Inhibitory Synapses Among Interneurons in the Glomerular Layer of Rat and Frog Olfactory Bulbs

MICHELINO PUOPOLO1 AND OTTORINO BELLUZZI2
1 Dipartimento di Biologia, Sezione di Fisiologia Generale, Università di Ferrara, 44100 Ferrara; and 2 Dipartimento di Biologia, Sezione di Fisiologia, Università di Modena, 41100 Modena, Italy

Puopolo, Michelino and Ottorino Belluzzi. Inhibitory synapses among interneurons in the glomerular layer of rat and frog olfactory bulbs. J. Neurophysiol. 80: 344–349, 1998. The patch-clamp technique was applied to periglomerular (PG) cells from slices of frog and rat olfactory bulbs to characterize whole cell and single-channel properties of inhibitory synaptic currents. Spontaneous and electrically driven bicuculline-sensitive synaptic events were recorded under ionic conditions that excluded any possible interference of excitatory synapses. The peak amplitude distribution of spontaneous events could be fitted by several Gaussians having the same interpeak distance. Spontaneous currents reversed polarity at the chloride equilibrium potential and were suppressed by 2 mM Co2+; the decay phase could be fitted with a single exponential having a time constant of ~10 ms at 0 mV. Bicuculline-sensitive monosynaptic responses could be evoked in PG cells by electrical stimulations delivered at the distance of several glomeruli within the glomerular layer. Finally, in excised outside-out patches, single-channel analysis revealed the presence of typical γ-aminobutyric acid-A receptor channels, with a single-channel conductance of 28 pS in symmetrical chloride and mean open times of 3 ± 4 ms. The simplest explanation of these data, effectively supported by pristine Rats (Wistar strain) from 8- to 20-days old were...
with solutions of the following composition (in mM): 1) symmetrical chloride (110 KCl, 0.5 CaCl₂, 5 K-EGTA, 10 K-HEPES, 2 Mg-ATP, and 8 glucose) and 2) \( E_C = -70 \text{ mV} \) (107 K-glucuronate, 7 KCl, 0.5 CaCl₂, 5 K-EGTA, 5 K-HEPES, 2 MgSO₄, and 8 glucose). pH was set to 7.8 with KOH and osmolarity was adjusted to 235 mOsm with glucose.

Drugs, obtained from Sigma (St. Louis, MO) and from Tocris Neuramin (Bristol, UK), were dissolved in standard bath solution and perfused the entire preparation. Effects of drugs were observed within 1–2 min after changing bath solution.

**ELECTROPHYSIOLOGY.** The tight-seal, whole cell recording technique in thin slices was used (Edwards et al. 1989). The cells were not cleaned before patching. Seal resistances were always >3 GΩ. The pipettes (borosilicate glass with internal filament, 1.5 OD, 0.86 ID; Hilgenberg), made with a Sutter P-97 puller, had tip resistances of 4–5 MΩ (5–8 MΩ in frog) when filled with a standard or Cs⁺ internal solutions and usually were not fire polished. Cells were accessed under direct visual control using a ×40 water immersion objective. Membrane currents were recorded with a patch amplifier (Axopatch 1D, Axon Instruments). The serial resistance in these experiments was ~15 MΩ (19 MΩ in frog); series resistance compensation of 60–70% was routinely used. Leakage current was digitally subtracted from the recorded current tracings following the P4 protocol (Armstrong and Bezanilla 1974). Corrections for liquid-junction potential (Neher 1992) were applied a posteriori.

In the frog electrical stimulation was achieved with suction electrodes applied to the ON. The stimulus width was 1–1.5 ms and its amplitude ranged from 2 to 9 V. In the rat stimulation was obtained with Teflon-coated silver (Ag) wires (100-μm diam).

Stimulation and data acquisition were controlled by a PC-386 computer using a 12 bit A/D–D/A converter (TL-1–125, Axon Instruments). Before acquisition the signals were filtered at 3 kHz by a low-pass four-pole Bessel filter and digitized with sample times ranging from 50 μs to 1 ms. Data are given as means ± SE.

