Transmembrane segment M2 of glycine receptor as a model system for the pore-forming structure of ion channels✪

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The glycine receptor belongs to the ligand-gated ion channel superfamily. It is a chloride conducting channel composed of four transmembrane domains. It was previously shown that the second transmembrane domain (M2) of the glycine receptor forms an ion conduction pathway throught lipid bilayers. The amino-acid sequence of the transmembrane segment M2 of the glycine receptor has a high homology to all receptors of the ligand-gated ion channel superfamily. In our report, we have used a synthetic M2 peptide. It was incorporated into a planar membrane of known lipid composition and currents induced by M2 were measured by the Black Lipid Membrane technique. When the planar lipid bilayer was composed of 75% phosphatidylethanolamine and 25% phosphatidylserine, the reversal potential measured in a 150/600 mM KCl (*cis/trans*) gradient was –19 mV suggesting that the examined **pore was preferential to anions, P_K/P_{Cl} = 0.25.** In contrast, when 75% phosphatidylserine and 25% phosphatidylethanolamine was used, the reversal potential was +20 mV and the **pore was preferential to cations, P_K/P_{Cl} = 4.36.** Single-channel currents were recorded with two predominant amplitudes corresponding to the main-conductance and sub-conductance states. Both conductance states (about 12 pS and 30 pS) were measured in a symmetric solution of 50 mM KCl. The observed single-channel properties suggest that the selectivity and conductance of the pore formed by the M2 peptide of the glycine receptor depend on the lipid composition of the planar bilayer.

Abbreviations: BLM, black lipid membrane; GABA$_A$R and GABA$_C$R, γ-aminobutyric acid A and C subtype receptors; GlyR, glycine receptor; 5-HT$_3$R, 5-hydroxytryptamine type 3 receptor; LGICS, ligand-gated ion channel superfamily; M2, transmembrane segment of glycine receptor; nAChR, nicotinic acetylcholine receptor; PE, phosphatidylethanolamine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine); PS, phosphatidylserine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]); TFE, 2,2,2-trifluoroethanol.

Nicotinic acetylcholine receptor (nAChR), γ-aminobutyric acid A and C subtype receptors (GABA$_A$R and GABA$_C$R), glycine receptor (GlyR) and 5-hydroxytryptamine type 3 receptor (5-HT$_3$R) are members of the ligand-gated ion channel superfamily (LGICS). Members of this ion channel superfamily share certain structural and functional properties (Leite & Cascio, 2001; Adcock *et al.,* 1998). Each channel molecule is composed of five subunits. Each subunit has four hydrophobic segments M1–M4. M2 forms a narrow region of the ion conduction pathway (Fig. 1A).

The best-characterized member of this family is the nicotinic acetylcholine receptor, which mediates neurotransmission at vertebrate neuromuscular junctions. It also plays a role at some synapses of the vertebrate central nervous system and in the nervous system of invertebrates (e.g. Nicholls *et al.,* 2001). Both functional and structural studies on nAChR indicate that it undergoes a conformational change upon binding two neurotransmitter (acetylcholine) molecules, switching from a closed to an open state. The open state allows passage of monovalent and divalent cations by way of a pore that runs through the center of the protein. Other LGICS, such as GlyR, are permeable to anions (Kuhse *et al.,* 1995). The anion-selective LGICS mediate inhibitory neurotransmission, whereas the cation selective ones are excitatory. Other members of the superfamily include the anion-selective GABA$_A$R (Sieghart & Sperk, 2002; Sieghart, 1995), GABA$_C$R (Bormann & Feigenspan, 1995) and the cation-selective 5HT$_3$ receptor (Reeves & Lummis, 2002).

The single-channel properties of the glycine receptor have been investigated in detail by patch clamp recordings from embryonic mouse spinal cord neurons in culture (Twyman & Macdonald, 1991). The channel is selective for monovalent anions and exhibits multiple conductance states (Keramidas *et al.,* 2002). A synthetic peptide, with a sequence corresponding to the M2 segment of GlyR,

forms an anion selective pore in lipid bilayers (Reddy *et al.,* 1993; Langosch *et al.,* 1991). The properties of M2 peptide modified by adding four lysine residues to the C-terminus are similar to those described for the parent sequence (Wallace *et al.,* 2000). Single-channel currents induced by the M2 peptide show two predominant amplitudes corresponding to a main conductance states of about 25 pS and 49 pS in symmetric 500 mM KCl with channel open lifetimes in the milliseconds time range (Reddy *et al.,* 1993).

