Baculovirus-driven expression and purification of glycine receptor α1 homo-oligomers

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Abstract The glycine receptor is a ligand-gated anion channel protein of postsynaptic membranes. We expressed a homo-oligomeric receptor composed of human α1 subunits in *Spodoptera frugiperda* cells by infection with a recombinant *Autographa californica* nuclear polyhedrosis virus. A substantial fraction of the recombinant receptor was incorporated as a functional channel protein into the cell's plasma membrane at expression levels 4- to 30-fold higher than in other eukaryotic heterologous expression systems or native rat spinal cord membranes, respectively. Upon detergent solubilization, the α1 receptor was found to exist in a predominantly monodisperse state and could be affinity-purified to near homogeneity. This preparation is a potential starting point for future crystallisation studies.

Key words: Glycine receptor; Baculovirus; Affinity purification; Heterologous expression

1. Introduction

The inhibitory glycine receptor (GlyR) is a member of the ligand-gated ion channel superfamily which includes nicotinic acetylcholine, γ-aminobutyric acid A-type and 5-HT_3 serotonin receptors. The intrinsic ion channel of these proteins is activated in response to agonist binding and thus mediates fast synaptic transmission. The GlyR can be detected by radioligand binding assays using the selective antagonist, [^{3}H]strychnine, and affinity chromatography on aminostrychnine agarose columns yields highly purified GlyR preparations. In analogy to other neurotransmitter receptors, the GlyR was found to be a pentameric molecule consisting of homologous ligand-binding α (α1–α4) and modulatory β-subunits (for review, see [1]).

Transient heterologous expression in eukaryotic cell systems, such as *Xenopus laevis* oocytes or HEK-293 cells, creates functional a homo-oligomeric or α/β heterooligomeric receptors depending on the type(s) of subunit(s) expressed. The anion channel of these recombinant receptors is gated by the agonists glycine, β-alanine or taurine; binding of these ligands is competitively inhibited by strychnine [2–5]. The recombinant receptors display a sedimentation behaviour indistinguishable from that of native GlyR [6], suggesting that a pentameric quaternary structure is preserved upon heterologous expression. Hydropathy analysis of α and β subunit primary structure predicts a transmembrane topology characterized by an extended N-terminal extracellular domain followed by four transmembrane segments (M1–M4; see [1]).

Here, we expressed α1 homo-oligomeric GlyR in *Spodoptera frugiperda* (Sf9) insect cells infected with a recombinant *Autographa californica* baculovirus, in which the GlyR α1 cDNA was placed under the control of the polyhedrin promoter. The baculovirus Sf9 cell system was previously used to overexpress the α1 GlyR [7] in order to produce preparative amounts of GlyR protein. Receptor purification described in that study involved solubilisation with digitonin/desoxycholate and resulted in a partially proteolysed protein. Here we used solubilisation of the receptor with the more economical conventional detergent Triton X-100 and affinity-purified it to a nearly homogenous and monodisperse state, which appears suitable for crystallization studies.

2. Materials and methods

2.1. Viruses and cells

Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were propagated in *Spodoptera frugiperda* (Sf9) cells at 27°C in IPL-61 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum, amphotericin (2.5 mg/ml) and gentamycin (50 mg/ml) [8]. The Sf9 cell line was maintained in monolayer culture and split for propagation every 6 days. For large-scale infection, cells were grown in suspension at 27°C with gently stirring in 1 litre glass bottles. For GlyR expression, cells were infected with recombinant AcNPV at a multiplicity of infection of 5–10. Cells were harvested for assays 2–4 days later. The AcNPV baculovirus, the insect Sf9 cells, and the transfer plasmid pVL1393 were purchased from Invitrogen.

2.2. Construction of the transfer vector

The baculovirus transfer vector, pVL1393 was modified as follows to yield the transfer vector pVLGlyRα1. A *Nhe*I restiction site was created immediately upstream from the initiator ATG of the human α1 GlyR cDNA [9] by site-directed mutagenesis (Amersham In Vitro Mutagenesis System II), using the oligonucleotide: 5′-GCACAAAGGAC-CGCTAGCATGTACAGC-3′. The coding region of the mutated α1 GlyR cDNA was excised by *Nhe*I/*Xba*I digestion and ligated into the *Xba*I site of pVL1393.

