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Evaluation of $GluR_2$ subunit involvement in AMPA receptor function of neonatal rat hypoglossal motoneurons

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Abstract

AMPA receptors (AMPAr) mediate fast synaptic responses to glutamate and, when they lack the $GluR_2$ subunit, are strongly Ca^{2+} permeable and may increase intracellular Ca^{2+} levels. Because hypoglossal motoneurons possess restricted ability to buffer internal Ca^{2+} and are vulnerable to Ca^{2+} excitotoxicity, we wondered if, in these cells, any significant Ca^{2+} influx could be generated via AMPAr activity. Using whole cell patch-clamp recording from neonatal rat hypoglossal motoneurons, we tested the AMPAr properties conferred by $GluR_2$ subunits, namely Ca^{2+} permeability, current rectification and sensitivity to pentobarbital or to the subunit-specific channel blockers, IEM-1460 and IEM-1925. We recorded membrane currents generated by the agonist, kainate, and compared them with those obtained from hippocampal pyramidal neurons (expressing $GluR_2$-containing AMPAr) and from striatal giant aspiny or hippocampal interneurons (with $GluR_2$-lacking AMPAr). Ca^{2+} vs. Na^+ permeability of motoneuron AMPAr was relatively low (0.25 $\pm$ 0.05), although higher than that of pyramidal neurons. With intracellularly applied spermine, significant inward rectification was absent from motoneurons. These data indicated the prevalence of functional $GluR_2$ subunits. However, the sensitivity of motoneuron AMPAr to pentobarbital did not differ from that of $GluR_2$-lacking AMPAr on interneurons. Motoneurons possessed sensitivity to IEM-1460 (IC_{50} = 90 $\pm$ 10 μM) approximately 10-fold lower than striatal interneurons, although 10-fold higher than hippocampal pyramidal cells. IEM-1925 also reduced the amplitude of excitatory synaptic currents in brainstem slice motoneurons. We hypothesize that hypoglossal motoneuron AMPAr (moderately Ca^{2+} permeable because they contain few GluR2 subunits) may contribute to intracellular Ca^{2+} rises especially if persistent AMPAr activation (or the pathological $GluR_2$ down-regulation) occurs.

Introduction

On most central neurons, the AMPA subtype of ionotropic glutamate receptors (AMPAr) mediates the fast component of excitatory synaptic transmission. As AMPAr are heterogeneous in terms of subunit structure, their various composition may be responsible for functionally distinct properties (for review see Dingledine *et al*., 1999). In particular, when AMPAr contain $GluR_2$ subunits, they display small channel conductance, small permeability to divalent cations and low sensitivity to cationic channel blockers like, for instance, spermine or some polyamines. AMPAr lacking $GluR_2$ subunits possess opposite properties, the most striking of which is high Ca^{2+} permeability. Naturally occurring polyamines (spermine, spermidine and putrescine) block $GluR_2$-lacking AMPAr intracellularly (at positive membrane potentials), thus, producing the inward current rectification typical of such channels (Bowie & Mayer, 1995; Donevan & Rogawski, 1995; Koh *et al*., 1995). Extracellularly applied polyamines (and related substances), and dicationic adamantane or phenylcyclohexyl derivatives selectively block inward currents through $GluR_2$-lacking AMPAr channels (Herlitze *et al*., 1993; Magazanik *et al*., 1997; Washburn *et al*., 1997; Tikhonov *et al*., 2000). Conversely, pentobarbital preferentially blocks $GluR_2$-containing rather than $GluR_2$-lacking AMPAr (Yamakura *et al*., 1995).

As expression of the $GluR_2$ subunit is cell specific within the mammalian brain (Geiger *et al*., 1995; Isa *et al*., 1996; Petralia *et al*., 1997), its differential topography might impart special characteristics to the intracellular Ca^{2+} homeostasis of certain neurons. When compared with other brain cells, the Ca^{2+} buffer capacity of hypoglossal motoneurons (HM) is very low (Lips & Keller, 1998), a factor which may confer them selective vulnerability to excitotoxic events (Krieger *et al*., 1994; Reiner *et al*., 1995). Glutamate excitotoxicity is Ca^{2+}-dependent and contributes to certain pathologies of the nervous system (for review see Pellegrini-Giampietro *et al*., 1997). In this sense Ca^{2+} permeability of HM AMPAr and, more generally, abundance of $GluR_2$ on HM can play a role in determining how much free Ca^{2+} enters through open AMPAr. Low staining levels with a $GluR_2$-specific antibody led Del Cano *et al*. (1999) to propose that, on HM, most AMPAr are Ca^{2+} permeable. Conversely, Paarmann *et al*. (2000), using RT-PCR methods, found strong expression of $GluR_2$ subunits in HM and concluded that these cells possess mainly Ca^{2+} impermeable AMPAr (Paarmann *et al*., 2000). Nonetheless, no direct measurement of Ca^{2+} permeability of HM AMPAr has been reported so far.

The present study functionally characterized the relative contribution played by $GluR_2$ subunit containing receptors to the overall currents mediated by AMPAr of freshly isolated HM. For this

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purpose we used four functional tests: (i), Ca^{2+} permeability; (ii), inward rectification; (iii), block by pentobarbital, and (iv), block by the dicationic compounds IEM-1460 and IEM-1925. The results of these tests were compared with those obtained from neurons with known AMPAr subunit composition, namely giant aspiny interneurons of the striatum or the hippocampus (expressing $GluR_2$-lacking AMPAr; Samoilova *et al*. 1999; Vorobjev *et al*., 2000), and hippocampal pyramidal neurons (expressing GluR2-containing AMPAr; Burnashev *et al*., 1992; Geiger *et al*., 1995). The action of IEM-1925 (50 μM) was also studied on synaptically evoked excitatory postsynaptic currents (EPSC) in hypoglossal slices.

