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Mesencephalic trigeminal neuron responses to γ-aminobutyric acid

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Abstract

Mesencephalic trigeminal neurons are primary sensory neurons which have cell somata located within the brain stem. In spite of the presence of synaptic terminals on and around the cell somata, applications of a variety of neurotransmitter substances in earlier studies have failed to demonstrate responses. Using intracellular recording in a brain slice preparation, we have observed prominent depolarizations and decreases in input resistance in response to applications of γ-aminobutyric acid (GABA) in most recorded mesencephalic trigeminal neurons. Those cells failing to respond were located deeply within the slice, and the low responsiveness was shown to be related to uptake of GABA in the slice. The responses were direct, since they remained during perfusion with a low calcium, high magnesium solution that blocks synaptic transmission. The responses were mimicked by the $GABA_A$ receptor agonist isoguvacine, and blocked by $GABA_A$ receptor antagonists. The $GABA_B$ receptor agonist baclofen evoked no changes in membrane potential or input resistance in neurons exhibiting depolarizations with GABA application. Tests of neuronal excitability during GABA applications indicated that the excitatory effects of the depolarization prevail over the depressant effects of the increase in membrane conductance. In situ hybridization histochemistry indicated that the $GABA_A$ receptors in Me5 cells are comprised of α_2, β_2 and γ_2 subunits.

Keywords: Mesencephalic trigeminal neuron; γ-Aminobutyric acid (GABA); $GABA_A$ receptor; In situ hybridization; Sensory neuron; GABA uptake

1. Introduction

The mesencephalic trigeminal (Me5) nucleus consists of a unique population of primary sensory cell somata located within the pontine and mesencephalic regions of the brain stem. Their sensory terminals are associated with muscle spindles of the jaw-closing muscles, and with periodontal mechanoreceptors. The cell somata are relatively large, and mostly pseudo-unipolar, though some bipolar/multipolar cells have been described [19,22,26]. They are therefore homologous to the large mechanosensitive neurons of the dorsal root ganglia (DRG), and trigeminal ganglia. The Me5 cells are unique in having their cell somata located within the central nervous system, and in having relatively large numbers of synaptic endings on cell somata [13,14,19,20], by comparison with the small numbers observed on DRG cell bodies [15,16].

In view of the presence of these synaptic endings, and the observations of modulation of Me5 neuronal excitation [17,24,27], it is surprising that these cells have been found to be unresponsive to neurotransmitters [6,12,33]. It is particularly unexpected in the case of γ-aminobutyric acid (GABA), which has well-known depolarizing actions on the homologous DRG neurons, and is thought to be responsible in part for presynaptic inhibition of the central terminals of these neurons. Since the only report on testing of Me5 neurons with GABA relied on extracellular recording and iontophoretic application of drugs in anesthetized animals [6], our study was designed to test whether mRNAs for GABA receptor subunits are present in Me5, whether GABA responsiveness could be observed in these neurons under different experimental conditions, and if such responsiveness might be a basis for modulation of excitability of these cells. A preliminary report of some of the findings has been presented earlier [25].

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[2] Professor Paul Feltz, a valued colleague and friend, died in January, 1996. We dedicate this report to his memory.

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2. Materials and methods

2.1. Electrophysiology

Transverse pontine brainstem slices of about 350 μm thickness were prepared from Wistar rats of either gender weighing 50–100 gms. The animals were given intramuscular ketamine (35 mg/kg) followed by anesthesia using ether or halothane in an oxygen-rich chamber, and subsequent rapid surgical decapitation. A block of tissue containing brainstem and cerebellum was removed from the skull and trimmed to form a small block of pons plus some overlying cerebellum. Slices were prepared using an Oxford Vibratome; this and all other steps following removal of brain from the skull were carried out in cold (about 4°C) artificial cerebrospinal fluid (ACSF) equilibrated with 95:5 O_2/CO_2, and with replacement of NaCl by sucrose (248 mM) [1], and containing (in mM) KCl 2, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, KH_2PO_4 1.25 and D-glucose 10. The selected slice was allowed to recover for 30 min in this solution and then transferred on to a nylon-mesh support in the recording chamber and perfused with regular (non-sucrose-containing) ACSF. The slice was stabilized by a surrounding U-shaped silver bar across which several very fine nylon threads had been glued to stabilize the slice. The bath was continuously perfused with ACSF maintained at about 30°C, pH 7.4 and a flow rate of 1.5 ml/min.

