Spatial Distribution and Subunit Composition of GABA_A Receptors in the Inferior Olivary Nucleus

ANNA DEVOR, JEAN-MARC FRITSCHY, AND YOSEF YAROM
1Department of Neurobiology, Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel; and 2Institute of Pharmacology and Toxicology, University of Zurich, CH-8057 Zurich, Switzerland

Received 27 September 2000; accepted in final form 18 December 2000

Devor, Anna, Jean-Marc Fritschy, and Yosef Yarom. Spatial distribution and subunit composition of GABA_A receptors in the inferior olivary nucleus. J Neurophysiol 85: 1686–1696, 2001. GABAergic inhibitory feedback from the cerebellum onto the inferior olivary (IO) nucleus plays an important role in olivo-cerebellar function. In this study we characterized the physiological, subunit composition, and spatial distribution of γ-aminobutyric acid-A (GABA_A) receptors in the IO nucleus. Using brain stem slices, we identified two types of IO neuron response to local pressure application of GABA, depending on the site of application: a slow desensitizing response at the soma and a fast desensitizing response at the dendrites. The dendritic response had a more negative reversal potential than did the somatic response, which confirmed their spatial origin. Both responses showed voltage dependence characterized by an abrupt decrease in conductance at negative potentials. Interestingly, this change in conductance occurred in the range of potentials wherein subthreshold membrane potential oscillations usually occur in IO neurons. Immunostaining IO sections with antibodies for GABA_A receptor subunits α1, α2, α3, α5, β2/3, and γ2 and against the postsynaptic anchoring protein gephyrin complemented the electrophysiological observation by showing a differential distribution of GABA_A receptor subtypes in IO neurons. A receptor complex containing α2β2/3γ2 subunits is clustered with gephyrin at presumptive synaptic sites, predominantly on distal dendrites. In addition, diffuse α3, β2/3, and γ2 subunit staining on somata and in the neuropil presumably represents extrasynaptic receptors. Combining electrophysiology with immunocytochemistry, we concluded that α2β2/3γ2 synaptic receptors generated the fast desensitizing (dendritic) response at synaptic sites whereas the slow desensitizing (somatic) response was generated by extrasynaptic α3β2/3γ2 receptors.

INTRODUCTION

The inferior olivary (IO) nucleus is highly innervated by GABAergic projections derived from various sources, including deep cerebellar nuclei (De Zeeuw et al. 1989), raphe nuclei (Walberg and Dietrichs 1982), nucleus prepositus hypoglossi, dorsal column nuclei, and reticular formations (Nelson and Mugnaini 1989). Most of the projections from the deep cerebellar nuclei, the largest GABAergic input to the olive (De Zeeuw et al. 1989), terminate in glomerular structures. Each olivary glomerulus usually contains 5–8 spines, derived mostly from distal dendrites of different IO neurons, coupled by gap junctions and interdigitated with both excitatory and inhibitory synapses of extrinsic origin (De Zeeuw et al. 1990a).

Coupling by gap junctions has a central role in the generation of subthreshold oscillations, a striking feature of IO neurons (Bleasel and Pettigrew 1992; Lampl and Yarom 1997; Linas and Yarom 1986; Manor et al. 1997; Yarom 1991). Subthreshold oscillatory activity of assemblies of olivary neurons was hypothesized to play a key role in olivo-cerebellar physiology by controlling the synchrony and rhythmicity of complex spikes in the cerebellar cortex (Lang et al. 1999). The coexistence of inhibitory terminals and gap junctions in IO glomeruli suggests that GABAergic inputs may control electrotonic coupling among IO neurons (Linas 1974) and may thereby control subthreshold oscillatory activity. Consistent with this possibility, physiological experiments in vivo have demonstrated that activation of γ-aminobutyric acid-A (GABA_A) receptors modulates the degree of synchronization of complex spike activity in Purkinje cells (Lang et al. 1996; Linas and Sasaki 1989).

The variability in the subunit composition of GABA_A receptors is well documented. Nineteen different mammalian GABA_A receptor subunit genes have been described: α1(1–6), β1(1–4), γ1(1–3), ε, θ, π and ρ1(1–3). A number of studies on recombinant receptors have shown that the biophysical and pharmacological properties of GABA_A receptors depend on their subunit composition (Mehta and Ticku 1999; Whiting et al. 1999). Moreover, synaptic and extrasynaptic receptors were shown to differ in subunit composition (Brickley et al. 1999; Nusser et al. 1998).

