bulk effects, the response data from FCs 2 and 4 were double referenced. First, the response data from FCs 1 and 3 were subtracted from the response obtained from FCs 2 and 4. Second, the response data for the vehicle of the analyte (HBS-EP) were subtracted from the response data from the analytes. All experiments was performed in duplicate and repeated twice on two separately coated surfaces. The evaluation was performed using BIAevaluation 3.2 (GE Healthcare). A two-state reaction (conformation change) model was used to analyze the association rate constant, k_a, and the dissociation rate constant, k_d. K_D was determined as the ratio of k_a and k_d.

Cytochemistry

hGlyRα1-expressing L(tk) cells or parental L(tk) cells were seeded in a 384-well plate (Greiner, Bio-One, Monroe, NC) and incubated overnight at 30 °C. The cells were washed in phosphate buffered saline (PBS) containing 1 µg/µL BSA and 2 mM $MgCl_2$ (buffer C). Fluorescein-tagged aptamers were diluted to a final concentration of 2.4 µM in buffer C. The cells were then incubated in 50 µL of the buffer solution for 30 min at 37 °C, followed by nuclear counterstaining with 12 µM Hoechst (Sigma-Aldrich). In some experiments, the red fluorescent Alexa594 wheat germ agglutinin (WGA, 5 g/liter in PBS; Molecular Probes, Eugene, OR) was used as a plasma membrane (PM) marker. For immunofluorescence, GlyRα1-expressing L(tk) cells and parental L(tk) cells were fixed in 2% paraformaldehyde for 20 min. The cells were permeabilized with PBS containing 0.05% Tween 20 for 30 min, and nonspecific binding was blocked using 5% goat serum together with 1% BSA (Sigma-Aldrich) for 30 min at room temperature (RT). The cells were then incubated with a rabbit polyclonal GlyRα1 antibody (0.20 g/l, dilution 1:10; Abcam, Cambridge, UK) overnight at 4 °C in blocking buffer. The unfixed cells were washed in PBS and blocked with 5% goat serum together with 1% BSA for 30 min at RT. The cells were then incubated with GlyRα1 antibodies for 1 h at RT. The viable and fixed cells were washed in PBS-0.05% Tween 20 or PBS, respectively, and subsequently incubated with a goat antirabbit secondary antibody, conjugated to Alexa594, together with 12 µM Hoechst, for 30 min. The cells were imaged using an ImageXpress 5000 automated microscope (Molecular Devices, Sunnyvale, CA). The images were processed and evaluated using MetaXpress imaging software 2.0.1.24 (Molecular Devices).

Fluorescent Membrane Potential Dye Assay

hGlyRα1-expressing L(tk) cells were seeded in poly-D-lysine-coated 384-well plates (17,000 cells/well). Following an 18 h incubation, the cells were washed with assay buffer containing 160 mM sodium D-gluconate, 4.5 mM potassium D-gluconate, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES at pH 7.4. The aptamers were diluted to 6 µM in assay buffer, in a separate plate. Aptamers were added to all wells at the same time using a robotic system (Biomek FX, Brea, CA). Cells were then immediately loaded with a red fluorescence imaging plate reader (FLIPR) membrane potential dye (R8123; Molecular Devices) and incubated for 30 min at 37 °C. The FLIPR assay started with a 30 s baseline, after which differences in fluorescence counts were measured during the application of 300 µM glycine (EC05) for a period of 5 min. All data analyses were performed using Graph Pad Prism 4 (San Diego, CA).

Patch-Clamp Electrophysiology

Ionic currents, from hGlyRα1 or hGlyRα1β expressing L(tk) cells, were recorded using a conventional whole-cell patch-clamp technique together with an EPC 10 amplifier (Heka Electronics, Lambrecht, Germany) and an inverted microscope (Nikon ECLIPSE TE2000-S; Nikon, Tokyo, Japan). All experiments were performed at RT (20–22 °C). Recording pipettes with resistances of 2–3 MΩ were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus Ltd., Edenbridge, UK) using a horizontal puller (model DMZ-Universal Puller; Zeiss Instrument, Jena, Germany). The membrane potential was held at −40 mV throughout the experiments. The extracellular bath solution was composed of 137 mM NaCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, and 10 mM HEPES (300–310 mOsm) at pH 7.4. The intracellular solution contained 140 mM KCl, 3 mM NaCl, 1 mM EGTA, and 10 mM HEPES (290 mOsm) at pH 7.2. To allow the exchange of the solutions, the cells were placed into a recording chamber that had a volume of 600 µL. The different extracellular solutions containing different concentrations of glycine or vehicle were applied using a DAD-12 superfusion system (ALA Scientific, Farmingdale, NY). In between control or glycine solution applications, the cell was continuously perfused with extracellular solution. In experiments using aptamers, cells were pre-incubated, with aptamers diluted in extracellular buffer to a final concentration of 4 µM, for 15 min at 37 °C. For quantification, currents induced by a given concentration of glycine were normalized to the maximal response (1 mM glycine, established in initial studies). All data analysis was performed using Graph Pad Prism 4 (San Diego, CA).

