Evidence for Inhibition Mediated by Coassembly of GABA<sub>A</sub> and GABA<sub>C</sub> Receptor Subunits in Native Central Neurons

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Fast inhibition in the nervous system is commonly mediated by GABA<sub>A</sub> receptors comprised of 2α/2β/1γ subunits. In contrast, GABA<sub>C</sub> receptors containing only p subunits (p1-p3) have been predominantly detected in the retina. However, here we present evidence that GABA<sub>C</sub> agonists can also modulate responses consistent with heteromeric formation (Qian and Dowling, 1993; Qian et al., 1997). Because responses in retina to GABA<sub>C</sub> receptors were highly similar to activation of these recombinant receptors, they were attributed to GABA<sub>C</sub> receptors (Feigenspan et al., 1993; Qian and Dowling, 1993; Zhang et al., 1995). In addition, coinjection of GABA<sub>C</sub> agonists into retinal cells produced GABA<sub>C</sub> responses attributed to GABA<sub>C</sub> receptors (Arakawa and Okada, 1988; Park et al., 1999; Delaney and Sah, 1999; Zhu and Lo, 1999). One explanation could be that GABA<sub>C</sub> receptors are expressed in the CNS, the characteristics of GABA<sub>C</sub> receptors in regions containing p subunits may not correspond to GABA<sub>C</sub> receptors (Arakawa and Okada, 1988; Park et al., 1999; Delaney and Sah, 1999; Zhu and Lo, 1999). One explanation could be that GABA<sub>C</sub> receptors assemble with subunits from other receptor subfamilies, but this hypothesis is contentious. Immunohistochemistry in the retina using an antibody recognizing all three GABA<sub>C</sub> subunits did not overlap with GABA<sub>A</sub> α1, β1, or glycine receptor subunits (Koulen et al., 1998). In addition, coinjection of p1 with GABA<sub>A</sub> α1, β1, and γ2s subunits in oocyte expression systems revealed no change in GABA<sub>C</sub> responses (Shimada et al., 1992). In contrast, coexpression of p1 with GABA<sub>A</sub> γ2 subunits has produced responses consistent with heteromeric formation (Qian and
Ripps, 1999; Pan et al., 2000; Ekema et al., 2002; Pan and Qian, 2003). Some of this contention may be attributable to the fact that the properties and pharmacology of GABA receptor channels in expression systems may differ to those in native tissue because of lack of associated intracellular proteins (Everitt et al., 2004). Indeed, such differences have also been observed between native and expressed nicotinic receptors because some nicotinic receptors assemble appropriately only in cells of neuronal origin (Wanamaker et al., 2003).

In this study we provide evidence that ρ1 subunits are expressed in native brainstem neurons in situ, where they may assemble with GABA receptor subunits. This suggests that the GABA and GABAc receptor subtypes are not always distinct molecular and functional entities and provides a new substrate for inhibition in the CNS.

Materials and Methods

RNA isolation and reverse transcription-PCR. Total RNA was extracted from freshly dissected brainstem using TRI reagent (Sigma, Poole, UK) according to the manufacturer’s instructions. Two micrograms of RNA were reverse-transcribed as described (Deuchars et al., 2001a). Oligonucleotide primers used to amplify the ρ1–3 genes were as follows: ρ1.71s, 5′-GGTGCTTCCGGAATACTGCA-3′; ρ1.273a, 5′-TTGGTGAGAGGCGACTTGGT-3′ (numbering according to GenBank accession number U21070); ρ2.62s, 5′-CAAGAAGGCAATCTTCCA-3′; ρ2.175a, 5′-TCTTGAGATTAGATCTGCC-3′ (GenBank accession number S57984); ρ3.1258s, 5′-GAAGATGGAGAAGCCTACA-3′; and ρ3.1505a, 5′-AGTAGATGATAGAGTGGT-3′ (GenBank accession number D50671). Cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min followed by a final extension step of 72°C for 10 min. Amplification products were subcloned into pGem-T Easy (Promega, Southampton, UK) and verified by sequencing. The specificity of the amplified sequences was determined by sequencing in situ, hybridization was performed on free-floating sections (see Methods). Cerebellar tissue was used as a positive control because ρ1 has been previously reported within this region (Boue-Grabot et al., 1998).