In view of the importance of GABA transmission in olivo-cerebellar function, and of the variety of GABA_A receptor subtypes, each with its own distinct kinetics and composition, it is essential to identify the type of receptors that mediate extrinsic inhibitory control of the IO nucleus. Both the location of the receptors and their functional characteristics, particularly their voltage dependence and kinetics, are likely to determine and limit the range of possible regulatory functions.

In the present study, we characterized the physiological properties of GABA_A receptors in the IO nucleus and analyzed their spatial distribution and subunit composition. Postsynaptic receptors were identified by their colocalization with the clustering protein gephyrin (Essrich et al. 1998; Kneussel et al. 1999; Sassoe-Pognetto and Fritschy 2000; Sassoe-Pognetto et al. 2000). Because gephyrin labels GABAergic and glycinergic synapses, and because glycinergic synapses were not found in
the inferior olivary nucleus (De Zeeuw et al. 1994), gephyrin was used in our study as a specific marker for GABAergic postsynaptic sites. The strength of this study lies in the comparison of electrophysiological with immunocytochemical findings, which enabled us to substantiate our conclusions.

METHODS

Slice preparation

Slices 300-μm thick were prepared from the brain stems of Wistar-derived Sabra strain rats (18–25 days old). The animals were anesthetized intra-peritoneally with 60 mg/Kg pentobarbitone and perfused through the heart with 100 ml cold (0–1°C) physiological solution (Table 1, solution A). After decapitation, the brain stem was quickly removed and sliced (752 M vibroslice, Campden Instruments) in cold sucrose solution (Table 1, solution C). The slices were incubated in the sucrose solution at room temperature for 60 min. During this time, the sucrose solution was slowly replaced by solution A and the brain stem slices were then kept at room temperature until they were transferred into the recording chamber. Use of the sucrose solution was critical for increasing the viability of IO neurons.

Recordings

The recording chamber was mounted on an upright microscope stage (Zeiss Axioskop), was maintained at a constant temperature of 35°C by a temperature control unit, and was continuously perfused with solution B (Table 1). Whole-cell patch recordings were performed under visual control using infrared differential interference contrast optics (DIC). Recordings were made throughout the IO nucleus from neurons whose cell bodies, which lie below the surface of the slice, were visually identified. The pipettes were filled with the intracellular solution (Table 1). In some experiments, chloride concentration in the pipette was increased to 20 mM by replacing K-glucuronate with KCl. Bicuculline (Sigma) was added to the recording solution (solution A) to reach a final concentration of 100 μM. In some experiments bicuculline was applied by pressure pulses through a micro-pipette (tip diameter ~2 μm) filled with 100 μM bicuculline in solution A locally over the cell body. GABA was applied by a micropipette (tip diameter some experiments bicuculline was applied by pressure pulses through a micropipette filled with 1 mM GABA in solution A locally over the cell body. GABA was applied by a micropipette (tip diameter

<table>
<thead>
<tr>
<th>TABLE 1. Solution composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgSO4</td>
</tr>
<tr>
<td>KH2PO4</td>
</tr>
<tr>
<td>NaHCO3</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>CaCl2</td>
</tr>
<tr>
<td>K-glucuronate</td>
</tr>
<tr>
<td>EGTA</td>
</tr>
<tr>
<td>Mg-ATP</td>
</tr>
<tr>
<td>HEPES</td>
</tr>
<tr>
<td>Values are given in mM. Solutions were aerated with 95% O2-5% CO2, pH 7.4. * pH = 7.2.</td>
</tr>
</tbody>
</table>

In control experiments, preadsorption of primary antibodies against GABA_A receptor subunits with 1–3 μg/ml of the corresponding peptide antigen resulted in a complete loss of specific staining (not shown).

In immunohistochemistry

Eighteen 21–30 day old Wistar rats were used for the immunolabeling of GABA_A receptor subunits α1, α2, α3, α5, γ2 (Fritschy and Mohler 1995), and β2/3 (bd17, Chemicon, Temecula, CA). An antibody body against the synaptic anchoring protein gephyrin (mAb7a, Connex, Martinsried, Germany) was used as a marker for GABAergic synapses (Sassoe-Poggetto et al. 2000). All of the animals were anesthetized intraperitoneally with pentobarbitone and were perfused with the physiological solution (Table 1, solution A).

