Pharmacological Evidence for Two Types of Postsynaptic Glycinergic Receptors on the Mauthner Cell of 52-h-Old Zebrafish Larvae

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Legendre, P. Pharmacological evidence for two types of postsynaptic glycinergic receptors on the Mauthner cell of 52-h-old zebrafish larvae. J. Neurophysiol. 77: 2400–2415, 1997. The presence of homooligomeric and heterooligomeric glycine receptors (GlyRs) on the Mauthner (M) cell in the isolated medulla of 52-h-old zebrafish larvae was investigated by analysis of the effects of picrotoxin on glycine-gated channels and on glycinergic miniature inhibitory postsynaptic currents (mIPSCs). Two functionally different GlyRs have been previously described on the M cell. The effects of picrotoxin on these two GlyRs were first analyzed by measuring the relative change in their total open probability (NP_o) with picrotoxin concentration. Picrotoxin had no significant effect on the glycine channel with a single conductance level of 40–46 pS. In contrast, picrotoxin application decreased the NP_o of the GlyR with multiple subconductance levels. On this GlyR, picrotoxin decreased in a concentration-dependent manner the occurrence of the 80- to 86-pS substate (median inhibiting concentration = 0.89 μM) and had no apparent effect on the 40- to 46-pS opening probability. Opening frequency and the mean open times of the 80- to 88-pS main conductance state were reduced in the presence of 10 μM picrotoxin, but their relative weight remained unchanged. These effects of picrotoxin were not voltage dependent. Picrotoxin also modified 40- to 46-pS kinetics. At 10 μM, picrotoxin evoked voltage-independent flickering during channel openings. Short and long mean open times were significantly decreased, whereas the relative proportion of long mean open times was increased. The medium closed time was decreased, whereas medium burst duration was increased. The burst frequency remained unchanged. Spontaneous glycinergic mIPSCs were recorded in the presence of 1 μM tetrodotoxin + 25 μM bicuculline (holding potential = −50 mV). Application of 10 μM picrotoxin did not change the frequency of the synaptic activity, whereas it decreased the amplitude of large mIPSCs. No effect was observed on the time to peak (0.8 ms) or the mean decay time constant (τ_d = 7.7 ms). Increasing picrotoxin concentration to 100 μM resulted in a decrease of mIPSC frequency (35.6%), amplitude (39.8%), and τ_d (from 7.7 to 5 ms). These results suggest that these two functionally different GlyRs correspond to α1 homooligomeric-like and α1/β-heterooligomeric-like GlyRs, and that both are synthetically activated. Variation in the proportions of GlyR subtypes from one synapse to another could partly account for the broad amplitude distribution of mIPSCs recorded from the zebrafish M cell.

INTRODUCTION

Glycine is the main inhibitory receptor in the spinal cord and brain stem (Curtis and Johnston 1974a; Curtis et al. 1968). As for other ligand-gated channels, it is now clearly established (Langosch et al. 1990) that glycine-gated chloride channels [glycine receptors (GlyRs)] exist in different forms depending on subunit combination (α1, α2, α3, and β). Alternative splicing of the mRNAs encoding the α1 and α2 subunits reinforces this GlyR heterogeneity (Langosch et al. 1990). The developmental and regional distributions of the different GlyR subunit mRNAs are complex. In the rat, α1 transcripts are detected by in situ hybridization in the spinal cord, the brain stem, and the colliculi, whereas α2 subunit mRNAs are only present in the olfactory bulb, the hippocampus, and the cerebellum (Betz 1991). α2 Subunit mRNAs are detected in several brain areas (Betz 1991), especially in developing neurons (Becker et al. 1988). The subunit stoichiometry of GlyR has been proposed to be three α and two β (Kuhse et al. 1993), although homooligomeric-like GlyR activity has been described in fetal and more mature mammalian CNS (Smith et al. 1989; Takahashi and Momiyama 1991).

GlyR subtypes have different functional and pharmacological properties. α1 Homooligomers are less sensitive to strychnine (Langosch et al. 1990). α1, α2, and α3 homoligomers are characterized by a variable proportion of several subconductance levels, whereas α/β-heterooligomers open with lower conductance states (Bormann et al. 1993). GlyRs also exhibit distinct pharmacological properties depending on subunit stoichiometry. α-Homoligomers are highly sensitive to picrotoxin, whereas a channel made of α- and β-subunits has a 100- to 500-fold reduced sensitivity to picrotoxin inhibition (Lynch et al. 1995; Pribilla et al. 1992).

At glycinergic synapses the functional consequences of this receptor heterogeneity have only been partly established on developing spinal cord and sympathetic preganglionic neurons (Krupp et al. 1994; Takahashi et al. 1992). In newborn rats, glycinergic synaptic events have a longer decay time than those recorded in more mature animals (Krupp et al. 1994; Takahashi et al. 1992), a phenomenon proposed to result from a switch between α2 and α1 subunits during brain maturation (Takahashi et al. 1992).

In the zebrafish (Brachydanio rerio) larva Mauthner (M) cell, the amplitude distribution of miniature inhibitory postsynaptic currents (mIPSCs) resulting from the activation of glycine-gated channels cannot be resolved by a single Gaussian curve. This distribution is skewed and even multimodal (Legendre and Korn 1994), as often observed at central synapses (Bekkers et al. 1990; Edwards et al. 1990; Manabe et al. 1992; Silver et al. 1992; Tang et al. 1994). Such histograms have been proposed to reflect morphofunctional heterogeneity of postsynaptic elements (Bekkers et al. 1990; Tang et al. 1994), although in some prepara-
tions they were thought to result from an unsaturation of postsynaptic receptors during exocytosis (Frerking et al. 1995).

Alternatively, the complex shape of the mIPSC amplitude distributions could also partly derive from the expression of variable proportions of functionally different receptors facing distinct synaptic sites. This can be the case in the zebrafish larva M cell, where two functionally different glycine-operated channels have been postulated (Legendre and Korn 1994). One of these channels has three subconductance levels with a main conductance state of 80–88 pS, and the other is characterized by a single open state of 40–46 pS. It is, however, not yet clear whether both GlyRs can be activated by synaptically released glycine.

Here, the hypothesis is evaluated that functionally different synaptic receptors contribute to the complex shape of the amplitude distributions of glycineric mIPSCs in zebrafish larva M cells. Functional discrimination between the two types of GlyRs was achieved through the analysis of their sensitivity to picrotoxin (Pribilla et al. 1992). Determination of the synaptic location of the GlyRs, and thus of their contribution to the large variation in mIPSC amplitude, was performed by the analysis of picrotoxin effects on spontaneously occurring glycineric mIPSCs.