2.3. Transfection and isolation of recombinant virus

To achieve homologous recombination between wild-type (wt) virus and the transfer vector, Sf9 cells were co-transfected with 1 μg of AcNPV DNA and 2 μg of the transfer vector pVLGlyRα1, using the calcium phosphate method [8]. Following a 7 day incubation at 27°C, recombinant baculoviruses were isolated by limited dilution. After saving the virus-containing supernatants, cell clones were solubilized with 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 20% (v/v) methanol, 2% (w/v) sodium desoxycholate and protease inhibitors as described [10], transferred onto a nitrocellulose membrane and analysed for GlyR-specific immunoreactivity using a dot assay with mAb4 [11,12]. In addition, GlyR α1 sequences were detected by dot-blot hybridisation with a randomly primed ^{32}P-labeled GlyR α1 cDNA fragment encoding the entire reading frame.

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; GlyR, glycine receptor; Sf9, *Spodoptera frugiperda* cell line 9; wt, wild-type.

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The virus supernatants of those samples that were immunopositive and hybridised with the ^{32}P-labeled probe, were subjected to three more rounds of dilution, infection and identification. Thus, singular clones of recombinant viruses were obtained, which were devoid of polyhedrin protein as detected by visual inspection.

2.4. Gel electrophoresis, Western blotting and immuno-fluorescence

SDS-PAGE on 12% gels was followed either by silver staining [13] or by Western blot analysis with monoclonal antibody mAb4 as described [6]. Immuno-fluorescence staining was done on Sf9 cells grown on 12 mm coverslips (Thermanox, Nunc) as described [14]. Cells were stained with mAb2 [11] in the unpermeabilised state, or fixed with methanol and stained with mAb4.

2.5. [^{3}H]Strychnine binding assay

Agonist displaceable binding of [^{3}H]strychnine to homogenates of crude Sf9 cell membranes or solubilised receptor preparations was determined as described [15].

2.6. Whole-cell recording

For electrophysiological experiments, Sf9 cells were grown on 12 mm glas coverslips in a 24-well plate. Three days after infection, glycine-gated whole cell currents were analysed in the voltage-clamp mode of the whole cell patch configuration as described previously [5].

2.7. Sucrose density gradient centrifugation

Detergent extract containing about 3 pmol [^{3}H]strychnine binding sites was sedimented through a 12.5 ml sucrose gradient (5–20%, w/w), and fractions were assayed for mAb4 immunoreactivity [6]. The following marker enzymes were analyzed in a parallel gradient: cytochrome *c* (1.8 S), malate dehydrogenase (4.32 S), lactate dehydrogenase (6.95 S), catalase (11.3 S), and β-galactosidase (15.9 S).

2.8. Purification by affinity chromatography

Purification of the GlyR from infected Sf9 cells was done according to the published procedure [10]. Briefly, cells grown in suspension were pelleted at $1000 \times g$ and homogenized using a glass homogeniser (10 strokes). The homogenate was centrifuged with a maximal acceleration of $10000 \times g$, which proved important for subsequent solubilisation. The membrane pellet was homogenised in 25 mM potassium phosphate buffer: pH 7.4, 0.2 M KCl, 5 mM dithiothreitol, and protease inhibitors (see [10]) at a protein concentration of 4 mg/ml and solubilised at 4°C for 1 h after adding 2% (w/v) Triton X-100 (Serva) and 1 M KCl. After centrifugation at $100000 \times g$ and filtering, the solubilisate was applied onto a 2-aminostrychnine agarose column and washed with 50 bed-volumes of 25 mM potassium phosphate buffer, pH 7.4, 1 M KCl, 1% (w/v) Triton X-100, 5 mM dithiothreitol and protease inhibitors. The purified GlyR was eluted with 2 bed-volumes of 200 mM glycine in the washing buffer.