The phospholipid composition of the lipid bilayer plays an important role in membrane transport (Sprong *et al.,* 2001). The charge of the lipid polar groups and ion absorption create electrostatic potential that modulates the concentration of protons, ions, and other charged substrates in the vicinity of the membrane (Nalecz *et al.,* 1980). Understanding these regulatory processes requires knowledge of the lipid polar group structure, organization, and the state of ionization. The membrane surface charge modifies the conductance of multi-state channels formed in planar lipid bilayers by the peptide antibiotic alamethicin (Aguilella & Bezrukov, 2001) and gramicidin A (de Godoy & Cukierman, 2001; Rostovtseva *et al.,* 1998). Fan and Makielski (1997) show that anionic phospholipids activate ATP-sensitive potassium channels. It has also been shown that the conductance of the K$^+$ channel of the sarcoplasmic reticulum increases with an increase in the number of negative charges in the membrane (Anzai *et al.,* 1994).

In our report, the channel activity of a synthetic M2 peptide from the glycine receptor was measured after reconstitution into lipid bilayers. We observed single-channel currents with two predominant amplitudes corresponding to the main-conductance and sub-conductance states. When the planar lipid bilayer was composed of uncharged phospholipids the reversal potential measurements suggested that the examined pore was preferential to anions. In contrast, when negative

phospholipids were used for bilayer formation, the pore induced by the M2 peptide was preferential to cations.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) were from Avanti Polar Lipids Inc. (U.S.A.). 2,2,2-Trifluoroethanol (TFE) and n-decane were from Sigma-Aldrich, Germany. All other chemicals were of the highest purity available commercially.

Peptide synthesis. The M2 peptide was synthesized by the solid-phase method using Fmoc chemistry. The crude peptide was purified on a semipreparative C8 HPLC column. The purity of the peptide was greater than 98%. The synthesized peptide gave the correct molecular mass as measured by mass spectroscopy using the MALDI-TOF technique. The amino-acid sequence of the synthetic M2 peptide used in this study is shown in Fig. 1B. The M2 peptide corresponds to the M2 segment (amino acids 250–272) of the α subunit of the strychnine-sensitive subunit of the inhibitory glycine receptor from human brain.

Black lipid membrane measurements. Planar phospholipid bilayers were formed in a 250 μm diameter hole drilled in a Delrin cup (Warner Instrument Corp., Hamden, CT. U.S.A.), which separated two chambers (*cis* 1 ml and *trans* 1 ml internal volume). The chambers contained 150/600 mM KCl (*cis/trans*), 20 mM Hepes, pH 7.0. The outline of the aperture was coated with a lipid solution and N$_2$ dried prior to bilayer formation to improve membrane stability. Planar phospholipid bilayers were painted using 3:1 or 1:3 mixed solutions of the synthetic lipids PE:PS in n-decane at a final concentration of 25 mg lipid/ml. Stock solutions (1 mg/ml) of M2 peptides in TFE were added to the *trans* compartment. The M2 peptide was incorporated into the black lipid membrane within a few min-

utes. All measurements were carried out at room temperature. Formation and thinning of the bilayer was monitored by capacitance measurements and optical observations. The final accepted capacitance values ranged from 110 to 180 pF. Electrical connections were made by Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. The current was measured using a Bilayer Membrane Amplifier (BLM-120, Bio-Logic).

Data analysis. Signals were filtered at 200 Hz, digitized (A/D converter PowerLab 2/20, ADInstruments) and transferred to a PC or digital tape recorder (DTR-1204, BioLogic) for off-line analysis by Chart v4.1.2 (PowerLab, ADInstruments) and pCLAMP8 (Axon Instruments). Conductance values were calculated from current histograms best fitted by a sum of two Gaussian distributions. The permeability ratios for K^+ and Cl^- were calculated according to the Goldman-Hodgkin-Katz *voltage* equation (Hille, 2001):

$$E_{rev} = -\frac{RT}{zF} \ln \frac{[Cl]_{cis} + {}^{P_K}/_{P_{Cl}}[K]_{trans}}{[Cl]_{trans} + {}^{P_K}/_{P_{Cl}}[K]_{cis}}, \quad (1)$$

