Functionally Distinct Chloride-Mediated GABA Responses in Rat Cerebellar Granule Cells Cultured in a Low-Potassium Medium

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INTRODUCTION

Type A receptors for the transmitter γ-aminobutyric acid (GABA) mediate most of the fast inhibitory synaptic transmission in the vertebrate CNS. They inhibit neuronal firing by activating a chloride conductance that hyperpolarizes cells (Sivilotti and Nistri 1991). At least 14 genes encoding the GABA_A receptor subunits have been cloned so far, suggesting an enormous number of possible subunit combinations that can form a receptor-ionophore complex (McKernan and Whiting 1996). Different combinations of these subunits may account for the functional diversity of native GABA_A-receptor-mediated responses (Schönrock and Bormann 1993). Recently, GABA-gated chloride channels with unusual pharmacology have been detected in vertebrate retina (Feigespan et al. 1993; Qian and Dowling 1993) or in hippocampal neurons during a restricted period of postnatal development (Martina et al. 1995; Strata and Cherubini 1994). These channels are bicuculline insensitive and therefore do not fit into the conventional scheme for GABA_A receptors. They belong to a new class of receptors named GABA_C, which are likely to be composed by the recently cloned ρ_1 or ρ_2 receptors. Transcripts for the ρ_1 subunit mRNA are highly expressed in the retina, whereas those for the ρ_2 subunit are expressed also in the CNS, particularly in the hippocampus, cortex, and cerebellum (Bormann and Feigenspan 1995).

In the present study we report that cerebellar granule cells cultured in low-potassium (5 mM) medium, a condition that favors the development of functional GABAergic synapses (Virginio et al. 1995), express both high- and low-sensitivity GABA responses. The receptors with apparent low affinity are less sensitive to bicuculline and in this respect they resemble those present in the retina or in the hippocampus during development, which are supposed to be mediated by GABA_C receptor types.

METHODS

Cell culture

Granule cells were prepared according to the procedure described by Levi et al. (1984). Cells were plated with Basal Eagle Medium (Irvin) on petri dishes and were kept in the incubator at 37°C. After 1 day in culture, the K+ concentration of the medium was decreased from 25 to 5 mM. Under these conditions the cells were maintained for ~15 days and were available for experiments starting from the 2nd day in culture.

Data acquisition and analysis

The whole cell configuration of the patch-clamp technique was used to characterize the electrophysiological properties of GABA-evoked currents. Borosilicate glass pipettes were fire-polished to a tip resistance of 5–10 MΩ in working solutions. The external (bath) solution contained (in mM) 137 NaCl, 3 KCl, 1.8 CaCl_2, 1 MgCl_2, 10 N-2-hydroxyethylpiperazine-N°-2-ethanesulfonic acid (HEPES)-NaOH, and 10 D-glucose. The pipette solution contained (in mM) 137 CsCl, 4 MgCl_2, 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N°,N°-tetraacetic acid, 1 CaCl_2, 2 ATP-Na_2, and 10 HEPES-tetraethylammonium-OH. The pH of both solutions was adjusted to 7.3. Granule cells were kept at room temperature (22–24°C) and continuously superfused with extracellular (control) solution applied by gravity (at 2 ml/min). GABA agonists and antagonists were applied by a fast superfusion system (Akaike et al. 1991). With this method a complete exchange of the external solution surrounding the neuron was achieved within 100 ms. GABA-evoked currents were recorded with a patch-clamp amplifier (EPC-7, List Medical Instruments, Darmstadt, Germany). The current signal, filtered at 10 kHz, was recorded on a video tape. Data were transferred to a microcomputer (ATARI 1040ST) after digitization with an analog-digital converter (ITC-16, Instrutech).
Whole cell GABA currents were sampled at 100 Hz and filtered at 1 kHz. Drugs used were GABA, isoguvacine, bicuculline methyliodide, and picrotoxin, all purchased from Sigma. GABA responses were normalized to peak currents induced by GABA (1 mM). Data points in the dose-response curves were fitted with the logistic Hill equation \( I_{\text{norm}} = \frac{I}{I_{\text{max}}} = \frac{1}{1 + (c/k)^n} \), where \( I_{\text{norm}} \) is equal to \( I/I_{\text{max}}, \) \( k \) is the agonist concentration activating one half of the receptors (EC\(_{50}\)), \( c \) is the agonist concentration, and \( n \) is the Hill coefficient. When two components were present, data points in the dose-response curves were fitted with the use of the sum of two Hill functions:

\[
I_{\text{norm}} = a \left[ \frac{1}{1 + (k'/c)^n} + \frac{1 - a}{1 + (k/c)^n} \right],
\]

where \( 0 \leq a \leq 1 \); \( I_{\text{norm}}, k, c, \) and \( n \) have the usual meanings; and \( k' \) and \( n' \) represent the half-maximum concentration and the Hill coefficient of the second component, respectively.

**Results**

Whole cell patch-clamp recordings were obtained from 38 neurons exhibiting similar morphological features. Cell capacitance measured between 3 and 14 days in culture was 2.28 ± 0.24 (SE) (\( n = 28 \)). Assuming a membrane specific capacitance of 1 pF per 100 mm² (Hille 1992), the surface area of individual cells was 228 ± 24 μm². This value was similar to that obtained in the same cells cultured in a high-potassium medium (Kilic et al. 1993). Plotting the peak amplitude of GABA currents versus agonist concentrations revealed three distinct populations of responses. In the first population (\( n = 8 \)) the dose dependence of the normalized current was described by a sigmoidal curve (Fig. 1A). The threshold for a detectable current was in the range of 0.5–1 μM and apparently saturating responses were obtained with GABA concentrations of ~100 μM. The estimated EC\(_{50}\) value, obtained by fitting data points with the empirical Hill equation, was 7.2 ± 3 μM (\( n = 8 \)). The Hill coefficient was equal to 1. In the second population (\( n = 11 \)), detectable currents were still obtained with low concentrations of GABA (<10 μM). However, the dose-response curve, after reaching a first plateau, increased again with higher GABA concentrations (Fig. 1B). Normalized peak current amplitudes were fitted by two sigmoidal curves with different apparent affinities for GABA. The EC\(_{50}\) value for the first component was 22 ± 18 μM (\( n = 11 \)). This value was not significantly (\( P > 0.5 \)) different from that found in the first population of GABA responses. The third group of cells (\( n = 3 \)) exhibited only low-affinity responses. The dose-response curve was fitted by a sigmoidal curve. The threshold for a detectable current was in the range of 10 μM and a saturating response was obtained with a GABA concentration of 1 mM.

A biphasic sigmoidal dose-response curve was obtained when results from the whole set of cells (\( n = 22 \)) were pooled together (Fig. 1D). EC\(_{50}\) values were 13 and 255 μM for the high- and low-sensitivity component, respectively. Hill coefficients were 1.4 for the first and 4 for the second response, suggesting increasing cooperativity in channel gating. Interestingly, the dose-response curve for isoguvacine, a structural analogue of GABA selectively acting on GABA\(_X\) receptors, was fitted by a single sigmoidal curve (Fig. 1C). The EC\(_{50}\) value was 16 μM. This suggests that at least one population of GABA responses was mediated by conventional GABA\(_X\) receptors. If we assume that, in some neurons, GABA activates both high- and low-sensitivity receptor types whereas isoguvacine only activates the high-sensitivity one, a saturating concentration of isoguvacine should give a response that should not occlude the response to coapplication of submaximal dose of GABA. As shown in the example of Fig. 2, when GABA (300 μM) was applied during the desensitizing phase of the response elicited by a saturating concentration of isoguvacine (100 μM), it was still able to induce a small response of ~40 pA. Full occlusion did occur, however, when the same concentration of isoguvacine was applied during the desensitizing phase of GABA-evoked current. Similar results were obtained in two other cells. In three additional neurons GABA responses were completely occluded by isoguvacine, suggesting that these neurons bore only the high-sensitivity receptor types.