Materials and methods

Slice preparation and cell isolation

Brainstem slices were prepared from 3–9 day-old Wistar rats. Slices of hippocampus and striatum were prepared from 7–14 day-old Wistar rats. Rats of both sexes were decapitated under urethane anaesthesia in accordance with the European Communities Council Directive (24th November 1986; 86/609/EEC) and approved by the Italian Veterinary Authority. The brain was removed, immersed in ice-cold medium and cut into slices (300 μm thick) with a vibratome (Vibroslice-752M, Campden Instruments). The slices were incubated in external solution saturated with 95% O_2 and 5% CO_2 for 1–6 h at room temperature (22–24 °C). Single slices were transferred to a recording chamber. The cells were isolated mechanically (without any enzymatic treatment of the tissue; Vorobjev, 1991) using a small vibrating (50–100 Hz) glass sphere placed close to the surface of the slice. This method allowed cell isolation from a restricted area of the slice under visual control.

Cell identification

Cells were identified on the basis of their morphological features. For isolated hippocampal neurons and striatal giant aspiny interneurons, the identification procedure was described previously (Samoilova *et al*., 1999). Cells isolated from the XII cranial nucleus were subdivided into two groups according to their morphological properties in analogy with the two types of hypoglossal motoneurons described recently as type I and II (Fukunishi *et al*., 1999). For our experiments we selected mainly the cells similar to type I HM.

Patch-clamp recording

The whole-cell configuration of the patch-clamp recording technique was employed with the use of an Axopatch 200A (Axon Instruments). Patch electrodes pulled from borosilicate glass (Clark Electromedical Instruments) had resistances of 2–5 MΩ. The current signals were filtered at 5 kHz, digitized and stored on a personal computer for 'on-line' and 'off-line' analysis using custom-made in-house programs (PATCH3, RAMP, RITM, made by D.B. Tikhonov, Sechenov Institute, St Petersburg).

Recording from brainstem slices

Thin (300 μm) slices of rat brain stem were prepared following the procedure described by Viana *et al*. (1994) and by Lape & Nistri (1999) and placed in a recording chamber superfused continuously (2–5 mL/min) with oxygenated solution as reported by Donato & Nistri (2000). Single hypoglossal motoneurons within the XII nucleus were visually identified in the slice by using an infrared video camera and whole-cell patch-clamped. Electrically evoked currents were elicited by extracellular stimulation (0.2 Hz; 0.2 ms; variable intensity) of afferent fibers by placing a single bipolar tungsten electrode in the lateral reticular formation (Donato & Nistri, 2000). After stabilization of the synaptic response, stimulus intensity was adjusted to obtain suprathreshold responses which were then stored in a PC as individual files and averaged.

Solutions and drugs

The medium for slice cutting and incubation contained (in mM): NaCl, 124; KCl, 5; $CaCl_2$, 1.3; $MgCl_2$, 1.5; $NaHCO_3$, 26; NaH_2PO_4, 1.24 and D-glucose, 10 (pH 7.4–7.5). The standard Na^+ solution for cells dissociation and recording had the following composition (in mM): NaCl, 143; KCl, 5; $CaCl_2$, 2.7; D-glucose, 18 and HEPES, 10 (pH adjusted to 7.4 with HCl). The Na^+-free, high Ca^{2+} solution contained (in mM): $CaCl_2$, 100; *N*-methylglucamine, 45; KCl, 5; D-glucose, 18 and HEPES, 10 (pH adjusted to 7.4 with HCl). The pipette solution for isolated neurons contained (in mM): CsF, 100; CsCl, 40; NaCl, 5; $CaCl_2$, 0.5; EGTA, 5 and HEPES, 10 (pH adjusted to 7.2 with CsOH). Spermine (100 μM) was added to the pipette solution only for the experiments carried out to test rectification in order to prevent 'washout' of this process. The pipette solution for motoneuron slice recording contained (in mM): K-methyl-SO_4, 110; KCl, 20; NaCl, 10; $MgCl_2$, 2; HEPES, 10; EGTA, 0.5; ATP-Mg, 2 (pH 7.2 adjusted with KOH). All experiments were carried out at room temperature (22–24 °C).

Drugs were applied using a fast perfusion technique (Vorobjev *et al*., 1996). Drugs used were kainate, GYKI-52466, spermine, pentobarbital, D-aminophosphonic valerate: all Sigma; cyclothiazide (Tocris), IEM-1460, which is the adamantane derivative [1-trimethylammonio-5-(1-adamantane-methylammoniopentane) dibromide], and IEM-1925, which is *N*-(5-aminopentyl)-1-phenylcyclohexylamine dibromide. The latter two compounds were synthesized by Dr V.E. Gmiro (Institute of Experimental Medicine, Russian Academy of Medical Sciences, St.-Petersburg). Stock solutions of GYKI-52466 (10 mM) and cyclothiazide (100 mM) were dissolved in dimethyl sulfoxide (DMSO, ICN). The maximal final DMSO concentration never exceeded 0.1% and control experiments showed any DMSO effect to be negligible.

Data analysis and statistics

Dose–response curves for agonist-mediated responses were fitted with the following logistic equation:

$$R = R_{max}/\{1 + (EC_{50}/[A])^n\} \quad (1)$$

where R_{max} is the response to a saturating concentration of agonist; EC_{50}, the agonist concentration producing half-maximal response; $[A]$, the agonist concentration; n, the Hill coefficient.