3. Results

3.1. Isolation of recombinant GlyR α1 baculovirus

For the construction of recombinant virus, the coding region of the human GlyR α1 subunit cDNA was inserted into the baculovirus transfer vector pVL1393 and co-transfected with wild-type AcNPV virus DNA into Sf9 cells. Several recombinant virus clones were identified that were positive in both DNA hybridization and immunoblot analysis. Here we describe the characterisation of clone G6, which showed the highest expression level.

3.2. Immunological and pharmacological characterisation

Western blot analysis with mAb4 revealed that membranes of Sf9 cells infected with recombinant baculovirus clone G6 contained an immunoreactive protein with an apparent molecular weight of 48 kDa, i.e. that of the GlyR a subunit detected in rat spinal cord membranes (Fig. 1A). However, staining intensities in the Sf9 membranes significantly exceeded that of

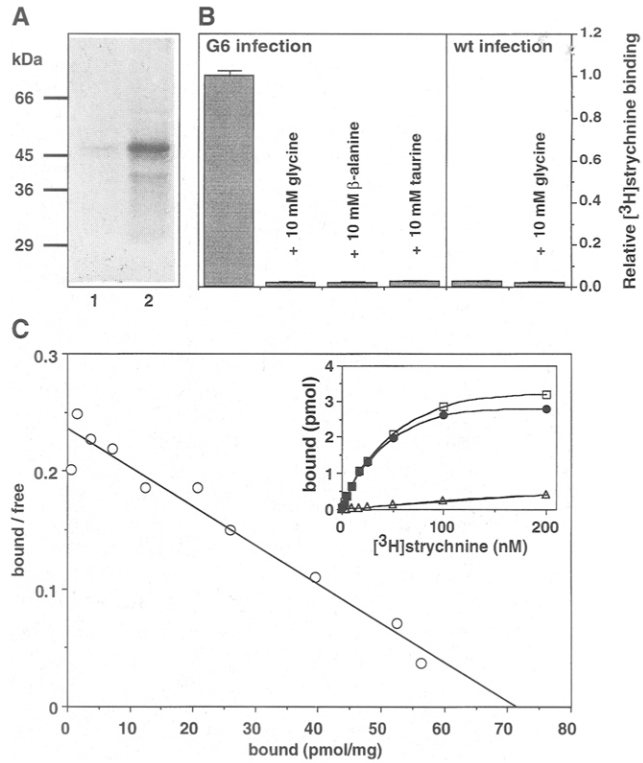

Fig. 1. Identification of α1 GlyR in Sf9 cells infected with an isolate of recombinant baculovirus (clone G6). (A) Western blot analysis of Sf9 and rat spinal cord (sc) membranes (5 μg of protein/lane). After SDS-PAGE and blotting, the protein was immuno-stained with mAb4, specific for GlyR α-subunits. The difference in staining intensity between spinal cord and Sf9 cell membranes reflects the different levels of GlyR expression. (B) [^{3}H]Strychnine binding to Sf9 cell membranes infected with G6 or wt virus. Binding of 25 nM [^{3}H]strychnine to the membranes was competed for by glycine (10 mM), β-alanine (10 mM) or taurine (10 mM). Cells infected with wt virus show no specific [^{3}H]strychnine binding. (C) Quantitation of radioligand binding. Upon incubation of G6 infected Sf9 cell membranes with [^{3}H]strychnine at concentrations ranging from 2 to 200 nM, saturable binding could be demonstrated (inset: saturation curves with and without 10 mM glycine; □ = total binding; △ = unspecific binding; ● = specific binding). Transformation of the data according to Scatchard yielded a dissociation constant (K_d) of 38.5 nM and a maximal number of [^{3}H]strychnine binding sites (B_{max}) of 71 pmol/mg protein. The data points are derived from the means of a measurement done in triplicate.

spinal cord, which is consistent with a higher GlyR expression level in the former (see below). Cells infected with wt AcNPV did not produce specific immunoreactivity.

For pharmacological analysis, membranes were subjected to a [^{3}H]strychnine radioligand binding assay. In contrast to wild-type AcNPV infected cells, membranes of G6 infected cells exhibited [^{3}H]strychnine binding displaceable by glycine, β-alanine and taurine, a characteristic feature of the native GlyR (Fig. 1B). Scatchard analysis of displaceable [^{3}H]strychnine binding revealed a dissociation constant (K_d) of 38.5 nM and a maximal number of [^{3}H]strychnine binding sites (B_{max}) of 71.0 pmol/mg protein (Fig. 1C).

