Agonist binding to purified glycine receptor reconstituted into giant liposomes elicits two types of chloride currents

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Using 'inside-out' membrane patches obtained from reconstituted giant liposomes containing purified glycine receptor from rat spinal cord, we have detected chloride currents elicited in response to the presence of the agonists glycine or β-alanine. Regardless of the agonist employed, two different patterns of single channel currents could be detected, which differ in their main conductance, complexity of substates and opening frequency. In agreement with the expectations of glycine receptor heterogeneity suggested recently at the mRNA and cDNA level, our results indicate the existence of functionally different glycine receptors in the adult rat spinal cord.

Ion channel-coupled receptor; Reconstitution; Patch-clamp method; Receptor heterogeneity

1. INTRODUCTION

Glycine is a major inhibitory neurotransmitter in the spinal cord and in other regions of the central nervous system [1]. Binding of glycine to specific receptors in the target neurons transiently increases the membrane permeability to chloride, thus producing membrane hyperpolarization. The glycine receptor (GlyR) purified from the spinal cord of different mammals, is an oligomeric transmembrane protein formed by two different types of glycosilated polypeptides (M_r 48 000 and 58 000) in an unknown stoichiometry [2,3]. Recent cloning of the cDNAs of the 48 kDa and 58 kDa subunits has revealed that they share significant sequence and structural homologies with subunits of the nicotinic acetylcholine receptor and the $GABA_A$ receptors [4,5].

The GlyR purified from cholate extracts of rat spinal cord has been recently reconstituted into small phospholipid vesicles [6] and shown to retain the pharmacological characteristics of native GlyR [7]. Furthermore, using an assay based on the fluorescence quenching of an anion-sensitive, vesicle-entrapped probe, we reported that the reconstituted GlyR retains the ability to promote chloride translocation in response to agonist binding [6]. Such ion flux measurements, however, have several drawbacks, including low sensitivity and time resolution, and do not allow the study of single ion channels. To overcome these limitations, we have reconstituted purified GlyR into giant liposomes, a preparation suitable for patch-clamp recording of single channel ion currents [8-10].

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Abbreviations: GlyR, glycine receptor

2. MATERIALS AND METHODS

2.1. *Reconstitution of the glycine receptor into giant liposomes*

The GlyR was purified from cholate extracts of rat spinal cord membranes by affinity chromatography on 2-aminostrychnine-agarose [2,6]. The specific activities of the purified preparations were ~3.2 nmol of [^{3}H]strychnine bound/mg protein. The purified GlyR was reconstituted into small diameter proteoliposomes (containing typically 30-50 pmol of [^{3}H]strychnine binding sites/ml) that retain the pharmacological properties of the native receptor, as determined by ligand binding [6]. The reconstituted preparation was frozen and used within 1-3 days. Giant liposomes were prepared by submitting a mixture of the reconstituted GlyR preparation (see above) and asolectin lipid vesicles to a partial dehydration/rehydration cycle, similarly to a recent report [10] on reconstitution of the acetylcholine receptor. Routinely, a 1 ml aliquot of reconstituted GlyR vesicles was mixed with 2.5 ml of a ~13 mM (in terms of lipid phosphorus) suspension of asolectin vesicles. After the partial dehydration/rehydration cycle [10], the resulting giant liposomes (mostly ranging from 5 to 50 μm in diameter) displayed a strychnine binding sites-to-phospholipid molar ratio of approximately 1:450 000, as determined by photoaffinity labeling of the reconstituted GlyR with [^{3}H]strychnine [6].

2.2. *Patch-clamp measurements*

Aliquots (3-15 μl) of giant liposomes were deposited into 3.5 cm Petri dishes, mixed with 200-300 μl of a DEAE-Sephadex A-50 suspension (~3 mg of dry gel/ml) previously equilibrated in 4 mM Hepes buffer, pH 7.4, containing 50 mM NaCl and 0.1 mM $CaCl_2$ and incubated for 15-30 min, at room temperature. This treatment anchors the liposomes to the gel beads, thus allowing extensive washing with the buffer of choice for electrical recording (bath solution), while leaving a high number of liposomes remaining into the dish [10]. Single channel recordings were obtained by using patch-