Recordings of intracellular activity were made using borosilicate glass electrodes containing 2 M potassium chloride (KCl) or potassium acetate (KAc) solutions, with tip resistance of 40–100 MΩ. Signals were amplified by an Axoclamp 2A amplifier, and digitized and analyzed using an A/D converter and pCLAMP software. Hard-copies of data were made with a Gould TA240 thermal recorder unit or Gould 2200 pen recorder. Input resistance of cells was estimated by measurement of voltage responses to repetitive constant-current hyperpolarizing pulses of 20 ms duration.

GABA and other agonists were normally applied by pressure ejection from a glass micropipette using a General Dynamics Picospritzer with pulse duration of 50–500 ms. Concentrations of agonists in the electrodes were GABA (10 mM), isoguvacine (5 mM), baclofen (10 mM). The micropipette tip was located above the slice surface, and directed toward the tip of the recording microelectrode. Antagonists were added to the perfusion line from a secondary reservoir containing ACSF.

Me5 neurons were located by tracking just lateral to the nucleus locus coeruleus (LC), which could be visualized as a bright area in the transilluminated slice. Electrophysiological identification was made on the basis of characteristics observed in earlier studies [12]. The depth of impaled cells below the slice surface was measured from the recording electrode manipulator for all neurons studied. In some cases, cells were dye-injected using 3% lucifer yellow in 0.2 M lithium chloride in the recording electrode. The slices were subsequently processed according to the method described by Grace and Llinas [11], and the slices were viewed and photographed in a fluorescence microscope.

2.2. In situ hybridization histochemistry

Oligodeoxynucleotide probes (45–47 bases in length) homologous to mRNA sequences coding for the amino acids in the putative cytoplasmic domains of the receptor subunits (except the α_2 probe which is homologous to the signal sequence) were synthesized using an Applied Biosystems 380A DNA synthesizer. All probes used in this study have been used before in other studies where their specificity and reliability have been demonstrated [23,30,31].

Young 125–175 g Sprague Dawley rats were killed by decapitation while under deep ether-induced anesthesia. The brain was rapidly removed and frozen on dry ice and stored at −80°C until sectioning. Coronal and parasaggital sections (12 μm in thickness) were prepared and thaw mounted on twice-coated porcine gelatine chromalum slides. Sections were then stored at −80°C until hybridization. At the start of each hybridization experiment sections were allowed to warm to room temperature and then were fixed (4% paraformaldehyde in sterile phosphate buffered saline). Acetylation, dehydration, delipidation and rehydration of the sections were then done as previously described [41].

Oligonucleotide probes were tailed using ^{35}S-dATP (New England Nuclear) and terminal transferase (Boehringer Manheim) to a specific activity of 3500–5000 μCi/mM [21]. Six to seven probes were labelled at the same time and used for hybridization immediately. Hybridization was performed overnight (approx. 18 h) with ^{35}S-labelled probes in hybridization buffer at a concentration of $1 \cdot 10^6$ dpm/50 μl. Following hybridization, slides were washed at moderately high stringency for 15 min four times at 55°C in 1 × standard saline citrate solution (SSC) (approximately 20° below the calculated melting temperature-Tm) followed by two lower stringency washes in 1 × SSC for 1 h at room temperature. Sections were rinsed in distilled water and dried. They were then exposed for 3–4 weeks to autoradiographic film (Amersham b max). Afterwards, slides were dipped in photographic emulsion (Amersham LM-1) and exposed for 2–4 weeks. Results reported below were obtained from experiments done on three separate occasions. Quantification of the graining intensity was done by counting the number of grains over a Me5 neuron and then dividing this number by the number of grains seen over an equivalent background area. In order for a neuron to be considered positive this ratio had to be greater than 2.

