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Research report

Heterogeneous current responses to GABA and glycine are present in post-natally cultured hippocampal neurons

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Abstract

In a patch-clamp study of cultured hippocampal neurons, heterogeneous desensitization responses were observed in all cells with GABA_A-gated channels, but in only 5% of cells with glycine-gated channels. GABA-and glycine-activated whole-cell currents from 'fast' and 'slow' cells had very similar amplitudes of about 2.0 nA, but different time-courses of desensitization. Single-channel main conductance states obtained from slow and fast cells both had values of about 27 ± 1 pS for GABA, and values of 24 ± 1.8 pS for slow and 19 ± 1.5 pS for fast desensitizing glycine-gated channels. For GABA, the channel open or burst frequency of fast desensitizing cells was about twice that of slow desensitizing ones, whereas for glycine, the opening frequency of slow desensitizing glycine-gated cells. Dose-response curves obtained for slow and fast desensitizing cells displayed similar degrees of cooperativity and antagonist affinity, but clearly greater GABA sensitivity for fast desensitizing cells. In contrast, fast desensitizing glycine-gated cells displayed low antagonist affinity, whereas both types of cells displayed similar agonist sensitivity and cooperativity. These results indicate a mosaic-like distribution of different GABA_A and glycine receptor isoforms in hippocampal neurons, with the possible existence of pre-natal-like glycine receptor subunits at this early stage of post-natal life.

Keywords: GABA; Glycine; Hippocampus; Post-natal; Tissue-culture; Whole-cell current; Single-channel current

1. Introduction

GABA- and glycine-activated Cl⁻ currents in cultured hippocampal neurons of new-born rat pups have already been studied in detail [14,15]. In addition, there have been some very detailed and comprehensive analyses of GABA and glycine currents in spinal neurons (e.g. [3,8]). Molecular biological studies of rodent GABA receptors have shown that receptor subunits are developmentally controlled. Recently, Northern blotting, in situ hybridisation, and quantitative polymerase chain reaction (PCR) techniques have demonstrated changes in levels of mRNA encoding GABA_A receptor subunits in rat brain during development. Levels of $\alpha 1$, $\alpha 6$, $\beta 2$, $\gamma 2$, and δ mRNAs have been shown to increase during development, whereas α 3 decreased. Several other subunits, including α 2, α 5, β 1, β 3, γ 1, and γ 3, demonstrate a postnatal peak followed by a decrease later in development [9,17,18,22,26]. Similarly, detailed mapping of glycine receptor-like immunoreactive (GRLI) structures in the rat central nervous system using a monoclonal antibody against the glycine receptor [29] have revealed GRLI structures in forebrain and diencephalon including the hippocampus. Further biochemical and expression studies have indicated developmental heterogeneity of the glycine receptor in the spinal cord and in other brain regions [7,1,6,21].

In these present studies, we have examined and compared the GABA_A- and glycine-activated Cl⁻ currents from hippocampal neurons cultured from 1-day-old rat pups, the cultured cells being patch-clamped during the first 2–4 days in order to gain information about the main characteristics of these channels at this stage of life. The GABA studies have both confirmed and extended the recent work of [36] and added single-channel measurements and kinetics data, whereas the glycine studies are totally new.

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2. Materials and methods

2.1. Tissue-culturing of hippocampal neurons

Hippocampal neurons of one-day-old rat pups were tissue-cultured as described earlier [14]. Briefly, one-day-old rat pups were quickly decapitated, and their hippocampi removed. The cells were mechanically isolated without the use of any dissociating enzyme in 3 ml ice-cold Puck's saline and incubated in EMEM (pH 7.4), at 37° C in an atmosphere of humidified air with 5% CO₂, and used from the second day of preparation onwards.

2.2. Electrophysiological recording

Cells were bathed in mammalian saline and maintained on the stage of an inverted microscope at room temperature (20-22°C). Whole-cell and outside-out single-channel currents were studied with the patch-clamp technique [20], using an EPC-7 patch-clamp amplifier (List-Medical, Darmstadt, Germany). Patch-clamp pipettes were pulled from borosilicate haematocrit tubing (Modulohm, 1/s, Vitrex, Herlev, Denmark), either on a vertical two-stage puller (Model 720, Kopf Instruments, Tujunga, CA, USA) in earlier measurements or on a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments Co., USA) in later ones. Patch pipettes, coated with Sylgard resin (Dow Corning), were heat-polished and filled with pipette solution and had resistances of 3-8 M Ω for whole-cell and 5-20 M Ω for single-channel recordings. Data were filtered at 10 kHz before being stored on video tape. Records were then digitised at 10 or 5 kHz and filtered at 2 or 1 kHz respectively for both analysis and display.

Occasionally, the series resistance compensation was set at 50% (particularly necessary for currents larger than 1 nA) while the cell and pipette capacitance were maximally compensated. Series resistance errors were minimised by using low resistance pipettes and by selecting small cells.