3.3. Subcellular localisation

To study the subcellular distribution of the recombinant GlyR protein, G6 infected Sf9 cells were analysed by fluores-

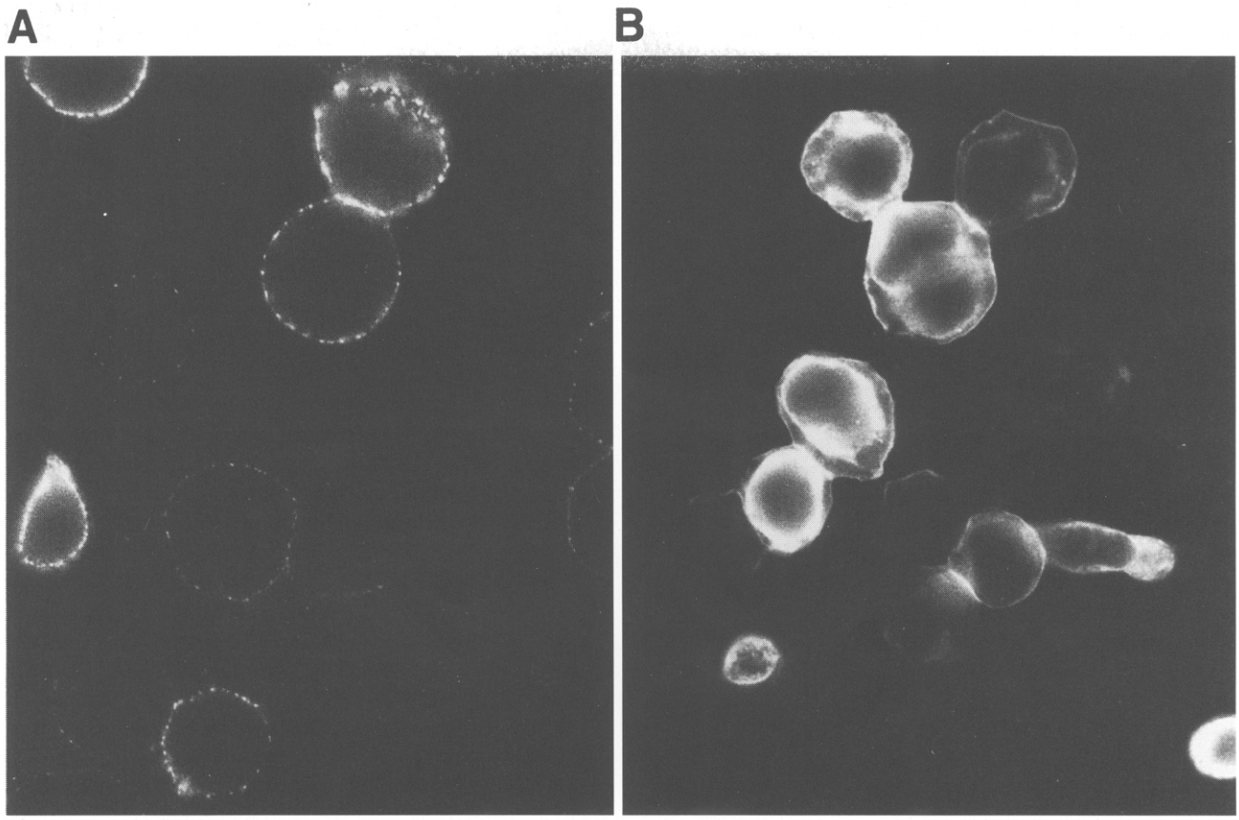

Fig. 2. Fluorescence microscopy of whole Sf9 cells infected with clone G6. Cells were incubated with mAb2 prior to fixation (A), or with mAb4 after methanol fixation (B). Non-infected cells as well as wt virus infected cells showed no staining above background level. The punctate staining pattern seen with mAb2 is likely to result from the formation of receptor aggregates by the divalent antibody.