**METHODS**

**Isolated brain preparation**

The isolated brain preparation was used as previously described (Legendre and Korn 1994). Briefly, brains of newly hatched larvae (52 h after fertilization) were dissected out in a Ca²⁺- and Mg²⁺-free Gey’s balanced salt solution (Gibco) and glued on glass coverslips with the use of a plasma thrombin embedding procedure (Gahwiler and Brown 1985). Before the experiments, preparations were stored for 15 min in an oxygenated (95% O₂-5% CO₂) bathing solution containing (in mM) 145 NaCl, 1.5 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose, osmolarity adjusted to 330 mosM, pH 7.2.

**Outside-out and whole cell recordings**

Outside-out and whole cell recordings (Hamill et al. 1981) were performed on M cells under direct visualization with the use of Nomarski optics (x40; immersion lens; Nikon Optiphot). The brain was continuously perfused at room temperature (20°C) with oxygenated bathing solution (2 ml/min) in the recording chamber (0.5 ml). Patch-clamp electrodes were pulled from thick-wall borosilicate glass, fire polished, and filled with (in mM) 135 CsCl, 2 MgCl₂, 4 NaATP, 10 ethylene glycol-bis-(β-aminoethoxy) ether-N,N,N',N'-tetraacetic acid, and 10 N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.2, osmolarity adjusted to 290 mosM.

Whole cell recordings were performed with the use of 1- to 3-MΩ electrodes and outside-out patches were obtained by slowly pulling the pipettes out of the brain with the use of 10- to 15-MΩ electrodes. Whole cell and single-channel currents were recorded with the use of an Axopatch 1D (Axon Instruments), with the current filtered at 10 kHz, and were stored with the use of a digital tape recorder (DAT DTR 1201, Sony). During whole cell recordings, the series resistance (4–10 MΩ) was monitored by applying 2-mV hyperpolarizing pulses and was compensated by 50–70%. To ensure cell dialysis, measurements were performed ≥3–5 min after the whole cell configuration had been obtained.

Input resistance and capacitance of the M cells (5-μm diam, 100-μm long) ranged between 80 and 100 MΩ and 20–30 pF, respectively (holding potential = –50 mV) (Legendre and Korn 1994).

**Drug delivery**

Drugs were applied to the zebrafish brain (2 ml/min) via an array of four flowpipes (400 μm) positioned 200 μm from the preparation. During drug applications the bath perfusion was stopped. With the use of this application system, a low concentration of strychnine (100 nM) evoked a decrease in the postsynaptic current amplitude that reached a steady state 30 s after the beginning of the application. Therefore to analyze the voltage-dependent properties of glycine-evoked chloride current during whole cell recordings, measurements were performed 1 min after the beginning of drug application.

Tetrodotoxin (1 μM), glycine (3–5 μM, Aldrish), bicuculline methiodide (25 μM, Sigma), and picrotoxin (0.1–100 μM, Sigma) were dissolved in a perfusate containing (in mM) 145 NaCl, 1.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH 7.2; osmolarity adjusted to 330 mosM. All solutions were prepared daily. Picrotoxin is a combination of two toxins, Picrotinin and Picrotoxinin (Curtis and Johnston 1974b). Because these two compounds are equally effective on glycine-gated channels (Lynch et al. 1995), picrotoxin was preferentially used in these experiments.

**Analysis of whole cell currents**

Ongoing synaptic activity was digitized off-line on a Macintosh 950 quadra computer at 25 kHz with the use of MMII software (GW instruments). The detection of synaptic events was automatically performed with homemade software (Ankri et al. 1994, Legendre and Korn 1994) written in Labview II0.2 (National Instruments). For display purposes, histograms were constructed with the use of Kaleidagraph 2.1 software.

**Single-channel analysis**

Low concentrations of glycine (3 μM) were applied to outside-out patches to minimize the number of overlapping single-channel events. As openings of multiple channels were systematically observed, the total open probability (Npₒ) was then the product of the number of channels (N) and the open probability (Pₒ). Npₒ was determined by constructing point-per-point amplitude histograms of data segments.

For the analysis of the gating properties of glycine-gated channels, overlap of events was avoided by selecting patches with a low open frequency and by discarding responses containing multiple conductance levels. Single-channel currents were filtered at 2 kHz with the use of an eight-pole Bessel filter (Frequency Devices), sampled at 20 kHz, and analyzed on an IBM AT-compatible computer with the use of Pclamp 6.02 software (Axon Instruments). Opening and closing transitions were detected with the use of a 50% threshold criterion. For each patch, the threshold was first set at a low level to include all conductance states. Thereafter the threshold was set at a level including only one conductance state. Events (openings or closures) briefer than 1 ms were ignored in compiling event lists.

Bursts of openings were defined as groups of openings separated by closed intervals shorter than a critical value τₖ, which was determined in two ways. τₖ was first determined from a closed time histogram with the use of the method of equal percentage of misclassified bursts (Colquhoun and Sakmann 1985; Colquhoun and Sigworth 1995). The optimum interburst interval was also determined by measuring the number of closings per burst as a function of a test interburst interval (Sigurdson et al. 1987). As the test interval becomes identical to the true value of the interburst interval...
interval, the number of closings per burst becomes relatively insensitive to further change in the test interval values (Sigurdson et al. 1987). Both methods gave similar results for data obtained in the presence of picrotoxin concentrations <100 μM. In the presence of 100 μM picrotoxin, the number of closings per burst became independent of test interburst intervals for values larger than the second closed dwell time. Therefore $\tau_c$ was determined with the use of only the second method.

For display purposes, burst duration histograms show the distributions as log intervals with the ordinate on a square root scale (Sigworth and Sine 1987). Histograms were fitted with the sum of several exponential curves with the use of the maximum likelihood method. Fits with a variable number of components were compared with the use of $F$ statistics. In some histograms, a first short component improved the goodness of the fit. The mean value of this first component was <0.4 ms, and it did not reflect a true kinetic component because the filter cutoff frequency used was 2 kHz and the sampling rate was 0.05 ms. Omitting this component did not significantly change dwell time measurements.

Results are presented as means ± SD.

**Results**

*Picrotoxin preferentially blocked the activity of glycine-gated channels with multiple subconductance levels*

The ability of 0.1–100 μM picrotoxin to modify the activation of glycine-gated chloride channels was first investigated by the analysis of the change in their activity ($NP_o$) in outside-out patches. Two types of glycine-gated channels are present on the M cell of the zebrafish larva (Legendre and Korn 1994). They were observed on different outside-out patches, some of them obtained from the same M cell. They were discriminated on the basis of their main conductance state and the number of subconductance levels (Legendre and Korn 1994). The glycine-gated channels with a single conductance level of 40–43 pS were dominant (82%) (Legendre and Korn 1994). The other type of glycine-operated channels had a main conductance of 80–88 pS and two substates of 60–66 and 40–46 pS, respectively (Legendre and Korn 1994).

The effect of picrotoxin (0.1–100 μM) on the activity ($NP_o$) of these two types of channels was first analyzed by constructing point-per-amplitude histograms. Glycine (3 μM) and picrotoxin were simultaneously applied on outside-out patches. The value of $NP_o$ obtained in the presence of glycine and different concentrations of picrotoxin was normalized to that of $NP_o$ obtained on the same patch exposed to glycine alone.