2.3. Solutions

The cells were bathed in normal mammalian Ringer which contained (in mM): NaCl 137, KCl 5.4, CaCl₂ 1.8, MgCl₂ 2, Na-HEPES 5 (pH 7.4). The pipette solution was (in mM): CsCl 120, CaCl₂ 1, MgCl₂ 2, TEACl 20, EGTA 11, HEPES 10 (pH 7.4). For whole-cell and single-channel measurements, neurotransmitters and their antagonists were added to the solution bathing the cell. The bath changeover time for drug application was not much greater than 100 ms. Most reagents were obtained from the Sigma Chemical Co. (St. Louis, MI, USA), with the exception of TEACl (Merck, Munich, Germany) and HEPES (Calbiochem-Novabiochem Corp., CA, USA).

2.4. Data analysis

All data were analysed on an 80386 IBM-PC compatible computer using the *PNSCROLL* [5] and *SigmaPlot* 5.0 (Jandel Scientific, Corte Madera, CA, USA) software packages. Generally, ASCII files from *PNSCROLL* were imported to Corel DRAW 3.0 (Corel Corporation, Salinas, CA, USA) for final presentation purposes.

All voltages have been corrected for liquid junction potentials using the program *JPCalc* [4] and have been expressed as the potential of the interior membrane surface with respect to the exterior one. Upward deflection of current in all figures represents current flow out of the cell (or from internal to external membrane surface in excised patches). The initial speed of desensitization of GABA and glycine receptor-channels was determined by measuring the time (t_{80}) at which their whole-cell currents were reduced to 80% of the peak value. The decay phase of the GABA and glycine response was best fit by the following double exponential equation:

$$I(t) = I_{ss} + I_{f} e^{-t/\tau_{f}} + I_{s} e^{-t/\tau_{s}}$$
(1)

where I_{ss} represents the steady-state current and I_f and I_s represent the magnitudes of fast and slow desensitizing current components respectively, with corresponding time constants τ_f and τ_s . GABA and glycine dose-response curves were constructed for applied GABA concentrations from 5 μ M to 0.5 mM and glycine concentrations from 5 μ M to 1 mM. In both cases, any higher concentrations did not produce larger responses. Peak current amplitudes were normalized with respect to their maximum values. The Cl⁻ currents for both agonists were fitted to the Hill version of Michaelis-Menten Kinetic equation:

$$I = I_{\max} C^n / (C^n + K_D^n)$$
⁽²⁾

Where I is the agonist-induced I_{CI} , C the agonist concentration, $K_{\rm D}$ the dissociation constant (agonist concentration to elicit half-maximal current), n the Hill coefficient and I_{max} , the maximum current response as C is increased. The affinity and pharmacological modulation of GABA and glycine receptors were estimated by comparing 'fast'and 'slow'-activated GABA and glycine whole-cell currents and their shift to the right by their respective antagonists. Agonist current responses with t_{80} values of about 3 s and residual non-desensitized current less than 20% of the I_{max} were classified as 'fast' and those with t_{80} values more than 20 s and a residual current of more than 50% of I_{max} as 'slow'. The duration of the drug application was constant for the two agonists for both 'fast' and 'slow' cells. Agonist-activated single-channel current conductances, open-time durations and channel open or burst frequencies were measured using the PNSCROLL [5] program. A burst was defined as the opening or group of openings separated by a relatively long closed-time 'tc' [12] with a value of 5 ms selected for 'tc', similar to [34].

3. Results

The effect of GABA and glycine in low concentrations with symmetrical Cl^- was studied in post-natal tissue-cultured hippocampal neurons from 2–5 days after plating, using whole-cell and single-channel patch-clamp configurations. There was no significant correlation in the size of agonist-induced currents with the number of days in culture.

3.1. GABA-activated whole-cell currents

Peak-currents (2 nA \pm 0.04) in response to 5 μ M GABA were observed for fast and slow desensitizing cells at the resting membrane potential (range: from -70 to -80 mV) with symmetrical chloride concentrations and Cs⁺ replacing K⁺, and 20 mM TEACl in the pipette. Heterogeneous whole-cell current desensitizing responses were evoked randomly from different cells during the continuous bath application of 5 μ M GABA. In some cells, chloride currents in response to 5 μ M GABA exhibited incomplete desensitization during the bath application period of 42 s with the final remaining current still at 80% of the initial peak value (Fig. 1a), whereas in others the current had dropped to the base line (Fig. 1b). The former GABA-gated whole-cell Cl⁻ currents took more than 20 s to reach 80% of the initial peak value in (Fig. 1a), whereas the latter took about 3 s (Fig. 1b), thus allowing them to be readily characterized as slow and fast desensitizing receptor-channels and correspondingly 'slow' and 'fast' cells respectively. Further analyses of their time-courses showed that both cell types exhibited double exponentials. Decay time constants for slow cells were $\tau_f = 7.5 \pm 2.5$ s and $\tau_s = 42 \pm 5.5$ s and for the fast cells were $\tau_f = 9.2 \pm 2.6$ s and $\tau_s = 23 \pm 6.1$ s (n = 30 in each case).

This heterogeneous desensitization behaviour could be due to the presence of functionally distinct $GABA_A$ receptor subtypes, with GABA able to activate different subtypes of chloride-conducting receptor channel complexes, as described previously [42].