where $E_{\rm rev}$ is the potential at which the current is zero, R is the gas constant, T is temperature in Kelvin, $z = -1$ (chloride anion charge), F is Faraday's constant, $P_{\rm ion}$ is the permeability of the ion, and [ion] is the concentration of the ion in the *cis* and *trans* chambers, respectively. We can rewrite Eqn. 1 as

$$ {}^{P_K}/_{P_{Cl}} = \frac{\xi[Cl]_{trans} - [Cl]_{cis}}{[K]_{trans} - \xi[Cl]_{cis}}, \quad (2)$$

where $\xi = \exp\left(-\dfrac{zFE_{rev}}{RT}\right)$.

RESULTS AND DISCUSSION

The glycine receptor is a chloride conducting channel (Twyman & Macdonald, 1991). The

channel molecule is composed of five subunits. Each subunit has four hydrophobic segments M1–M4. The M2 segment forms an ion conduction pathway through lipid bilayers (Reddy *et al.,* 1993; Langosch *et al.,* 1991). It is known that the amino-acid sequence of M2 influences the properties of the pore. Reddy *et al.* (1993) postulated that two arginine residues in the M2 peptide (Fig. 1B) determine

served. Current-time traces were recorded in the 150/600 mM KCl gradient (*cis/trans*) at different voltages (Fig. 2A) with the planar lipid bilayers composed of PE:PS=3:1 phospholipids (number of observations, n = 5). Fig. 2B shows current-voltage curves for single channel openings at different voltages under the same conditions. Solid and dashed lines indicate the main-conductance and sub-con-
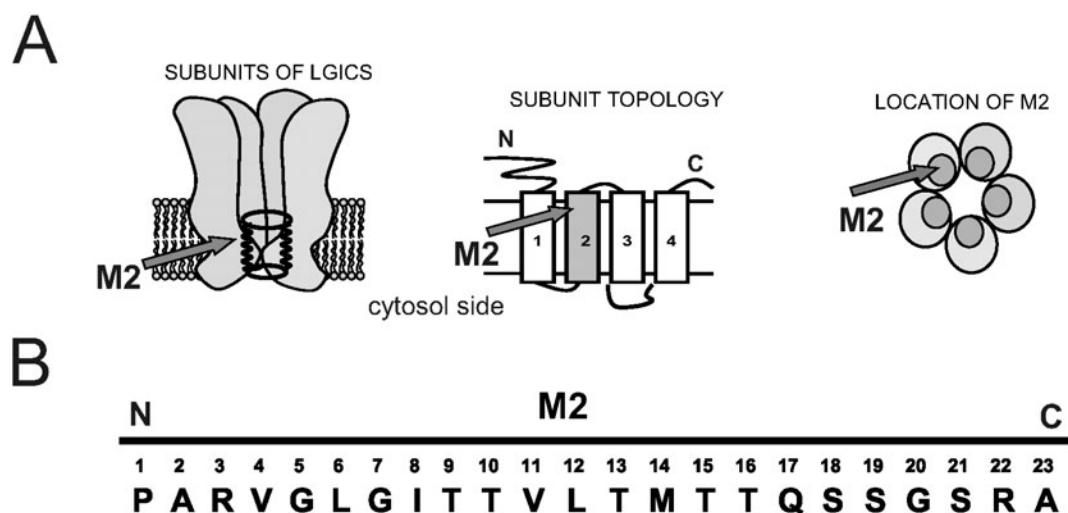

Figure 1. Structure of the ligand-gated ion channel superfamily and amino-acid sequence of M2 peptide.

(A) Five protein subunits of LGICS, the classic model showing transmembrane subunit topology of hydrophobic segments M1–M4 and schematic structure showing the location of M2 lining the pore. (B) Amino-acid sequence of the synthetic M2 peptide of the glycine receptor.

the ion-selectivity of the M2 pore. It has been shown that two arginine residues at the N and C termini of M2 (positions 3 and 22) contribute to the anion selectivity of the channel. M2 forms a cation selective pore when these arginine residues are substituted with glutamic acid (Reddy *et al.,* 1993).