Light staining of the cell bodies was accomplished with a 0.1% solution of toluidine blue. Photos were taken using phase contrast optics.

3. Results

In an attempt to demonstrate the presence and identity of $GABA_A$ receptor subunits in Me5 neurons, in situ hybridization histochemistry studies were carried out. The results revealed that Me5 neurons express GABA receptor subunit mRNAs. Twelve different radio-labelled probes complimentary to the mRNAs encoding the receptor subunits α_{1-6}, β_{1-3}, or γ_{2-3} and δ were used. Only three different subunit mRNAs could be detected; these being α_2, β_2 and γ_2 (splice variant unknown). Each subunit mRNA's signal could be detected at greater than 2–14 times the background signal (Fig. 1). These results indicate that the Me5 nucleus expresses mRNAs that if translated, code for a fully functional GABA receptor [37].

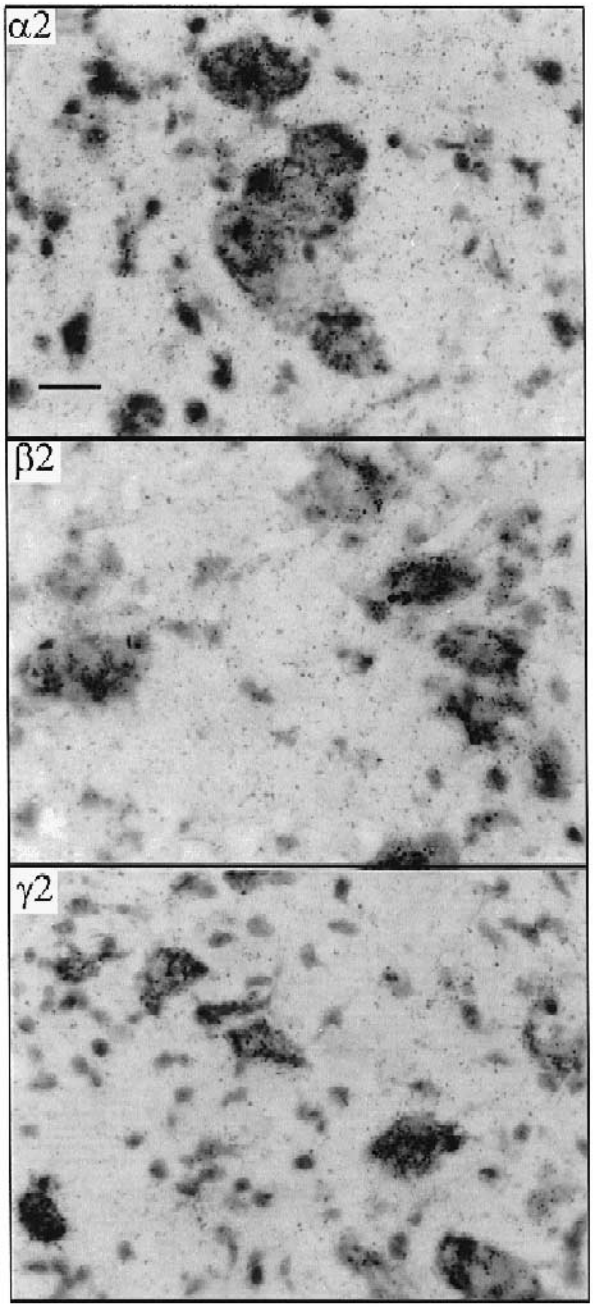

Fig. 1. Expression of $GABA_A$ receptor subunit mRNAs in the Me5 nucleus (scale bar equals 21 μm).

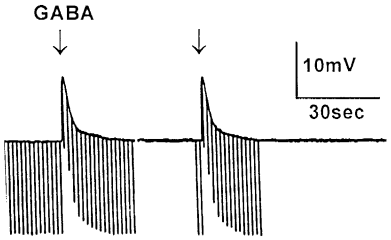

Fig. 2. Depolarizing response to pressure applied GABA. Vertical deflections are voltage responses to hyperpolarizing constant current pulses to monitor changes in input resistance. Applications of GABA are indicated by arrows.