**RESULTS**

Recordings were obtained from 50 neurons in vitro rat OB slice preparations. Neurons in the glomerular layer were identified as PG cells on the basis of the location around the glomerular neuropile, value of membrane capacity (6.9 ± 2 pF, \( n = 50 \) ), and single action potential in response to the injection of depolarizing current pulses. We rule out any possibility of misidentifying PG cells and external tufted cells for the following two reasons: 1) the two populations had quite distinct membrane capacity (7.2 ± 0.2 pF, \( n = 109 \) for PG cells; 20.3 ± 0.66 pF, \( n = 119 \) for external tufted cells) and 2) they responded in entirely different ways to direct injection of depolarizing currents; PG cells responded with a single action potential (Fig. 1C), and external tufted cells responded with trains of spikes (Fig. 1D). The identification was confirmed by Lucifer yellow injection in ~10 cells in this study (Fig. 1A and B) and in ~20 in a previous study (Puopolo and Belluzzi 1996).

For a more extensive description of the fundamental electrophysiological properties, we refer to previous papers (Bardoni et al. 1995; Puopolo and Belluzzi 1996). Basically, in rat PG cells the resting membrane potential was ~52 ± 7 mV, input resistance was 750 ± 63 MΩ, and membrane charging could be fitted by a single exponential function, suggesting that PG cells usually behave as a single, electrically compact compartment (Johnston 1981; Rall 1977).

In the rat both spontaneous kynurenate-sensitive and bicuculline-sensitive postsynaptic events were observed in 20% of the PG cells under current- or voltage-clamp conditions, whereas in the frog only GABAergic events were observed. For brevity, we shall refer to GABAergic synaptic events as inhibitory postsynaptic currents (IPSCs; see Discussion). In spite of the variety of neurotransmitters described in this region (Shipley and Ennis 1996; Trombley and Shepherd 1993), we have never observed spontaneous events that could not be blocked by either bicuculline or kynurenate.

Spontaneous excitatory postsynaptic currents (EPSCs) were blocked by 1 mM kynurenate (Fig. 2A), were insensitive to bicuculline, reversed ~0 mV, and had a reversal potential independent of \( E_C \). An analysis of the amplitudes of EPSCs is shown in Fig. 2B for a 5-min recording. The amplitude distribution histogram could be fitted by three or four equally spaced Gaussian curves with the mean centered at multiples of 18 pA at ~70 mV. The EPSCs occurred with frequencies ranging from 2 to 4 Hz and decayed according to a single exponential with a time constant of 5 ms. These events, provisionally ascribed to spontaneous activity from the on terminals (Bardoni et al. 1996; Berkowicz et al. 1994), were not analyzed further in the current study.

In ~30% of the PG cells studied in the rat (50% in frog), spontaneous GABAergic postsynaptic currents were observed under voltage-clamp conditions. Spontaneous IPSCs were first studied in the frog (Fig. 3). In some cases the basic properties of these events were analyzed at the holding potential of ~70 mV, with solutions having an \( E_C = 0 \) mV (Fig. 3A). In most of the experiments, however, we adopted the condition shown in Fig. 3C (i.e., \( E_C = -70 \) mV, holding potential 0 mV) corresponding to the EPSCs’ equilibrium potential, even if in the frog excitatory spontaneous events were never observed. Spontaneous IPSCs could be reversibly blocked by the GABAₐ antagonist bicuculline (10 mM; Fig. 3, A and C), were insensitive to kynurenate 1 mM and reversed at the chloride equilibrium potential. An example of amplitude distribution is shown in Fig. 3B for a 5-min recording. The amplitude distribution histogram could be fitted by three equally spaced Gaussian curves with the mean centered at multiples of ~50 pA, and the IPSCs decayed following a single exponential with time constants of 38 ± 2 ms.

The second of the two protocols used in the frog (\( E_C = -70 \) mV, \( V_{\text{hold}} = 0 \) mV) was chosen to study IPSCs in the rat because the holding potential set at the equilibrium potential of the glutamatergic synapses excluded any possible interference caused by spontaneous activity of excitatory terminals.