It has been shown that the membrane surface charge modifies the conductance of multi-state channels formed in planar lipid bilayers by the peptide antibiotic alamethicin (Aguilella & Bezrukov, 2001). In our studies, we focused on the influence of the bilayer composition on M2 pore properties.

The synthetic M2 peptide was reconstituted into black lipid membranes and the current characteristic for an ion channel was ob-

ductance states of the M2 pore. The reversal potential measured in the 150/600 mM KCl gradient is equal to –19 mV and this shift proves that the examined pore is anion-selective. The permeability ratio for P_K/P_{Cl} is 0.25. The current-time traces were also recorded in the 150/600 mM KCl gradient (*cis/trans*) at different voltages (Fig. 3A) for membranes containing phospholipids PE:PS = 1:3 (n = 10). Fig. 3B shows current-voltage curves for single channel openings at different voltages under these conditions. Solid and dashed lines indicate the main-conductance and sub-conductance states of the M2 pore. The reversal potential measured in the 150/600 mM KCl gradient is equal to 20 mV and this shift proves that the
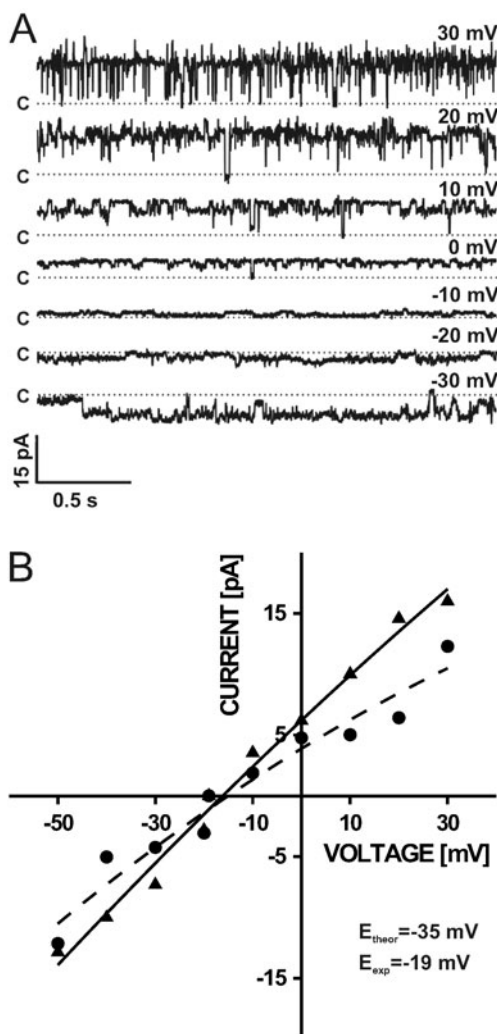
Figure 2. Single-channel recordings of segment M2 pore in 75% PE and 25% PS planar lipid bilayers.

(A) Recordings were made in 150/600 mM KCl gradient (*cis/trans*), 20 mM Hepes, pH 7.0, at different voltages (n = 5). C indicates the closed channel state. (B) Current-voltage characteristics of single-channel events in the M2 pore. Solid and dashed lines indicate substates of the M2 pore. Recordings were low pass filtered at 200 Hz.

examined pore, in contrast to the results obtained from the data in Fig. 2, is cation-selective. The permeability ratio for P_K/P_{Cl} is equal to 4.36. The single-channel currents were recorded with two predominant amplitudes corresponding to the main-conductance and sub-conductance states. Both conductance states (about 12 pS and 30 pS) were measured in a symmetric solution of 50 mM KCl. The planar lipid bilayer was com-
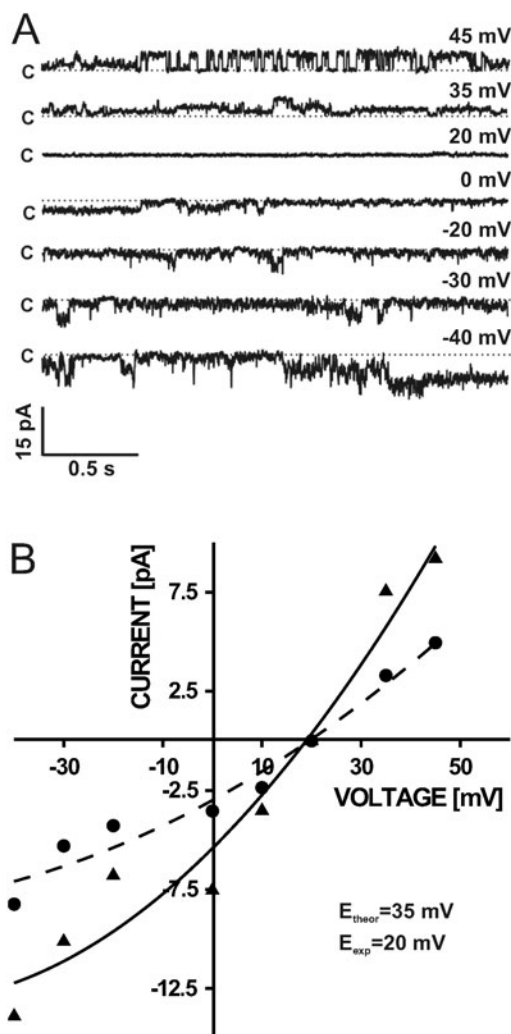