Recording in the regions just lateral to the LC [28], neurons were located having stable membrane potentials of −52 to −62 mV, with no rhythmic activity or spontaneous discharge, and no apparent synaptic potentials. Brief depolarizing constant current pulses passed through the recording electrode evoked single, short-duration action potentials (0.4–0.7 ms measured at threshold), or with larger currents, high-frequency repetitive spikes. Longer duration hyperpolarizing pulses revealed a prominent time and voltage-dependent inward rectification, followed by an off-spike on recovery at the end of the hyperpolarizing pulse. These characteristics are very similar to those observed by Henderson et al. [12], for Me5 neurons.

Focal pressure application of GABA in the region of neurons having these properties frequently resulted in a depolarization of the membrane potential by up to 15 mV, accompanied by a decrease in cell input resistance of up to 70%, as measured from the amplitude of repetitive hyperpolarizing constant current pulses (Fig. 2). The presence of responses to GABA was found to be related to the location of the cell within the slice. Cells impaled within 75 μM of the slice surface were invariably depolarized by GABA ($n = 78$), whereas neurons below 125 μM seldom showed responses. (Fifty-one of the depolarized neurons were recorded with KAc-filled electrodes, and 27 with KCl-filled electrodes.) The $GABA_A$-selective agonist isoguvacine was also tested, and found to evoke depolarizing responses in the recorded cells. However, isoguvacine was also able to evoke responses in deeper cells that did not respond to GABA applications. In tests on four of these cells, addition of the GABA uptake inhibitor nipecotic acid to the perfusion solution resulted in the appearance of responses to GABA, where no GABA responses had been observed before this treatment (Fig. 3).

The responses to GABA were characterized by several additional means. Repetitive pressure pulse application showed the depolarizing response to be strongly desensitizing, and full responsiveness was restored only after about 40–60 s (Fig. 4). A further indication of desensitization

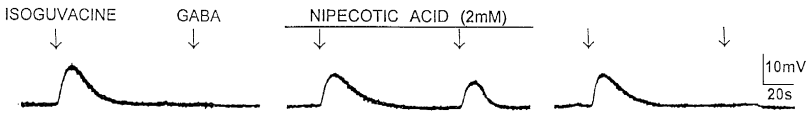

Fig. 3. Deep-lying neuron which gave depolarizing response to isoguvacine, but not to GABA. Addition of the GABA uptake blocker nipecotic acid to the perfusion solution resulted in appearance of a response to GABA (middle trace: 12 min after nipecotic acid). The effect was reversible after washout of the nipecotic acid (right hand trace 15 min after beginning of wash).

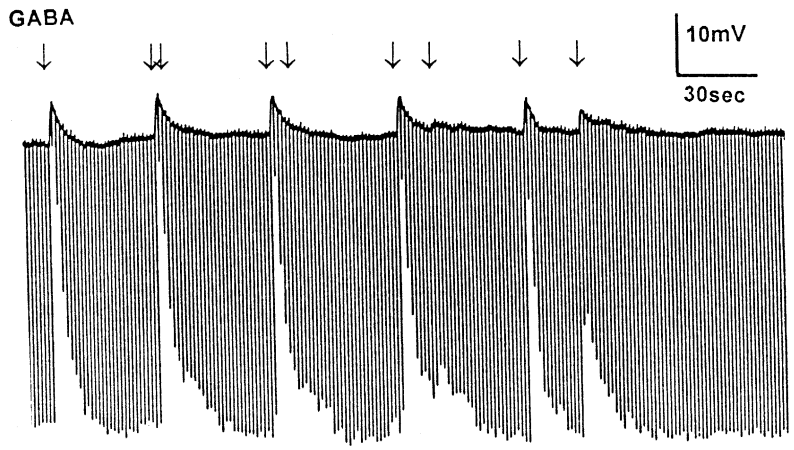

Fig. 4. Desensitization of GABA responsiveness. From left is shown a control response, and four pairs of applications with increasing separation. With rapid sequential applications of GABA (arrows), the response to the second application is hardly discernible. Even with separation of pulses by 20 s, the second response is diminished.