Results

Expression of hGlyRα1 in *Pichia pastoris*

Binding of the antagonist strychnine to purified hGlyRα1 suggests that a protein with similar protein folding to the native form of the protein was expressed in *P. pastoris* membranes (**Fig. 1A**). Using the mild detergent dodecylmaltoside, GlyRα1 was solubilized and purified using two consecutive affinity chromatography steps to >90% purity (**Fig. 1B**). The purified GlyRα1 is homogeneous in its N-terminal, as verified by protein sequencing showing that the α-mating factor is cleaved off in vivo during expression.

Aptamer Selection

Eight high-affinity, 2′-fluoro pyrimidine modified aptamers were generated for the hGlyRα1. Five aptamers were derived from automated SELEX against the purified GlyRα1 target protein (C3, C13, C20, C7, and C9) (**Fig. 1C**). Three aptamers were derived from two different types of cellular SELEX experiments against GlyRα1 expressing L(tk) cells (C2, C4, and C15) (**Fig. 1D**). The aptamers C2 and C4 were selected in a cellular SELEX experiment by using AZ1.

The Selected Aptamers Interact with the hGlyRα1 Using SPR

The K_D for two of the aptamers, C2 and C9, was determined to be 3±1 nM (C2) and 4±1 nM (C9), respectively, using a nitrocellulose retention filter assay. In the same assay, the estimated K_D for the other aptamers was also in the low-nanometer range. To further characterize the binding for aptamers to purified hGlyRα1, SPR was applied. SPR is an optical technique for detecting the interaction of two different molecules in which one is mobile and one is fixed on a thin gold film.[18] In the work described here, binding of the aptamers to the GlyRα1 produced a small change in the refractive index at the gold surface, which can be quantified with precision.[19] An overview of the K_a, k_d, and K_D for all aptamers is given in **Table 1**. As shown in **Figure 2**, representative sensograms for aptamers C2 (A) and C9 (B) reveal high-affinity ligand binding and a clear concentration-dependent binding to the immobilized GlyRα1 protein (K_D: 4.4 nM and 4.6 nM, respectively), similar to what was determined using the filter retention assay. Aptamers C2 and C9 have a relatively slow on-rate and slow off-rate (**Table 1**). Because the C2 aptamer was eluted with the positive modulator AZ1 compound during selection, a competition experiment was set up in which the binding of C2 to GlyRα1 was displaced using an equimolar concentration (250 nM) of AZ1. The compound did partially reduce C2 binding to the GlyRα1 chip, indicating that AZ1, at a higher

Table 1. Kinetic Properties of GlyRα1-Binding Aptamers Determined by SPR.

	Selection Strategy										
	Protein					Panning Heat		Panning Compound			
	C3	C7	C9	C9-Fluorescein	C13	C20	C15	C2	C2-OH	C2- Fluorescein	C4
k_a (10^5 1/ms)	3.4	6.0	1.2	2.5	1.4	0.40	8.9	1.3	5.9	3.3	20
k_d (10^{-4} 1/s)	0.80	9.2	5.7	360	5.0	2.4	5.6	5.5	450	620	350
K_D (nM)	24	1.5	4.6	150	3.6	6.7	0.6	4.4	87	190	18

k_a, association rate constant; k_d, dissociation rate constant; K_D, the ratio of k_a and k_d; SPR, surface plasmon resonance.

concentration, indeed would be able to elute the C2 aptamer (**Fig. 2G**).

To study the importance of the modified nucleic acids, with the protective 2′-fluoro-groups at the 5′-end, or fluorescein-labeled aptamers, for binding, the C2 aptamer was produced without the 2′-flouro groups. In SPR experiments, a similar binding pattern was observed when using the unprotected C2 aptamer (C2-OH) (**Fig. 2F**). The fluorescein-labeled aptamers (C2-Fl and C9-Fl) had a faster off-rate compared to unlabeled aptamers. The fluorescein-labeled RNA molecules had a lower affinity (**Fig. 2C, Fig. 2D**, and **Table 1**) for the hGlyRα1 protein than the unlabeled aptamers (**Fig. 2A, Fig. 2B**, and **Table 1**). The kinetics differed mostly for the dissociation from the GlyRα1 (**Table 1**). However, the on-rate was still similar for the unlabeled aptamers compared to the fluorescein-labeled aptamers (**Table 1**).