cence microscopy after staining with the monoclonal antibodies mAb2 or mAb4. In contrast to mAb4, which only binds denatured GlyR a subunits, mAb2 recognises the protein also in its native state [16] and can therefore be used for the staining of unfixed samples. Fig. 2A shows Sf9 cells 48 h post-infection that had been incubated with mAb2 prior to fixation to visualise the GlyR exposed on the cell surface. Intense fluorescence is observed at the cell perimeter that is typical of plasma membrane surface staining. Fig. 2B shows infected cells incubated with mAb4 after methanol fixation, i.e. after permeabilisation of the cell membrane. Now, intracellular perinuclear membranes were stained more intensely than the plasma membrane. Together, these data indicate that the receptor is efficiently incorporated into the plasma membrane; most of the recombinant protein, however, is found intracellularly, i.e. presumably within the endoplasmic reticulum and Golgi compartments.

3.4. Functional analysis

Application of 5–100 mM glycine to infected Sf9 cells elicited inward currents of up to 9 nA at a holding potential of −70 mV. The half-maximal activating glycine concentration (EC_{50}) was 18.6 ± 8.1 mM (mean ± S.D., $n = 9$ cells), and the corresponding Hill coefficient 4.2 ± 1.0 (Fig. 3A). Transmembrane currents could be reversibly blocked by strychnine (Fig. 3B), with a half-maximal inhibitory concentration (IC_{50}) of 85.4 ± 60 nM ($n = 5$). The channels formed were activated not only by glycine, but also by micromolar concentrations of β-alanine and taurine (Fig. 3B). Uninfected or wild-type AcNPV infected Sf9 cells were glycine-insensitive. Thus, expression of the human GlyR α1 subunit in insect cells generates glycine-gated chloride channels, which display a pharmacology typical of the mammalian GlyR (compare [2–5]).

3.5. Solubilisation and purification

The recombinant GlyR was solubilised from Sf9 cell membranes using the non-ionic detergent Triton X-100 according to the original purification protocol [10]. The recovery of [^{3}H]strychnine binding sites after solubilisation was about 50%. To analyse its aggregation state, the solubilized receptor was subjected to sedimentation analysis using sucrose gradient centrifugation. The majority of the receptor protein migrated at an apparent sedimentation coefficient of 7.5 S (Fig. 4A). No indications were found for α1 subunit monomers, but some immunoreactive material sedimented at 11 S, which may represent receptor dimers. Additionally, some large aggregates were found at the bottom of the gradients. We conclude that most of the recombinant GlyR exists in a monodisperse solution upon Triton X-100 solubilisation.

The recombinant GlyR was purified from the detergent extract by affinity chromatography on 2-aminostrychnine agarose. In a typical purification experiment, about 30% of the [^{3}H]strychnine binding sites applied to the column were recovered in the eluate, and the final yield of the binding sites in the glycine eluate was about 10% of the sites present in unsolubil-