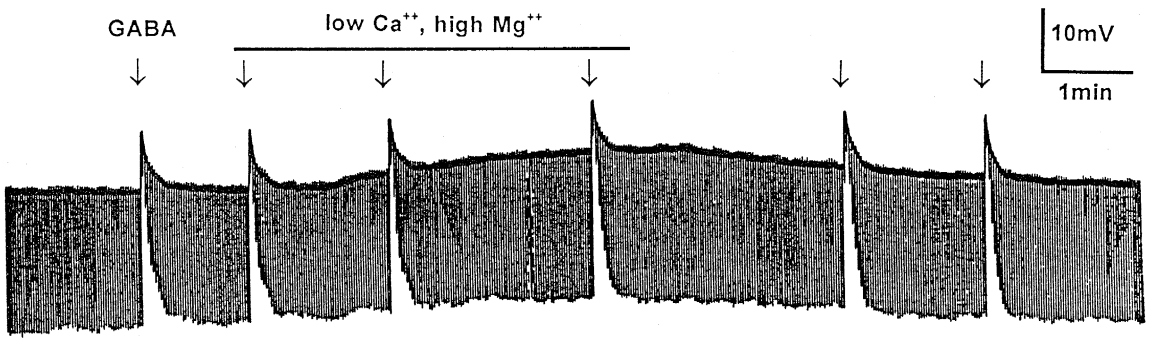

Fig. 5. GABA responses remain constant during replacement of standard perfusion ACSF with one containing low Ca^{2+} and high Mg^{2+} (time indicated by horizontal bar) to block Ca^{2+}-dependent transmitter release.

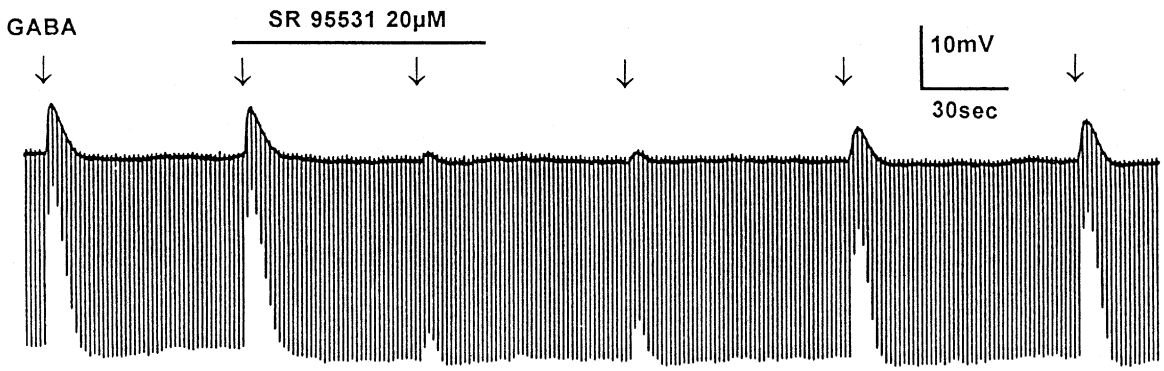

Fig. 6. GABA responses are reversibly antagonized when the $GABA_A$-selective blocker SR95531 is added to the perfusion solution. Time of perfusion with antagonist-containing ACSF is indicated by the horizontal bar.