In situ hybridization. Single-stranded digoxigenin (DIG)-UTP-labeled (Roche Diagnostics, Lewes, UK) sense (control) and antisense riboprobes were transcribed from a 202 bp ρ1 DNA template, a 113 bp ρ2 DNA template, or from a 247 bp ρ3 DNA template inserted in opposite orientations into pGEM-T Easy (Promega) using linearized templates and T3 or T7 SP6 RNA polymerase (Ambion, Austin, TX). The BLAST database was used to verify the specificity of these probes to their targets, and these were further verified by sequencing. Probe concentrations were determined by dot-immunoblotting. In situ hybridization was performed on coronal brainstem sections as described (Deuchars et al., 2001a), with the alteration that 30 μm free-floating sections were used here.

Western blotting and immunohistochemistry. The specificity of the rabbit antisera to GABAc (Enz et al., 1996) and the ρ1 subunit (Boue-Grabot et al., 1998) has been described previously. The ρ1 (1:1000), goat anti-α1 antisera (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA) and guinea pig anti-β2 (1:500; a gift from Dr. Jean Marc Frischy, Institute of Pharmacology, University of Zurich, Zurich, Switzerland) were verified to detect proteins of appropriate molecular weights in brainstem by Western blotting as described previously (Deuchars et al., 2001a). Western blots were also performed on non-transfected JTC-19 cells using the above antisera. After electrophoresis of FITC-19 cell membranes, the gel was fixed and stained successively with 0.1% Coomassie Brilliant Blue R250 (Sigma) in 10% acetic acid. The gel was initially stained with Coo- massie Blue solution for 1 hr and destained gradually with several changes of destaining solution (25% methanol and 10% acetic acid) for 60 min until bands showed the appropriate color.

For immunohistochemistry, rats were anesthetized with Sagatal (60 mg/kg, i.p.; Rhône Merieux, Harlow, Essex, UK), perfusion-fixed with 0.05–0.25% glutaraldehyde and/or 4% paraformaldehyde, and 50 μm brainstem sections were prepared as described previously (Deuchars et al., 2001a). For immunofluorescence, primary antibodies (GABA-c1: 100; ρ1–1:200, α1–1:300) were detected with donkey anti-rabbit Cy3 (1:1000; Stratech Scientific, Luton, UK) and donkey anti-goat Cy2 (1:1000; Stratech Scientific) as appropriate. For immunoelectron microscopy, anti-GABAα1 (1:100) and anti-ρ1 (1:200) were localized with biotinylated donkey anti-rabbit (1:500; Stratech Scientific), detected with extravidin-peroxidase (1:1500; Sigma) and visualized with diaminobenzidine. In some cases anti-ρ1 was localized with a 1 nm gold-conjugated secondary antibody raised in donkey (1:200; British Biocell International, Cardiff, UK) and visualized by silver intensification. For dual-labeling electron microscopic procedures, anti-ρ1 was first visualized using the peroxidase procedure and anti-α1 subsequently with the pre-embedding gold procedure. Control sections were incubated with omission of one or both primary antisera. Tissue processing for electron microscopy is described elsewhere (Deuchars et al., 2001a).

Immunoprecipitation. Brainstem dissected from male Wistar rats was homogenized in radioimmunoprecipitation assay buffer containing 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 5% sodium deoxycholate, 0.1% Nonidet P-40, and protease inhibitor with 10 strokes of a glass–glass homogenizer. Twenty microfilters of this sample were used as an input lane to verify the presence of ρ1, α1, or ψ2 subunits in the lysate. Pre-cleared supernatant was incubated in ρ1 antibody (1:200), α1 antibody (1:200), or ψ2 antibody (1:250; guinea pig, a gift from Dr. Jean-Marc Frischy, specifications published in Somogyi et al., 1996) for 4 hr at 4°C. After incubation, 20 μl of protein G-Sepharose beads (Sigma) was added, and samples were tumbled for 4 hr at 4°C. The resin was collected by centrifugation and washed four times in buffer. For control purposes, brainstem lysates were incubated in IgG from the appropriate species. The bound proteins were eluted by boiling the resin in 20 μl of 2× SDS-PAGE loading buffer and analyzed by SDS-PAGE and Western blotting using enhanced chemiluminescence.