The SELEX starting library N47Nβ was used as a negative control. This library is a mixture of 2′-fluoro-pyrimidine modified nucleic acids that are of the same size as the finally identified aptamers. The N47Nβ control did not show specific binding to the GlyRα1 protein (**Fig. 2E**).

Aptamers Bind to Both the Cytoplasm and Plasma Membrane

To determine the cellular localization of hGlyRα1 in L(tk) cells, an antibody that binds residues within its large globular extracellular N-terminal domain[20] was used to stain both fixed (**Fig. 3A**) and viable cells (**Fig. 3B**). As shown in **Figure 3A**, the majority of antibody staining was found in the cytosol of fixed cells. No staining was found in the nucleus. In viable cells, the GlyRα1 antibody gave a more punctuate distribution in the plasma membrane, sometimes with a clear capping reaction. Again, no staining was detected in the nuclei. When viable cells were stained with C2-Fl and C9-Fl (**Fig. 3D and 3E**), the pattern differed from the corresponding antibody staining. The majority of fluorescein-labeled aptamers was found in the cytoplasm. The staining was similar but not identical to the antibody staining of fixed cells. Fluorescence appeared to accumulate in the cytosol and frequently displayed shaded intracellular areas, which may correspond to the endoplasmic reticulum (ER) and Golgi apparatus (arrow; **Fig. 3D**). These shaded areas were not seen in fixed cells stained with antibodies (**Fig. 3A**), which may indicate that the newly synthesized pool of GlyRα1 is not available to the C2-Fl and C9-Fl aptamers. The apparent absence of aptamer staining of plasma membrane was surprising and indicated rapid cellular uptake. Co-staining with the plasma membrane marker wheat germ agglutinin (WGA) was performed to demonstrate possible low-intensity aptamer staining in the plasma membrane (**Fig. 3F**). As shown in **Figure 4H**, cells co-stained with WGA together with the C2-Fl aptamer (**Fig. 3G**) did indeed show some co-expression close to or on the plasma membrane. Note that parental L(tk) cells incubated with fluorescent C2-Fl (**Fig. 3C**) stained cells very weakly and did not show the intracellular accumulation of fluorescence as seen for hGlyRα1-expressing L(tk) cells (**Fig. 3A**).

Aptamers Have a Positive Modulatory Effect on Membrane Potential

A fluorescent membrane potential assay with capacity to detect the antagonistic, agonistic, and modulatory activity of aptamers was used to capture the functional properties of the eight GlyRα1-binding aptamers. Following initial experiments, which were performed to determine optimal conditions, fluorescently labeled aptamers were pre-incubated with GlyRα1-expressing L(tk) cells for 30 min in a low-Cl^- solution. During application of 300 μM glycine (EC05), the cells were therefore depolarized instead of hyperpolarized to enable detection of minute changes in activity. The increased Cl^- efflux caused a change in membrane potential, and the resulting fluorescence changes were monitored in a FLIPR using a red fluorescent membrane potential sensitive dye. **Figure 4A** shows a typical response in hGlyRα1-expressing L(tk) cells following exposure to the C2aptamer. Neither an agonistic nor antagonistic effect was detected when the cells were activated with aptamers alone or aptamers together with high concentrations of glycine. However, in the presence of a low concentration of glycine, the C2 aptamer caused a pronounced increase in