On the 10 patches tested, picrotoxin at concentrations ranging from 0.1 to 100 μM did not suppress the activity of glycine-gated channels having a single conductance state ($P = 0.01$ paired $t$-test, Fig. 1B). In the presence of 0.1, 1, 10, and 100 μM picrotoxin, single-channel activity represented 1.07 ± 0.09 (SD, $n = 9$), 1.08 ± 0.29 ($n = 9$), 1.09 ± 0.44 ($n = 9$), and 1.16 ± 0.18 ($n = 9$) of the control $NP_o$, respectively.

In contrast, the application of picrotoxin on patches displaying multiple subconductance levels reduced glycine-gated channel activity. This effect was concentration dependent, but it was not use dependent, because the effect of this antagonist on $NP_o$ did not increase with the duration of the application (1–3 min). $NP_o$ was slightly affected by the application of 0.1 μM picrotoxin (0.92, $n = 2$), but it decreased to 0.67 ± 0.18 ($n = 4$) of the control value in the presence of 1 μM picrotoxin. $NP_o$ was decreased to 0.42 ± 0.18 ($n = 5$) by the application of 10 μM picrotoxin and to 0.26 ± 0.04 ($n = 5$) in the presence of 100 μM picrotoxin.

To estimate the median inhibiting concentration (IC$_{50}$) for picrotoxin’s action on the activity of glycine-gated channel with a main conductance state of 80–88 pS, the data were first fitted with a single binding isotherm of the form

$$NP_o(\text{picrotoxin}) = NP_o(\text{control}) \times (1 - \{\text{picrotoxin}/[(\text{picrotoxin}) + \text{IC}_{50}]\})$$

where $NP_o(\text{control})$ is $NP_o$ measured in the presence of 3 μM glycine alone, $NP_o(\text{picrotoxin})$ is $NP_o$ in the presence of glycine.
plus picrotoxin, and IC$_{50}$ is the concentration of picrotoxin that inhibited 50% of the glycine-gated channel activity. This fit produced a value of 5.7 μM for IC$_{50}$.

The decrease of NP$_o$ observed in the presence of 100 μM picrotoxin (74%) was, however, significantly lower than its predicted theoretical value (94.6%) when a single binding isotherm was used. This observation suggests that effect of picrotoxin on NP$_o$ cannot be simply described by a single binding isotherm function, possibly because the picrotoxin effect involved more than one binding site.

The effect of picrotoxin on the relative contribution of subconductance levels to NP$_o$ was then analyzed (Fig. 2). The relative contributions of large subconductance levels (80–88 and 60–66 pS) and small conductance states (40–46 pS) were compared with the use of point-per-point amplitude histograms. As shown in Fig. 2A, picrotoxin selectively decreased the relative proportion (A$_i$) of large conductance levels. This effect was concentration dependent (Fig. 2B), and, in contrast to what was observed for NP$_o$, a nearly complete inhibition was observed in the presence of 100 μM picrotoxin (A$_{i1} = 0.021 \pm 0.015$ of control values, n = 5; Figs 2, B and C).

To estimate the IC$_{50}$ for picrotoxin’s action on the large conductance substates (Fig. 2C), the data were fitted with a single binding isotherm of the form

$$A_{i1(\text{picrotoxin})} = A_{i1(\text{control})} \times (1 - ((\text{picrotoxin})/(1(\text{picrotoxin}) + IC_{50})))$$

where $A_{i1(\text{control})}$ is the integral of the current due to the opening of large conductances levels in the presence of 3 μM glycine alone, $A_{i1(\text{picrotoxin})}$ is the integrated current due to the openings of large conductance levels in the presence of glycine plus picrotoxin, and IC$_{50}$ is the concentration of picrotoxin that decreased $A_{i1(\text{picrotoxin})}$ to 50% of $A_{i1(\text{control})}$. The IC$_{50}$ was 0.89 μM, which was 6 times smaller than that obtained for picrotoxin effects on NP$_o$. This difference can be explained by the effects of picrotoxin application on the glycine-gated channel of 40– to 46-pS conductance level.

As shown in Fig. 2, A and D, picrotoxin applied at concentrations ranging from 0.1 to 10 μM slightly enhanced the relative area (A$_{i2}$) of small subconductance levels. But on the five patches tested this effect was not statistically significant ($P = 0.05$, paired t-test). In the presence of 10 μM and 100 μM picrotoxin, A$_{i2}$ reached 1.74 ± 0.77 and 1.65 ± 1.04 of control values, respectively (n = 5).

These results suggest that the decrease in NP$_o$ evoked by the application of ≈100 μM picrotoxin on patches displaying multiple subconductance levels may be incomplete. The IC$_{50}$ value for picrotoxin’s inhibitory effect on NP$_o$ (Fig. 1B) was therefore recalculated with the use of the single binding isotherm of the form

$$NP_{o(\text{picrotoxin})} = NP_{o(\text{control})} \times (1 - ((\text{picrotoxin})/(1(\text{picrotoxin}) + IC_{50})))$$

where $D_{\text{max}}$ represents, as a first approximation, the maximum decrease in NP$_o$ evoked by the application of picrotoxin. Adding this variable increased the goodness of the fit and $D_{\text{max}}$ was 0.28. IC$_{50}$ was 1.6 μM, close to the IC$_{50}$ obtained for the decrease in the relative proportion of large subconductance levels.

Overall, these results suggest that the inhibitory effect of picrotoxin observed on patches displaying multiple subconductance levels was mainly due to the disappearance of large conductance states.

**Effect of picrotoxin on the gating properties of 80- to 88-pS substate glycine-gated channels**

The effect of picrotoxin on large subconductance levels could result from a change in their opening frequency and/or mean open times. The frequency of occurrence of 80- to 88-pS openings was decreased by picrotoxin application (Fig. 3). It was 53% reduced (41–69%, n = 3) in the presence of 10 μM picrotoxin (Fig. 3).

This effect of picrotoxin on 80- to 88-pS channels limited our ability to perform detailed kinetic analysis. Only changes in mean open time were analyzed. In the three patches tested that displayed a sufficient number of large openings at intermediate picrotoxin concentrations, open time histograms could be fitted with the sum of two exponential curves in control conditions (3 μM glycine). As previously described (Legendre and Korn 1994), the two open times ($\tau_{o1}$ and $\tau_{o2}$) had mean values of 1.25 and 7.22 ms, respectively (n = 3). Their relative weights were 0.66 and 0.34, respectively. Adding 10 μM picrotoxin to glycine solution decreased $\tau_{o1}$ to 0.9 ms and $\tau_{o2}$ to 5.53 ms (n = 3), whereas relative weights remained unchanged (0.68 and 0.32). In the presence of 100 μM picrotoxin, large subconductance levels were rare, never occurred in bursts, and had a short lifetime (Fig. 3D).