In seven animals in which GABA_A receptor subunits and gephyrin were code-tected by double immunofluorescence staining, the following procedure was used: the brains were removed and frozen in dry ice immediately after perfusion and were stored at −80°C for at least three days. Cryostat sections (12 μm) were defrosted for 30 s at room temperature and then were fixed with 0.5% paraformaldehyde in a 150 mM phosphate buffer (PBS, pH 7.4) during 30 s of microwave irradiation (Fritschy et al. 1998b). The cryostat sections were rinsed with PBS and then were incubated overnight at 4°C with primary antibodies for GABA_A receptor subunits and gephyrin in PBS containing 4% normal goat serum. The following antibody dilutions were used: α1, 1:40,000; α2, 1:4,000; α3, 1:6,000; α5, 1:3,000; β2/3, 1:20,000; γ2, 1:2,000; gephyrin, 1:300. The sections were again rinsed with PBS and were incubated for 30 min in a mixture of the corresponding secondary antibodies conjugated with Alexa 488 (Molecular Probes, Eugene, OR), Cy3, or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). After incubation, the sections were washed and coverslipped with buffered glycerol.

In the remaining 11 animals, IO neurons were triple-labeled with neurobiotin, antibodies to GABA_A receptor subunits, and antibodies to gephyrin. In this group, the brain stem was dissected after perfusion and was kept in cold (0–1°C) oxygenated sucrose solution (Table 1, solution C). The IO nucleus was pressure injected through a glass micropipette with 4.5% neurobiotin in sucrose solution. Within minutes, 300-μm brain slices were cut on a vibratome, fixed for 4 min with 4% paraformaldehyde in 150 mM phosphate buffer (pH 7.4), rinsed with PBS, and re-cut on a cryostat in 25-μm sections. The thin sections were then processed for immunofluorescence staining as described in the previous paragraph. Streptavidin conjugated with Cy2 (Vector Laboratories, Burlingame, CA) was used as a marker for neurobiotin.

In control experiments, preadsorption of primary antibodies against GABA_A receptor subunits with 1–3 μg/ml of the corresponding peptide antigen resulted in a complete loss of specific staining (not shown).

Both morphological procedures gave identical staining of the GABA_A receptor subunits. Therefore, the results of labeling produced by both techniques were pooled. Fluorescent images were captured with a confocal laser-scanning microscope (Leica, TCS SP) at a magnification of 60–75 nm per pixel by using simultaneous acquisition of double- and triple-labeled images in separate channels. Care was taken to sample images over the entire dynamic range of the photodetectors and to individually adjust the intensity of the excitation lines (488, 568, and 640 nm) to avoid cross-excitation of the fluorochromes. Images were preprocessed with ImageJ image analysis software (Birtplane, Zurich, Switzerland). Colocalization of GABA_A receptor subunits and gephyrin in presumptive postsynaptic clusters was assessed in raw images as described previously (Sassoe-Poggetto et al. 2000) by using a “colocalization” algorithm that selects and marks

Electrical signals were stored on videocassette (Neurocorder DR-484) for off-line analysis by the LabVIEW data acquisition and programming system (National Instruments). All results are expressed as means ± SD.
double-labeled pixels above a user-defined threshold. Clusters were defined based on size (larger than 0.04 μm², which corresponds to three adjacent pixels at the magnification used) and relative staining intensity (>30%). Colocalization between different GABA_A receptor subunits was assessed using the same approach but without the size criterion of labeled structures, thus including diffuse staining of neuronal somata and the neuropil. The results are derived from a total of 10 measurements in three sections from three different animals and are expressed as means ± SD.

RESULTS

Two types of response of IO neurons to local GABA application

The response to local application of GABA was analyzed in 34 olivary neurons recorded under voltage clamp conditions. At resting potential (~52.1 ± 2.6 mV), 20-ms pulses of GABA, applied near the cell body, elicited an outward current (Fig. 1A). The response appeared after a delay of 6.1 ± 5.1 ms (n = 9, measured from application onset) and reached an averaged peak amplitude of 89.3 ± 45.6 pA with a rise time of 38.1 ± 13.1 ms. These parameters depended, to different degrees, on the distance between the GABA-filled pipette and the cell body.

