Comparative Biochemistry and Physiology, Part C 149 (2009) 275–280

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Characterization of ionotropic glutamate receptors in insect neuro-muscular junction

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ARTICLE INFO

Article history:
Received 4 June 2008
Received in revised form 28 July 2008
Accepted 29 July 2008
Available online 31 July 2008

Keywords:
Calliphora vicina
Ionotropic glutamate receptors
AMPA receptors

ABSTRACT

Pharmacological properties of ionotropic glutamate receptors from *Calliphora vicina* larvae neuro-muscular junction (*C. vicina* iGlurs) were studied by two-electrode voltage-clamp technique. Characteristics of the ion channel pore were analyzed using a 26-member series of channel blockers, which includes mono- and dicationic derivatives of adamantane and phenylcyclohexyl. Structure–activity relationships were found to be markedly similar to the Ca^{2+}-permeable AMPA receptors (AMPAR) but not NMDA receptors (NMDAR) channel subtype seen in vertebrates. Like AMPARs the channels of *C. vicina* iGlurs are sensitive mainly to dicationic compounds with 6–7 spacers between hydrophobic headgroup and terminal aminogroup. Study of the voltage dependence of block demonstrated that, like AMPARs, the *C. vicina* iGlur channels, are permeable to organic cations with dimensions exceeding 10 Å. Concentration dependence of block suggests the presence of two distinct channel populations with approximately 20-fold different sensitivity to cationic blockers. The recognition domain properties are more complex. Besides glutamate, the channels can be activated by kainate, quisqualate and domoate. Competitive antagonists of AMPAR and NMDAR are virtually inactive against the *C. vicina* iGlurs as well as allosteric modulators GYKI 52466 and PEPA. Surprisingly, the responses were potentiated 3 times by 100 mkM of cyclothiazide. We conclude that the channel-forming domain of *C. vicina* iGlurs is AMPAR-like, whereas the recognition domain is specific.

1. Introduction

Ionotropic glutamate receptors (iGlurs) play a key role in excitation processes in the CNS of vertebrates. It is well known that different molecular forms of glutamate receptors possess markedly different physiological and pharmacological properties (see Dingledine et al., 1999 for review) that in their turn, significantly affect synaptic characteristics. For example, voltage-dependent block of NMDA receptor (NMDAR) channels by Mg^{2+} (Nowak et al., 1984; Mayer et al., 1984) is important for triggering long-term potentiation (Herron et al., 1986). Differences in Ca^{2+} permeability between GluR2-lacking and GluR2-containing AMPA receptors (AMPAR) are also important for synaptic plasticity (Jia et al., 1996; Liu and Cull-Candy, 2000). EPSC decay and thus, the carried charge (Lomeli et al., 1994; Mosbacher et al., 1994), depend on whether the AMPAR contains the flip or flop versions of subunit (Sommer et al., 1990). Characteristics of glutamate receptors of vertebrates were studied intensively and large progress was achieved due to combined efforts of many groups employing methods of electrophysiology, molecular pharmacology, molecular and structural biology, and computer modeling.

In contrast, much less is known about properties of glutamate receptors of invertebrates. The genome of the fruit fly contains 30 putative glutamate receptors (Littleton and Ganetzky, 2000). According to the modern view, receptors of neuro-muscular junction are composed of the DGluR3, DGluR2D, DGluR2E subunits and either DGluR2A or DGluR2B subunit (review by DiAntonio, 2006). DGluR2A-containing and DGluR2B-containing iGluRs differ in desensitization and sensitivity to polyamine toxin, but molecular determinants of this difference remain unknown (DiAntonio et al., 1999). Heteromeric subunit composition of *Drosophila* iGlurs differs markedly from vertebrate iGlurs which are homomers or "dimers of dimers" (Safferling et al., 2001). This could determine some of the difference in properties between invertebrate and vertebrate iGlurs.

Comparison of vertebrate and invertebrate receptors is a promising approach for understanding molecular evolution of the receptor protein and evolution of synaptic transmission mechanisms. Such comparison may also help to obtain more detailed insights into functioning of ligand-gated channels. There are some intriguing differences between amino-acid sequences of *Drosophila* and vertebrate iGlurs, in the ion channel as well as in the recognition domain. For instance, DGluR3 subunit contains the TA motif instead of the QQ motif typical for the AMPARs selectivity filter. DGluR2B subunit contains a Lys residue in the M3 segment, which is atypical for pore-lining segments of cation channels. In DGluR2A subunit the M3 segment contains a unique deletion.

The main aim of present study was to characterize post-synaptic glutamate receptors in the neuro-muscular junction of *Calliphora vicina* larvae and to compare them with the vertebrate AMPAR and NMDAR. Our laboratory developed a series of organic channel blockers that discriminate NMDAR and AMPAR channels and allow us to characterize topography of the binding sites in these channels (Bolshakov

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doi:10.1016/j.cbpc.2008.07.010

et al., 2005). With the help of these tools we demonstrated that the ion channel structure of *Calliphora* iGlurs is remarkably similar to that of Ca^{2+}-permeable AMPAR channels. In contrast to this, recognition domain of the receptor demonstrates specific properties with no direct analogs in vertebrate receptors.