3.2. Single-channel currents

Excised patches were obtained from the same cells used for recording whole-cell currents. These membrane patches were bathed in symmetrical chloride solutions (pH 7.3), and showed no current activity in control solutions without GABA. In the presence of 2 μ M GABA in the bath, these outside-out excised patches from both slow and fast cells showed outward current rectification (Cl⁻ influx > Cl⁻ efflux for equal magnitude driving forces in both directions). The channel open probability increased with depolarization and the pronounced outward rectification at positive potentials seemed to be related to the total number of higher conductance levels exhibited at that potential (see later in Fig. 5, top panel).



Fig. 1. Two types of GABA-activated current responses, with different time courses of desensitization, from two different cells. Whole-cell current responses are shown in the upper panels and their corresponding outside-out single-channel records in the lower panels, following bath application of 5 μ M GABA in symmetrical Cl⁻ solutions at resting membrane potential, $V_{\rm m}$, = -70 mV. Panel a: a slow desensitizing current response (maximum whole-cell current, $I_{\rm max}$, = 2.2 nA; $t_{80} > 20$ s). Panel b: a fast desensitization response ($I_{\rm max}$ of 2.4 nA; $t_{80} = 2.9$ s).

GABA-gated single-channel conductances were very similar for both fast and slow cells. In excised outside-out patches, the main single-channel conductance state at -70 mV was 27 ± 1 pS for both cell types. In addition, there was no significant difference between the open-times of the single-channel events (7.4 ± 2.4 ms (n = 25) for fast cells and 8.1 ± 1.3 ms (n = 25) for slow cells; see Fig. 1, lower panels).

Further careful examination of GABA-activated singlechannel currents revealed that the channel open or burst frequency of the fast desensitizing cells was about twice that of the slow cells and with a much greater fraction of the burst time spent in the open state. The frequency of channel opening for fast cells was 5.4 ± 0.03 s⁻¹, compared to 2.6 ± 0.01 s⁻¹ for slow, with both having very similar conductance values (n = 25 in each case).

3.3. GABA dose-response curve

The amplitude of the GABA-induced whole-cell peakcurrent response was found to vary sigmoidally with agonist dose and the data fitted to the Hill Equation (Eq. (2)). In the presence of GABA alone, the two sets of data points for fast (hollow triangles) and slow (filled circles) cells exhibited two sigmoidal curves (Fig. 2a) giving a K_D value of 7 μ M for fast and 54 μ M for slow desensitizing currents, suggesting the expression of two functionally distinct receptors in our preparations. For currents from both cell types a Hill coefficient of 2.2 was obtained, implicating multiple binding of GABA molecules with a single molecule of GABA_R. These results demonstrated a similar degree of cooperativity but a different GABA_R affinity for both cell types. Bicuculline methiodide, an antagonist of the GABA_A receptor, at a concentration of 10 μ M shifted the GABA dose-response curve of both slow and fast cells towards the right, increasing the threshold-dose from 5 to 10 μ M and the K_D from 54 to 126 μ M for slow (Fig. 2b) and from 7 to 13 μ M (Fig. 2c) for fast desensitizing whole-cell currents with the Hill coefficient still remaining close to 2 (2.1 and 2.3, respectively). Similarly, the maximum current response (6.6 nA) remained almost the same (6.3 nA) for the slow and from 6.4 nA to 5.8 nA for the fast, indicating that bicuculline is a competitive antagonist of the GABA binding site in both cell types.

3.4. Number of functional GABA_R-channels

An estimate of 3200 functional channels per cell activated by GABA was obtained from (1) the average singlechannel current response of 1.9 pA and (2) the whole-cell current response (about 6 nA) at -70 mV for a saturating concentration (0.5 mmol/l) of GABA. The cell capacitance, determined by capacity neutralization during experiments, was approximately 15 pF, implying a cell area of about 1500 μ m² (assuming a specific membrane capacitance of 1 μ F cm⁻²). This implied an approximate receptor-channel density of about 2 μ m⁻² for both slow and fast cells.

3.5. Glycine-activated whole-cell currents

The average peak amplitude of whole-cell currents induced by the continuous bath application of 5 μ M glycine (resting membrane potential, $V_{\rm m}$, = -70 mV; symmetrical Cl⁻ concentrations), was 2.0 ± 0.7 nA. The whole-cell



Fig. 2. Dose-response curves for GABA-activated fast and slow desensitizing whole-cell currents ($V_m = -70$ mV), and Hill Equation fits. Panel a: data from fast (closed triangles; n = 10) and slow (closed circles; n = 16) cells, indicating a Hill coefficient = 2 for both curves (see text) and K_D values of 54 and 7 μ M for the slow and fast cells, respectively. Panel b: dose-response curves for GABA for slow cells in absence (filled circles) and presence (open circles) of 10 μ M bicuculline, the antagonist changing K_D from 54 to 126 μ M, without significantly affecting the maximum current, I_{max} , or Hill coefficient. Panel (b) is similar to Fig. 4a (top) of [14], but is now based on more data. Panel c: GABA-activated currents in the absence (open triangles) and presence (closed triangles) of bicuculline in fast cells. Again, 10 μ M bicuculline changed the K_D for GABA from 7 to 13 μ M, without significantly changing I_{max} or the Hill coefficient. The error bars in each panel represent the s.e.s.