Because bicuculline is a selective GABA\(_X\) receptor antagonist, known to compete with GABA for the same binding site, in the following experiments the sensitivity of GABA responses to this antagonist was tested. In a small number
could be due to the fact that in that plot are included cells showing different degrees of sensitivity to bicuculline. Moreover, the threshold for activation of GABA currents recorded in the presence of bicuculline varied consistently from cell to cell (between 100 and 300 μM), leading to a shallower slope of the dose-response curve. The bicuculline-resistant GABA currents, like the bicuculline-sensitive ones, were chloride mediated, because they reversed at a potential very close to that predicted by the Nernst equation for chloride permeant channels (3.2 ± 2 mV, n = 5, not shown).

**DISCUSSION**

Despite the morphological homogeneity of granule cells cultured in low-potassium medium (see Virginio et al. 1995), we found that when data obtained from the entire cell population were pooled together the dose-response curve for GABA had a bimodal behavior. This suggests that in our culture conditions, cerebellar granule cells express at least two binding sites for GABA, having high- or low-sensitivity for the ligand. The EC₅₀ value of the first component was very close to that found in cells grown in a high-

![Fig. 2. Cross-desensitization between isoguvacine and GABA-evoked currents. When a submaximal concentration of GABA was applied during the desensitizing phase of the response induced by a saturating concentration of isoguvacine, this aminoacid was still able to induce a small current (top trace). On the contrary, no response was detected when isoguvacine was applied during the desensitizing phase of GABA response (bottom trace). Holding potential: -50 mV.](image)

of cells (3 of 25), bicuculline (10 μM) completely abolished responses evoked by GABA (100 μM); in the majority of the neurons (22 of 25), the blocking effect of bicuculline (100 μM) was 64 ± 4% (Fig. 3A). The bicuculline-resistant response was abolished by picrotoxin (100 μM). The blocking effect of bicuculline (3–30 μM) versus responses elicited by GABA (10 μM), supposed to selectively activate the high-sensitivity receptor type, was also tested and compared with those evoked by GABA (100 μM), supposed to activate both the high- and low-affinity receptor types. As shown in Fig. 3B, bicuculline (1 μM) reduced the current evoked by GABA (10 μM and 100 μM) to 87 ± 9% and 84 ± 14%, whereas bicuculline (10 μM) reduced these currents to 45 ± 5% and 70 ± 18%, respectively. Fitting regression lines to the linear portion of this plot produced slope values of –0.26 and –0.52 for 10 and 100 μM GABA, respectively, giving support to the hypothesis that at least two different GABA receptors having different sensitivities for the antagonist were activated by different concentrations of GABA. In bicuculline (100 μM), the dose-response curve for GABA was fitted by a sigmoidal curve with an EC₅₀ value of 209 μM (Fig. 3C). This value is similar to that calculated for the second component of the dose-response curve obtained in the absence of bicuculline. The Hill coefficient was 1.7. The discrepancy between this value and that for the second component of the plot obtained in the absence of bicuculline from the entire population of cells (Fig. 1D) could be due to the fact that in that plot are included cells showing different degrees of sensitivity to bicuculline. Moreover, the threshold for activation of GABA currents recorded in the presence of bicuculline varied consistently from cell to cell (between 100 and 300 μM), leading to a shallower slope of the dose-response curve. The bicuculline-resistant GABA currents, like the bicuculline-sensitive ones, were chloride mediated, because they reversed at a potential very close to that predicted by the Nernst equation for chloride permeant channels (3.2 ± 2 mV, n = 5, not shown).