The inhibition of agonist-activated currents by IEM-1460 or pentobarbital were fitted with the equation:

$$I = I_{max}/\{1 + (IC_{50}/[B])^n\} \quad (2)$$

where I_{max} is the current inhibition by a saturating concentration of the blocking drug; IC_{50}, the blocking drug concentration producing 50% inhibition; $[B]$, the concentration of the blocking drug; n, the Hill coefficient.

The relative Ca^{2+} permeability was determined from the reversal potential (V_{rev}) values obtained in standard Na^+ or Na^+-free (100 mM Ca^{2+} containing) solutions using the following equation (Geiger *et al*., 1995):

$$P_{Ca}/P_{Na} = 0.25 \times (a_{Na}/a_{Ca}) \times \{\exp[(2V_{revCa} - V_{revNa}) \times F/RT] + \exp[(V_{revCa} - V_{revNa}) \times F/RT\} \quad (3)$$

TABLE 1. Characterization of AMPA receptors of isolated hypoglossal motoneurons, hippocampal pyramidal neurons and striatal giant aspiny or hippocampal interneurons

Cell type	P_{Ca}/P_{na}	(*n*)	Rectification index (*RI*)	(*n*)	Inhibition (%) with 100 μM pentobarbital	(*n*)	IEM-1460 IC_{50} (μM)	(*n*)	IEM-1925 IC_{50} (μM)	(*n*)
Hypoglossal motoneurons	0.25 ± 0.05†	(6)	1.1 ± 0.1‡	(8)	33 ± 5	(11)	90 ± 10	(6)	51 ± 4	(7)
Hippocampal pyramidal neurons	0.1 ± 0.01**	(22)	1.29 ± 0.06**	(22)	81 ± 4	(5)	1102 ± 596*	–	640 ± 200***	–
Striatal and hippocampal Interneurons	1.01 ± 0.14**	(16)	0.37 ± 0.03**	(16)	37 ± 12	(5)	2.6 ± 0.4*	–	3.0 ± 0.7***	–

†$P < 0.02$, ‡$P > 0.5$. *Data from Buldakova *et al*., (1999), **data from *et al*., (1999), ***data from Tikhonov *et al*., (2000). Values represent means ± SEM from *n* cells as indicated in parentheses. Note that the P_{Ca}/P_{Na} value of hypoglossal motoneurons is significantly different from the one of pyramidal neurons while the rectification index is similar for both cell types.

where a_{Na} and a_{Ca} represent the activities of Na^+ and Ca^{2+} in the extracellular solutions, respectively, and R, T and F have their conventional meaning. Standard values for activity coefficients were used (NaCl, 0.76; $CaCl_2$, 0.518).

The rectification index (RI) was calculated according to the following equation (Isa *et al*., 1996):

$$RI = [I_{+40}/(40 - V_{revNa})]/[I_{-60}/(-60 - V_{revNa})] \quad (4)$$

where $I_{+40\ and}\ I_{-60}$ are the amplitudes of agonist-induced currents at +40 and –60 mV, respectively, and V_{revNa} is the reversal potential in standard Na^+ solution. Data are presented as mean ± SEM for *n* experiments. Statistical significance of the effects was tested by one-way ANOVA with P = 0.05.

Results

Activation of AMPA receptors

Steady-state responses of AMPAr to AMPA are small due to their profound desensitization (Partin *et al*., 1993). For this reason we used the agonist kainate as a relatively low-desensitizing agonist on AMPAr (Kiskin *et al*., 1986; Mayer & Vyklicky, 1989; Huettner, 1990). On acutely isolated HM (clamped at –80 mV) kainate induced concentration dependent inward currents (Fig 1A and B). Fitting data with Eqn 1 gave an EC_{50} value of 110 ± 11 μM with an apparent Hill coefficient of 1.7 ± 0.2 (*n* = 7). These currents were due mainly to the activation of AMPAr rather than kainate receptors, because GYKI-52466 (30 μM), the selective AMPAr antagonist (Paternain *et al*., 1995, Wilding & Huettner, 1995; Paternain *et al*., 1996) blocked them by 92 ± 0.7% (*n* = 6) (see example in Fig. 1A). Co-application of 100 μM kainate and 100 μM cyclothiazide increased the kainate current amplitude by 3.5 ± 0.7 times (*n* = 8; not shown), confirming that the pharmacology of these responses was typical of currents mediated by AMPAr (Paternain *et al*., 1995, 1996) comprising the flop version of these receptors (Partin *et al*., 1993). For our further experiments, we used 100 μM kainate on neurons clamped at –80 mV except for experiments with *I–V* relations.