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additional data

(b)

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inward current responses, from two different cells (Fig. 3a and b), due to the efflux of Cl⁻ ions, again displayed two different rates of desensitization. Fast desensitizing glycine currents, unlike the GABA currents, were observed in only 5% of the total cells studied (3 out of 60 cells). The two whole-cell current responses displayed in Fig. 3a and b were of similar amplitudes but different time courses of desensitization. In 95% of the cells studied, glycine-gated whole-cell Cl⁻ currents decreased to 80% of their peak value in 35 s and remained at more than 50% of the initial value at the end of a 1.6 min application of glycine (Fig. 3a). However in 5% of the cells, t_{80} was attained in 3.8 s with 17% of the initial current remaining at the end of the glycine application for the same period of time (Fig. 3b), allowing characterization of the two cell types as 'slow' and 'fast' respectively. The distribution of decay times was fitted by double exponentials with time constants of $\tau_{\rm f}$ = 2.5 ± 1.0 s and $\tau_s = 19 \pm 3.5$ s for the fast cells and $\tau_{\rm f} = 4.2 \pm 2.6$ s and $\tau_{\rm s} = 20 \pm 4.1$ s for slow cells (n = 23in each case).

3.6. Single-channel glycine currents

(a)

Unlike GABA-gated single-channel currents, glycinegated single-channel currents exhibited a number of differences. First of all, 'fast' desensitizing currents were rarely present (only in 3 out of 60 cells).

Secondly, single-channel currents of fast cells did not display any outward rectification but rather a very linear current–voltage relationship.

In the presence of 5 μ M glycine in the bath (with $V_{\rm m} = -70$ mV), single-channel conductances for glycine

Fig. 4. A dose-response curve for glycine in the absence (filled circles) and presence (open circles) of strychnine, and Hill Equation fits. The average whole-cell current values (n = 16) include both fast and slow cells, since I_{max} from fast cells (5.9±0.5; n = 3) had similar values to slow cells (6.5±1.5; n = 50), although the two cell types were antago-

nised by different strychnine concentrations, μM and nM respectively. The panel is an extended version of Fig. 4a (bottom) of [14] with

receptor-channels were 24 ± 1.8 pS for slow and 19 ± 1.5 pS for fast cells, and in contrast to the heterogeneous single-channel population of the GABA receptors there was a significant difference between the single-channel burst open-times of 560 ± 10 ms for slow (n = 25) and 130 ± 9 ms for fast cells (n = 3) (Fig. 3, lower panels). Note also the cooperative opening of two different type of channels in that figure with longer closed periods for the



Fig. 3. Two types of whole-cell (upper panels) and corresponding outside-out single-channel (lower panels) current responses of glycine receptor-channels in two different cells activated by bath application of 5 μ M glycine ($V_m = -70$ mV; symmetrical Cl⁻ concentrations). The slow glycine whole-cell current response (panel a) exhibited partial desensitization ($I_{max} = 2.0$ nA; $t_{80} = 35$ s). A fast glycine whole-cell current, observed for only 5% of cells, is shown in Panel b under similar experimental conditions to those of Panel a ($I_{max} = 2.0$ nA; $t_{80} = 3.8$ s). See text for further details.

fast cells. The channel opening and burst frequency of slow cells were three times greater than those of the fast cells. The channel opening frequency for slow cells was $6.8 \pm 0.04 \text{ s}^{-1}$ compared to $2.3 \pm 0.02 \text{ s}^{-1}$ for the fast cells.

3.7. Glycine dose-response curve

Whole-cell currents through glycine receptor-channels for both slow and fast cells, showed a steep dependence on external glycine concentration ($V_m = -70 \text{ mV}$; with symmetrical Cl⁻ concentrations), with the currents becoming detectable in 5 μ M glycine. The peak amplitude of glycine-activated whole-cell currents, which varied sigmoidally with agonist dose, was fitted well by the Hill Equation (Eq. (2)) and is shown in Fig. 4 (with all responses being normalized to the peak current produced by 1 mM glycine).

The average glycine concentration required for half-

GABA - 100 mV

maximal activation, K_D , was 63 μ M and the mean Hill coefficient was about 2.0; suggesting that at least two glycine molecules bind to activate the glycine receptor. In slow cells, the addition of 10 nM of strychnine increased the K_D to 520 μ M and the Hill coefficient to about 2.1, without significantly affecting the maximum response of 7 nA, suggesting that strychnine is a competitive inhibitor for glycine binding sites in these cells. In contrast, concentrations of more than 1 μ M strychnine were needed to block the glycine-gated currents in the fast cells, thus indicating a much lower affinity for strychnine for these glycine receptors.

3.8. Number of functional glycine_R-channels

An estimate of about 4800 functional glycine-activated channels per cell was obtained for slow cells from (1) the average single-channel current response of 1.47 pA and (2) the average whole-cell current response (about 7.0 nA) at

1 pA



Fig. 5. Examples of single-channel currents at positive and negative membrane potentials with 2 μ M GABA (top 2 panels) or 5 μ M glycine (2 lower panels) in the bath, with symmetrical chloride concentrations, demonstrate outward rectification. The values of the main (most frequently occurring) conductance state are shown along with the membrane potential for each record. The brief closures of the channel and the presence of an intraburst state during high and low conductance states, readily seen at -100 mV, suggest that this is more likely to represent subconductance states of a single-channel, rather than the cooperative opening of multiple channels. See text for further details.