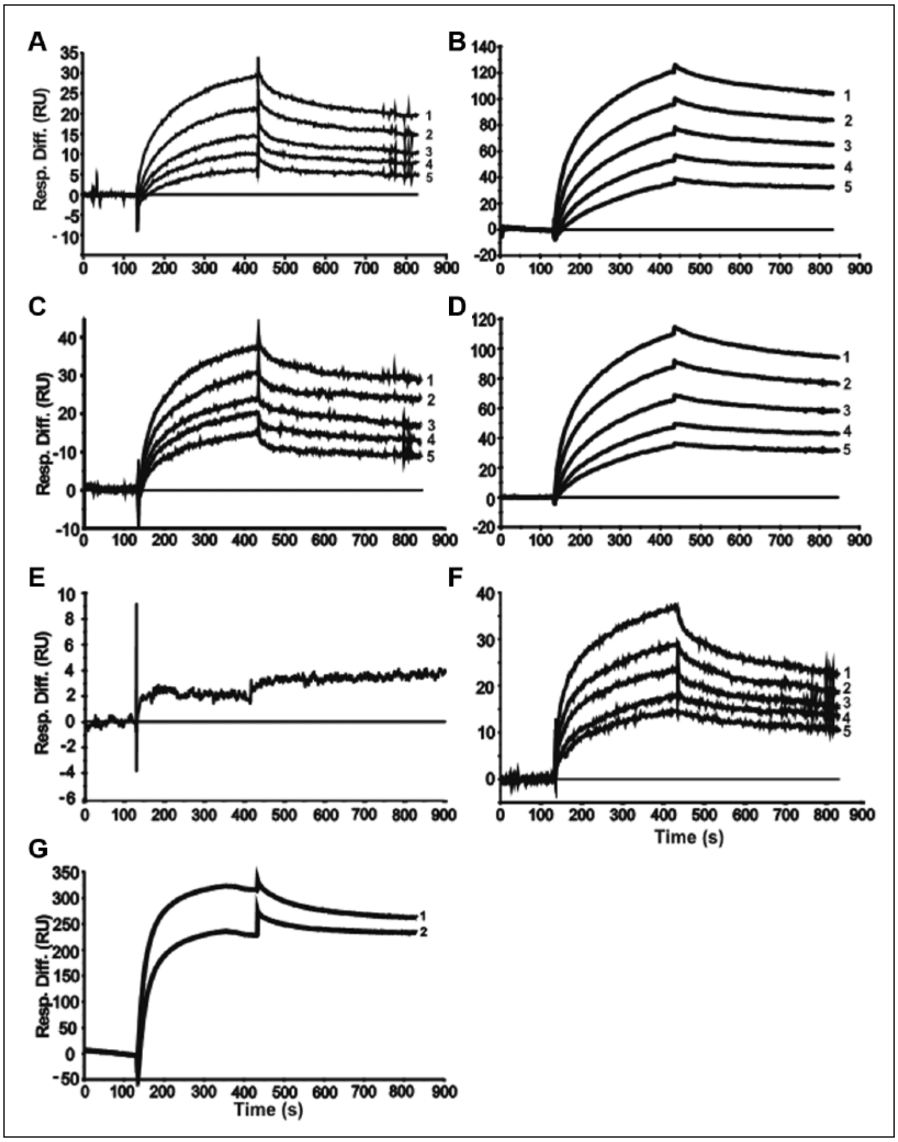

Figure 2. Surface plasmon resonance interaction of aptamers to GlyRα1. Aptamers with different concentrations [(1) 125 nM; (2) 62.5 nM; (3) 31.3 nM; (4) 15.6 nM; and (5) 7.8 nM] were injected over a CM5 sensor chip with immobilized recombinant GlyRα1. The sensogram shows the binding curves for (**A**) aptamer C2 and (**B**) aptamer C9, fluorescein-tagged (**C**) aptamer C2 and (**D**) aptamer C9, (**E**) negative control N47Nbetta nucleic acid, and (**F**) aptamer C2 without 2′-F protective groups. All aptamers shown in the figure were run on the same GlyRα1-coated surface. Aptamer C2 (1) at a concentration of 250 nM and aptamer C2 together with AZ1 (2) at the same concentration were injected over a CM5 sensor chip with immobilized recombinant GlyRα1. The sensogram shows partial inhibition of the C2 binding to the GlyRα1 surface with approximately 100 RU (**G**).

fluorescence compared to controls. The negative control N47Nβ nucleic acid gave a similar fluorescence peak value in the presence of 300 μM glycine compared to the control. **Figure 4B** shows the average peak-value fold change from GlyRα1-L(tk) cells treated with 6 μM of aptamers, and N47Nβ negative control, as compared to the 300 μM vehicle control. Aptamer binding to GlyRα1 resulted in a positive modulation ranging from a 1.2- to 2.0-fold change, whereas the negative control N47Nβ nucleic acid did not affect the cells. The statistical analysis shown in **Figure 4C** shows that aptamers C2, C4, C7, C9, and C13 gave a significant positive modulation of hGlyRα1 compared to the negative control nucleic acids (N47Nβ, $p < 0.05$), whereas three of the aptamers (C3, C15, and C20) did not show a significant difference in average peak values compared to the negative control and are therefore considered to be nonfunctional binders.