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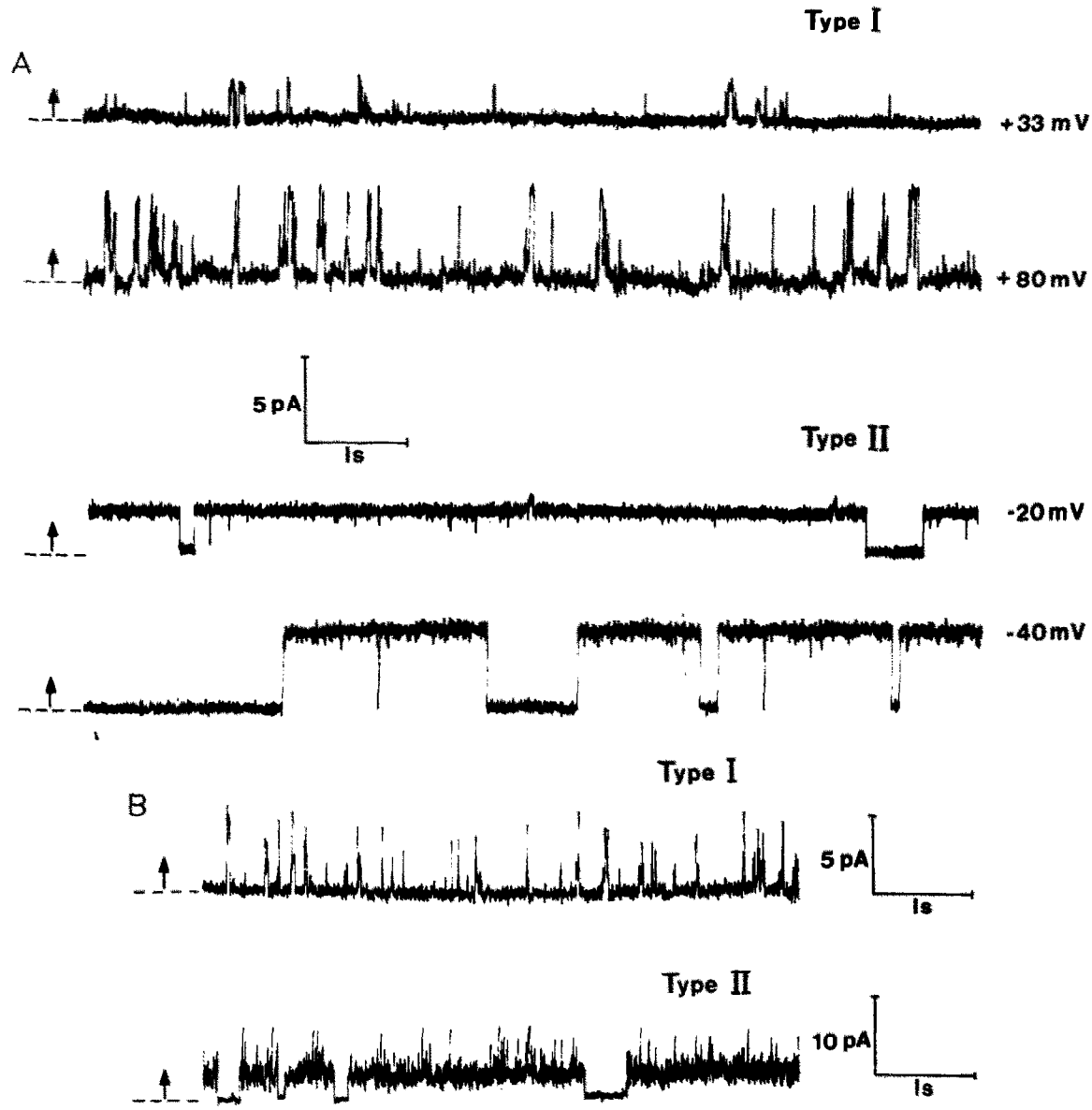

Fig. 1. Ion channel activities exhibited by excised, 'inside-out' patches of giant liposomes containing purified GlyR, in response to the presence of glycinergic agonists. Zero current level is indicated by the dashed line and channel openings correspond to upward deflections, as indicated by the arrows. (A) Shows records of type 'I' and type 'II' currents obtained at different holding potentials (indicated within the figure). The bath solution was 4 mM Hepes, pH 7.4, containing 100 mM NaCl and 0.1 mM $CaCl_2$. The pipette solution was 4 mM Hepes, pH 7.4, containing 100 mM KCl, 0.1 mM $CaCl_2$ and 5 μM glycine. To illustrate better the differences in channel opening frequency, panel B shows records of type 'I' and type 'II' currents obtained at the same holding potential (+60 mV). In the latter experiments, the bath solution was 4 mM Hepes, pH 7.4, containing 145 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The pipette solution was 4 mM Hepes, pH 7.4, containing 145 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 μM glycine.

clamp techniques as described [11]. Giga seals (10–20 GΩ) were formed on giant liposomes with regular patch pipette microelectrodes (10–20 MΩ resistance for the open electrode). After sealing, withdrawal of the pipette from the liposome surface resulted in an excised path ('inside-out' configuration). An EPC-5 patch amplifier (List Medical Electronics) was used, at a gain of 20 mV/pA and a filter setting of 10 kHz. The holding potential was applied to the interior of the patch pipette and the bath electrode was maintained at virtual ground. The signal from the clamp was visualized on an oscilloscope, digitized and stored on video tape. All measurements were made at room temperature.