Electrophysiology. Rats aged 17–21 d were anesthetized with Sagatal (60 mg/kg, i.p.) and perfused with ice-cold sucrose artificial CSF (aCSF) containing (in mM): sucrose (217); NaHCO3 (26); KCl (3); MgSO4 (2); NaH2PO4 (2.5); CaCl2 (1); and glucose (10). The brain was removed, the brainstem was isolated, and the dura was removed. Slices of 250 μm were cut on a Vibrislice and superfused at a rate of 3–5 ml/min with aCSF at room temperature [composition in mM: NaCl (124); NaHCO3 (26); KCl (3); MgSO4 (2); NaH2PO4 (2.5); CaCl2 (2); glucose (10)]. Low Ca2+– high Mg2+ solutions contained 0.3 mM CaCl2 and 4 mM MgSO4 and were applied in place of normal aCSF until responses to electrical stimulation were abolished. Patch electrodes (tip diameter 3 μm, resistance 4–6 MΩ) were filled with (in mM) K-gluconate (110); EGTA (11); MgCl2 (2); CaCl2 (0.25); and 10 mM hepes (10). AP-5 (0.5 mM), TTX (50 μM), and GABA C (100 μM) were included and diffused into the neuron during recording. Whole-cell clamp patch recordings were performed in current-clamp mode using an Axopatch 1D. Electrophysiological characterization of neurons was performed using methods as before (Deuchars et al., 2001b), and responses of neurons to drugs applied to the bathing medium were determined. Antagonists and modulators of GABA receptor activities were preincubated for 5–10 min before reapplications of the agonists. IPSPs were elicited in dorsal vagal nucleus (DVC) neurons by stimulation (2× threshold) of the NTS and isolated by applications of the excitatory amino acid agonists, 1,2,3,4-tetrahydro-6-nitro-2-dioxobenzoz-[1]quinazoline-7-sulfonamide disodium (NBQX) and n-(2-amino-5-phosphonovaleroyl)-L-aspartate (AP4). Spontaneous IPSPs were recorded at holding potentials of 10–0 mV, and bath applications of (1,2,5,6-tetrahydropyridin-4-yl)methylphosphonic acid (TPMPA) and bicuculline determined which receptors were involved in spontaneous synaptic transmission.

Drugs used were GABAa, muscimol, the GABAc agonist cis-4-acetomicronic acid (CACA; Sigma and Tocris Cookson, Bristol, UK), the GABAa antagonist TPMPA, the GABAa antagonism bicuculline methochloride, and the GABAa antagonist (2S)-3-[(1S)-1-(3,4-dichlorophenyl)ethyl][amino-2-hydroxypropyl][phenylmethyl]phosphonic acid (CGP55845; dissolved in DMSO, used to counteract any effects on TPMPA on GABAa receptors). The GABAa modulators sodium pento-barbital, N,N6,trimethyl-2-(4-methylphosphonic acid)[imidazol]-1,2-epaljine-3-acetamide (zolpidem, dissolved in ethanol) and 5-[aminosulfonyl]-4-
Expression and localization of the $\rho_1$ subunit in brainstem. $\rho_1$ (A), $\rho_2$ (Aii), $\rho_3$ (Aiii) subunit PCR products in brainstem (lane 1) and the positive control cerebellum (lane 3). RNA (lane 2) and water controls (lane 4) are negative. Bands correspond to the calculated size for all PCR products, and their veracity was confirmed by sequencing. The positive control hprt brainstem (lane 1) and the positive control cerebellum (lane 3). RNA (lane 2) and water controls (lane 4) are negative. Bands

Results

Neuronal expression and synaptic localization of $\rho_1$ subunits

To test for expression of $\rho$ subunits in the brainstem, we performed reverse transcription (RT)-PCR with primers specific to cDNA to $\rho_1$, $\rho_2$, and $\rho_3$ (Fig. 1A), which revealed that all three $\rho$ subunits are expressed in the rat brainstem. However, in situ hybridization with riboprobes complementary to and selective for the mRNA encoding each subunit revealed mRNA for the $\rho_1$ subunit (Fig. 1B) but not $\rho_2$ and $\rho_3$, suggesting that the latter two subunits are expressed at much lower levels. The control sense probe (identical to the $\rho_1$ cellular mRNA) did not result in hybridization, indicating that staining was specific to the antisense probe (Fig. 1C). A BLAST database search indicated that the sequence detected with the antisense probe was specific to the $\rho_1$ subunit. In the DVN, 100% of neurons contained $\rho_1$ mRNA, but in the nucleus tractus solitarius (NTS) ~70% of neurons were labeled (Fig. 1B). mRNA expression is consistent with the presence of protein, because similar populations of cells were labeled using antiseraum directed to an epitope common to all three $\rho$ subunits.
subunits (Fig. 1D) and also with an antiserum selective for the $\rho$1 subunit (Fig. 1E).