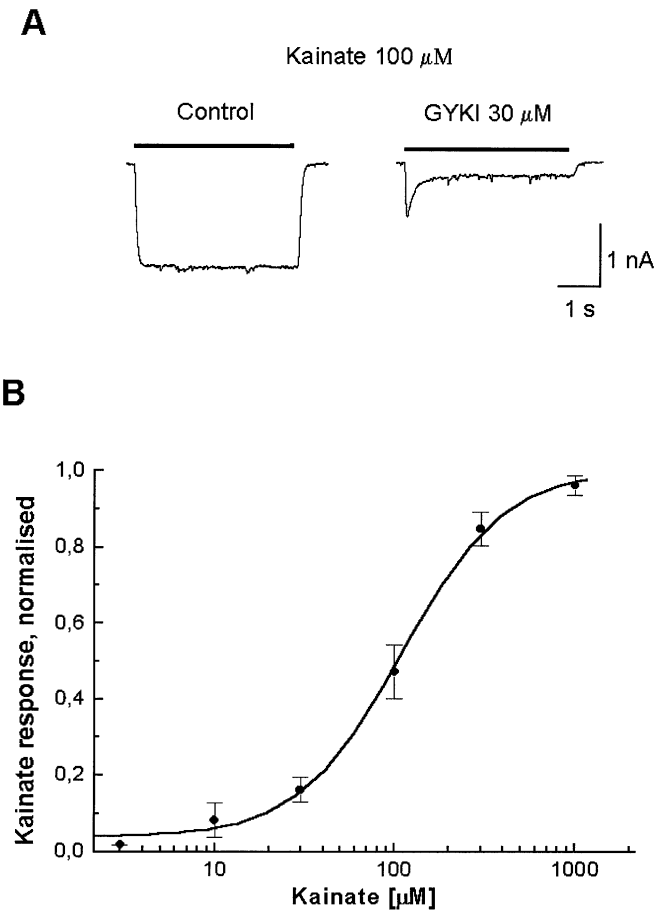

FIG. 1. Kainate induced responses of HM AMPAr. (A) Responses evoked by application of kainate (100 μM) are blocked by selective AMPA antagonist, GYKI-52466 (30 μM). (B) Concentration-dependence of kainate-activated whole-cell currents from isolated HM. Data fitting with Eqn 1 gave an EC_{50} = 110 ± 11 μM (mean ± SEM) with an apparent Hill coefficient of 1.7 ± 0.2 (mean ± SEM; *n* = 7).

Tests for the presence and relative contribution of $GluR_2$ subunits

The results from four tests for the presence of $GluR_2$ subunits in AMPAr of HM are summarized in Table 1. For comparison, our previous data (Buldakova *et al*., 1999; Samoilova *et al*., 1999; Tikhonov *et al*., 2000) from hippocampal pyramidal cells (with $GluR_2$-containing AMPAr), and from hippocampal and striatal giant cholinergic interneurons (lacking $GluR_2$ type AMPAr) are also shown in Table 1. Because responses of striatal and hippocampal interneurons were very similar, they were pooled together. Additionally,

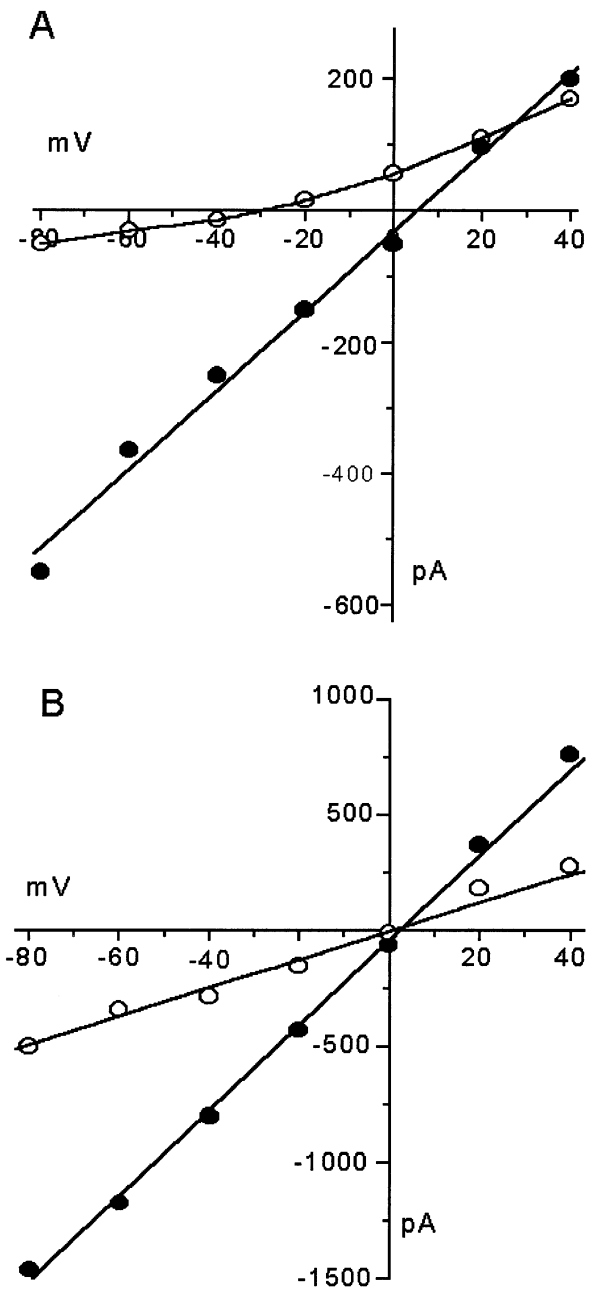

FIG. 2. Electrophysiological evidence for $GluR_2$ subunit containing AMPAr in HMs. (A) Representative example of *I–V* relations of kainate-evoked currents in Na^+-containing (filled circles) and in Na^+- free/100 mM Ca^{2+} external solution (open circles). Pronounced negative shift of V_{rev} in the latter case suggests low Ca^{2+} permeability. (B) Representative example of *I–V* relations of kainate-evoked currents in the presence of intracellular spermine (100 μM). In control conditions (filled circles) as well as in the presence of 100 μM pentobarbital (open circles), *I–V* relations are apparently linear.