3. RESULTS AND DISCUSSION

Cell-size, giant liposomes containing reconstituted GlyR were adequate to produce seals of high resistance (>10 GΩ) and excised patches in an 'inside-out' configuration. In the absence of glycinergic agonists in the patch pipette solution, the patches had no detectable electrical activity at pipette holding potentials ranging from −150 to +150 mV ($n=6$). A lack of electrical ac-

tivity was also characteristic of patches from giant liposomes lacking GlyR, i.e. made only from asolectin lipids ($n=4$).

In patches from GlyR-containing liposomes, the presence of 5 μM glycine or β-alanine in the pipette solution resulted in recordings exhibiting complicated electrical activity during the first seconds following seal formation. Thereafter, the number of events decreased and it was possible to obtain recordings showing discrete transient fluctuations between two or more current levels. In different patches, such recordings corresponded always to what we arbitrarily called type 'I' or type 'II' channels (Fig. 1A,B). These ion channel activities in response to the presence of glycinergic agonists were found in 11 (type 'I', $n=5$; type 'II', $n=6$) out of a total of 21 patches recorded (i.e. 52% of the cases). In most of the experiments shown in this report, we used bath and pipette solutions having an identical concentration of chloride but different concentrations of sodium and potassium (symmetrical chloride solutions, see legend to Fig. 1). Since channel currents under these conditions reversed at zero potential (see Fig. 2), it can be concluded that the charge is carried by chloride ions. A similar conclusion was also reached from determinations of the reverse potential for these currents in asymmetrical chloride solutions. No channel activity was detected when liposome patches were exposed to simultaneous presence of 5 μM glycine and 50 μM strychnine in the pipette solution. It is worth noting that the probability of channel opening for the dominant and subconducting current levels in both type 'I' and type 'II' single channel responses, was increased as the holding potential was made more positive (Fig. 1A), in agreement with previous reports on GlyR electrical recordings in cultured cells [12].

Type 'I' recordings exhibited highly reproducible, short-lived openings of a main conductance and of a large variety of complex subconducting states. Curren versus voltage plots obtained from the amplitudes o the dominant current level at different holding poten tials, were linear within the voltage range studied (Fig. 2A). Using 145 mM chloride solutions at both sides of the membrane patch, the estimated value for such main conductance was 91.6 ± 2.8 pS.

Type 'II' recordings presented much longer-lived channel opening events than type 'I' recordings (Fig. 1A,B). Also, the number of different classes of substates associated to the main conductance was seemingly lower than that present in type 'I' activity (Fig. 1A,B). Furthermore, type II recordings were characterized by a level of electrical fluctuation associated to the open channel state, which always surpassed that observed in the closed state (the latter of which should be mostly due to instrumental noise). This finding is somewhat similar to previous observations made in other chloride channels [13] and became more evident when type II recordings were obtained at positive holding potentials (Fig. 1B). At the present time, however, we do not have a satisfactory explanation for this phenomenon. Current versus voltage plots for the dominant current level in the type 'II' activity were not linear and presented a downward curvature at positive potentials (Fig. 2B). Moreover, we found a considerable variability from experiment to experiment in the amplitude of the dominant current level. Such variability was reflected in that the estimates of the