To determine whether the $\rho$1 subunit was present at synaptic junctions, we performed immunoelectron microscopy. Because antibodies to GABA receptors do not readily penetrate synaptic junctions during pre-embedding immunohistochemical procedures (Nusser et al., 1995), we tried a post-embedding approach to localize the $\rho$1 subunit. However, the $\rho$1 antigen did not survive the embedding procedures, resulting in absence of immunoreactivity. We therefore examined tissue prepared with pre-embedding techniques, which limited us to a more qualitative analysis. However, we reasoned that if we could detect immunoreactivity at synaptic junctions even with this reduced probability, it would provide an indication of potential for synaptic localization of the $\rho$1 subunit. In nine blocks from the dorsal vagal complex, three each from three rats, 240 synapses were identified that had $\rho$1 immunoreactivity adjacent to the postsynaptic membrane (Fig. 1G–I, see Fig. 7A–D). Similar ultrastructural examination of tissue reacted with the GABA$\text{C}$ antibody also revealed reaction product adjacent to the postsynaptic membrane (Fig. 1F) ($n = 30$ terminals). Because the ultrastructural localization with both antibodies was identical and in situ hybridization could detect only the $\rho$1 subunit, $\rho$1 appears to be the predominant $\rho$ subunit present in the brainstem. Although $\rho$ subunits have been localized within cell groups in the CNS, these first ultrastructural observations of postsynaptic $\rho$1 subunits indicate a possible involvement in central synaptic transmission.

**Atypical GABA receptor properties**

We investigated whether these $\rho$1-containing receptors are functional using whole-cell current clamp recordings from both DVN and NTS neurons in rat brainstem slices. Current clamp was the preferred mode of recording because the DVN cells in particular have large dendritic trees that are maintained in the slice, hindering effective space clamp. Bath application of the GABA$\text{C}$-selective agonist CACA (Chebib and Johnston, 1999; Zhang et al., 2001) at concentrations of $100–800 \mu\text{M}$ depolarized all DVN neurons ($10.5 \pm 0.9 \text{ mV}; \text{mean } \pm \text{SEM}; n = 41$) (Figs. 2A, 3A,B) and 56% of NTS neurons ($8.6 \pm 1.7 \text{ mV}; n = 9/16$) (see Fig. 5B). To reduce the possibility of CACA contamination, we used two sources and several batch numbers, with identical results. CACA acted directly on the postsynaptic membrane of the recorded neuron because responses were maintained in tetrodotoxin ($1 \mu\text{M}; n = 3$) (Fig. 2A) and/or blockade of excitatory synaptic transmission with NBQX ($20 \mu\text{M}$) and AP-5 ($50 \mu\text{M}$; data not shown). Furthermore, responses to CACA ($n = 5$) were maintained in low Ca$^{2+}$–high Mg$^{2+}$, which totally blocked synaptic responses to electrical stimulation (Fig. 2B). Responses to GABA ($500 \mu\text{M}; n = 5$) (Fig. 2C, see Fig. 5C) and muscimol ($0.5–2 \mu\text{M}; n = 5$) (Fig. 3C) were also depolarizing, consistent with the responses to CACA arising from activation of GABA receptors on the recorded neuron. Such depolarizations are commonly observed after intense activation of GABA receptors (Staley and Proctor, 1999), which may induce a bicarbonate efflux, especially with intracellular chloride levels as in our experiments (Kaila and Voipio, 1987). To test this we made microinjections of GABA ($n = 7$) near the cell soma at $-50 \text{ mV}$, and these elicited a biphasic response comprised of a transient hyperpolarization followed by a depolarization ($n = 7$) (Fig. 2D). At more hyperpolarized potentials a sustained depolarization similar to that of bath application of agonists was observed (Fig. 2D). In the same DVN neurons that were depolarized by bath application of CACA, monosynaptic responses to electrical stimulation of the NTS were hyperpolarizing ($n = 7$) (Fig. 3B). Furthermore, spontaneous IPSPs recorded at more depolarized potentials were also hyperpolarizing ($n = 5$) (Fig. 4B), confirming that depolarizations are likely caused by prolonged activation of GABA receptors (Staley and Proctor, 1999).