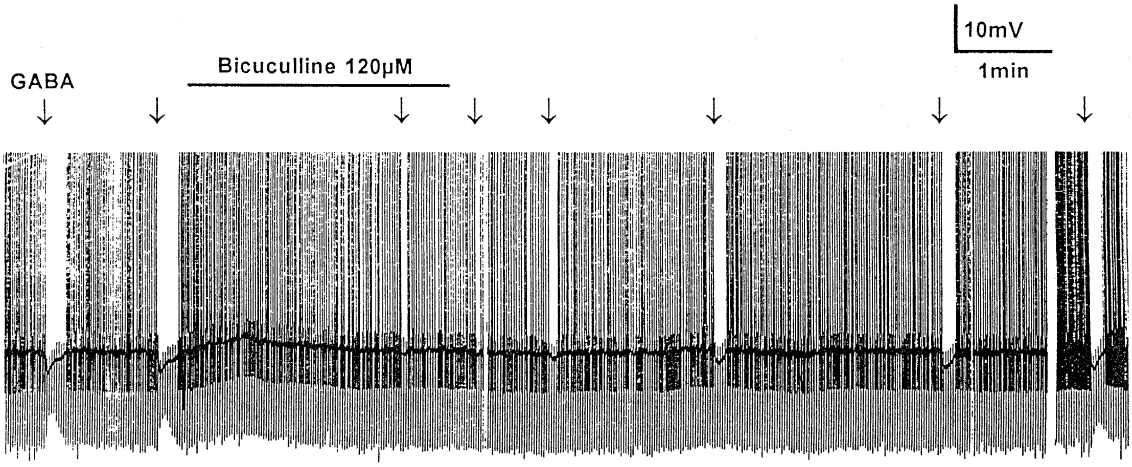

Fig. 7. Intracellular recording from spontaneously firing LC neuron shows hyperpolarizing response to GABA accompanied by decrease in input resistance. The response is reversibly blocked by addition of bicuculline to the perfusion solution. A short break in the record at the right (6 min) permitted complete recovery of the GABA response.

was observed with bath application of 1 mM GABA, during which responses to pressure applications of GABA were almost abolished (not illustrated). Because GABA is known to depolarize some nerve terminals, the depolarizing responses might have resulted from release by GABA of an excitatory neurotransmitter onto Me5 neurons. For that reason, GABA responses were tested in a low Ca^{2+}, high Mg^{2+} solution (0.1 mM, 16 mM respectively) in which chemical neurotransmission is blocked. Perfusion with this test solution consistently resulted in a moderate depolarization of the resting membrane potential, but little or no change in the GABA response was observed (Fig. 5).

Antagonists selective for $GABA_A$ receptors were also tested. Both bicuculline (up to 120 μM; $n = 3$) and 2-

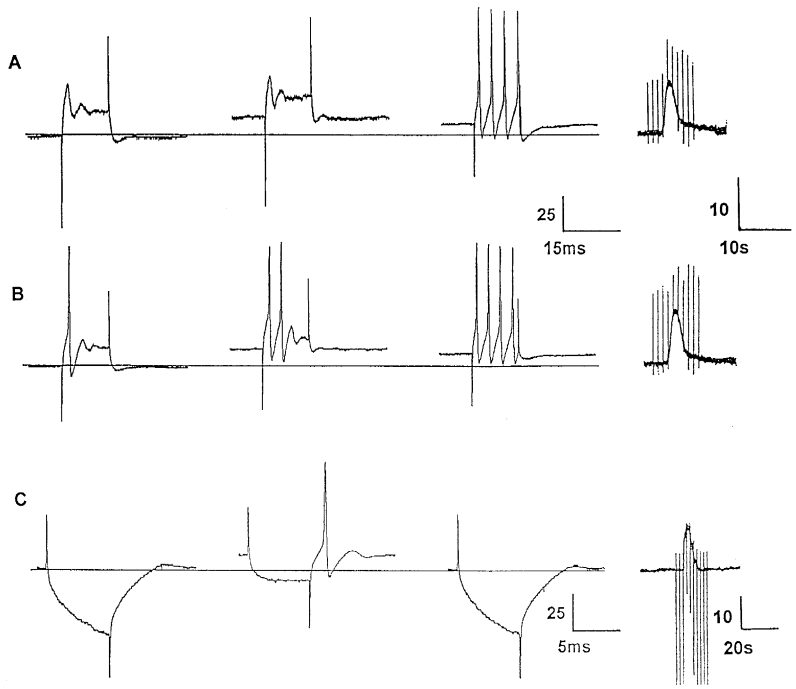

Fig. 8. Tests of excitability of Me5 neuron during GABA application; all excitability tests were made using KAc electrodes. Individual fast-sweep records at left show responses to constant current pulse injections prior to and during depolarizing responses to GABA. (Action potentials are not apparent on these traces due to slow response character of recorder.). Slow time-base records at right show membrane potential response to GABA. A and B show, from left to right, responses to first, fifth and seventh depolarizing pulses during series shown at right. A: depolarizing pulses during control period were just sub-threshold for spike generation. B: depolarizing pulses were just super-threshold for spike generation during control period. Resting membrane potential of this cell was −62 mV. C: different Me5 neuron responses to first, fifth and ninth hyperpolarizing constant current pulses of series shown at right. Resting membrane potential for this cell was −57 mV.