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Various parameters	of fast and slow	cells for both GABA	and glycine-gated	receptor-channels
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Parameters	GABA		Glycine	
	fast	slow	fast	slow
Whole-cell current (nA)	≈ 2	≈ 2	≈ 2	≈ 2
$t_{80}(s)$	≈ 3	≈ 20	≈ 3.8	≈ 35
Non-desensitized % of peak current	20%	> 50%	17%	50%
$\tau_{\rm f}(\rm sec)$	9.2 ± 2.6	7.5 ± 2.5	2.5 ± 1.0	4.2 ± 2.6
$\tau_{\rm s}(\rm sec)$	23 ± 6.1	42 ± 5.5	19 ± 3.5	20 ± 4.1
$\vec{K}_{\rm D}(\mu {\rm M})$	7	54	60	63
Bicuculline (conc.)	10 µM	10 µM		_
Strychnine (conc.)	_	_	$> 1 \ \mu M$	10 nM
Single-channel conductance	$27 \pm 1 \text{ (pS)}$	$27 \pm 1 \text{ (pS)}$	$19 \pm 1.5 (\text{pS})$	$24 \pm 1.8 (\text{pS})$
Single-channel open-time (ms)	7.4 ± 2.4 ^a	8.1 ± 1.3^{a}	130 ± 9 (burst) ^b	560 ± 10 (burst) ^c
Opening frequency (s^{-1})	2.6 ± 0.01 ^a	5.4 ± 0.03^{a}	2.3 ± 0.02 ^b	6.8 ± 0.04 °
Single-channel rectification	outward at	outward at	no rectification	outward at
c	positive potentials	positive potentials	(linear I/V curve)	positive potentials
No of receptor-channels per cell	3200	3200	5300	4800
Channel density (μm^{-2})	2	2	3.5	3.0
% receptor types present	about 50%	about 50%	about 5%	about 95%

^a Mean values obtained from 27 pS conductance state openings.

^b Mean values obtained from 19 pS conductance state openings.

^c Mean values obtained from 24 pS conductance state openings.

-70 mV for a saturating concentration (1 mM) of glycine. From the measured cell capacitance of approximately 15 pF (determined by capacity neutralization procedures), which implied a cell area of about 1500 μ m², the channel density was estimated to be at least 3 μ m⁻² for these slow cells in these early post-natal cultured hippocampal cells. In contrast for fast cells, the average single-channel current value was 1.33 pA, with a similar saturating whole-cell current response of about 7.0 nA, thus implying the opening of about 5300 receptor-channels and hence (using capacitance measurements for these cells) an approximate channel density of about 3.5 μ m⁻².

3.9. Rectification in GABA and glycine-gated currents

Except for the few fast glycine-activated cells, singlechannel currents showed pronounced outward rectification for both GABA and glycine receptor-channels (Fig. 5, lower panel). At hyperpolarized membrane potentials, the open probability of low conductance channel states was greater than that of high conductance channel states. This distribution of subconductance states could help to determine the normal resting membrane potential. In addition to the main conductance state at that membrane potential, several other less frequently occurring states were also observed for both GABA- and glycine-gated single-channels in excised patches. A selection of recordings shown in Fig. 5 illustrates the multi-conductance state behaviour of GABA and glycine receptor-channels at +100 and -100mV. For example, for GABA-gated channels at -100 mV, in addition to the main current level of 2.3 pA, other levels of 3.1 pA, 2.6 pA, 2.1 pA, 1.9 pA and 1.7 pA were also evident. Nevertheless, the main conductance state for

GABA was consistently found to be 23 pS for more than 65% of the time in the open channel state. Similarly, GABA-gated channels at +100 mV spent more than 70% of their time in the main conductance state of 48 pS. Likewise for slow cells, the glycine-gated single-channel main conductance state at -100 mV was 18 pS with other substates of 23 pS and 13 pS and at +100 mV, in addition to the main state of 42 pS, there were conductance states of 30 pS and 20 pS. In contrast, glycine-gated fast desensitizing currents did not show any current rectification (traces not shown).

All the various parameters for $GABA_A$ - and glycinegated receptor-channels for fast and slow cells are summarized in Table 1.

4. Discussion

We have observed heterogeneity for both GABA- and glycine-activated currents in cultured hippocampal neurons, exhibiting two different types of whole-cell currents, i.e. slow desensitizing and fast desensitizing currents. Glycine-activated heterogeneity of whole-cell currents has not been observed before in the hippocampal neurons, whereas the GABA-activated currents in our cells basically confirmed the work of [36] with a few differences, which may be due to different culture induction conditions (e.g. chemical dissociation instead of mechanical separation of hippocampal neurons). The two types of GABA-gated whole-cell currents in our cultured cells had similar conductance values, but differed from each other with respect to both rate and extent of desensitization (Fig. 1a and b, top panels). The single-channel currents underlying both types of GABA-gated whole-cell currents had similar channel conductances, open durations, outward rectification and subconductance states but different frequencies of channel openings during burst, and different GABA sensitivities. These properties are unlikely to be due to the presence of $\alpha 1 \beta 2$, $\alpha 1 \gamma 2$ or $\alpha 1 \beta 2 \gamma 2$ subunits [41,28] or any combination with $\alpha 6$ GABA-receptor subunits [22] at this stage of life, but may be due to the presence of a GABA/benzodiazepine receptor subunit [32] with a different variant of the α subunit [24], which could account for the complex bursting properties of the receptor channel [34]. It is interesting that photo-affinity labelling of GABA_A receptors by benzodiazepine ligands occurs via the α subunits [16].