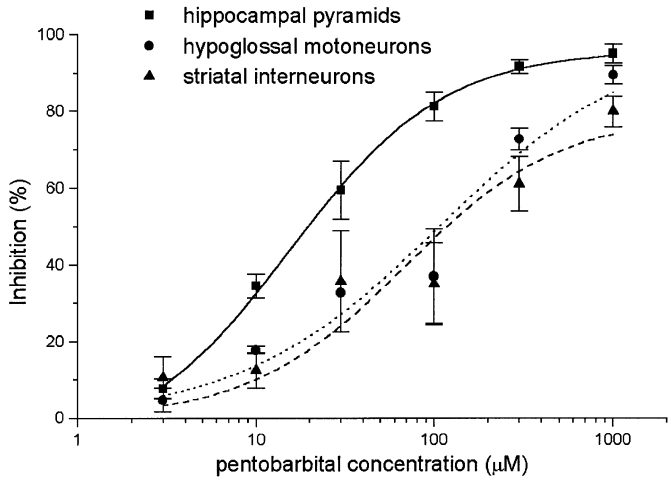

FIG. 3. Sensitivity of isolated HM, hippocampal pyramidal neurons or striatal giant aspiny interneurons to the blocking action of pentobarbital. Note that the dose–response curve for hippocampal pyramidal cells could be adequately fitted by Eqn 2. Conversely, the concentration-dependence of pentobarbital effect on striatal interneurons and HM could not fitted by the Eqn 2 and displayed a biphasic profile. Sensitivity of HM to pentobarbital is close to that of striatal interneurons. Data are means ± SEM; n = 3–5 neurons.

we also tested the inhibition by pentobarbital of AMPAr in hippocampal pyramidal cells and in striatal interneurons as this information was not available. Because all the data presented in Table 1 were obtained using the same electrophysiological methods under the same experimental conditions, it was possible to make quantitative comparisons amongst various cell types.

Relative Ca^{2+} permeability (P_{Ca}/P_{Na}) of HM AMPAr was determined from the V_{rev} shift of kainate-induced currents recorded in standard Na^+ or Na^+-free solutions. In six out of seven cells, a significant negative shift (from 4 ± 2 to –24 ± 2 mV; n = 6) of V_{rev} was detected as demonstrated by the example in Fig. 2A. The calculated P_{Ca}/P_{Na} value was closer to that obtained for pyramidal cells than for interneurons (see Table 1). Thus, according to our results, AMPAr of HM have relatively low Ca^{2+} permeability although significantly larger than the one found in pyramidal neurons.

In standard extracellular solution, all HM had linear or slightly rectifying *I–V* dependence in spite of the presence of spermine in the patch pipette as exemplified in Fig. 2B. Like the result concerning Ca^{2+} permeability, the rectification index for HM came closer to that of hippocampal pyramidal cells than of interneurons (Table 1). Taken together, these two tests argue for the presence of $GluR_2$ subunits in AMPAr of HM.

Pentobarbital induced a concentration dependent inhibition of kainate-evoked currents in all three cell types (Fig. 3). The action of pentobarbital was not voltage-dependent as shown in Fig. 2B. The IC_{50} value of pentobarbital block on hippocampal pyramidal cells was 21 ± 2 μM (n = 5). If we compare cell sensitivity to the same concentration of pentobarbital (100 μM), normally used for testing AMPAr properties, Table 1 indicates that AMPAr of HM did not differ from $GluR_2$-lacking AMPAr of striatal interneurons while they differed from $GluR_2$-containing AMPAr of hippocampal pyramidal cells. Thus, the results of this test show a relatively larger difference between HM and pyramidal cells than that shown by the previous two tests (Ca^{2+} permeability and rectification index).

It is worth noting that, as shown in Fig. 3, the data points pertaining to the inhibitory action by pentobarbital on striatal interneurons and HM were poorly fitted by a single sigmoidal function (using Eqn 2). This observation might suggest either distinct subpopulations of AMPAr, with differential sensitivity to pentobarbital, or a complex mechanism of action by pentobarbital on the AMPAr expressed in HM and striatal interneurons.

If receptor heterogeneity due to pleiotropic subunit compositions were indeed present, other AMPAr antagonists should likewise help to differentiate between distinct subpopulations and generate multi-sigmoidal dose–response plots. To examine this issue, we studied, on

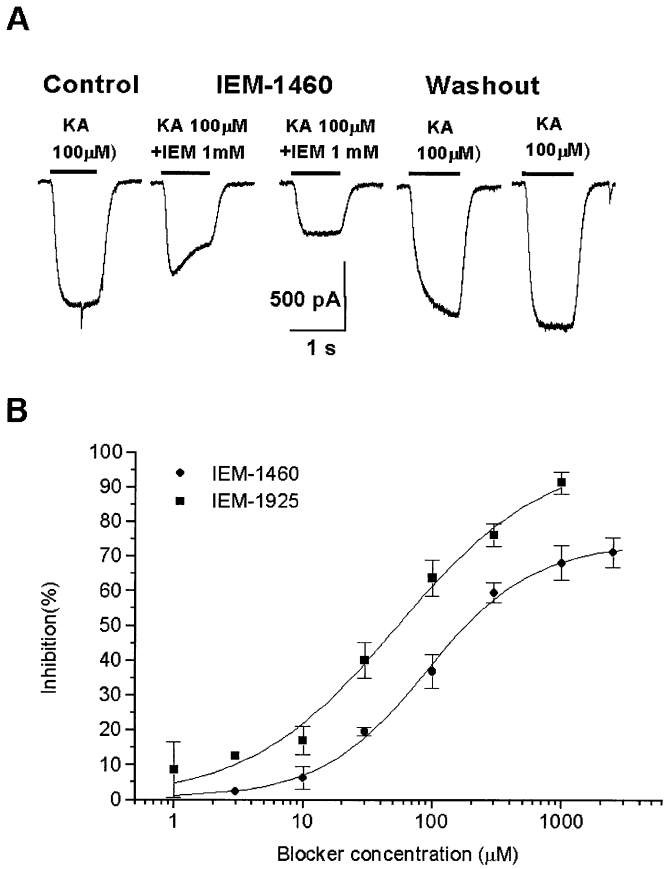

FIG. 4. Sensitivity of isolated HM to the blocking action of IEM-1460 and IEM-1925. (A) The onset of (and recovery from) blocking effect of IEM-1460 (1 mM) on currents evoked by application of 100 μM kainate. Steady-state block and complete drug washout are reached with 3–5 consecutive applications spaced at 10 s intervals. (B) Concentration dependence of IEM-1460 and IEM-1925 block of kainate-evoked responses in HM. Data are means ± SEM; n = 3–6 neurons.