**Effect of picrotoxin on the gating properties of glycine-gated channels with a single conductance level**

Kinetics of 40- to 46-pS substates was modified by the application of picrotoxin, in contrast to the apparent absence of picrotoxin effect on the NP$_o$ of these substates (Fig. 4). At a holding potential of −50 mV, the application of 100 μM picrotoxin increased the open channel noise (Fig. 4). At a filter cutoff frequency of 2 kHz, the flickering was prominent and included complete closures to the baseline. Gating behavior of the channels was then characterized by long bursts of short openings. Flickering was still observed at positive holding potentials (Fig. 4). Moreover, depolarization did not suppress the effects of picrotoxin on large subconductance levels described above.

The action of picrotoxin on the gating properties of 40- to 46-pS conductance levels was analyzed in detail on patches displaying only one conductance state (see Table 1). In the presence of 3 μM glycine, open time histograms of this main conductance state could be fitted by the sum of two exponential curves (Fig. 5A). In the 10 patches tested, the two mean open times were $\tau_{o1} = 0.86$ ms and $\tau_{o2} = 5.9$ ms in control conditions (3 μM glycine alone). Adding 1 or 10 μM picrotoxin to the glycine solution had no significant effect on the mean open time values (Table 1). However, $\tau_{o2}$ was markedly decreased (1.6 ms) in the presence of 100 μM picrotoxin, as expected in the presence of flickering (Fig. 5, B and C). At this concentration, $\tau_{o1}$ was also decreased (0.5 ms; Table 1, Fig. 5B).

In control conditions, the relative areas of the two exponential components (a$_1$ and a$_2$) were 0.66 and 0.34, respectively (Table 1). As shown in Fig. 5D, changes in a$_1$ and a$_2$ evoked by picrotoxin application appeared to be concen-
Fig. 2. A: superimposed point-per-point amplitude histograms of data segments (20-s epoch) with multiple subconductance levels in the presence of 3 μM glycine (thin curves) or in the presence of 3 μM glycine + 100 μM picrotoxin (thick curves) (Vh = −50 mV; filter 2 kHz; bin = 0.1 pA). Note that 100 μM picrotoxin completely abolished 86-pS substates, whereas in this case open probability (P_o) of 44-pS subconductance states was increased. B: in control conditions (3 μM glycine), the contribution of large conductance substates to N_Po was close to 80% (n = 5; ●). In the presence of 0.1–100 μM picrotoxin, the relative P_o of 40- to 46-pS (○) and 80- to 88-pS (●) substates were normalized to those obtained in control conditions. Note that picrotoxin selectively decreased the relative P_o of large conductance substates [median inhibiting concentration (IC_50) = 0.9 μM], whereas the relative P_o of 40- to 46-pS substates was not significantly modified (P < 0.05; paired t-test). Each point is the average of 2–5 measurements (n = 5 patches). C and D: plots of the relative weight of 80- to 88-pS (C) and 40- to 46-pS (D) substates in the presence of 1–100 μM picrotoxin normalized to that obtained in control conditions. Note that the relative weight of 40- to 46-pS substates had a tendency to increase with picrotoxin concentrations. Data points were not significantly different (P < 0.05, paired t-test).

The relative frequency of the long mean open time (τ_o2) increased in the presence of 10 μM picrotoxin and a_1 and a_2 shifted to 0.52 and 0.48, respectively (Fig. 5D and Table 1). In the presence of 100 μM picrotoxin, the long openings became more frequent and a_1 and a_2 reached 0.37 and 0.63, respectively (Table 1). At least three mean closed times have been observed for M cell glycine-gated channels, the shortest being considered as gaps within a burst (Legendre and Korn 1994, 1995). In control conditions, mean closed time values τ_c1, τ_c2, and τ_c3 were 0.52, 19.6, and 105.7 ms, respectively (Table 1). τ_c2 and τ_c3 were not constant between patches. Because all patches contained more than one channel, these were not considered true kinetics values. However, the application
FIG. 3. Picrotoxin selectively decreased the open time duration of 80- to 88-pS subconductance level. A: behavior of glycine-gated channels with a main conductance state of 82 pS in the presence of 3 μM glycine and 3 μM glycine + 100 μM picrotoxin. (Filter cutoff frequency = 2 kHz; Vh = -50 mV.) Examples of single-channel currents plotted as a function of open time in the presence of 3 μM glycine (B), 3 μM glycine + 10 μM picrotoxin (C), and 3 μM glycine + 100 μM picrotoxin (D). Note that picrotoxin application decreased the frequency and the open time duration of 82-pS subconductance levels (Vh Å 0.50 mV).

of picrotoxin decreased τc2 and τc3, whereas τc1 remained unchanged (Fig. 6, Table 1). For picrotoxin concentrations ≤100 μM, the apparent change in τc3 (Fig. 6) was not statistically significant (P = 0.05, paired t-test), whereas τc2 decreased in a concentration-dependent manner (Fig. 6D).

In the presence of 100 μM picrotoxin, τc2 was close to 3 ms and this value became less variable (3.01 ± 0.79 ms, n = 10) than that observed in control conditions (19.62 ± 17.1 ms; n = 10).

The relative areas of the three exponential curves (a1, a2, and a3) were greatly affected by the application of 100 μM picrotoxin. At this concentration, τc2 became significantly more frequent (P = 0.05, paired t-test) and the relative area of τc3 was decreased. a1, a2, and a3 reached 0.45, 0.41, and 0.14, respectively (in control conditions: a1 = 0.55, a2 = 0.12, a3 = 0.33).

The long bursts of short openings evoked by high picrotoxin concentration suggest that the effect of this antagonist requires a shift in the channel opening conformation. To address this issue and to determine whether picrotoxin had any effect on the number of bursts, records were analyzed by ignoring a critical closed interval value (see METHODS).

In the stationary condition (3 μM glycine), kinetic analysis of glycine-operated channels revealed three mean burst durations (τb1, τb2, and τb3, Fig. 7A), the two shorter ones having values similar to the mean open times τo1 and τo2 (Legendre and Korn 1994, 1995). In the 10 patches tested, τb1, τb2, and τb3 were close to 0.5, 4, and 34 ms, respectively (Table 1). At 1 μM, picrotoxin had no significant effect on mean burst duration, whereas the application of 10 μM picrotoxin increased τb1 and τb2 to 0.7 and 7.3 ms, respectively (see Table 1 and Fig. 7).

During application of 100 μM picrotoxin, the number of closings per burst became independent of test interburst inter-
FIG. 4. Picrotoxin-induced flickering of open channel was voltage independent. In control conditions, only few brief interruptions of the 42-pS open state were observed at holding potentials of +50 and −50 mV (A). In the presence of 100 μM picrotoxin (B), single-channel current induced by glycine (3 μM) showed flickering at positive and negative holding potentials. Filter cutoff frequency ≈ 2 kHz.

vals for values larger than $t_{c2}$ only (see METHODS). Burst duration histograms could be fitted by the sum of three exponential curves (Fig. 7B). At this concentration, picrotoxin increased significantly only $t_{b2}$ ($P < 0.01$, paired t-test), and had no effect on $t_{b1}$ and $t_{c3}$ (Fig. 7, C–E). In these conditions, $t_{b1}$, $t_{b2}$, and $t_{b3}$ were close to 0.6, 8, and 39 ms (Table 1).