2. Materials and methods

2.1. Electrophysiology

Late third stage larvae of *C. vicina* (Diptera: Calliphoridae) were used. After dissection, the internal organs were removed, so that the preparation consisted only of muscles attached to the cuticle. The ventral ganglion was excised and the segmental nerves were stimulated through the suction electrode. Recordings were made from ventral longitudinal fibers. To eliminate electrical contacts of the recorded fiber with its neighbors, the latter were dissected. This resulted in significant increase of input resistance in the recorded cell. The preparation was perfused with a saline solution containing (in mM): 172 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 8.0 $MgCl_2$, 2.4 $NaHCO_3$, 0.3 H_2PO_4, and 52 sucrose. pH was adjusted to 7.2 with NaOH or HCl. Experiments were performed at room temperature (20–24 °C). The excitation post-synaptic currents (EPSC) were evoked by nerve stimulation and recorded by a conventional two-electrode voltage clamp using Axoclamp 2B (Axon Instr.) amplifier. The data were filtered at 2 kHz and stored on the computer. Drugs were purchased from Sigma and Tocris. The IEM compounds were synthesized by Dr. V. Gmiro at the Institute of experimental medicine RAMS, St. Petersburg.

2.2. Data analysis and statistics

To avoid signal-to-signal fluctuations, signals were digitally averaged from 10 consecutive responses before analysis. To minimize the influence of fluctuations in synaptic delay, the signals were adjusted by the midpoint of their rise. This provided good agreement between the shape (rise and decay) of individual and averaged signals. The concentration dependence of block was analyzed by the classical Hill equation

$$\text{Block}(\%) = 100/(1 + (C/\text{IC}_{50})^n) \quad (1)$$

or by the model of binding of drug molecules to two distinct populations of receptor

$$\text{Block}(\%) = A/(1 + C/\text{IC}_{50A}) + (100-A)/(1 + C/\text{IC}_{50B}). \quad (2)$$

The concentration dependencies were analyzed separately for each cell, and fitting data were then averaged. This was necessary because of cell to cell variations of relative abundance of the receptor populations. Pooling of experimental data significantly distorted the results.

All data are presented as means ± SD from at least five experiments. Significance of the effects was assessed by one-way ANOVA test and considered significant when the p value was less than 0.05.

3. Results

Ionotropic receptors are composed of two principal domains, an intramembrane pore-forming domain and an extracellular recognition domain that controls the opening of the pore. Properties of these domains are largely independent and can be studied by use of specific series of ligands.

3.1. Characterization of the ion channel

An important feature of the NMDAR ion channel is the voltage-dependent block by Mg^{2+}. This effect causes non-monotonous behavior of *I–V* curve at negative voltages (Nowak et al., 1984; Mayer et al., 1984). However, in our experiments with *C. vicina* iGlurs, this effect was not observed. *I–V* curve deviated from linearity only at holding potentials more negative than −100 mV but still remained monotonous.

Organic open-channel blockers serve as powerful tools for analysis of spatial structure of ion channels. Our studies of the structure–activity relationships (Bolshakov et al., 2000, 2003, 2005) in series of channel blockers made possible to develop topographical and molecular models of AMPAR and NMDAR channels (Tikhonov et al., 2002; Tikhonov, 2007). We selected 26 representative mono- and dicationic derivatives of adamantane and phenylcyclohexyl for the present comparative study. Blocking activities of these compounds were estimated by constructing concentration dependencies of their action as described in the Materials and methods section. The results are summarized in Fig. 1. Quantitatively, *C. vicina* iGlurs sensitivity to blockers differs significantly from both NMDAR and Ca^{2+}-permeable AMPAR. However, there is a strong correlation between *C. vicina* iGlurs and AMPAR sensitivity to the compounds used. Moreover, all key fingerprint features of AMPAR (see Bolshakov et al., 2005) are well reproduced. First, dicationic compounds are much more effective than their monocationic analogs. Second, the most active dicationic compounds have 6–7 spacers between hydrophobic head and terminal ammonium group. Third, trimethylammonium terminal group provides better activity than aminogroup. These results show strong similarity in molecular organization of the channel binding site in vertebrate AMPARs and *C. vicina* iGlur.

It is known that pentobarbital effectively inhibits Ca^{2+}-impermeable (GluR2-containing) AMPAR of vertebrates whereas GluR2-lacking receptors are much less sensitive to it (Taverna et al., 1994; Yamakura et al., 1995). However, it is still unclear if pentobarbital acts as a

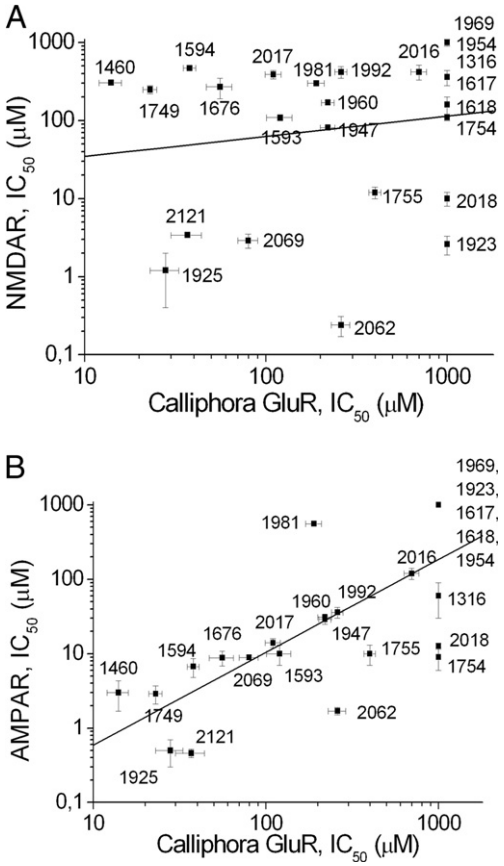

Fig. 1. Correlations between sensitivity of *C. vicina* iGlurs and vertebrate NMDA (A) and Ca^{2+}-permeable AMPA (B) receptors to mono- and dicationic blockers. Significant correlation (0.78) is observed only with AMPA receptors.