HM, how the AMPAr subunit selective drugs, IEM-1460 and IEM-1925, affected kainate-induced currents. Figure 4A shows the blocking action by IEM-1460 (used at the receptor saturating concentration of 1 mM; Magazanik *et al*., 1997; Samoilova *et al*., 1999) when it was coapplied with kainate. The first response in the presence of the antagonist was less reduced than the second one when the current reached plateau level. Rapid recovery was obtained on washout of the drug. The concentration-dependent inhibition by IEM-1460 and IEM-1925 is depicted in Fig. 4B. Note that, unlike pentobarbital (Fig. 3), either drug produced a similarly shaped sigmoidal plot characterized by analogous Hill coefficients of 0.94 ± 0.21 (for IEM-1925) and 1.07 ± 0.14 (for IEM-1460). As shown by the IC_{50} values in Table 1, the sensitivity of HM AMPAr to IEM-1460 and IEM-1925 was intermediate between the one of interneurons and the one of hippocampal pyramidal cells. IEM-1925 was about twice more active than IEM-1460. A similar difference between activities of IEM-1925 and IEM-1460 has been obtained by Tikhonov *et al*. (2000) for forebrain neurons.

Our current results show that AMPAr of HM functionally differed from those on interneurons or hippocampal pyramidal cells. Additionally, the homogeneous blocking action of IEM-1460 and IEM-1925 suggests that the bimodal plot of pentobarbital antagonism was probably caused by the complex mechanism of action of the latter drug. This issue was further explored with experiments combining pentobarbital and IEM-1460, with the aim of testing the possibility that HM possessed both $GluR_2$-lacking and $GluR_2$-containing AMPAr.

Pentobarbital and IEM-1460 possess contrasting receptor selectivity, because 100 μM pentobarbital strongly blocked $GluR_2$-containing AMPAr of hippocampal pyramidal cells (Fig. 3) while 100 μM IEM-1460 almost completely blocks $GluR_2$-lacking AMPAr of striatal interneurons (Magazanik *et al*., 1997; Buldakova *et al*., 1999). It follows that, in the case of a mixed AMPAr population, if we first blocked a certain receptor group with one selective blocker, the antagonistic action of the second blocker selective for the residual subpopulation of AMPAr should become more apparent. On HM, after continuously applying 100 μM IEM-1460, pentobarbital (100 μM) induced 27 ± 3% (n = 6) inhibition of kainate currents, a result that did not differ significantly from its antagonistic action in the absence of IEM-1460 (33 ± 5%; n = 11). We also measured rectification of I–V relation in the presence of 100 μM pentobarbital (n = 4) and found it linear (Fig. 2B). Hence, all these data did not support the hypotheses regarding a mixed population of $GluR_2$-containing and $GluR_2$-lacking AMPAr on HM.

The effect of IEM-1925 on synaptically evoked EPSCs

To test the applicability of results obtained on isolated cells for more complex physiological systems, the action of IEM-1925 was also examined on HM of brainstem slices. The average amplitude of EPSC in HM (clamped at –60 mV) was –79 ± 12 pA (n = 26). These currents were mediated primarily by AMPAr because application of 10 μM CNQX reduced the amplitude of evoked EPSCs by 85 ± 7% (n = 3). Bath application of IEM-1925 (50 μM) produced a slowly developing reduction in the EPSC amplitude (see example in Fig. 5A and B) which, on average, amounted to 35 ± 4% (n = 7) decrease after approximately 7 min This effect of IEM-1925 was readily reversible on washout of the drug (Fig. 5B). The action of IEM-1925 was not changed in the presence of the competitive NMDA antagonist, aminophosphonic valerate (APV, 50 μM, n = 2). The degree of block by IEM-1925 on slice neurons was compatible with the one obtained with isolated HM (see Table 1) if we take into account the voltage dependence of the drug block (Tikhonov *et al*., 2000). Previous experiments have shown that the IC_{50} value at –60 mV is twice as large than at –80 mV (S.Buldakova & M.Samoilova, unpublished data). Thus, 50 μM IEM-1925 at –60 mV should produce about a 30% block, a result that agrees with the data obtained from hypoglossal slices.

Discussion

The principal goal of the present study was to demonstrate, with functional studies, the presence of $GluR_2$ subunits in HM AMPAr and to determine (by electrophysiological and pharmacological criteria) their role in AMPAr activity. By comparing AMPAr mediated responses of HM with those recorded from hippocampal pyramidal cells and interneurons, we concluded that the relative contribution of $GluR_2$ subunits to AMPAr function of HM was intermediate between the high contribution to hippocampal pyramidal cell and the low contribution to interneuronal currents.

Functional and pharmacological properties of AMPAr determined by $GluR_2$ subunits

To activate AMPAr we used the agonist kainate rather than AMPA itself because it is less desensitizing. As kainate-evoked currents of HM were suppressed strongly by the selective AMPAr antagonist,