Although the application of picrotoxin modified the gating behavior of 40- to 46-pS substates, it had no significant effect on the overall burst frequency ($P > 0.05$, paired t-test). In the presence of 10 and 100 μM picrotoxin, the burst frequencies were 1.2 ± 0.28 ($n = 6$) and 1.01 ± 0.45 ($n = 10$) of control values, respectively. In contrast, the relative frequencies of $t_{b1}$, $t_{b2}$, and $t_{b3}$ ($a_1$, $a_2$, and $a_3$) were modified in a concentration-dependent manner. As shown in Fig. 7F, the proportion of $t_{b3}$ increased when picrotoxin concentration was increased, whereas the relative frequency of $t_{b1}$ decreased. In the presence of 3 μM glycine alone, $a_1$, $a_2$, and $a_3$ were close to 0.48, 0.34, and 0.18, respectively, and reached 0.34, 0.34, and 0.32 in the presence of 3 μM glycine plus 100 μM picrotoxin (Table 1). This change in the relative proportion of the mean open times and the mean burst duration of the 40- to 46-pS sub-

### Table 1. Effects of picrotoxin on the kinetics of the glycine receptor main conductance state of 40–46 pS

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<td>1.6 ± 0.3</td>
<td>$a_2$</td>
<td>0.34 ± 0.07</td>
<td>0.37 ± 0.1</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>$t_{a3}$</td>
<td>0.52 ± 0.12</td>
<td>0.46 ± 0.12</td>
<td>0.56 ± 0.19</td>
<td>0.58 ± 0.18</td>
<td>$a_3$</td>
<td>0.55 ± 0.5</td>
<td>0.61 ± 0.05</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>$t_{c1}$</td>
<td>19.6 ± 17.1</td>
<td>13.5 ± 7.6</td>
<td>5.3 ± 3.9</td>
<td>3.1 ± 0.8</td>
<td>$a_1$</td>
<td>0.12 ± 0.05</td>
<td>0.19 ± 0.09</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>$t_{c2}$</td>
<td>107.5 ± 67.7</td>
<td>87.2 ± 47.5</td>
<td>81.3 ± 50.7</td>
<td>61.5 ± 37.5</td>
<td>$a_1$</td>
<td>0.33 ± 0.08</td>
<td>0.2 ± 0.09</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>$t_{c3}$</td>
<td>0.46 ± 0.15</td>
<td>0.5 ± 0.28</td>
<td>0.77 ± 0.3</td>
<td>0.59 ± 0.22</td>
<td>$a_1$</td>
<td>0.48 ± 0.07</td>
<td>0.44 ± 0.15</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>$t_{b1}$</td>
<td>4.3 ± 1.2</td>
<td>4.3 ± 1.9</td>
<td>7.3 ± 2.8</td>
<td>8.1 ± 3.1</td>
<td>$a_2$</td>
<td>0.35 ± 0.06</td>
<td>0.32 ± 0.08</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>$t_{b2}$</td>
<td>34.1 ± 9.6</td>
<td>34.9 ± 16.3</td>
<td>35.2 ± 2.8</td>
<td>39.4 ± 9.1</td>
<td>$a_3$</td>
<td>0.18 ± 0.07</td>
<td>0.24 ± 0.09</td>
<td>0.25 ± 0.05</td>
</tr>
</tbody>
</table>

Results from outside-out patches exposed to 3 μM glycine and to 3 μM glycine + 1, 10, and 100 μM picrotoxin are presented as means ± SD. Open time constant ($t_{o1}$), closed time constant ($t_{c1}$), burst duration ($t_{b1}$), and relative area ($a_1$) were obtained from exponential components fitted to histograms, as shown in Figs. 5–7.
Two types of glycine-gated channels on zebrafish larvae M cell

FIG. 5. Open time duration histograms of 40- to 46-pS subconductance level obtained in the absence (A) and in the presence of 100 μM picrotoxin (B) are shown as a function of log intervals with the ordinate on a square root scale. Histograms were better fitted with the sum of 2 exponential curves (see METHODS). In the presence of 3 μM glycine alone (A), mean open times were $\tau_{o1} = 0.9$ ms and $\tau_{o2} = 4.7$ ms. Mean open times were decreased in the presence of 100 μM picrotoxin (B). In this example, $\tau_{o1}$ and $\tau_{o2}$ were equal to 0.7 and 2.1 ms, respectively. C: summary of the results obtained in 6 ± 10 patches exposed to 1, 10, and 100 μM picrotoxin. Note that picrotoxin had no effect on mean open times when it was applied at a concentration <100 μM. D: averaged relative area of the 2 mean open times in function of picrotoxin concentration. Note that relative areas of the 2 exponential components ($a_1$ and $a_2$) varied with picrotoxin concentrations ($V_h = -50$ mV).

states suggests that picrotoxin application can also increase glycine-gated channel activity, because the relative proportion of $\tau_{o1}$ or $\tau_{o2}$ depends on glycine concentration (Legendre and Korn 1994). It is unlikely that picrotoxin acted as an agonist at GlyRs because, at concentrations of 10–100 μM, it never evoked any single-channel openings on the three patches tested (data not shown).

**Picrotoxin decreased the mean amplitude of glycineric mIPSCs**

Because the main conductance state of one receptor type (80–88 pS) is twice the value of the main conductance state of the other (40–46 pS), classes in mIPSC amplitude distribution (Legendre and Korn 1994) may reflect the activation of clusters of one or the other receptor type facing different synaptic terminals. This possibility was investigated by comparing mIPSC amplitude distribution in the absence and in the presence of 10 and 100 μM picrotoxin.

Glycinergic mIPSCs were recorded in the presence of $10^{-6}$ M tetrodotoxin. Bicuculline methiodide (25 μM) was added to the bath solution to block any spontaneously occurring GABAergic mIPSCs. Picrotoxin was applied for 10 min. The isolated brain preparation was washed for 8 min
and mIPSCs were then collected for 3 min to control for the recovery from effects induced by picrotoxin.

A recording period of 25–30 min was needed before picrotoxin application to ensure stationary conditions. It was observed that the decay time constant ($\tau_d$) of mIPSCs increased 20 min after the beginning of the recordings. Thereafter it remained stable. It was close to 4 ms (4.1 ± 0.7 ms, $n = 10$) during the first 15–20 min and then reached 8 ms (7.72 ± 1.18, $n = 10$). This change in $\tau_d$ was not associated with a loss of the voltage-dependent properties of mIPSC duration (Legendre and Korn 1995) and was not correlated with an increase of the access resistance. It may suggest a change in the phosphorylation processes of GlyRs attributable to intracellular dialysis during whole cell recordings.