CACA-mediated responses were significantly reduced in amplitude by TPMPA ($40–160 \mu\text{M}$), a GABA$\text{C}$ antagonist (Bornmann, 2000; Zhang et al., 2001) ($13.3 \pm 2.5$ to $3.5 \pm 0.2 \text{ mV}; n = 14$) (Fig. 3A) and in keeping with the $\rho$ subunits forming a GABA$\text{C}$ receptor. However, responses to CACA were also significantly reduced by the classical GABA$\text{A}$ receptor antagonist bicuculline ($10 \mu\text{M}; 12.6 \pm 1.7$ to $1.6 \pm 0.6 \text{ mV}; n = 10$) (Fig. 3A). This was unexpected because, in immature hippocampal (Strata and Cherubini, 1994) and pelvic ganglion (Akasu et al., 1999) neurons, CACA at concentrations of up to $1 \text{ mM}$ could elicit responses that were resistant to very high bicuculline concentrations ($100 \mu\text{M}$) and were mediated by GABA$\text{A}$ receptors. Nevertheless, we observed a similar profile of responses with low concentrations of GABA ($5–20 \mu\text{M}; n = 4$) and muscimol ($0.5–2 \mu\text{M}; n = 5$) (Fig. 3C), which preferentially activate GABA$\text{C}$ receptors (Schmidt et al., 2001; Zhang et al., 2001). Although the highest concentration of TPMPA used could abolish CACA responses (Fig. 3B1), monosynaptic IPSPs were only reduced in amplitude by TPMPA (-8.7 $\pm$ 1.9 to $-4 \pm 0.5 \text{ mV}; n = 7$) (Figs. 3B2, 4A). After recovery from TPMPA, bicuculline abolished the entire IPSP ($-7.9 \pm 1.8$ to $-0.3 \pm 0.1 \text{ mV}; n = 7$) (Figs. 3B2, 4A). This indicated that TPMPA at the concentrations used did not affect all GABA receptors in a nonselective manner. More importantly, these data show that IPSPs elicited in DVN neurons by stimulation of the NTS were likely to be mediated not only by activation of GABA$\text{A}$ receptors but also by pharmacologically distinct GABA receptors that contain $\rho$ subunits.
Further evidence for an unusual receptor was evident because responses to CACA were significantly increased by pentobarbitone (300 μM; 7.6 ± 1.4 to 16.4 ± 2.5 mV; n = 4) (Fig. 3A2), which does not modulate GABA<sub>C</sub> receptors but enhances effects of GABA on GABA<sub>A</sub> receptors (Bormann, 2000; Zhang et al., 2001). In the cerebellum, CACA-elicited responses were mediated by activation of α6-containing GABA<sub>C</sub> receptors (Wall, 2001), and α6 subunit transcripts can be detected using RT-PCR in the brainstem (T. F. C. Batten, personal communication). However, furosemide (100 μM), a selective modulator of α6-containing heteromers (Korpi and Luddens, 1997), had no effect on CACA-mediated responses (Fig. 3A2) (n = 5). Although the pharmacology of putative receptors that may contain α6 and β subunits is not known, these data suggest that the ρ1-containing receptor is unlikely to contain α6 subunits. Responses to CACA were also abolished by picrotoxin (100 μM; n = 3) (Fig. 3A), showing that they were mediated by activation of GABA-gated chloride channels.

Because electrical stimulation may result in neurotransmitter release sufficient to activate extrasynaptic receptors, we tested whether ρ1-containing receptors become activated during spontaneous transmitter release. Spontaneous IPSPs were recorded in DVN neurons at depolarized potentials (−10 to 0 mV) to enhance their amplitude by increasing the chloride driving force. These spontaneous IPSPs were significantly reduced in amplitude by TPMPA (40–80 μM; 5.7 ± 0.5 to 3.7 ± 0.5 mV; n = 5) (Fig. 4B). This indicates a likely participation of ρ1-containing receptors in synaptic transmission. Once more, application of bicuculline then abolished the spontaneous IPSPs (n = 5) (Fig. 4A), indicating that spontaneous and evoked IPSPs were mediated by GABA<sub>A</sub> receptors with a similar profile. The TPMPA-sensitive component of IPSPs, elicited by ρ1-containing receptors, could not be studied in isolation because bicuculline completely abolished responses. Therefore we were clearly unable to determine whether this TPMPA-sensitive component was influenced by other GABA<sub>A</sub> receptor modulators because any change in IPSP amplit-

Figure 3. CACA responses in brainstem neurons display unusual pharmacology. A, A1, CACA-mediated responses in this DVN neuron were reduced by preincubation of the GABA<sub>C</sub> antagonist TPMPA. After recovery from TPMPA, bicuculline also antagonized the CACA response. Furthermore, after partial recovery from bicuculline, application of picrotoxin also abolished the response to CACA. A2, Pooled data: CACA responses were decreased to a similar degree by TPMPA and bicuculline, antagonized by picrotoxin, and enhanced by sodium pentobarbitone, but not affected by furosemide. B, In this DVN neuron, 160 μM TPMPA abolished CACA-mediated responses (B1) but only partially decreased the amplitude of IPSPs elicited by NTS stimulation, whereas bicuculline (10 μM) abolished the IPSP (B2). IPSPs were elicited by double pulse stimulation (arrows) of the NTS and responses shown are averages of 10 sweeps for each condition. C, Responses to low doses of muscimol (1 μM) were also reduced by preincubation of TPMPA at 80 μM.