**DISCUSSION**

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![Fig. 3. Different sensitivity to bicuculline of GABA-evoked currents. A: original traces from 2 representative neurons showing bicuculline-sensitive (top) or -insensitive (bottom) GABA-mediated responses (100 μM). Bicuculline was coapplied with GABA. Horizontal bars: period of drug application. Holding potential: –50 mV. B: bicuculline inhibition of responses evoked by GABA at 10 μM (●, n = 16) or 100 μM (■, n = 9), supposed to activate only the high-affinity or both the high- and low-affinity receptors, respectively. Slope values of the regression lines were –0.26 (r = 0.99) and –0.52 (r = 0.99) for 10 or 100 μM GABA, respectively. C: dose-response relationship for normalized currents evoked by GABA in the presence of bicuculline. Data points (from 6 experiments) could be fitted with a single sigmoidal curve. Estimated EC₅₀ value was 209 μM.](image)
potassium medium (Kilić et al. 1993), a condition in which only high-affinity binding sites for GABA are expressed (Meier and Schousboe 1982). Evidence in favor of two different receptor populations is also given by the occlusion experiments in which responses to a submaximal concentration of GABA could still be obtained during receptor desensitization caused by a prolonged application of a saturating concentration of isoguvacine, which is a selective ligand for GABA<sub>A</sub> receptors (Sivilotti and Nistri 1991). Responses with lower affinity for GABA were also found by Zhu et al. (1995) in granule cells maintained in 12.5 µM extracellular potassium. However, these authors did not test the sensitivity of GABA-evoked currents to bicuculline.

In the present experiments GABA responses were highly heterogeneous regarding their sensitivity to bicuculline. The results ranged from a complete inhibition to a very low sensitivity of the responses for the antagonist. Interestingly, when the dose-response curve for GABA obtained in the presence of bicuculline was plotted, an EC<sub>50</sub> value similar to that found for the second component of the entire population response (obtained in the absence of bicuculline) was found. This suggests that bicuculline binds preferentially to receptors showing high affinity for GABA. A different bicuculline sensitivity for the high- or low-affinity GABA receptor is further supported by the experiments in which the dose-response relation to bicuculline was tested on currents evoked by two different concentrations of GABA, supposed to activate only the high-sensitivity or both the high- and low-sensitivity receptors, respectively. If only one type of receptor was present, a parallel shift in the regression lines fitted to the experimental data should be found, because of the fact that an increasing concentration of bicuculline just causes a reduction in the effective GABA concentration. As expected for the case of more than one receptor, a clear difference in the slope of the regression lines was found, indicating the existence of at least two different GABA receptors. Like GABA-mediated responses with apparent high affinity, the low-sensitivity ones gated chloride-permeable channels, as shown by their reversal potential close to the equilibrium potential for Cl<sup>-</sup> and by their sensitivity to picrotoxin. In this respect, the low-sensitivity GABA responses resemble those described in the optic tectum (Sivilotti and Nistri 1989), in the retina (Feigespán et al. 1993; Qian and Dowling 1993), or in the developing hippocampus (Martina et al. 1995; Strata and Cherubini 1994) and supposed to be mediated by a new receptor type, named GABA<sub>C</sub>. In the retina, when bicuculline-sensitive GABA receptors are co-expressed with the bicuculline-insensitive ones, the latter exhibit higher affinity for the agonist (Bormann and Feigespán 1995). Similarly to the present experiments, however, in the developing hippocampus, the bicuculline-insensitive responses had a lower affinity for GABA in comparison with the bicuculline-sensitive ones, indicating the involvement of different receptor subtypes. In conclusion, we have demonstrated that lowering the extracellular potassium concentration, a condition that favors the development of functional GABAergic synapses, switches on the expression of low-sensitivity GABA receptors. Interestingly, in previous work in which binding techniques were used, it was shown that additional low-affinity GABA receptors could be expressed by exposing cerebellar granule cells to GABA or GABA<sub>A</sub> agonists (Meier et al. 1984).

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