Aptamers Have a Positive Modulatory Effect on Cl^- Currents

Fluorescent membrane potential assays are an indirect measurement of ion channel activity.[21] Therefore, whole-cell patch-clamp electrophysiology was used to confirm the C2-aptamer-dependent positive modulation of hGlyRα1-expressing L(tk) cells. Pre-incubation of cells was reduced to 15 min to maintain cell viability. Also, the concentration of the C2 aptamer was reduced to 4 μM, a concentration that in the fluorescent membrane potential assay produced a robust positive modulation. Initial experiments determined that 1 mM was sufficient to reach saturating concentrations of glycine on GlyRα1- and GlyRα1/β-expressing L(tk) cells, in line with previously published data.[22] This concentration of glycine was used to normalize the recorded data throughout the

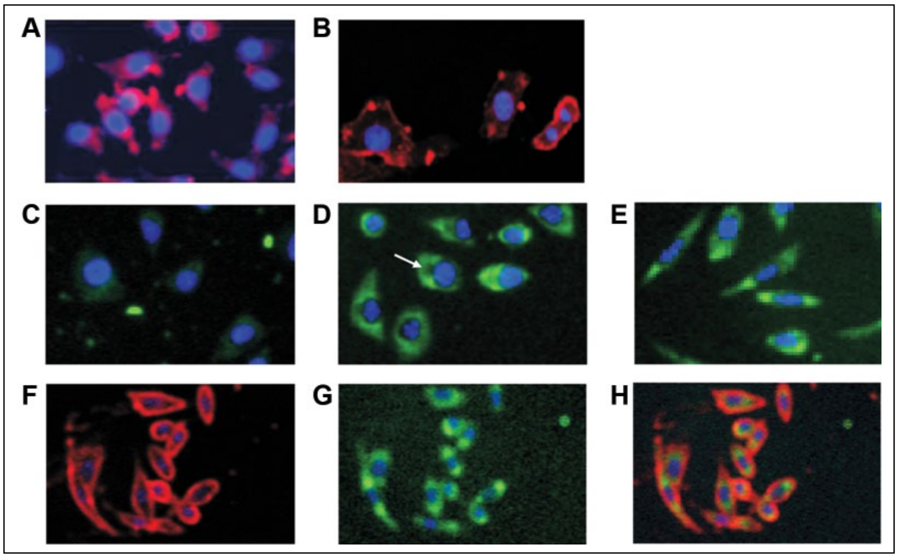

Figure 3. Aptamers bind to GlyRα1-expressing cells. GlyRα1 immunoreactivity in GlyRα1-expressing L(tk) cells in (**A**) fixed and (**B**) viable cells. Green fluorescein-labeled aptamers were used to stain viable GlyRα1-expressing L(tk) cells (C2: **D** and **G**; C9: **E**) or parental L(tk) cells (C2: **C**). (**F**) Plasma membrane staining with red fluorescent wheat germ agglutinin (WGA) was used together with (**G**) fluorescein-labeled C2 aptamers to stain GlyRα1-expressing L(tk) cells. (**H**) A merged micrograph of the two stainings. The instrument settings were the same throughout all experiments.

experiments on both the GlyRα1- and GlyRα1β-expressing L(tk) cells. As shown in **Figure 5A and 5C**, the Cl^- currents induced by 50 and 100 µM glycine were significantly increased in cells pre-incubated with C2 aptamer compared to vehicle-treated control cells. The positive modulatory effect of C2 aptamer was seen in five out of nine cells. Cells that did not show a positive modulation following pretreatment with C2 and after addition of glycine showed the same current amplitudes as cells pretreated with vehicle after the addition of glycine. The native glycine receptor is a heteropentamer between the α1- and β-subunits. Hence, whole-cell patch-clamp electrophysiology was used to study C2-aptamer-treated GlyRα1β-expressing L(tk) cells to verify that positive modulation also could be observed when using receptors in their native conformation. As shown in **Figure 5B and 5D**, Cl^- currents induced by 50 and 100 µM glycine were significantly increased, in four out of six cells, after pre-incubation with the C2 aptamer compared to control cells. The two cell lines expressing either GlyRα1 or GlyRα1β gave similar results in terms of positive modulation after pre-incubation with the aptamer. A slightly larger fraction of cells expressing the GlyRα1β showed this positive modulatory effect after exposure of the aptamer. Therefore, negative control N47Nβ nucleic acid was tested on the latter cell line. No cell showed any positive modulation of glycine responses following pre-treatment with N47Nβ (n= 5). The glycine responses were, as expected, similar to those of cells pre-incubated with vehicle (n= 4).