An increase in the injection pressure (using 20 ms application) was followed by an increase in the amplitude without an effect on the rise time of the response (Fig. 1A), which suggests that larger numbers of GABA receptors were activated. Increasing the duration of the application increased both the amplitude and the duration of the response (Fig. 1B). A lack of increase in the amplitude for durations longer than 100 ms indicates either that 1 mM GABA saturated the GABA_A receptors present or that the dynamics of receptor activation and receptor desensitization reached an equilibrium steady state. Because prior reports have shown that 1 mM GABA is not enough to saturate the receptors (Hutcheon et al. 2000; Jones and Westbrook 1995; Maconochie et al. 1994), we conclude that an equilibrium was reached in our experiments because of the slow application of GABA.

When GABA was applied for more than 1 s, the amplitude of the response started to decrease before the end of the stimulus (Fig. 1B). An exponential fit to the decay showed a time constant of 0.98 ± 0.11 s (n = 3). This decay could result either from a change in the driving force for Cl− ions or from desensitization of the receptors. To rule out the former possibility, we analyzed the time course of conductance change by introducing −15-mV pulses before and during GABA application (Fig. 1C). The response to voltage pulses alone was subtracted from the response to voltage pulses during GABA application. The amplitude of the current induced by each pulse, which tracks the change in conductance, was plotted as a function of time. As shown in Fig. 1C, the time course of conductance change followed the GABA-induced current almost exactly. Therefore, the decrease in response in the presence of GABA must be explained by slow desensitization of the receptors. However, the desensitization was incomplete. As follows from Fig. 1B, when GABA was applied for 3 s and longer, a residual nondesensitized current was clearly observed. Consistent with this, when 1 mM GABA was added to the recording chamber a permanent small increase in conductance was measured (not shown).

When GABA was applied in the dendritic area, the response (to 20-ms pulses) had lower amplitude (19.0 ± 8.4 pA, n = 4), faster rise time (19.8 ± 1.7 ms), and shorter duration (Fig. 1D) than was observed during somatic application. An increase in stimulus duration failed to prolong the dendritic response, which decayed rapidly with a time constant of 0.11 ± 0.03 s. Instead, a prolonged stimulus activated a slow and delayed outward current that resembled the somatic response. A further increase in the duration of the stimulus increased the amplitude and duration of the second response component without affecting the first. Furthermore, analyzing the time course of con-
ductance change (see the previous paragraph) during the
dendritic response revealed that, in this case, it also followed the
time course of GABA-induced current. The second component
resembled the response to somatic application except for its
much longer delay (150 ms in Fig. 1D). Both components were
reversibly blocked by 100 μM bicuculline, which indicates that
both were mediated by GABAA receptors (Fig. 1E, n = 3).
Moreover, when bicuculline was applied locally over the cell
body, only the second component was blocked (Fig. 1F).

To further characterize the response of IO neurons to so-
matic and dendritic GABA application, we used trains of 10
consecutive pulses (20 ms duration, 200 ms interpulse interval)
(Fig. 2). This paradigm further distinguished between the two
types of responses. Near the soma (Fig. 2A), responses sum-
mated to an almost steady-state level. An increase of injection
pressure increased this steady state level, which was followed
by a moderate decrease, as was observed with a single pro-
longed injection (Fig. 1). In contrast, at dendritic locations,
only the first GABA pulse elicited a response, which was
almost unaffected by an increase in pressure (Fig. 2B). The
inability to follow a train of pulses indicates that desensitiza-
tion at the dendritic location occurred much faster than at the
somatic location.

The spatial distribution of the two types of response was
further confirmed by measuring reversal potentials. To elicit
both responses, we used prolonged (up to 500 ms) injections in
the dendritic area of IO neurons and measured GABA-induced
current at different membrane potentials. As shown by the
individual traces in Fig. 3A, the compound outward current at
−50 mV was almost a mirror image of the compound inward
current observed at −100 mV. Both components were clearly
distinguishable, which indicates the activation both of somatic
and of dendritic receptors. However, the biphasic appearance
of the response at −68 mV indicated that the late (somatic)
component reversed at a less negative potential.

FIG. 2. The rate of desensitization distinguished between the dendritic and
somatic responses. The neuron was clamped at the somatic level to −50 mV.
A train of 10 GABA pulses (20 ms duration, 200 ms interval, dashed lines
below the traces) was applied near the cell body (A) or in the dendritic area (B)
of the inferior olivary (IO) neuron. Low and high pressure (top and bottom traces,
respectively) was used at each location. Note the different behaviors of the
two modes of application and the insensitivity of the dendritic response to
the applied pressure.