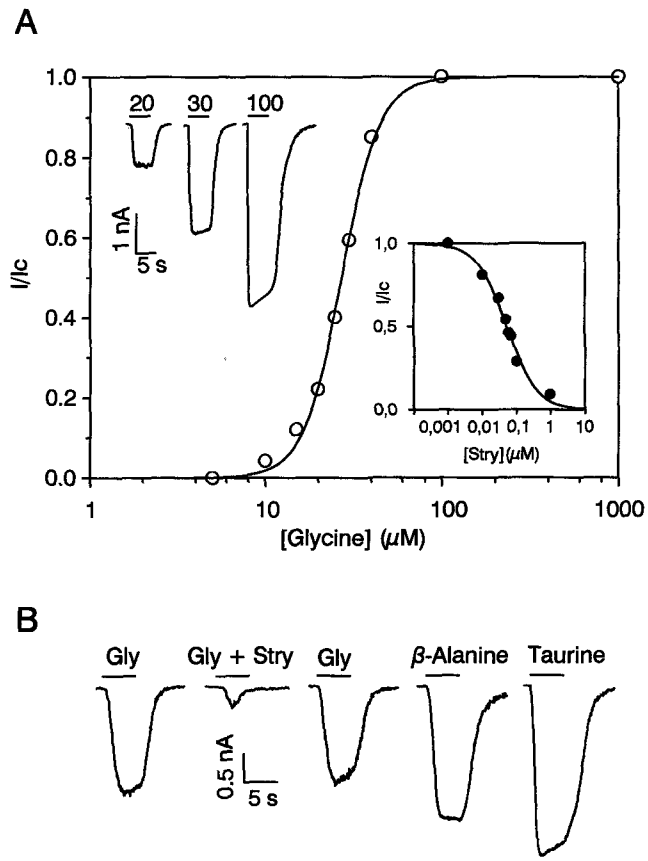

Fig. 3. Functional analysis of G6 infected Sf9 cells. (A) Glycine elicited whole-cell currents at a half-maximal concentration (EC_{50}) of 27.2 μM and with a Hill coefficient of $h = 4.0$. Strychnine inhibited these currents with a half-maximal inhibitory concentration (IC_{50}) of 51.0 nM and a Hill coefficient of 1.0 (inset). The bars above the exemplary traces given denote the duration of glycine application, and the numbers correspond to glycine concentrations in μM. I/I_c denotes relative current. (B) Current traces showing the activation of receptor by glycine (Gly, 20 μM), its reversible inhibition by strychnine (Stry, 0.3 μM), and the activation by β-alanine (100 μM), and taurine (100 μM).

ized membranes. This is comparable to the yield obtained with GlyR solubilized from mammalian spinal cord membranes [10,17]. In absolute numbers, one liter of cell culture containing about 1×10^9 cells, yielded about 400 pmol of affinity purified [^{3}H]strychnine binding sites.

SDS-PAGE of the purified preparation followed by silver staining revealed a predominant protein band at an apparent molecular weight of 48 kDa. Western blotting using mAb4 identified the protein as GlyR α subunit (Fig. 4B). Thus, recombinant α1 homo-oligomeric GlyR can be purified to near homogeneity from infected Sf9 cells.

4. Discussion

Our data show that Sf9 cells infected with a recombinant baculovirus carrying the GlyR α1 subunit cDNA express a functional homo-oligomeric glycine receptor. Immunocytochemistry with intact cells visualised the recombinant receptor on the cell surface, indicating its incorporation into the plasma membrane. With permeabilised cells, however, accumulation of receptor protein within intracellular organelles was also apparent.

Electrophysiological analysis of the infected Sf9 cells demonstrated that the recombinant GlyR responded to the agonists glycine, β-alanine and taurine in a manner sensitive to the glycinergic antagonist strychnine. This confirmed that at least a fraction of the channel protein was correctly inserted into the plasma membrane. The affinity for glycine (EC_{50} = 18.6 μM) and the degree of agonist cooperativity as expressed by the Hill coefficient of channel activation (h = 4.2) were similar to values previously found for GlyR α1 receptors expressed in HEK-293 cells (EC_{50} = 29–100 μM, h = 1.6–4.2; see [4,5,18]). Preliminary single channel analysis in the outside-out patch clamp configuration revealed a main elementary conductance of 88 pS accompanied by subconductance states of 70 pS, 54 pS and 35 pS (N. Rundström, unpublished results). These values are similar to the conductances of GlyR α1 homo-oligomers in HEK-293 cells (86, 64, 46, 30, and 18 pS; see [5]).

Our electrophysiological data derived from intact cells are complemented by radioligand binding studies using Sf9 cell membrane homogenates. A dissociation constant (K_d) for [^{3}H]strychnine of 38 nM is within the range of values found for native spinal cord membranes and HEK-293 cells transiently expressing α1 GlyR (2–64 nM; see [19–21,18,15]). The maximal number of strychnine binding sites (B_{max} = 71 pmol/mg) obtained with the Sf9 cell system, however, significantly exceeds the values found for spinal cord membranes and transfected HEK-293 cells (2.4–17 pmol/mg) by 4- to 30-fold (compare: [19,20,18,15]). Thus, infected Sf9 cells express the GlyR to much higher levels than native neurons or even transiently expressing mammalian cells.