Discussion

Eight RNA aptamers, with nanomolar affinity for the purified, recombinant hGlyRα1, have been identified using the SELEX approach. No obvious similarities between the alignments of the aptamers, identified via different approaches (S118–S120), could be observed, neither between the identified aptamers using different approaches nor between the identified aptamers using the same approaches. Modifications of the aptamers, by removing protective fluoro-groups or fluorescein end labeling, resulted in only minor changes in binding properties. By using SA in the purification scheme, only receptors adopting a native-like conformation are purified. This conclusion was further substantiated by the fact that purified hGlyRα1 behaves like a pentamer in size exclusion chromatography. Based on the described quality measurements, the preparation of recombinant hGlyRα1 was judged to be suitable for the identification of GlyR-binding aptamers. Importantly, the C2 aptamer is shown to (1) selectively bind hGlyRα1, as demonstrated in a nitrocellulose filter binding assay as well as in a SPR assay; (2) bind to hGlyRα1 on cells, as demonstrated with cytochemistry; and (3) give a positive modulation of the hGlyRα1 and hGlyRα1β receptors, as demonstrated in the membrane potential assay and by using patch-clamp electrophysiology. There are only a few positive modulators for the Glyr described.[23] A potent positive modulator of GlyRα1 was characterized in house: the compound AZ1 (AstraZeneca). AZ1 was used for elution of the C2 aptamer in the SELEX process. Also, a competition experiment was set up in which the binding of C2 to the hGlyRα1 protein was to some extent displaced by using an equimolar concentration of AZ1. Possibly, this partial displacement could be explained by the slow off-rate of C2 aptamers and suboptimal conditions in the competition experiments. It should be noted that, during the selection, C2 aptamer was eluted from living L(tk) cells using a 4× higher concentration of AZ1 (1 mM), and due to the non-specific binding and aggregation of AZ1 to the sensor chip, we were unable to use a higher concentration than 250 nM in the BIAcore system. Another explanation to the partial displacement may be that the relatively long aptamer ligands typically bind via several footprints to their target. The