(carboxy-3[1]-propyl)-amino-6-paramethoxy- phenylpynazidium bromide (SR 95531) (20 μM; $n = 5$) were found to reversibly block the GABA-induced depolarizations (e.g., Fig. 6). The presence of functional $GABA_B$ receptors was tested by pressure application of the selective $GABA_B$ agonist baclofen. In four cells showing clear responses to GABA, application of baclofen gave no detectable change in either membrane potential or input resistance (data not shown).

GABA was also pressure applied to intracellularly recorded LC neurons, using KAc-containing recording electrodes, in the same slices from which Me5 neurons were recorded. The responses observed in these cells were hyperpolarizing, with decreased input resistance, and were evident even in cells located deep within the slice. These hyperpolarizing responses were also blocked by the $GABA_A$ antagonist bicuculline (Fig. 7).

In order to identify the morphological features of cells depolarized by GABA, several of these neurons were dye-labelled by injection of lucifer yellow from the recording electrode. Of seven cells marked in this way, all had large round or ovoid cell somata; in six of these, a single process was connected to the soma, and the seventh had an apparent bipolar configuration, with two processes leading from opposite sides of the soma.

The effects of GABA applications on neuronal excitability were examined during passage of depolarizing or hyperpolarizing constant current pulses through the recording electrode. The generation of spikes by depolarizing pulses was used as a measure of excitability. Spikes were generated with a higher probability during the response to GABA than in control periods (Fig. 8A,B). The strongest excitatory effect occurred shortly after the peak of the depolarizing response to GABA, after partial recovery of the input resistance. On the rising phase of the depolarization, during the large conductance increase, there appeared to be only a small increase in excitability, but a decrease in excitability was observed on only one occasion, of more than 20 cells tested. Off-spikes evoked following hyperpolarizing current pulses also exhibited the same pattern of increased firing probability during GABA responses (Fig. 8C).

4. Discussion

The electrophysiological properties of neurons observed in this study are similar to those described earlier for Me5 neurons [12] and to those of some cells of dorsal root ganglia [39] and the trigeminal ganglion [32]. The Me5 cells are homologous to the larger mechanoreceptive neurons of DRG and trigeminal ganglion, and their electrophysiological properties are correspondingly similar. The results of our intracellular staining tests confirm that the recorded cells have the morphological characteristics of Me5 neurons.

The depolarizing responses to GABA accompanied by increased conductance are also similar to those observed in neurons of the DRG [9,10,40] and trigeminal ganglion [32]. We have demonstrated that the depolarizations are direct responses of Me5 cells, mediated by receptors having properties appropriate for $GABA_A$ subtypes—mimicking by isoguvacine, antagonism by bicuculline and SR 95531, and rapid desensitization. The presence of $GABA_A$ responses in these cells is correlated with the results of binding studies [3], and with the observations of a dense network of GABA-immunoreactive fibres and terminals in and around the Me5 nucleus [5]. These cells showed no changes in membrane potential or input resistance with applications of the $GABA_B$-selective against baclofen. While $GABA_B$ receptor binding has been demonstrated in Me5 [3], the absence of responses corresponds with similar observations in larger DRG neurons [7,40]. Even in the smaller DRG neurons, the $GABA_B$ receptor-mediated effect is primarily the reduction of a voltage-sensitive calcium potential, rather than changes in membrane potential or resistance. Me5 neurons and the large DRG cells display little if any of the calcium action potential components found in the small DRG neurons [7]. On this basis, and the virtually complete blocking action of $GABA_A$-selective antagonists, we conclude that the depolarizing responses are mediated exclusively through $GABA_A$ receptors.