The plot in Fig. 3B shows the currents, measured at the times
denoted by the dashed lines, as a function of the holding
potential. Two points are of interest. First, as expected from
cable theory (Rall 1989), the reversal potential of the somatic
response was 13 mV more depolarized than that of the den-
dritic response (−63 and −76 mV, respectively). The average
reversal potentials of the somatic and dendritic responses were
−58.1 ± 4.2 mV and −67.9 ± 6.0 mV, respectively (n = 8).
In one cell, we further confirmed this difference by indepen-
dently measuring the reversal potentials of the somatic and
dendritic responses using short (20 ms) GABA pulses applied

FIG. 3. The dendritic and somatic GABA-induced currents are voltage
dependent. A: currents evoked by the dendritic application of GABA (200 ms,
bar) were recorded at different holding potentials. Membrane potential values
are shown on the left. Note two distinct components of the response that
reversed at different potentials. B: the amplitude of GABA-induced current as
a function of holding potential. Amplitudes of both components were measured
at each membrane potential at the peak of the dendritic response (squares) and
at the peak of the somatic response (triangles). The reversal potential of the
dendritic and somatic responses was −75 mV and −63 mV, respectively. The
significant voltage dependence of GABA-induced current was characterized by
two conductance states. Each conductance state was fitted with a line. The
intersection between these two lines (arrow) denotes a breaking point: the
temperature at which a transition between these two states occurred. C: the voltage
dependence of GABA-induced current persists after removal of voltage-de-
pendent conductances. Voltage pulses (5 mV, 300 ms) were followed by application of 30 ms GABA. Recordings were made at different holding
potentials, denoted on the left. D: the amplitude of GABA-induced current as
a function of holding potential (squares) and slope membrane conductance
(triangles). The slope conductance was calculated by averaging responses to
voltage pulses of both polarities at each holding potential.
locally at the soma or at a distance of 40 μm from the soma. The results fell well within the range that was calculated for the compound response (−58 mV for somatic application and −70 mV for dendritic application).

Second, both responses, somatic and dendritic, showed voltage dependence with an abrupt decrease in conductance at a membrane potential of approximately −65 mV (n = 6). We fitted the experimental data with two linear segments corresponding to two conductance states and defined a break point as the intersection of these two linear segments (Fig. 3B, arrows). The ratio between the two conductance states varied in different cells (from 6.08 to 19.35, n = 6). This apparent voltage dependence may be caused by an inadequate voltage clamp, by a nonsymmetric flow of Cl− caused by a concentration difference between inside and outside (Goldman rectification), or by voltage dependence of the receptor channel. To rule out the first possibility, we linearized the membrane by adding Cs (5 mM), harmaline (0.1 mg/ml), tetraethylammonium (10 mM), and 4AP (0.1 mM) and by replacing Ca2+ with Co2+ in the bath solution (Yarom and Llinas 1987). The linearity of the membrane was examined by 300-ms, 5-mV voltage steps of both polarities and the resultant currents were used to calculate the membrane conductance at each level of membrane potential. Indeed, as shown in Fig. 3D, the slope membrane conductance under these conditions was about 2 nS throughout the entire range of holding voltages. The GABA-induced current, on the other hand, still showed a pronounced decrease in conductance near the break point of about 50 mV for dendritic components and −56.0 ± 7.6 mV for the dendritic components, n = 4). However, the value of the break point was unaffected by the change in Cl− concentration (Fig. 4B).

We therefore concluded that GABA-induced current in olivary neurons is voltage dependent. This finding is especially interesting because the abrupt decrease in conductance occurred in the range of potentials wherein subthreshold oscillations usually occur in IO neurons.

Nonhomogeneous distribution of GABA<sub>A</sub> receptor subunits in IO neurons

To further support the conclusion that the slow- and fast-desensitizing responses to GABA application are separated spatially, we studied the subunit distribution of GABA<sub>A</sub> receptors in different subcellular compartments of IO neurons. Immunostaining of olivary sections was performed for the α1, α2, α3, α5, β2/3, and γ2 GABA<sub>A</sub> receptor subunits. The α1 and α5 subunits were not detected in IO neurons although they were intensely stained in other brain stem neurons in the same section. The α3 subunit staining was weak and diffuse and was most apparent on IO somata. Finally, the α2, γ2, and β2/3 subunits exhibited prominent puncta, irregularly distributed in the neuropil of the IO, as well as some diffuse staining. In comparison, gephyrin staining revealed only brightly stained puncta with a distribution similar to that of the α2, β2/3, and γ2 subunits.