Figure 4. Evoked and spontaneous IPSPs are reduced in amplitude but never blocked by TPMPA. A, IPSPs elicited by stimulation of the NTS region of the brainstem were reduced in amplitude by TPMPA at 40 μM. Full recovery was observed on washout. This IPSP was then abolished by bicuculline. B, Spontaneous IPSPs recorded at 0 mV were reduced in amplitude by TPMPA, indicated by the raw data and the shift to the left of the histogram. After washout of TPMPA, these IPSPs were abolished by bicuculline.
tude could have been caused by an effect on either or both of the components of the IPSPs.

Responses to GABA$_\mathrm{C}$ agonists require $\rho 1$ subunits

Although responses to CACA at concentrations of up to 1 mM have been shown to be resistant to bicuculline and therefore not mediated by GABA$_\mathrm{A}$ receptors (Strata and Cherubini, 1994; Akasu et al., 1999), we further tested whether CACA only has an effect when $\rho$ subunits are present. During electrophysiological recordings, neurons were filled with Lucifer yellow contained within the patch pipette and subsequently processed for $\rho 1$ immunofluorescence. In slices that were successfully stained for the $\rho 1$ subunit, 100% of DVN neurons responding to CACA were $\rho 1$-immunopositive ($n = 16$) (Fig. 5A). In contrast, only NTS neurons that responded robustly to CACA were $\rho 1$-immunopositive ($n = 3$) (Fig. 5B). NTS neurons that were not affected by CACA but responded to GABA did not contain $\rho 1$ ($n = 4$) (Fig. 5C). These data, together with the selective blockade of CACA responses with TPMPA, show that the $\rho 1$ subunit is necessary for the action of CACA in brainstem neurons, and therefore it is unlikely that CACA is activating GABA$_\mathrm{A}$ receptors.

GABA receptors contain $\rho 1$ and $\alpha 1$ subunits

Because CACA-mediated responses were dependent on the presence of the $\rho 1$ subunit yet were affected by both GABA$_\mathrm{A}$ and GABA$_\mathrm{C}$ receptor modulators, we hypothesized that the $\rho 1$ subunit combines with GABA$_\mathrm{A}$ receptor subunits. We focused on DVN neurons because our immunohistochemistry and in situ hybridization indicated that they all contain $\rho 1$ subunits and they all responded to CACA. In electrophysiological experiments, CACA-mediated responses were significantly enhanced by zolpidem (500 nM; $7.8 \pm 1.2$ to $14.7 \pm 1.7$ mV; $n = 5$) (Fig. 6A,B), a GABA receptor modulator that binds in vitro with high affinity to GABA$_\mathrm{A}$ receptors expressing $\alpha 1$ subunits but with relatively low affinity to receptors expressing $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits (Pritchett and Seeburg, 1991; Kralic et al., 2002). These data also suggest the presence of $\gamma 2$ subunits in the receptor because GABA receptors with mutant $\gamma 2$ subunits are not responsive to zolpidem (Cope et al., 2003).

We therefore tested for the possibility of coexpression of $\rho 1$ with $\alpha 1$ or $\gamma 2$ subunits in DVN neurons. Immunofluorescence was conducted using antibodies verified with Western blotting as
detecting proteins of the appropriate molecular weights in the brainstem (see Fig. 8A–C) (n = 3), but not in cultured JTC-19 cells (see Fig. 8D). The p1 subunit was detected in DVN neurons that were also immunoreactive for α1 (Fig. 6C,D) or γ2 (Fig. 6E,F). To determine whether the p1 and α1 subunits could be colocalized ultrastructurally, we performed dual labeling pre-embedding immunohistochemistry. In tissue from the dorsal vagal complex of three rats, immunoreactivity for p1 and α1 was localized postsynaptically (Fig. 7A–D). Examination of 60 synaptic junctions that contained labeling for either subunit postsynaptically revealed that 12% were immunoreactive solely for p1 and 30% only for α1. The remaining 58% were immunoreactive for both p1 and α1 which were located at an identical site on the postsynaptic membrane. Although such calculations indicate that colocalization of p1 and α1 subunits is not a chance occurrence, the absolute values may be affected by factors such as unequal access of primary or secondary antibodies to different antigens or restriction by conjugation to gold particles. These data therefore indicate that p1 GABA receptor subunits can be coexpressed in individual neurons with α1 and γ2 subunits. Furthermore, we have shown that p1 and α1 subunits may be present at an identical site at synapses.