The mean amplitude of mIPSCs was close to 150 pA (150.1 ± 29.9 pA, $n = 10$) in control conditions. Continuous application of 10 $\mu$M picrotoxin by bath perfusion resulted in a 14.9 ± 6.2% decrease of mIPSC mean amplitude ($n = 5$). Cumulative amplitude histograms (Fig. 8B) show that this reversible effect results largely from an apparent suppression of large events. The mean frequency of mIPSCs was not significantly modified by 10 $\mu$M picrotoxin application: it represented 98.9 ± 7.2% of the control values ($n = 5$). This suggests that 10 $\mu$M picrotoxin did not abolish large synaptic currents but rather reduced their amplitude.

Increasing picrotoxin concentration to 100 $\mu$M resulted in a more pronounced effect on mIPSCs (Fig. 8C). Both their amplitude and their frequency were strongly modified. Amplitude and frequency were reduced to 35.6 ± 8.5% and 39.8 ± 9.1% of control values, respectively ($n = 10$). These effects were fully reversible when the preparation was washed for >7 min (Fig. 8C).

To determine whether all synaptic events were equally affected by 100 $\mu$M picrotoxin, the amplitude histogram
obtained during picrotoxin application was compared with a theoretical distribution (Fig. 9, A and B) obtained as follows. The amplitude of mIPSCs recorded in the absence of picrotoxin was multiplied by a scaling factor to obtain a histogram range similar to that observed in the presence of 100 μM picrotoxin (Fig. 9, A and B). This scaling factor was empirically estimated: in the example shown in Fig. 9 it was set to 0.55. The theoretical and the picrotoxin-resistant mIPSC amplitude distributions were then subtracted from that obtained in control condition and the two resulting histograms were compared. As shown in Fig. 9, C and D, the subtracted histogram differed from that obtained with picrotoxin data set. In the example of Fig. 9, the number of picrotoxin-resistant small inhibitory postsynaptic currents (10–80 pA) is smaller than expected if the amplitudes of all mIPSCs were equally reduced. Similar results were obtained on three other cells. This might suggest that, in the presence of picrotoxin, there was a failure to detect a significant number of mIPSCs ranging from 10 to 80 pA. Visual inspection of mIPSCs with amplitudes >30 pA obtained in the presence of 100 μM picrotoxin suggested that this was not the case. Moreover, as shown in Fig. 9, C and D, the number of picrotoxin-sensitive mIPSCs ranging from 80 to 150 pA was decreased to a larger extent than that predicted by scaling the amplitude of mIPSCs recorded in control conditions. Altogether, these results suggest that picrotoxin at 100 μM had distinct effects on mIPSCs of different amplitudes.

**Effects of picrotoxin on mIPSC kinetics**

The time to peak of mIPSCs was not significantly modified by 10–100 μM picrotoxin ($P = 0.05$, paired $t$-test). The mean time to peak was 0.86 ± 0.07 ms in control conditions and 0.8 ± 0.06 ms in the presence of 100 μM picrotoxin ($n = 10$). As previously described, the decay
FIG. 8. A: glycinergic miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of 1 μM tetrodotoxin + 25 μM bicuculline before (left), during (middle), and 7 min after (right) 100 μM picrotoxin application. For each trace, 10 continuous 500-ms epochs are superimposed (filter = 2 kHz, V_h = −50 mV). B: cumulative amplitude histograms of mIPSCs obtained before (n = 807 per 180 s), during (n = 705 per 180 s), and 7 min after application of 10 μM picrotoxin (n = 754 per 180 s). Note that picrotoxin preferentially decreased large mIPSCs. Mean amplitudes measured before, during, and after picrotoxin were 114.3 ± 80.9 (SD) pA, 105 ± 74.8 pA, and 115.1 ± 87.3 pA, respectively. C: cumulative amplitude histograms of mIPSCs obtained before (n = 952 per 180 s), during (n = 605 per 180 s), and 7 min after application of 100 μM picrotoxin (n = 1,028 per 180 s). Note that picrotoxin had a pronounced effect on the mIPSC amplitude. Mean amplitudes measured before, during, and after picrotoxin were 140.9 ± 125.5 pA, 82.5 ± 64.1 pA, and 141.4 ± 129.1 pA, respectively.

phase of mIPSCs was fitted with a single exponential curve (Legendre and Korn 1994, 1995). The decay of mIPSCs was reversibly decreased by the application of 100 μM picrotoxin only (Fig. 10A) and the τ_d distribution became skewed when compared with that obtained in control conditions (Fig. 10B). The mean value of τ_d was 7.72 ± 1.18 ms (n = 10) in control conditions and 4.98 ± 0.83 ms (n = 10) in the presence of 100 μM picrotoxin. As shown in Fig. 10, C and D, the τ_d and the amplitude of mIPSCs were not well correlated (r < 0.25) in the presence or in the absence of picrotoxin.

In control conditions there was a poor correlation between the time to peak and τ_d (<0.2). It was not modified by application of 100 μM picrotoxin (data not shown). These results suggest that filtering due to cable properties of cell membrane does not account for the skewed nature of mIPSC amplitude histograms (Legendre and Korn 1994; Rall 1969).

DISCUSSION

Our results show that two functionally different glycine-operated channels are present on the M cell of 52-h-old zebrafish larva. The analysis of the effects of picrotoxin on glycinergic mIPSCs recorded in the M cell is in favor of a noncompetitive mode of action for the toxin and suggests that the two GlyRs can be synaptically activated on the same neuron.

GlyR subtypes in zebrafish M cell

The two types of identified GlyRs differed in the number of their subconductance levels, their mean conductance state...
TWO TYPES OF GLYCINE-GATED CHANNELS ON ZEBRAFISH LARVAE M CELL

FIG. 9. A: amplitude histograms of tetrodotoxin-resistant inhibitory postsynaptic currents in the absence (open bars) and in the presence (shaded bars) of 100 μM picrotoxin. (Binwidth = 0.4 ms, $V_h = -50$ mV, epoch duration = 3 min.) Note that the amplitude histogram of picrotoxin-resistant mIPSCs remained skewed. B: to determine whether mIPSCs were equally affected by the application of picrotoxin, the amplitude histogram of tetrodotoxin-resistant inhibitory postsynaptic currents was compared in the absence of picrotoxin (open bars) with that obtained by multiplying the amplitude of mIPSCs by 0.55 with the use of the same data set. This scaling factor was adjusted to obtain a histogram range similar to that observed in the presence of picrotoxin. C: amplitude histogram of mIPSCs blocked by picrotoxin. The histogram was calculated by subtracting the amplitude distribution of picrotoxin-resistant mIPSCs from that obtained in control conditions shown in A. D: amplitude histogram obtained by subtracting the amplitude distribution of picrotoxin-resistant mIPSCs from that of scaled mIPSCs obtained in control conditions shown in B. Note that the number of small synaptic events would be considerably larger if picrotoxin equally decreased mIPSCs of all amplitudes as shown in D.

values, and their sensitivity to picrotoxin. The comparison of our results with data obtained on transfected cells (Bormann et al. 1993; Lynch et al. 1995; Pribilla et al. 1992) revealed that these two GlyRs are likely to correspond to $\alpha$-homooligomers and $\alpha/\beta$-heterooligomers.