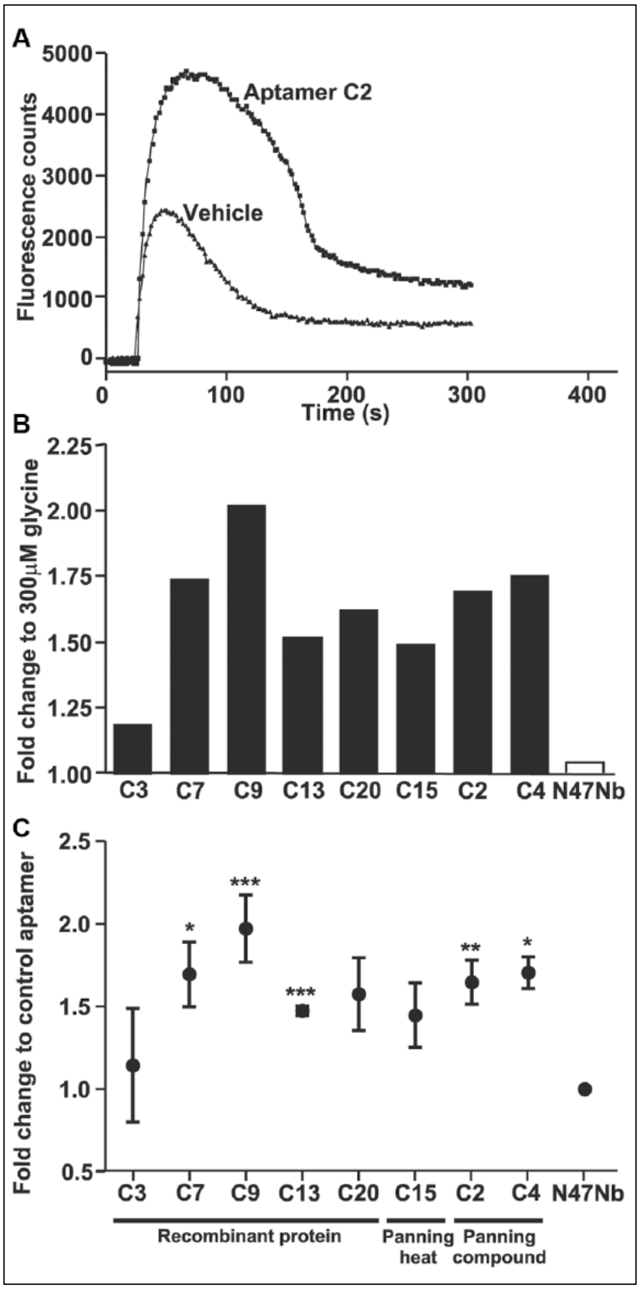

Figure 4. Aptamer-dependent positive modulation of GlyRα1. Fluorescence traces were recorded with a fluorescence imaging plate reader (FLIPR) in single cells using FLIPR membrane potential (FMP) red dye. (**A**) A representative trace showing the fluorescent membrane potential of cells incubated with the C2 aptamer compared to cells incubated with the vehicle. (**B, C**) Fold change of peak fluorescence membrane potential values for GlyRα1-expressing L(tk) cells pre-incubated with the different aptamers and the negative control N47Nbetta nucleic acid, compared to cells incubated with the vehicle at a concentration of 6 μM ($n = 3$). Data are presented as mean ± SEM, using Student's *t*-test. $***p < 0.001$; $**p < 0.01$; $*p < 0.05$.

disruption of only one of these footprints by a small-molecule competitor will therefore not automatically disrupt the binding of the entire aptamer. In any case, it is possible that the C2 aptamer binds to the same site on the GlyRα1 homopentamer/GlyRα1β heteropentamer as AZ1. Therefore, the C2 aptamer could be used for further exploration of the modulatory site on GlyRs.

One of the limitations to using aptamers in vivo is the poor stability of natural nucleic acids in biological media and fluids. To increase stability, one approach, as taken in this study, is to select aptamers from nucleic acid libraries in which the pyrimidine bases have been substituted for the corresponding 2′-fluoro, 2′-amino variants, or to substitute the purine bases for the respective 2′-*O*-alkyl variants, post SELEX.[24] For example, the US Food and Drug Administration (FDA)-approved aptamer drug Macugen, which specifically binds to vascular endothelial growth factor-165 ($VEGF_{165}$), is an oligonucleotide prepared by the SELEX processes that contains modified RNAs involving 2′-fluoropyrimidine nucleotides (U, C) and natural purine nucleotides (A, G), in which the 2′-position of ribose is a nuclease attack site.[25] Because the folding of the single-stranded oligonucleotide regions may change when these modifications are introduced, the binding properties of aptamers containing standard nucleotides are different.[24,26] Our interpretation of these data is that unprotected C2 aptamers have similar GlyRα1-binding properties. This suggests that genetic constructs could be designed to express functional C2 aptamers.[27]

It is interesting that the cytochemistry studies shows an aptamer-dependent internalization of the C2/GlyRα1 complex. It is, however, unclear from these studies whether the positive modulation of C2 aptamers is caused by the direct interaction of the C2 aptamer with GlyRα1 exposed on the cellular surface or if receptor internalization and subsequent events are required for the positive modulation to occur. Because of their size and negative charges, it is unlikely that aptamers passively enter cells.[28] Possibly, the diffuse background staining with C2-Fl aptamers to parental L(tk) cells is caused by spontaneous uptake of RNA molecules. The distinct aptamer uptake by hGlyRα1-expressing cells may be a result of receptor-dependent mechanisms, similar to those described for other membrane receptors.[29] Limited information is available for the mechanisms whereby GlyRs are internalized. Huang et al.[30] described that GlyRs endocytosis occurs via a dynamin-dependent receptor-mediated endocytosis in GlyRα1-expressing HEK293 cells. This internalization of GlyRα1-binding aptamers requires further study.

The functional assays are fundamentally different in that the changes in membrane potential are detected by a fluorescent dye in the fluorescent membrane potential assay, whereas in manual patch-clamp electrophysiology, a direct measurement of membrane potential is recorded. Despite differences in maximal concentrations in glycine (30 mM versus 1 mM, and the differences in buffer composition), the C2 aptamer was shown to potentiate both fluorescent