The depolarizing response to GABA in Me5 neurons is distinct from the responses to GABA observed in the neighbouring LC, and almost all other central neurons in adult animals. In the LC, hyperpolarizing responses are found to be mediated by both $GABA_A$ and $GABA_B$ receptor activation [36]. The characteristic depolarizing response in DRG neurons has been attributed to the presence of an inwardly directed chloride pump [2], and the same basis might be expected in Me5. It is interesting that similar depolarizing responses and mechanisms are observed in the centrally located Rohon-Beard sensory neurons of Xenopus [34].

Our difficulty in recording responses to GABA in deeper-lying Me5 neurons was not found in tests of LC neurons. This may be explained by the extensive dendrites of LC neurons which are likely to reach closer to the slice surface than the location of the recorded soma. The absence of dendrites in most of the Me5 may therefore at least partially explain the low sensitivity to GABA application; it is also possible, however, that the density of $GABA_A$ receptors is different in the two types of neurons. The observation that a GABA uptake blocker can confer responsiveness on a previously unresponsive cell suggests that sufficient GABA is being taken up by elements within the slice that sub-threshold concentrations are reaching the deeper cells. The heavy investment of GABA-immunoreactive nerve terminals [5] and of enveloping astrocytes [4] in the region of Me5 neurons may provide the basis for this effect. Nipecotic acid blocks uptake into

neural elements as well as astrocytes [35] and would not distinguish between these types of uptake.

In view of our results, it seems surprising that responses to iontophoretically applied GABA were not observed in a previous study [6]. However, we observed that the threshold for action potential generation is generally quite remote from the normal resting membrane potential in these cells. For that reason, responses detectable by extracellular recording may require very strong activation by exogenously applied neurotransmitters. In our experiments, GABA-evoked depolarizations did not by themselves result in action potential generation. This together with the presence of active GABA uptake mechanisms and rapid receptor desensitization may all contribute to difficulties in observing these responses using the methods employed in the earlier study.

Some clue as to the properties of the GABA receptors expressed in Me5 neurons can be implied from the in situ hybridization data. We have found that these neurons express α_2, β_2 and γ_2 subunit mRNAs. To date all in situ hybridization studies of DRGs have shown that both α_2 and γ_2 subunit mRNAs are the predominant subtypes found in DRG and therefore their presence in Me5 neurons is not surprising [23,29]. However, there are discrepancies as to the identity of the subunit mRNA expressed in DRG. In general agreement with our findings, Persohn et al. [29] have found that DRG neurons express β2 subunit mRNA. However, Ma et al. [23] have found that adult DRGs express β3 subunit mRNA. It is not clear as to why this discrepancy arises. We have used the same probes as Ma et al., so it is not attributable to anomalies arising from differing probe sensitivity. It is possible that the anatomical location of the DRG used was different in those two studies, and this may account for the difference in expression. Persohn et al. [29] used DRGs from the cervical region whereas Ma et al. [23] may have used DRGs collected from a different region. However, a change in subunit expression would not be predicted to alter appreciably the characteristics of the expressed receptors. Ducic et al. [8] have shown that there is only a small increase in the EC50 of GABA when the β3 subunit is substituted for a β_2 subunit (1.7–4 μM). The presence of the α_2 subunit predicts an EC50 value for GABA of about 10 μM [18]. It is likely that α subunits play an important role in determining the kinetics of $GABA_A$ receptors [38] however, detailed information about the activation, inactivation and desensitization kinetics of this particular subunit combination still remains to be determined.

The question remains whether GABA-mediated responses might explain the type of modulation of Me5 activity observed by Kolta et al. [17]. In most central neurons, GABA is strongly inhibitory as a result of the hyperpolarization moving the membrane potential away from threshold levels, and the shunting action of the large conductance increase. In Me5, the GABA-induced depolarization will enhance excitability, but this is balanced to some extent by the shunting action of the conductance increase. Our studies indicate that GABA would confer increased excitability in Me5 cells in most cases, though our lack of understanding of the nature of synaptic release of GABA onto these cells leaves this uncertain. It seems unlikely, therefore that GABA mediates the predominantly inhibitory modulation of Me5 neurons reported by Kolta et al. [17].

Acknowledgements

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