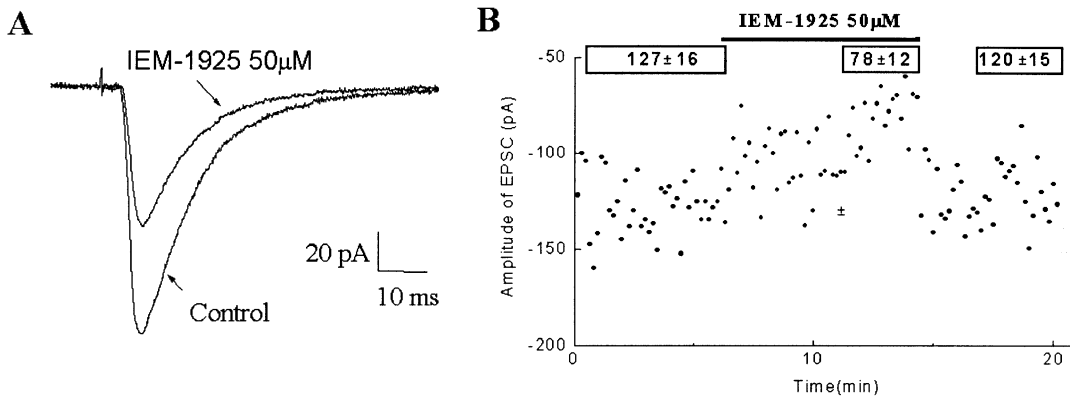

FIG. 5. Effect of IEM-1925 on HM EPSC. (A) Synaptically evoked currents in control solution and in the presence of 50 μM IEM-1925. Average responses are superimposed to aid comparison. (B) Time course of changes in amplitude of EPSCs following application of IEM-1925 (50 μM) and drug washout. Individual data points were collected at 10 s intervals and averaged. Mean values shown above open horizontal bars indicate averages for corresponding sampling periods.

GYKI-52466 (Wilding & Huettner, 1995; Paternain *et al*., 1996), they were mediated by AMPAr. The presence of $GluR_2$ subunits on AMPAr confers minimal Ca^{2+} permeability (for review see Dingledine *et al*., 1999), negligible current rectification (Donevan & Rogawski, 1995; Koh *et al*., 1995), pronounced block by pentobarbital (Yamakura *et al*., 1995) and low sensitivity to block by IEM-1460, or IEM-1925 (Magazanik *et al*., 1997; Tikhonov *et al*., 2000). Even a single $GluR_2$ subunit within the AMPAr is suggested to be sufficient to minimize both Ca^{2+} permeability and current rectification (Geiger *et al*., 1995). There are conflicting molecular biological and immunocytochemical results on the expression of $GluR_2$ subunits in HM motoneurons (together with a dearth of electrophysiological data). For instance, Del Cano *et al*. (1999) and Laslo *et al*. (2001), using $GluR_2$ antibodies, reported very low level of such subunits and inferred high Ca^{2+} permeability of such AMPAr. Conversely, Paarmann *et al*., (2000), using RT-PCR methods, reported strong expression of $GluR_2$ subunits in HM. Turmann *et al*., (2000), using a range of antibodies against $GluR_{1,2,3}$ and $_{4c}$ AMPAr subunits, reported that brainstem motoneurons of the neonatal and adult rat contained all those subunits at somatic and dendritic level.

The experimental evidence available so far left, however, unclear the issue of how the presence/absence of $GluR_2$ subunits determined the functional properties of HM AMPAr, especially because some receptors observed with immunocytochemistry might be nonfunctional or not accessible to synaptically released glutamate. Furthermore, detection of mRNA levels indicates the cell potential for synthesizing such receptors rather than actual receptor efficiency to transduce agonist binding into an electrical signal. For these reasons, we compared our electrophysiological data with those obtained from hippocampal pyramidal neurons characterized by large abundance of $GluR_2$ subunits and from interneurons of striatum and hippocampus expressing $GluR_2$-lacking AMPAr. We also performed experiments on excitatory synaptic transmission in slices containing HM exposed to the $GluR_2$-lacking receptor blocker IEM-1925.

Absence of significant inward rectification suggested that, in neonatal rat HM, AMPAr were poorly sensitive to the intracellular blocking action of spermine as a result of the presence of $GluR_2$ subunits. However, AMPAr also possessed a small, though clearly demonstrable Ca^{2+} permeability, indicating that not all those receptors contained $GluR_2$ subunits or, if they did, $GluR_2$ subunits were scantly present in some of them. Why did the tests on rectification and Ca^{2+} permeability not provide identical results in terms of $GluR_2$ presence on HM? Washburn *et al*. (1997) have reported that, in recombinant AMPAr, divalent ion permeability is more sensitive to $GluR_2$ mRNA abundance than rectification, and provided a model for this phenomenon based on differential interaction by Ca^{2+} and polyamines with the Q/R site of the AMPAr. Ca^{2+} permeability, therefore, appears to be a more sensitive diagnostic tool to quantify $GluR_2$ subunit activity.

Further data supporting a restricted presence of $GluR_2$ subunits on HM came from their block by pentobarbital which was close to that of $GluR_2$-lacking AMPAr of striatal interneurons. As the sensitivity of HM AMPAr to IEM-1460 and IEM-1925 was intermediate between the one of interneurons of striatum and hippocampal pyramidal cells, this result provides additional evidence for a population of $GluR_2$-lacking receptors on HM.

Comparison of the criteria for $GluR_2$ subunit presence and relative preponderance in AMPAr

It has been suggested by Geiger *et al*. (1995) that low Ca^{2+} permeability merely indicates presence of $GluR_2$ subunits rather than their stoichiometry within AMPAr because even a single $GluR_2$ subunit can reduce Ca^{2+} permeability through such receptors. Washburn *et al*. (1997) have later provided additional support for this hypothesis.