GlyR α1 receptors from expressing Sf9 cells were solubilised with the non-ionic detergent Triton X-100. This is an advantage to the digitonin/desoxycholate mixture reported in [7] to be required for solubilizing the GlyR from Sf9 cell membranes, since Triton X-100 is much more economical for large-scale receptor preparations. Velocity gradient centrifugation of the solubilized material on sucrose gradients revealed that the majority of the receptor migrates as a peak of 7.5 S. This corresponds to the sedimentation behaviour of native GlyR and that expressed in HEK-293 cells (7.7–7.9 S; [6,10]), which indicates that the predominant fraction of the receptor exists as α1 homopentamer in a monodisperse state. One-step affinity purification yielded a major protein band identical in molecular weight (48 kDa) and immunoreactivity to native GlyR a subunits, with our preparation being much less contaminated by proteolytic degradation products as reported previously [7]. This might reflect a stabilisation of the protein when using Triton X-100 as a solubilising agent. For a better understanding of the fine structure of the GlyR, we plan to grow 2- and 3-dimensional crystals for electron microscopy and X-ray diffraction analysis. The growth of protein crystals has been shown to be seriously hampered by autoaggregation phenomena [22]. The monodispersity of the solubilized recombinant GlyR demonstrated here thus may prove an important prerequisite for future high-resolution structure determination.

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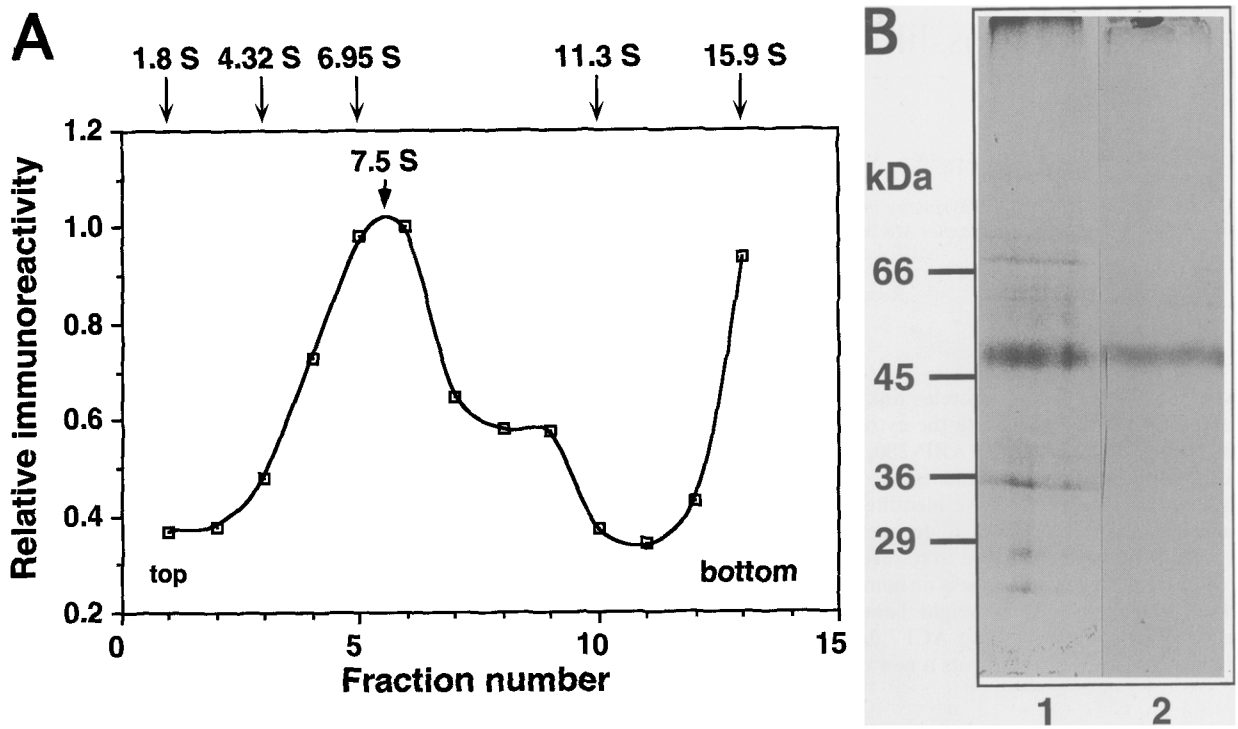