Immunolabeling of the α2 subunit in neurobiotin-stained IO neurons revealed that the strong punctate staining was concentrated around distal dendrites; cell bodies and proximal dendrites were virtually devoid of punctate α2 subunit staining (Fig. 5). In previous studies (Fritschy et al. 1998b; Giustetto et al. 1998; Sassone-Pognetto et al. 2000), punctate labeling of GABA<sub>A</sub> receptor subunits was attributed to GABA<sub>A</sub> receptors aggregated postsynaptically with gephyrin. Therefore, to determine the synaptic specificity of the α2 subunit puncta, sections were triple-labeled with gephyrin (Fig. 6). Similar to the α2 subunit, gephyrin-positive clusters were concentrated along dendrites and only a few were apposed to neurobiotin-positive IO somata. Gephyrin and the α2 subunit were extensively colocalized (Fig. 6, A and A’, and Table 2) in both double- and triple-labeled sections, which indicates the almost exclusive localization of α2 subunits at synaptic sites.

To further unravel the subunit composition of olivary GABA receptors, sections were processed for codetection of the α2 and β2/3 subunits along with neurobiotin. The results showed that the α2 subunit was highly colocalized with β2/3 (Table 2 and Fig. 6B), which indicates that a complex of α2β2/3 subunits clustered with gephyrin at presumptive synaptic sites. Immunoreactivity of the β2/3 subunit was more extensive than was the α2 subunit labeling and was, in addition, diffusely distributed throughout the neuropil of the IO and on neuronal somata.

Like the β2/3 subunit, staining for the γ2 subunit exhibited both intensely labeled clusters on dendrites of IO neurons and diffuse staining of the neuropil and of individual somata (Fig. 7A). A majority of the γ2 subunit-positive clusters was double-labeled with gephyrin (Fig. 7, B–D), but the incidence of colocalization was lower than that for the α2 subunit (Table 2). Moreover, clusters of γ2 and β2/3 subunits were largely colocalized throughout the IO nucleus (Table 2).

Finally, double immunofluorescence staining of the IO for the α3 subunit (Fig. 8, left) and gephyrin (Fig. 8, right) revealed weak and diffuse α3 subunit immunoreactivity on cell somata and proximal dendrites, with an occasional cluster...
colocalized with gephyrin (Fig. 8, arrowheads). Together these results indicate that a GABA_A receptor subtype comprising the α2β2/3γ2 subunits was clustered with gephyrin on distal dendrites of IO neurons. This receptor subtype is likely to correspond to the fast desensitizing receptors that were identified electrophysiologically. A second major subtype that contains the subunit complex α3β2/3γ2 is distributed extrasynaptically on IO somata, presumably corresponding to the slow desensitizing receptor population.

**DISCUSSION**

In this work we characterized two types of responses of olivary neurons to local application of GABA: a slow desensitizing response at the soma and a fast desensitizing response at the dendrites. Both responses were mediated by GABA_A receptors and both were reversibly blocked by bicuculline. These two types of responses differed in their kinetics as well as in their spatial distribution. These observations were complemented by immunocytochemistry, which showed that a complex containing α2β2/3γ2 subunits was clustered with gephyrin at presumptive synaptic sites, predominantly on distal dendrites, whereas a complex containing α3β2/3γ2 was distributed extrasynaptically on IO somata, presumably corresponding to the slow desensitizing receptor population.

During prolonged stimulation at dendritic locations, first the dendritic and then the somatic responses were evoked in sequential order with a noticeable delay between them. This sequential activation strongly suggests that two spatially separated and kinetically different populations of GABA receptors coexist in olivary neurons. The spatial segregation of the two responses was further confirmed by the somatic application of bicuculline, which specifically and reversibly blocked the somatic response. We conclude that the activation of dendritic receptors gave rise to the first, fast desensitizing response component whereas somatic receptors generated the delayed, slow desensitizing response.