The single-channel burst frequency of fast cells was double that of slow cells (Fig. 1, lower panels) and the increased whole-cell steady-state current of fast cells, with increased burst frequencies of underlying single-channel currents, is in agreement with the benzodiazepine studies of mouse spinal neurons in culture [34], implicating the possible expression of $\beta 2$ subunits along with γ subunits in fast cells [41]. Dose-response curves obtained from these cells showed that fast cells have low $K_{\rm D}$ values (7) μ M) compared with slow cells (54 μ M; Fig. 2a) implying that the fast cells are more sensitive to GABA. GABA, receptor affinity and the degree and rate of desensitization was not seen to be related to the morphology of the cells in our preparation. Presumably the multiple GABA response arises from the activation of different GABA_A receptor subtypes which vary with the age of the cells. In this process, some GABA receptors may mature earlier (possibly due to the expression of different α subunits) as differential regulation of α polypeptide isoforms may lead to (1) changes in GABA_A receptor function during ontogeny as well as (2) distinct changes in cellular responses to GABA and GABA-related drugs [26]. There was no difference in either the value of single-channel conductance obtained or in the channel density estimated for either type of cell. The average conductance value obtained for single-channel currents (with $V_{\rm m} \approx -70$ mV) from either type of cell was 27 pS and the estimated value of the receptor density was 2 μ m⁻². This suggested that the multiple GABA response was due to the different subunits of the GABA receptors. It is clear that multiple $GABA_A$ subtypes exist in the brain, that they have different physiological and pharmacological properties, and that different subtypes coexist in some brain regions [30]. However, other possible explanations for this heterogeneity may be considered. The receptors responsible for slow and fast desensitizing currents may be identical in structure but may have different modulatory influences, for example, due to different internal calcium ion levels [27] or phosphorylation states [11,23]. A second alternative to different receptor subtypes is that the slow decay component results from the longer channel open-time of a doubly-liganded receptor [25].

In contrast, glycine-gated whole-cell currents were essentially homogeneous, except for a few examples of fast desensitizing cell responses with neonatal characteristics (low strychnine affinity and linear current voltage relationship), implicating the presence of a differential expression of glycine receptor α and β subunits [19] or the assemblage of neo-natal $\alpha 2$ glycine receptor subtypes in the hippocampus at this stage of development, which would agree with the expression studies in rat spinal cord [6]. The glycine-gated channel density estimated in our preparation was greater than that of GABA-gated channels.

GABA-gated currents in our cells were competitively blocked by bicuculline in micromolar concentrations showing that bicuculline competes for the same receptor region that binds GABA with moderate affinity (μ M). Glycineactivated currents were competitively blocked by strychnine in nanomolar concentrations (except for the fast cells). This may simply mean that glycine and strychnine bind to the same receptor site with very different affinities. Alternatively, as may be more likely from other evidence [38], it has been suggested that the glycine post-synaptic receptors may be a complex possessing a site that binds strychnine with a very high affinity (nM) together with a second receptor site that binds glycine with moderate affinity (μM) . Binding to each site in both cases may partially or completely obscure the other site to give a competitive blocking effect.

Recent research has not only indicated that glycine acts as a transmitter in many supraspinal regions, such as the cerebellum, the cortex [2] and the hippocampus etc, but its effect on the NMDA receptor suggests that glycine or related substances may influence synaptic transmission throughout the brain. Knowledge about this latter effect will undoubtedly be exploited in attempts to develop new therapeutic strategies for neurological diseases.

4.1. Future direction

GABA_A and glycine are highly regulated receptorchannels and are composed of several homologous subunits, each the product of a distinct gene. Preliminary evidence suggests that some of the subunit-encoding genes are clustered, allowing for concerted gene regulation in neuronal populations (e.g. [10]). Until now, large numbers of GABA and glycine receptor subunits have been characterized by structure and functional expression. Although these subunits can be grouped into distinct sequence classes (e.g. α , β , γ and δ), upon recombinant expression each subunit seems to form oligohomomeric receptor/channels that can be gated and potentiated by GABA and its modulatory drugs [31,33,37] and gated by glycine [39]. However, certain allosteric interactions are known to occur in natural agonist receptors. For example for GABA, at least two different subunits are needed for co-assembly of the receptor [2]. Although channel properties and pharmacological characterization of simple subunit combinations should prove invaluable for studying the contribution of individual subunits to these agonist-gated receptors, the number of synthetic receptors that could be recombinantly expressed in cell lines from the cloned cDNAs is immense. Thus, to narrow down these combinations of subunits, the results of in situ hybridization, immunocytochemical studies [13], analysis by reverse transcription polymerase chain reaction [35] and electron imaging [40] will be needed.