Our electrophysiological and anatomical data are consistent with the formation of heteromers of p1 with α1 subunits, with involvement of γ2 subunits. To test for possible association of p1 with α1 or γ2 subunits in native tissue, we performed coimmunoprecipitation studies from brainstem. We first verified that p1, α1, and γ2 were present in nonprecipitated supernatant (Fig. 8E–G, input lanes). Protein complexes precipitated from brainstem cell lysates using the p1 antibody contained α1 (Fig. 8E) (n = 5), and γ2 (Fig. 8H) (n = 3), whereas p1 was detected in complexes precipitated with α1 (Fig. 8F) (n = 3) or γ2 (Fig. 8G) (n = 3) antibodies. Such association of p1 with γ2 subunits is consistent with recent observations (Ekema et al., 2002; Pan and Qian, 2003). Thus, when considered in conjunction with anatomy and pharmacological findings, these data suggest that in native brainstem neurons p1 subunits may form heteromers that also contain at least one α1 and a γ2 subunit.

Discussion

Ionotropic GABA receptors are critical mediators of synaptic inhibition in the brain. Each pentameric GABA-γ gated ion channel may be comprised of different subunits, and the exact composition defines the properties of the receptor (McKernan and Whiting, 1996; Rudolph et al., 2001). Whereas subunits from the GABAA and GABAC receptor subfamilies are widely regarded as forming separate receptors, the data from this current study strongly support the formation of heteromeric GABA receptors that contain subunits from the GABAA and GABAC receptor subfamilies.
First, we show by RT-PCR that all three $\rho$ subunits are expressed in the brainstem. However, the only $\rho$ subunit that we could detect with *in situ* hybridization was the $\rho_1$ subunit. Because RT-PCR can detect lower mRNA levels than *in situ* hybridization, this implies that $\rho_1$ is expressed at higher levels than $\rho_2$ and $\rho_3$ in the brainstem. This suggestion is also supported by immunohistochemical labeling because labeling using an antisem to an epitope common to all three $\rho$ subunits was similar to that with a $\rho_1$-specific antibody. This suggests that the receptors labeled with the pan $\rho$ antisera were likely to contain $\rho_1$ subunits. Because $\rho_1$ was the only subunit detectable with *in situ* hybridization, it seems likely that $\rho_1$ is the predominant $\rho$ subunit in the brainstem.

Although presynaptic GABA receptors with pharmacology consistent with homomeric GABA$_C$ receptors have been described in the retina (Matthews et al., 1994; Shields et al., 2000) and superior colliculus (Roller and Schmidt, 2001; Kirischuk et al., 2003), our evidence favors direct activation of $\rho$-containing receptors in the postsynaptic membrane for several reasons: (1) the presence of the $\rho_1$ subunit is necessary to elicit responses in DVN and NTS neurons with CACA, consistent with the view that this agonist acts preferentially on $\rho$ subunit containing GABA receptors (Chebib and Johnston, 1999; Zhang et al., 2001), because only neurons that were $\rho_1$-immunopositive responded to this compound, (2) the actions of CACA persist in the presence of TTX and blockade of excitatory amino acid receptors, as well as when synaptic transmission is blocked in a low Ca$^{2+}$–high Mg$^{2+}$ solution, (3) $\rho$ subunits were also localized postsynaptically using two different antibodies at the ultrastructural level. However, these responses were highly unusual because they were blocked by low concentrations of bicuculline (10 $\mu$m) as well as the GABA$_C$ receptor antagonist TPMPA. This contrasts strikingly with previous studies in which responses to CACA at concentrations of up to 1 mm were resistant to very high concentrations of bicuculline (up to 100 $\mu$m) and were therefore regarded as mediated by GABA$_C$ (comprising only $\rho$ subunits) and not GABA$_A$ receptors (Strata and Cherubini, 1994; Akasu et al., 1999). Because the CACA responses we observed were reliant on the $\rho_1$ subunit, but were not caused by activation of a receptor comprising only $\rho$ subunits, as indicated by bicuculline sensitivity, we tested the hypothesis that the $\rho_1$ subunit was present in a receptor that also contained GABA$_A$ subunits.