Indeed, M cell GlyRs with multiple conductance levels had a main conductance state of 80–88 pS and were dramatically inhibited by picrotoxin application, as described for $\alpha$-homooligomers (Bormann et al. 1993; Lynch et al. 1995; Pribilla et al. 1992). The IC$_{50}$ for picrotoxin inhibition was very close to the value described for $\alpha$-homooligomers (5–9 μM) (Lynch et al. 1995; Pribilla et al. 1992). Moreover, the inhibitory effect of 100 μM picrotoxin was not complete (75–80% of control value), as observed for glycine $\alpha$-homooligomeric receptors (Pribilla et al. 1992). In transfected cells, picrotoxin concentrations >1 mM are required for a nearly complete inhibition (Pribilla et al. 1992).

In our experiments, residual single-channel activity observed in the presence of 100 μM picrotoxin resulted from openings of 40- to 46-pS subconductance levels. Their relative frequency was slightly increased by application of 1–10 μM picrotoxin and their kinetics was modified in a fashion similar to that observed for heterooligomeric-like GlyRs (see next paragraph). The second type of zebrafish GlyRs have a single conductance state of 40–46 pS as observed for $\alpha/\beta$-heterooligomers (Bormann et al. 1993). The $\beta$-subunit has been shown to confer little picrotoxin sensitivity to $\alpha/\beta$-heterooligomers (Pribilla et al. 1992). Accordingly, in our preparation picrotoxin applications did not significantly change the NP$_\text{o}$ of GlyRs with a main conductance state of 40–46 pS.
Zebrafish GlyRs are functionally similar to the mature form of glycine-gated channels. The main conductance state of homoooligomeric-like M cell GlyRs (80–88 pS) was identical to that of the mature $\alpha_1$ homoooligomeric receptors (Bormann et al. 1993), whereas the main conductance state of embryonic $\alpha_2$ homooligomers is close to 100–110 pS (Bormann et al. 1993). $\alpha_2/\beta$-Heterooligomers still open with large conductance levels (Bormann et al. 1993), which is rarely the case for $\alpha_2/\beta$-heterooligomers (Bormann et al. 1993) or for heterooligomer-like M cell GlyRs. Moreover, synaptically activated immature $\alpha_2$ GlyRs lead to long synaptic events (15–20 ms) (Krupp et al. 1994; Takahashi et al. 1992), whereas mIPSCs recorded in 52-h-old zebrafish larvae had short decay times (4–7 ms), similar to glycineergic mIPSCs recorded in adult goldfish (Faber and Korn 1982) and in mature mammalian neurons (Takahashi et al. 1992).

Mechanisms of action of picrotoxin on zebrafish GlyRs

Two opposite hypotheses have been proposed to explain the mechanism of action of picrotoxin on $\alpha$-homoooligomers (Lynch et al. 1995; Pribilla et al. 1992). The first one supposes that picrotoxin acts as a noncompetitive antagonist (Pribilla et al. 1992), as it generally does on $\gamma$-aminobutyric acid-A (GABA$_A$) receptors (Takeuchi and Takeuchi 1969;
Yakushiji et al. 1987; Yoon et al. 1993; but see Inoue and Akaike 1988). Amino acid exchanges for the corresponding residues of the M2 domain of the GlyR α1 subunit in the M2 domain of the β-subunit restore picrotoxin sensitivity of α/β-heterooligomers (Pribilla et al. 1992). This observation has led to the proposal that the noncompetitive action of picrotoxin is due to its open channel blocker properties (Pribilla et al. 1992). The second hypothesis states that picrotoxin is an “allosterically” active competitive antagonist on human GlyR α1 homooligomers because it modifies the transduction process between agonist binding and channel activation (Lynch et al. 1995).

Picrotoxin inhibition of M cell glycine-gated channels was not use dependent or voltage dependent. This suggests that it did not act as an open channel blocker (see also Lynch et al. 1995). Moreover, picrotoxin binding sites on GlyRs are likely to be different from those present on GABA A receptors. In contrast to their actions on GABA A receptors, picrotin and picrotoxin, the two molecular components of picrotoxin (Curtis and Johnston 1974b), are equally potent in blocking GlyR α1 homooligomers (Lynch et al. 1995). It is also unlikely that picrotoxin acts as a classical competitive antagonist on M cell GlyRs and on GlyR α1 homooligomers. Indeed, picrotoxin decreased both the mean open time and the relative frequency of the 80- to 88-pS main conductance states of M cell GlyRs, whereas these two parameters are not concentration dependent (Legendre et al. 1994), and, as mentioned by Lynch et al. (1995), picrotoxin does not displace bound [3H]strychnine and does not interfere with glycine displacement of bound [3H]strychnine on GlyR α1 homooligomers.

Our results rather support the hypothesis that picrotoxin acts as a noncompetitive antagonist on M cell homooligomeric-like GlyRs and that its binding site is located in the extracellular domain of GlyR subunits. The action of picrotoxin was very similar to the effect of zinc reported on N-methyl-D-aspartate (NMDA) receptors (Legendre and Westbrook 1990). As does zinc on NMDA receptors, picrotoxin may evoke or promote a desensitization state linked to the open state. Because openings were shortened by picrotoxin application, the dissociation rate constant of this antagonist must be slower than the closing rate constant of the channel (Colquhoun and Hawkes 1995). The most probable escape from this desensitization state is a conformational change of the GlyR in response to the agonist dissociation from its binding site.

The lack of significant change in the NP A of GlyRs with a main conductance state of 40–46 pS resulted from two opposite actions on channel activation properties. First, 1-100 μM picrotoxin increased the relative frequency of longer openings. This might result from an increase in glycine binding site affinity, as previously proposed (Lynch et al. 1995), because the relative weights of the mean open times were dependent on glycine concentration (Legendre and Korn 1994; Twyman and Macdonald 1991). Second, picrotoxin, at concentrations >10 μM, decreased the mean open time, induced flickering, and increased the medium burst duration. This picrotoxin effect is similar to that described for NMDA receptor blockade evoked by Mg2+ (Ascher and Nowak 1988) but, in contrast to the action of Mg2+, flickering evoked by picrotoxin was not voltage dependent. Again, these results rule out the possibility that picrotoxin binds within the pore of the channel. Occurrence of flickering suggests that recovery from inhibition is fast (Ascher and Nowak 1988). The brief closures observed during channel openings could reflect a fast dissociation rate constant for binding of this toxin, consistent with the IC50 value (1 mM) obtained for picrotoxin inhibition on α1/β-heterooligomers (Pribilla et al. 1992). Although picrotoxin had different effects on NP A of homo- and heterooligomer-like GlyRs, it had similar effects on the 40- to 46-pS openings of both types of GlyRs. The β-subunit does not therefore seem to be involved in picrotoxin effects on the 40- to 46-pS substate kinetics, suggesting that the toxin allosteric binding sites are located on GlyR α1-subunits.