In conclusion, we would like to suggest that in addition to differences in subunit composition, the function of these receptors may also be modified by drugs, which alter the rates of binding of these agonists, modify the gating of the channel or block the channel. It is also likely that phosphorylation of the receptor subunits may also modify the biophysical properties, stability or assembly of these receptor-channels.

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References

- Akagi, H., Patton, E. and Miledi, R., Discrimination of heterogenous mRNAs encoding strychnine-sensitive glycine receptors in Xenopus oocytes by antisense oligonucleotides, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 8103–8107.
- [2] Araki, T., Yamano, M., Murakami, T., Wanaka, A., Betz, H. and Tohyama, M., Localization of the glycine receptors in the rat central nervous system: an immunocytochemical analysis using monoclonal antibody, J. Neurosci., 25 (1988) 613–624.
- [3] Barker, J.L., McBurney, R.N. and MacDonald, J.F., Fluctuation analysis of neuronal amino acid responses in cultured mouse spinal neurons, J. Physiol., 322 (1982) 365-387.
- [4] Barry, P.H., JPCalc, a software package for calculating liquid junction potential corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correcting junction potential measurements, J. Neurosci. Methods, 51 (1994) 107-116.
- [5] Barry, P.H. and Quartararo, N., PNSCROLL, a software package for graphical interactive analysis of single-channel patch clamp currents and other binary file records: under mouse control, *Comput. Biol. Med.*, 20 (1990) 193–204.
- [6] Becker, C.M., Hoch, W. and Betz, H., Glycine receptor heterogeneity in rat spinal cord during postnatal development, *EMBO J.*, 7 (1988) 3717–3726.
- [7] Betz, H. and Becker, C.M., The mammalian glycine receptor: biology and structure of a neuronal chloride channel protein, *Neurochem. Int.*, 13 (1988) 137–146.
- [8] Bormann, J., Hamill, O.P. and Sakmann, B., Mechanism of anion permeation through channels gated by glycine and GABA in mouse cultured spinal neurons, J. Physiol., Lond., 385 (1987) 243–286.
- [9] Bovolin, P., Santi, M., Memo, M., Costa, E. and Grayson, D.R., Distinct developmental patterns of expression of rat α1, α5, γ2 and γ1 GABA_A receptor subunit mRNAs in vivo and in vitro, J. Neurochem., 59 (1992) 62-72.
- [10] Buckle, V.J., Fujita, N., Ryder-Cook, A.S., Derry, J.M.J., Lebo, R.V., Schofield, P.R., Seeburg, P.H., Bateson, A.N., Darlison, M.G.

and Barnard, E.A., Chromosomal localization of $GABA_A$ receptor subunit genes: Relationship to human genetic disease, *Neuron*, 3 (1989) 647–654.

- [11] Chen, Q.X., Stelzer, A., Kay, A.R. and Wong, R.K., GABA_A receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurons, J. Physiol. (Lond.), 420 (1990) 207-221.
- [12] Colquhoun, D. and Hawkes, A.G., On the stochastic properties of bursts of single ion channel openings and clusters of bursts, *Philos. Trans. R. Soc. B.*, 300 (1982) 1–59.
- [13] Etwert, M., Shivers, B.D., Luddens, H., Mohler, H. and Seeburg, P.H., Subunit selectivity and epitope characterization of monoclonal antibodies directed against the GABA_A /benzodiazepine receptor, *J. Cell Biol.*, 110 (1990) 2043–2048.
- [14] Fatima-Shad, K. and Barry, P.H., A patch-clamp study of GABAand strychnine-sensitive glycine-activated currents in post-natal tissue-cultured hippocampal neurons, *Proc. R. Soc. Lond. B.*, 250 (1992) 99–105.
- [15] Fatima-Shad, K. and Barry, P.H., Anion permeation in GABA- and glycine-gated channels of mammalian cultured hippocampal neurons, *Proc. R. Soc. Lond. B.*, 253 (1993) 69–75.
- [16] Fuchs K., Adamiker, D. and Sieghart, W., Identification of α 2-and α 3-subunits of the GABA_A-benzodiazepine receptor complex purified from the brains of young rats, *FEBS Lett.*, 261 (1990) 52–54.
- [17] Gambarana, C., Pittman, R. and Siegal, R.E., Developmental expression of the GABA_A receptor α1 subunit mRNA in the rat brain, J. *Neurobiol.*, 21 (1990) 1169–1179.
- [18] Garrett, K.M., Saito, N., Duman, R.S., Abel, M.S., Ashton, R.A., Fujimori, S., Beer, B., Tallman, J.F., Vitek, M.P. and Blume, A.J., Differential expression of GABA_A receptor subunits, *Mol. Pharma*col., 37 (1990) 652–657.
- [19] Grenningloh, G., Pribilla, I., Prior, P., Multhaup, G., Beyreuther, K., Taleb, O. and Betz, H., Cloning and expression of the 58 kD beta subunit of the inhibitory glycine receptor, *Neuron*, 4 (6) (1990) 963–970.
- [20] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflügers Archiv*, 391 (1981) 85–100.
- [21] Kuhse, J., Schmeiden, V. and Betz, H., A single amino acid exchange alters the pharmacology of neonatal rat glycine receptor subunit, *Neuron*, 5 (1990) 867–873.
- [22] Laurie, D.J., Wisden, W.T. and Seeburg, P.H., The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain 111. Embryonic and postnatal development, J. Neurosci., 12 (1992) 4151-4172.
- [23] Lin, Y.F., Browning, M.D., Dudek, E.M. and Macdonald, R.L., Protein kinase C enhances recombinant bovine α1 β1 γ2L GABA_A receptor whole-cell currents expressed in L929 Fibroblast, *Neuron*, 13 (6) (1994) 1421–1431.
- [24] Luddens, H., Pritchett, D.B., Kohler, M., Killisch, I., Keinanen, K., Monyer, H., Sprengel, R. and Seeburg, P. H., Cerebellar GABA_A receptor selective for a behavioural alcohol antagonist, *Nature*, 346 (1990) 648–651.
- [25] Macdonald, R.L., Rogers, C.J. and Twyman, R.E., Kinetic properties of GABA_A receptor main conductance state of mouse spinal cord neurones in culture, J. Physiol. (Lond.), 410 (1989) 479–489.
- [26] Maclennan, A.J., Brecha, N., Khrestchatisky, M., Sternini, C., Tillakaratne, N.J., Chiang, M.Y., Anderson, K., Lai, M. and Tobia, A.J., Independent cellular and ontogenetic expression of mRNAs encoding three α polypeptides of the rat GABA_A receptor, *Neuro-science*, 43 (1991) 369–380.
- [27] Mody, I., Tanelian, D.L. and Maciver, M.B., Halothane enhances tonic neuronal inhibition by elevating intracellular calcium, Brain Res., 538 (1991) 319–323.
- [28] Mohler, H., Malherbe, P., Draguhn, A. and Richards, J.G. GABA_A receptors: structural requirements and sites of gene expression in mammalian brain, *Neurochem. Res.*, 15 (2) (1990) 199–207.