As far as pentobarbital is concerned, its precise action is incompletely understood. In the hippocampus, pentobarbital is more potent in depressing glutamatergic responses than enhancing those mediated by $GABA_A$ receptors (Sawada & Yamamoto, 1985). While part of the glutamate receptor antagonism by pentobarbital may be due to open channel block (Marszalec & Narahashi, 1993), recombinant glutamate receptor experiments indicate that assemblies containing $GluR_2$ subunits were the most sensitive to this drug (Hollmann *et al*., 1991; Taverna *et al*., 1994). Nevertheless, non-$GluR_2$ receptors also displayed measurable sensitivity to pentobarbital. In this context, it is important to note that the pharmacology of native AMPA receptors expressed by various neurons (including those in the brainstem) can differ considerably from the one of recombinant glutamate receptors (Weigand & Keller,

1998). In fact, $GluR_2$ subunit-lacking transgenic mice, whose AMPAr may be expected to be very insensitive to pentobarbital, have been found to be paradoxically most sensitive to pentobarbital effects, a phenomenon not accounted for by compensatory changes in $GABA_A$ receptor activity (Joo *et al*., 1999). *In vivo* experiments on rat abducens motoneurons indicate that coapplied pentobarbital and GYKI-53784 have unexpected contrasting effects on kainate-induced responses, although each agent per se has a depressant action (Ruiz & Durand, 1999). Our own results on HM confirm the presence of a complex mechanism underlying the effect of pentobarbital as indicated by the nonsigmoidal dose–response plots. In terms of sensitivity to pentobarbital, AMPAr of HM were similar to Ca^{2+} permeable AMPAr of striatal interneurons and differed significantly from Ca^{2+} impermeable AMPAr of hippocampal pyramidal cells. Caution is, however, necessary before concluding that pentobarbital sensitivity can be taken as a potent predictor for AMPAr structure, especially if not accompanied by additional functional tests.

One further, useful tool to differentiate between $GluR_2$ and non-$GluR_2$ receptors was the activity of dicationic compounds such as IEM-1460 and IEM-1925. Their blocking action probably depends on the actual number of $GluR_2$ subunits in the AMPAr oligomer (Magazanik *et al*., 1997; Buldakova *et al*., 1999; Samoilova *et al*., 1999). We found no evidence for multiple AMPAr subpopulations on HM because the plot for the antagonism by IEM-1460 or IEM-1925 had unitary slope (see Fig. 4B) and because combined application of pentobarbital and IEM-1460 did not manifest differential receptor antagonism. The simplest interpretation for the moderate sensitivity of HM receptors to these substances seems to be the possibility that the majority of AMPAr contained a restricted number of $GluR_2$ subunit only.

Previous *in vivo* (Ouardouz & Durand, 1994) or *in vitro* (Launey *et al*., 1999) studies of brainstem motoneurons have indicated that the AMPA selective blocker, GYKI-53784, completely suppresses the fast glutamatergic synaptic potential, demonstrating that this response is mediated by AMPAr. Our present investigation enquired whether HM AMPAr activated by synaptically released glutamate comprised subunits other than $GluR_2$. The channel blocker, IEM-1925, could also antagonize HM AMPAr in the brainstem slice preparation, showing that IEM-1925 sensitive, $GluR_2$-deficient receptors were involved in evoked synaptic transmission mediated by glutamatergic contacts which are believed to be distributed widely over soma and dendrites of brainstem motoneurons (Robinson & Ellenberger, 1997; Turman *et al*., 2000). This observation also helps to exclude the possibility that studying neurons isolated by vibrodissociation (lacking most dendritic arbor) provided functional information regarding AMPAr of somatic location only.

Taken together, the evidence collected in the present study favours limited expression of $GluR_2$ subunits in native AMPAr of neonatal rat HM. Further insight into this issue should come from fluorescence imaging studies of the intracellular Ca^{2+} increase induced by AMPAr activity. This phenomenon should be compared with the one induced by other receptors (for instance, NMDA) to assess its relative importance to HM function. Notwithstanding the future availability of such data, our hypothesis agrees with the observation that spinal motoneuron death induced by exposure to kainate is dependent on extracellular Ca^{2+} and is completely inhibited by the polyamine neurotoxin, Joro spider toxin, a very specific blocker of $GluR_2$-lacking AMPAr (Van Den Bosch *et al*., 2000).

Comparing the various criteria for detecting functional $GluR_2$ subunits shows that one simple and very reliable method is based on the action of externally applied channel blockers such as polyamine toxins and organic dications because it may reveal AMPAr assembled with a small number of $GluR_2$ subunits.

Functional implications of AMPAr stoichiometry on HMs

Expression of the $GluR_2$ subunit is down-regulated selectively by ischaemia (Pellegrini-Giampietro *et al*., 1997) and epilepsy (Friedman, 1998), or up-regulated by intense synaptic activity (Liu & Cull-Candy, 2000) or even modulated according to the level of spontaneous activity (Liu & Cull-Candy, 2002). In general, moderate down-regulation of $GluR_2$ subunits should not necessarily enhance Ca^{2+} permeability of AMPAr which contain more than one $GluR_2$ subunit. However, for HM even a small decrease in their $GluR_2$ expression might lead to the appearance of a significant pool of Ca^{2+} permeable receptors if AMPAr, indeed, possessed a restricted number of $GluR_2$ subunits. Enhanced Ca^{2+} influx due to $GluR_2$ subunit down-regulation might therefore contribute, together with the low endogenous Ca^{2+} buffer capacity of HM (Lips & Keller, 1998) and relatively high AMPA receptor current density typical for motoneurons (Vandenberghe *et al*., 2000), to the selective vulnerability of such cells to Ca^{2+}-dependent glutamate toxicity (Krieger *et al*., 1994; Reiner *et al*., 1995).

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAr, glutamate receptor of AMPA type; EPSC, excitatory postsynaptic current; $GluR_2$, AMPA receptor subunit; HM, hypoglossal motoneuron; IEM-1460–1-trimethylammonio-5-(1-adamantane-methylammoniopentane) dibromide IEM-1925, N-(5-aminopentyl)-1-phenylcyclohexylamine dibromide.

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