The weak antagonist effect observed on α1/β-heterooligomeric-like GlyR appears to result from the very small probability for this channel to open with a conductance state τ44 pS. Such a possibility is supported by previously published data obtained on α1/β-, α1/β-, and α1/β-heterooligomers. Picrotoxin is a less potent inhibitor of the activation of α1/β- and α1/β-heterooligomers (IC50 τ 1 mM; Pribilla et al. 1992) displaying a maximum conductance state of 40 pS (Bormann et al. 1993) than of the activation of α1/β-GlyR (IC50 = 0.3 mM) (Pribilla et al. 1992) displaying 112- and 80-pS subconductance levels (Bormann et al. 1993).

Our data are consistent with the proposal that the multiple effects of picrotoxin result from its capacity to modify the transduction process between agonist binding and channel activation (Lynch et al. 1995). In this scheme, picrotoxin would only be effective in certain GlyR conformations. The events intervening between agonist binding and channel activation critically depend on amino acids located on the extracellular loop between the M2 and M3 transmembrane domains (Lynch et al. 1995). This extracellular domain is important for conformational changes corresponding to distinct subconductance levels (Bormann et al. 1993). For example, Glu-290 of β-subunits is essential to prevent openings of 80- to 88-pS substates (Bormann et al. 1993). Moreover, Arg/Leu-271 or Arg/Gln-271 α1 subunit mutations associated with the human startle disease (Rajendra and Schofield 1995; Shia et al. 1993) decrease the average conductance of the glycine-gated channel (Rajendra et al. 1995; Vandenburg et al. 1992) and disrupt transduction processes between agonist binding and channel activation (Lagosch et al. 1994; Rajendra et al. 1994). Interestingly, this mutation also modifies the effect of picrotoxin on α1 homooligomeric human GlyRs (Lynch et al. 1995); it converts picrotoxin into an allosteric potentiator, whereas its antagonist properties remain unaffected. This is closely similar to the picrotoxin effects observed on 40- to 46-pS subconductance levels. Arg-271 is also present on zebrafish GlyR α1-subunits (C. Goblet and B. David-Watine, personal communication), but it is unlikely that this residue contributes to the picrotoxin binding site, because Arg/Leu-271 or Arg/Gln-271 α1 subunit mutations do not significantly change picrotoxin IC50 (Lynch et al. 1995).

**Physiological significance of GlyR subtypes**

The effect of picrotoxin applications on glycine-gated mIPSCs strongly suggests that both types of GlyRs ex-
pressed by the M cell can be synaptically activated. The large mIPSCs were more sensitive than the small synaptic events to picrotoxin, suggesting that the large mIPSCs depended mainly on the activation of homooligomeric-like GlyRs. It cannot, however, be excluded that all postsynaptic GlyR clusters possess a variable proportion of homo- and heterooligomeric-like GlyRs. Application of 100 μM picrotoxin decreased the decay time constant of all mIPSCs and increased the skewness of their distribution, which suggests that, at this concentration, picrotoxin modifies to a different extent the activation properties of all postsynaptic GlyRs. The mean open times of 40–46 ps are the main limiting factors for mIPSC duration (Legendre and Korn 1994, 1995) and, at this concentration, picrotoxin might also modify the kinetics of synaptically activated heterooligomeric-like GlyRs. This decrease in the decay time constant could therefore reflect shortening of 40- to 46-ps openings, although their burst durations were slightly increased. Analysis with the use of fast flow application techniques on outside-out patches (Hestrin 1992; Jonas 1995; Lester et al. 1990) is necessary to resolve this issue.

The presence of different types of GlyRs on the same cell could be the reflection of the immaturity of the neuron. For instance, somatic GlyRs from spinal cord neurons of newborn rats have subconductance levels closely similar to those of α, homooligomers (Takahashi and Momiyama 1991), whereas on more mature animals they are functionally identical to GlyR α/β-heterooligomers (Takahashi et al. 1992). However, it is now well established that a mature neuron can express several subunit isoforms of the same receptor, leading to the presence of functionally different postsynaptic receptors (Levey et al. 1995; Niedzielski and Wenthoud 1995; Santi et al. 1994; Vallano et al. 1996). In hippocampal neurons, GABA synapses evoke short or long inhibitory synaptic events depending on their somatodendritic location (Pearce 1993). In the adult goldfish, glycineric synapses asposed to the lateral dendrite are functionally more complex than those in contact with the soma (Korn et al. 1993). Likewise, differences in the main conductance state of the two glycine-gated channels imply that the efficacy of inhibition varies from one synapse to another in the zebrafish larva M cell. Lateral and ventral dendrites are not fully developed on the 52-h-old zebrafish larva M cell (Kimmel et al. 1981) and the presence of homo- and heterooligomeric-like GlyRs at somatic glycineric synapses might reflect a distinct stage during differentiation of this cell. Differences between proximal and distal neuronal inhibition could play a role in the integrative function of glycineric synapses of the more mature M cell, as proposed in other systems (Vu and Krasne 1992). For example, with respect to the electrotonic length of the cell, large inhibitory events from dendritic glycineric synapses could influence somatic membrane potential, whereas smaller inhibitory events originating at somatic glycineric synapses will have little effect on distal dendrites. In this case, functional somatodendritic heterogeneity of glycineric synapses could control the polarity of neuronal signal transmission. Electrophysiological analysis of glycineric mIPSCs in the adult zebrafish M cell may allow these issues to be resolved (Korn et al. 1996).

The functional receptor heterogeneity appears, however, unlikely to be the single contributor to the variability of the mIPSCs size because, in the presence of a high picrotoxin concentration, the amplitude distribution of mIPSCs remained skewed. The high number of neurotransmitter molecules released after an exocytosis (Faber et al. 1992) does not necessarily imply that neurotransmitters bind to all available receptors if the neurotransmitter diffusion is restricted and postsynaptic density is broad enough (Wahl et al. 1996). A variation in neurotransmitter quantity has been recently proposed to account for the skewedness of GABA mIPSC amplitude histograms in cultured retinal amacrine cells (Ferking et al. 1995). Finally, postsynaptic parameters such as the number of available receptors and the extent of desensitization (Tang et al. 1994) may, in addition to receptor heterogeneity, control the quantal size at central synapses. Consequently, quantal variability might originate either from or postsynaptically depending on the morphofunctional characteristics of the postsynaptic receptor matrix.

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