Several lines of evidence support this hypothesis. First, $\rho_1$ and $\alpha_1$ subunits were colocalized at light and electron microscopic levels. Second, although CACA responses require the $\rho_1$ subunit, they were also enhanced by zolpidem and sodium pentobarbital, which do not affect receptors comprising only $\rho$ subunits (Bormann, 2000; Zhang et al., 2001), but which do modulate receptors with GABA$_A$ subunits (Pritchett and Seeburg, 1991; Kralic et al., 2002). Third, $\rho_1$ is associated with $\alpha_1$ and $\gamma_2$ subunits, as revealed by coimmunoprecipitation. The association of $\rho_1$ and $\gamma_2$ subunits has recently been confirmed in yeast two hybrid studies (Ekema et al., 2002), coimmunoprecipitation from rat brain tissue (Ekema et al., 2002), and from expression systems (Ekema et al., 2002; Pan and Qian, 2003), as well as by observations that coexpression of $\gamma_2$ with $\rho_1$ subunits results in trafficking of $\gamma_2$ to the cell surface, whereas $\gamma_2$ subunits expressed alone remain intracellularly (Pan and Qian, 2003). Taking these results together supports the hypothesis that the $\rho_1$ subunit in these brainstem neurons is part of a GABA receptor that also contains subunits traditionally from the GABA$_A$ subfamily.

Possible stoichiometry of these GABA receptors

Based on the stoichiometry of other GABA receptors and our current data, the GABA receptor reported here is likely to contain at least one $\rho_1$, one $\alpha_1$, and one $\gamma_2$ subunit. Evidence that $\alpha_1$ is involved is the enhancement of GABA$_C$ agonist responses by zolpidem, a benzodiazepine that acts with high affinity on GABA receptors containing $\alpha_1$ subunits (Pritchett and Seeburg, 1991). Furthermore, $\rho_1$ and $\alpha_1$ are present in the receptor complex because they coimmunoprecipitate and are colocalized within neurons. $\gamma_2$ subunits are also likely to be present because they also precipitate with $\rho_1$ subunits (Ekema et al., 2002) and are necessary for the action of zolpidem on $\alpha_1$-containing receptors (Pritchett and Seeburg, 1991). Thus, in native neurons, $\rho_1$ subunits can form heteromers that also contain at least one $\alpha_1$ and a $\gamma_2$ subunit. The $\rho$ subunits have the potential to function like $\beta$ subunits because they share highest sequence homology with $\beta$ subunits (Whiting et al., 1999), which in some cases can form homomeric channels similarly to $\rho$ subunits (Moss and Smart, 2001). The remaining subunits are likely to be an $\alpha_1$ and either another $\rho_1$ or a $\beta$ subunit if the subunit composition is similar to the majority of GABA$_A$ receptors. Such heteromeric GABA receptors may account for some of the GABA receptors throughout the CNS that exhibit atypical characteristics (Arakawa and Okada, 1988; Delaney and Sah, 1999; Park et al., 1999; Zhu and Lo, 1999; Semyanov and Kullmann, 2002; Dean et al., 2003) and so provide a new substrate for inhibitory neurotransmission.

Localization of $\rho_1$ subunits

GABA$_C$ receptors comprising only $\rho$ subunits have a higher sensitivity to GABA than GABA$_A$ receptors (Chebib and Johnston, 1999; Bormann, 2000; Zhang et al., 2001). Interestingly, GABA$_A$ subunits with relatively higher GABA sensitivities have been located extrasynaptically where they mediate tonic inhibition, such as the $\delta$ subunit in the cerebellum (Wisden and Farrant, 2002). Because bath applications of compounds may activate both synaptic and extrasynaptic receptors on individual neurons, we considered whether the $\rho$ subunits were located at synapses. Three lines of evidence support this possibility. First, electron microscopy revealed $\rho_1$ immunoreactivity at synaptic junctions. Second, spontaneous IPSPs were partially antagonized by TPMPA, suggesting that they were mediated in part by $\rho_1$-containing receptors because the presence of the $\rho_1$ subunit is essential for TPMPA-mediated reductions of GABA responses in mouse spinal cord (Zheng et al., 2003). Third, $\rho_1$ and $\gamma_2$ subunits coimmunoprecipitate (Fig. 7) (Ekema et al., 2002; Pan and Qian, 2003) and $\gamma_2$ subunits are targeted to synaptic receptors (Somogyi et al., 1996), where they may be necessary for synaptic clustering of GABA receptors (Schweizer et al., 2003). Together these data suggest that the $\rho_1$-containing receptors may be located at the synaptic junction where they are involved in mediating inhibitory synaptic transmission.

The evidence we show here therefore favors the formation of GABA receptors that contain subunits from both GABA$_A$ and GABA$_C$ subfamilies, and these receptors display some unique properties. Because modulation of GABA-mediated neuronal inhibition is one of the most potent therapeutic approaches for the treatment of CNS diseases (Keverne, 1999), precise targeting of such treatments depends on identification and characterization of the different subunit complexes that exist. These heteromers of GABA$_A$ and GABA$_C$ subunits may therefore provide an important target for future therapeutic investigations.
References