Fig. 4. Solubilisation and purification. (A) Velocity gradient centrifugation. A Triton X-100 extract from infected Sf9 cell membranes was subjected to sedimentation on a 5–20% sucrose gradient. The major peak of mAb4 immunoreactivity migrated at an apparent sedimentation constant of 7.5 S. Arrows indicate the position of marker proteins as specified in section 2. (B) Purification of the recombinant α1 GlyR. Solubilized GlyR was affinity-purified on an 2-aminostrychnine agarose column. SDS-PAGE and silver staining (lane 1), or Western blotting with mAb4 (lane 2), identified the major polypeptide of the column eluate as the 48 kDa GlyR α subunit.

References

[1] Betz, H. (1992) Q. Rev. Biophys. 25, 381–394.
[2] Schmieden, V., Grenningloh, G., Schofield, P.R. and Betz, H. (1989) EMBO J. 8, 695–700.
[3] Schmieden, V., Kuhse, J. and Betz, H. (1992) EMBO J. 11, 2025–2032.
[4] Sontheimer, H., Becker, C.-M., Pritchett, D.B., Schofield, P.R., Grenningloh, G., Kettenmann, H., Betz, H. and Seeburg, P.H. (1989) Neuron 2, 1491–1497.
[5] Bormann, J., Rundström, N., Betz, H. and Langosch, D. (1993) EMBO J. 12, 3729–3737.
[6] Langosch, D., Kirsch, A., Herbold, A., Bormann, J., Schmieden, V. and Betz, H. (1993) FEBS Lett. 336, 540–544.
[7] Cascio, M., Schoppa, N.E., Grodzicki, R.L., Sigworth, F.J. and Fox, R.O. (1993) J. Biol. Chem. 268, 22135–22142.
[8] Luckow, V.A. and Summers, M.D. (1988) Biotechnology 6, 47–55.
[9] Grenningloh, G., Schmieden, V., Schofield, P.R., Seeburg, P.H., Siddique, T., Mohandas, T.K., Becker, C.-M. and Betz, H. (1990) EMBO J. 9, 771–776.
[10] Pfeiffer, F., Graham, D. and Betz, H. (1982) J. Biol. Chem. 257, 9389–9393.
[11] Pfeiffer, F., Simler, R., Grenningloh, G. and Betz, H. (1984) Proc. Natl. Acad. Sci. USA 81, 7224–7227.
[12] Becker, C.-M., Hoch, W. and Betz, H. (1989) J. Neurochem. 53, 124–131.
[13] Merril, C.R., Goldman, D. and vanKeuren, M. (1982) Electrophoresis 3, 17–23.
[14] Kirsch, J., Wolters, I., Triller, A. and Betz, H. (1993) Nature 366, 745–748.
[15] Langosch, D., Laube, B., Rundström, N., Schmieden, V., Bormann, J. and Betz, H. (1994) EMBO J. 13, 4223–4228.
[16] Schröder, S., Hoch, W., Becker, C.-M., Grenningloh, G. and Betz, H. (1991) Biochemistry 30, 42–47.
[17] Graham, D., Pfeiffer, F., Simler, R. and Betz, H. (1985) Biochemistry 24, 990–994.
[18] Rajendra, S., Lynch, J.W., Pierce, K.D., French, C.R., Barry, P.H. and Schofield, P.R. (1994) J. Biol. Chem. 269, 18739–18742.
[19] Young, A. and Snyder, S.H. (1973) Proc. Natl. Acad. Sci. USA 70, 2832–2836.
[20] Vandenberg, R.J., French, C.R., Barry, P.H., Shine, J. and Schofield, P.R. (1992) Proc. Natl. Acad. Sci. USA 89, 1765–1769.
[21] Vandenberg, R.J., Handford, D.A. and Schofield, P.R. (1992) Neuron 9, 491–496.
[22] Zulauf, M. and D'Arcy, A. (1992) J. Crystal Growth 122, 102–106.