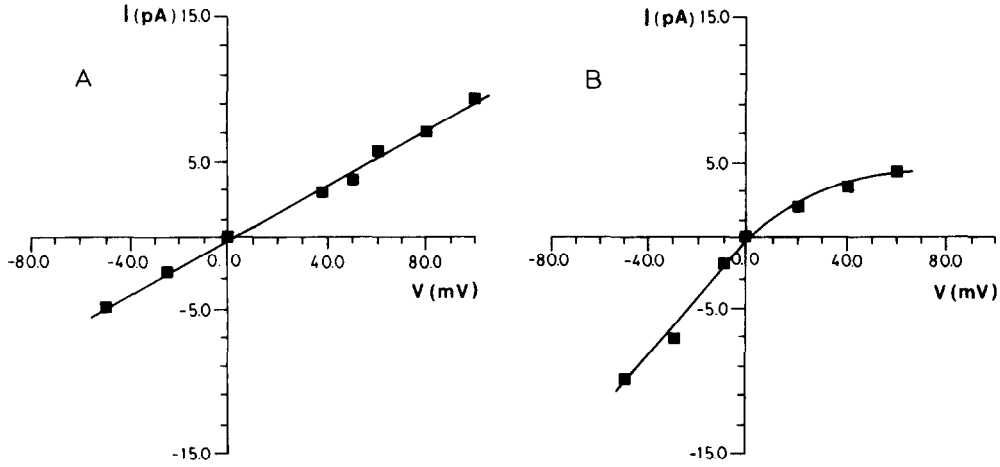

Fig. 2. Representative channel current versus voltage plots for the main current levels observed in type 'I' (panel A) and type 'II' (panel B) activities. For computer analysis of current amplitude data, the recordings were filtered at 1–2 kHz and digitized by a Cambridge Electronic Design 1401, at a sampling interval ranging from 400 to 600 μs. Routinely, for each holding potential, current amplitude values of the dominant channel opening event were plotted to produce gaussian amplitude histograms (number of events vs current amplitude), and used to determine the mean amplitude value. These amplitude values at the different holding potentials were used to calculate the open channel conductance. Pipette and bath solutions identical to those indicated in the legend to Fig. 1B were used in these experiments.

single channel conductance, which can be obtained from the slope of the linear regions of current versus voltage plots (i.e. only at negative potentials, see Fig. 2B), ranged from 55 to 115 pS in 100 mM chloride symmetrical solutions.

Patches from GlyR-containing giant liposomes which were unresponsive to the presence of agonist in the pipette, or patches not previously exposed to agonist, never responded to addition of glycine to the bath solution, suggesting that glycine binding sites were not exposed to the bath solution in our 'inside-out' excised patches. This is consistent with previous strychnine binding data showing a predominant 'right-side-out' orientation of GlyR in the starting reconstituted vesicles [6], and which must have been preserved during giant liposome formation.

The observation that approximately 48% of the membrane patches investigated were unresponsive to the presence of agonist, could be due to either (i) low density of ion channels in the reconstituted giant liposomes, thus resulting in silent liposome patches lacking functional GlyR or (ii) sensitization of GlyR caused by prolonged exposure of the patch to the agonist [14]. Despite the low agonist concentration used in our experiments (considered by other authors [12,14] as non-desensitizing), the possibility of receptor desensitization (or other processes of receptor inactivation) in our system is more likely than the former explanation, as suggested by the observation of a very high activity in the initial period following seal formation and by the rapid disappearance of initial ion channel activity from several of the patches classified as 'unresponsive'.

Our results showing that glycinergic agonists open chloride channels in giant liposomes containing the GlyR further confirm our previous report [6] that the purified protein contains all the necessary molecular components for ligand binding and channel activation. Moreover, liposome patches responsive to the presence of agonist exhibited an intricate electrical behaviour with multiple conductance states, in agreement with the complex patterns of channel activity previously described in cultured spinal and cerebellar neurons, except that the single channel main conductance values reported in this paper are seemingly larger than most described in vivo [12,14,15].

The striking finding of well-defined type 'I' and type 'II' activities suggest that functionally distinct populations of GlyR could be present in the spinal cord of adult rats. This functional diversity has not been previously reported in electrophysiological measurements made in cultured spinal cord cells, which is probably due to the fact that primary cell cultures tend to preferentially express a neonatal isoform of the GlyR [16]. It can be speculated that the different ion channel activities observed in our reconstituted GlyR system could arise from differences in posttranslational modifications (such as phosphorylation, etc.), or in the arrangement of the 48 kDa and 58 kDa subunits, or in subunit composition (variable subunit stoichiometry or presence of different subunit isoforms) [17]. For instance, the 48 kDa polypeptide is able to form homomeric channels when expressed in oocytes or mammalian cells [18,19], and has also been shown to be phosphorylated in vitro by protein kinase C [20]. Furthermore, recent reports indicate the existence of several different mRNAs encoding subtypes of the 48 kDa GlyR subunit in both the neonatal and the adult rat spinal cord [16,21,22] and two different 48 kDa subunit genes located in different chromosomes have been recently detected in humans [23]. This expected molecular heterogeneity would be similar to the receptor subtype diversity found for other ion channel-linked neuroreceptors homologous to the GlyR, such as the $GABA_A$ and the neuronal nicotinic acetylcholine receptors (see [24] for a review). Further experiments are needed to ascertain the molecular basis of the existence of functionally different forms of the glycine receptor and to fully characterize the electrophysiology of the ionic channels involved.

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