Both the fast and the slow desensitizing response components showed clear voltage dependence. This conclusion was reached after we eliminated the possibilities that nonlinear membrane or Goldman-like rectification generated the apparent voltage dependence. The abrupt decrease in conductance, which occurred close to the membrane resting potential, reflected a low conductance state at hyperpolarized potentials and a high conductance state at depolarized potentials. This arrangement is expected to result in a much weaker inhibitory effect at hyperpolarized than at depolarized membrane potentials. Although voltage dependence of GABA channels has been described (Kerrison and Freschi 1992; Weiss 1988), an approximately 10-fold change in conductance over the 15-mV range of potentials described here has been demonstrated regarding glycine receptors only (Faber and Korn 1987). Moreover, the switch between the two conductance states took place in the voltage range wherein subthreshold oscillations usually occur in olivary neurons (Lampl and Yarom 1997). Therefore, one is tempted to speculate that voltage dependence serves as an autoregulatory mechanism that ensures a constant effect of transient inhibitory inputs. That is, the increased excitability during the depolarizing phases of the voltage oscillations is compensated for by an increase in the efficacy of GABA inhibition caused by its voltage dependence.

The significant delay between the dendritic and somatic responses, as well as their distinct kinetics, implies spatial separation of the two types of receptors. This implication was complemented by the morphological part of this study, which demonstrated the existence of GABA_A receptors composed of α2β2/3γ2 subunits located mainly on distal dendrites as well as the existence of receptors composed of
The α2β2/3γ2 subunit composition is likely to form a predominant receptor subtype because these subunits appeared to be extensively colocalized with each other and with gephyrin within individual clusters. There was considerable variability in staining intensity among individual clusters (Fig. 6A'), which suggests that the number and/or density of GABA_A receptors per synaptic site is variable. In contrast to this receptor subtype, the diffuse staining not colocalized with gephyrin that was observed for the α3, β2/3, and γ2 subunits on IO somata and in the neuropil might correspond to one or several subtypes of extrasynaptic receptors. Indeed, colocalization of the diffuse γ2 and β2/3 subunit staining was only partial (Table 2). Unfortunately, the faint α3 subunit immunoreactivity precluded a detailed analysis of its codistribution with the β2/3 subunits.

If indeed α2β2/3γ2 and α3β2/3γ2 GABA_A receptor compositions generate the dendritic and somatic responses, respectively, they should correspond to the fast and slow desensitizing responses that were observed electrophysiologically. A number of previous studies showed that the kinetics of native as well as recombinant ligand-gated ion channels depend on subunit composition (Brussaard et al. 1997, 1999; Gingrich et al. 1995; Hutcheon et al. 2000; Liu and Cull-Candy 2000;
TABLE 2. Colocalization of GABA<sub>α</sub> receptor subunits and gephyrin

<table>
<thead>
<tr>
<th></th>
<th>α2</th>
<th>β2/3</th>
<th>γ2</th>
<th>Gephyrin</th>
<th>β2/3</th>
<th>γ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2</td>
<td>—</td>
<td>81.0 ± 3.8</td>
<td>—</td>
<td>89.4 ± 3.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β2/3</td>
<td>97.8 ± 3.1</td>
<td>—</td>
<td>95.6 ± 3.0</td>
<td>—</td>
<td>—</td>
<td>63.2 ± 5.8</td>
</tr>
<tr>
<td>γ2</td>
<td>—</td>
<td>93.3 ± 2.4</td>
<td>—</td>
<td>90.7 ± 2.9</td>
<td>59.6 ± 6.4</td>
<td>—</td>
</tr>
<tr>
<td>Gephyrin</td>
<td>78.0 ± 4.2</td>
<td>—</td>
<td>64.0 ± 2.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Clusters were identified by threshold segmentation based on size (>0.04 μm<sup>2</sup>) and intensity (>30% of maximal intensity) (see MATERIALS AND METHODS). The threshold for diffuse staining was set at 10% of maximal intensity (corresponding to a gray value of 25 on the 0–255 scale) with no assumption about size. Each column indicates the extent of colocalization (percentage ± SD) of the selected protein with the other markers investigated.