Figure 3. Single-channel recordings of the segment M2 pore in 25% PE and 75% PS planar lipid bilayers.

(A) Recordings were made in 150/600 mM KCl gradient (*cis/trans*), 20 mM Hepes, pH 7.0, at different voltages (n = 10). C indicates closed channel state. (B) Current-voltage characteristics of single-channel events of the M2 pore. Solid and dashed lines indicate substates of the M2 pore. Recordings were low pass filtered at 200 Hz.

posed of 100% phosphatidylserine (not shown).

In summary, we used the M2 peptide of the glycine receptor as a model system for the pore-forming structure of an ion channel. We observed an ionic current and two predominant amplitudes corresponding to the main-conductance and sub-conductance states. The permeability for cations, calculated from the reversal potential measure-

ments with concentration gradients of KCl, is equal to 0.25, indicating that the species carrying most of the current is Cl⁻. In contrast, in negatively charged planar membranes, the M2 pore is a cation-selective channel. The permeability for cations over anions is equal to 4.36. We have shown that the M2 segment incorporated into a lipid bilayer forms a pore with two conducting substates. It was also shown that the selectivity of the pore critically depends on lipid composition. The more net negatively charged lipid molecules are in the bilayer the lower anion and higher the cation concentration is near the membrane surface. The increased concentration of cations near the entrance to the pore increase the probability of cations flowing through the pore. Changing the lipid composition from PE:PS = 3:1 to PE:PS = 1:3 increases the permeability ratio of potassium cations over chloride anions 17.5-fold.

An electrophysiological study in which purified glycine receptor was incorporated into a planar bilayer consisting of a neutral phospholipid (Cascio *et al.,* 2001) and studies of native glycine receptors by the patch-clamp technique (Bormann *et al.,* 1987) showed a functional glycine-gated ion channel. The properties of the ion channel, it is preferential permeability to anions and sub-conductance levels were very similar to our observations.

In conclusion, the results provide evidence that the composition of the lipid bilayer is an important factor determining the ion selectivity of the M2 peptide. The electrostatic effect of charged lipid molecules on the pore formed by M2 seems to be similar to the effect of charged amino-acid residues on the ion selectivity of channels of the LGICS family, i.e. net negatively charged channels are cation specific and net positively charged channels are anion specific. In support of this hypothesis, the net charge of the cation specific nAChR-gated is (−69) elementary charges (Karlin, 1993), the anion specific GABA$_A$R-gated is (+25) (Tyndale *et al.,* 1995),

and the anion specific GlyR-gated channel is (+60) (Langosch, 1995).

It was previously shown that anion channel blockers, 9-anthracene carboxylic acid (9-AC) and influmic acid (NFA), blocked the activity of the anion selective T$_4$M2 pore but the anesthetic lidocaine (QX-222), a cation channel blocker (Reddy *et al.,* 1993), did not. It will be important to establish whether 9-AC and NFA influence the channel activity of the M2 peptide under anion selective and cation selective conditions. Moreover, it was shown, using circular dichroism spectra, that the M2 peptide was able to change its structure in various mixtures of trifluoroethanol and phosphate buffer (Langosch *et al.,* 1991). It is likely that changes in the secondary structure of the M2 peptide in lipid bilayers are able to influence the pore selectivity.

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