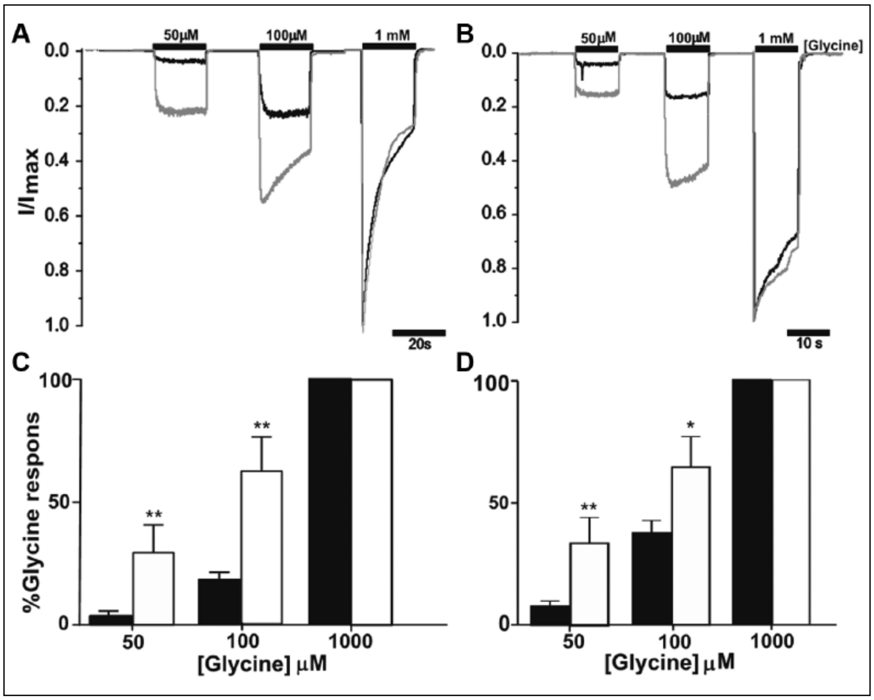

Figure 5. The GlyRα1 selected aptamers potentiate Cl^- currents. (**A, C**) GlyRα_1-expressing L(tk) cells or (**B, D**) GlyR$\alpha_1\beta$-expressing L(tk) cells were pre-incubated for 15 min with 4 µM C2 aptamer before addition of glycine as indicated, and they were analyzed by patch-clamp electrophysiology. (**A**) A representative trace showing a GlyRα_1-expressing L(tk) cell pre-incubated with the C2 aptamer, a cell incubated with the vehicle, and the response to glycine. (**B**) A representative trace showing a GlyRα1β-expressing L(tk) cell pre-incubated with the C2 aptamer, a control cell incubated with the vehicle, and the response to glycine. (**C**) The average normalized response in relation to the maximal response (1 mM glycine) for GlyRα1 L(tk) cells. Vehicle (black bar), $n = 10$; and C2 aptamer (open bar), $n = 5$. (**D**) The average normalized response in relation to the maximal response (1 mM glycine) for GlyRα1β L(tk) cells. Vehicle (open bar), $n = 4$; C2 aptamer (black bar), $n = 4$. Data are presented as mean±SEM, using Student's *t*-test; $^{**}p < 0.001$; $^{*}p < 0.05$.

membrane potential and the Cl^- currents in the presence of low concentrations of glycine. Although the majority of cells showed positive modulation with the C2 aptamer, a fraction of cells did not show this response. This may depend on some heterogeneity in the cell lines because the reference compound AZ1 also has shown a fraction of non-responding cells. Also, cells were washed in a non-aptamer-containing buffer prior to recording of the glycine-induced currents, which could reduce aptamer binding in a nonhomogeneous way.

Taken together, the results from the current study demonstrate that functional hGlyRα1 aptamers, which positively modulate this ion channel, could be selected by automated SELEX against soluble targets as well as by cellular SELEX approaches. To our knowledge, these are the first described aptamers acting as positive modulators for an ion channel. Clearly, more studies are required to understand the specific molecular mechanisms behind aptamer binding to GlyR and subsequent positive modulation of the channel. However, we believe that these aptamers will be valuable tools for the further exploration of GlyR biology.

Authors' Contributions

NDS designed research; produced aptamers; carried out experiments with surface plasmon resonance, cytochemistry, and fluorescent membrane potential assays; and drafted the manuscript. EA designed and carried out patch-clamp electrophysiology studies. MBla designed research to select aptamers, identified aptamers, produced aptamers, and analyzed data. JM designed research and prepared recombinant protein. EN participated in the design of the fluorescent membrane potential studies. MBli participated in the experimental design and data analysis for aptamer selection. MAD participated in the design of the studies for fluorescent membrane potential assays and patch-clamp electrophysiology. CVA participated in the experimental design and data analysis for surface plasmon resonance. KS conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Declaration of Conflicting Interests

At the time that this study was performed, NDS, EA, EN, CVA, MAD, and KS were employees of the Department of Neuroscience, AstraZeneca R&D, Södertälje, Sweden. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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