FIG. 7. Triple labeling of IO neurons with neurobiotin (blue), GABA<sub>α</sub> receptor γ2 subunit (red), and gephyrin (green). The images represent the superposition of four confocal sections spaced by 250 nm. A: γ2 subunit staining is partially clustered on neurobiotin-labeled dendrites and is partially diffuse on the soma and in the neuropil. B: in contrast, gephyrin staining is exclusively clustered on dendrites. C and D: gephyrin and γ2 subunit clusters are extensively colocalized (yellow puncta) whereas only the γ2 subunit is detected on the soma. Scale bar, 10 μm.
Verdoorn 1994). One of the most consistent observations is that the presence of the \( \alpha_3 \) subunit slows the activation, deactivation, and desensitization processes. Although the slow GABA application used in our study prevented us from fully characterizing the kinetics of desensitization, our results regarding the involvement of the \( \alpha \) subunit in receptor kinetics are in agreement with previous reports (Gingrich et al. 1995; Hutcheon et al. 2000; Verdoorn 1994).

Additional or alternative determinants may contribute to receptor kinetics, such as specific interactions with the proteins of the postsynaptic apparatus (Rosenmood and Westbrook 1993) or differences in posttranslational regulation such as receptor phosphorylation levels (Jones and Westbrook 1997; Moss et al. 1992; Wan et al. 1997). Therefore, synaptic and extrasynaptic receptors are likely to have distinct interactions with cellular cytoskeletons and to be differentially exposed to cytosolic proteins. Receptor location may also contribute to the difference in kinetics that was observed in our study. Interestingly, CA1 hippocampal pyramidal neurons were demonstrated to have fast synaptic receptors and slow extrasynaptic receptors (Banks and Pearce 2000).

Despite the different subunit compositions of the dendritic and somatic receptors, both somatic and dendritic responses showed rectification around the resting potential. Subunit composition per se is unlikely to contribute to this behavior because voltage-dependent rectification has not been reported for recombinant \( \alpha_2 \) and \( \alpha_3 \) receptors.

The observation of clusters immunoreactive for the \( \gamma_2 \) and \( \beta_2/3 \) subunits but lacking gephyrin was unexpected. Previous studies reported consistent colocalization of \( \gamma_2 \) subunit immunoreactivity with gephyrin (Essrich et al. 1998; Sassoe-Pognetto et al. 2000). Moreover, gephyrin knockout mice were shown to lack \( \gamma_2 \) clustering (Kneussel et al. 1999). Therefore, to explain gephyrin-independent \( \gamma_2 \) and \( \beta_2/3 \) clusters, one has to assume either local aggregation of extrasynaptic receptors or the presence of gephyrin isoforms not recognized by the antibody used in our study (Bedford et al. 1999). Either way, most gephyrin-independent clusters of \( \gamma_2 \) and \( \beta_2/3 \) subunits are not associated with the \( \alpha_2 \) subunit, which suggests that other subunits, such as \( \alpha_4 \) (Chang et al. 1995), are expressed in the IO, which adds to the heterogeneity of \( \text{GABA}_A \) receptors in this nucleus.

Assuming that antibody 7a labels gephyrin in a majority of postsynaptic inhibitory synapses, the soma of olivary neurons must be largely devoid of inhibition. This conclusion is consistent with the electron microscopic studies of De Zeeuw et al. (1989) who demonstrated that most of the inhibitory projections to the IO nucleus terminate on olivary glomeruli on distal dendrites and that only few of them terminate on cell bodies. Moreover, the majority of the glomerular inhibitory synapses was derived from the deep nuclei of the cerebellum whereas the somatic inhibitory synapses had a noncerebellar origin. The lack of strong synaptic inhibition on the soma of olivary neurons contradicts the classical view of neuronal integration wherein the most effective target of inhibition is the cell soma. However, it was shown that olivary neurons are endowed with axonal spines that form gap junctions and receive GABAergic inhibition (De Zeeuw et al. 1990b).

Close apposition of GABAergic synapses to gap junctions suggests that GABA released in the glomeruli may disrupt (uncouple) electrotonic connections and therefore alter subthreshold activity in the olivary network. The fast desensitization of the dendritic receptors found in our study implies that if GABA induces functional uncoupling it would be restricted to less than one cycle of oscillatory activity. Alternatively, brief interruption of electrotonic coupling at certain phases of the oscillatory cycle may be sufficient to induce a long-lasting blockade of oscillatory activity. A similar phenomenon was demonstrated in cardiac myocytes where a brief electric stim-
This study was supported by the US–Israel Binational Science Foundation and by the European Commission.

REFERENCES