- [29] Murakami, T., Araki, T., Yamano, M., Wanaka, A., Betz, H. and Tohyama, M., Localization of the glycine receptors in the rat central nervous system: An immunocytochemical analysis, *Adv. Exp. Med. Biol.*, 236 (1990) 71–80.
- [30] Olsen, R.W. and Tobin, A.J., Molecular biology of GABA_A receptors, FASEB J., 4 (1990) 1469–1480.
- [31] Pritchett, D.B., Sontheimer, H., Gorman, C.M., Kettenmann, H., Seeburg, P.H. and Schofield, P.R., Ligand-gating and allosteric potentiation of human GABA_A receptor subunits in a transient mammalian cell expression system, *Science*, 242 (1988) 1306–1308.
- [32] Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H., Schofield, P.R. and Seeburg, P.H., A novel GABA_A receptor subunit is important for benzodiazepine pharmacology, *Nature*, 86 (1989) 582–585.
- [33] Puia, G., Santi, M.R., Vicini, S., Pritchett, D.B., Purdy, R.H., Paul, S.M., Seeburg, P.H. and Costa, E., Neurosteroids act on recombinant human GABA_A receptors, *Neuron*, 4 (1990) 759–765.
- [34] Rogers, C.J., Twynan, R.E. and Macdonald, R.L., Benzodiazepine and β-carboline regulation of single GABA_A receptor channels of mouse spinal neurones in culture, J. Physiol., 475.1 (1994) 69–82.
- [35] Santi, M.R., Vicini, S., Eldadah, B. and Neale, J.H., Analysis by polymerase chain reaction of $\alpha 1$ and $\alpha 6$ GABA_A receptor cubunit mRNAs in individual cerebellar neuron after whole-cell recordings, *J. Neurochem.*, 63 (6) (1994) 2357–2360.

- [36] Schönrock, B. and Bormann, J., Functional heterogeneity of hippocampal GABA_A receptors, *Eur. J. Neurol. Sci.*, 5 (1993) 1042– 1049.
- [37] Shivers, B.D., Killisch, I., Sprengel, R., Sontheimer, H., Kohler, M. Schofield, P.R. and Seeburg, P.H., Two novel GABA_A receptor subunits exist in distinct neuronal subpopulations, *Neuron*, 3 (1989) 327-337.
- [38] Snyder, S.H., The glycine synaptic receptor in the mammalian central nervous system, Br. J. Pharmacol., 53 (1975) 473-484.
- [39] Sontheimer, H., Becker, C.M., Pritchett, D.B., Schofield, P.R., Grenningloh, G., Kettenman, H., Betz, H. and Seeburg, P.H., Functional chloride channels by mammalian cell expression of rat glycine receptor subunit, *Neuron*, 2 (1989) 1491–1497.
- [40] Unwin, N., Acetylcholine receptor channel imaged in the open state, *Nature*, 373 (1995) 37–43.
- [41] Verdoon, T.A., Drauguhn, A., Ymer, S., Seeburg, P.H. and Sakmann, B., Functional properties of recombinant rat GABA_A receptors depend upon subunit composition, *Neuron*, 4 (1990) 919–928.
- [42] Yasui, S., Ishizuka, S. and Akaike, N., GABA activates different types of chloride-conducting receptor-ionophore complexes in dosedependent manner, *Brain Res.*, 344 (1985) 176–180.