In the rat at 30°C, spontaneous IPSCs occurred at a frequency ranging from 1 to 4 Hz and their decay phase could be fitted by a single exponential with time constant of 11 ± 2 ms (Fig. 4C). The amplitude distribution histogram could be fitted by two, occasionally three, equally spaced Gaussian curves with the mean centered at multiples of ~17 pA at 0 mV (Fig. 4B). The spontaneous activity was Ca²⁺ dependent and could be reversibly blocked by Co²⁺ 2 mM or by elimination of Ca²⁺ ions in the extracellular solution. The spontaneous IPSCs were not sustained by autapses because their frequency was not increased by depolarization (not shown).

To characterize GABA-activated currents in greater detail, outside-out patches from PG cells were obtained (\( n = 6 \)). Figure 5A shows an example of single-channel opening acti-
vated by the addition of 10 μM GABA to the solution. GABA-activated single-channel currents revealed amplitudes of 2.9 pA at ±100 mV with the use of symmetrical chloride. The conductance obtained from the current-voltage relationship was 28 ± 1 pS (5 patches, Fig. 5B), and examination of the gating properties revealed open times of 3–4 ms (3 patches; Fig. 5C).

In addition to the spontaneous events, electrically driven IPSCs could also be observed in four cases in response to electrical shocks applied in the glomerular layer with a couple of Teflon-coated Ag wires. The tracings shown in Fig. 6 represent pure GABAergic responses evoked in PG cells by stimuli delivered through an electrode placed in the middle of the glomerular layer. A test shock followed a conditioning shock (0.5-ms duration by a 2-s interval) to test for the presence of paired-pulse depression (see next paragraph and DISCUSSION). The recordings in the example shown were taken from a PG cell located rostrally to the point of stimulation at a distance of ~0.5 mm, corresponding to about three diameters of an average glomerulus in rat.

These responses were completely and reversibly blocked by bicuculline (10 μM) and were insensitive to kynurenic acid. The delay between the stimulus artifact and the rising phase of the synaptic response was 3–4 ms, suggesting a monosynaptic response. The evidence that the presynaptic element involved was not an ON terminal is both pharmacological (sensitivity to bicuculline) and electrophysiological; no sign of paired-pulse depression could be observed with paired pulses, a peculiar feature of the ON stimulation (Gusel’nikova et al. 1973; Freeman 1974; personal observations).

FIG. 1. Morphology and basic properties of a rat periglomerular cell and of an external tufted cell. A: Camera lucida reconstruction of Lucifer yellow-filled periglomerular cells. B: external tufted cell. Calibration bars for A and B = 25 mm. C: voltage responses of a typical PG cell (7.1 pF) to injected currents (from −65 to 45 pA in steps of 20 pA) from a resting membrane potential of −80 mV. D: voltage responses of an external tufted cell (17 pF) to injected currents from a membrane potential of −80 mV. Represented are the responses to five current pulses from −100 to +10 pA in steps of 30 pA and to a pulse of 140 pA, the first giving rise to action potentials. C and D are represented in the same scale.

FIG. 2. Excitatory postsynaptic currents (EPSCs) in rat. A: voltage-clamp recordings of spontaneous activity in rat PG cell in the presence of 10 μM bicuculline. Recordings in symmetrical chloride, holding potential ~70 mV. B: histogram of the amplitude distribution of the EPSCs in control conditions (5 min). The histogram was fitted with 3 Gaussian curves with peaks at multiples of 18 pA. Bin width 5 pA, 560 events.
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FIG. 3. GABAergic spontaneous activity in frog. A: voltage-clamp recordings of spontaneous activity in frog PG cell in the indicated experimental conditions. Recordings in symmetrical chloride, holding potential −70 mV. B: histogram of the amplitude distribution of the IPSCs in control conditions (5 min). The histogram was fitted with 3 Gaussian curves (interpeak distance 48 pA). Bin width 10 pA, 342 events. C: voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (IPSCs) in frog PG cell. Recordings in $E_{Cl} = −70$ mV, holding potential 0 mV. D: fit with a single exponential function of the decay of one of the IPSCs shown in C; $t = 38.5$ ms.

DISCUSSION

This study provides the first physiological evidence that both spontaneous and electrically driven GABAergic synaptic input can be observed in PG cells.

We are confident that the cells studied in this work were actually PG cells and did not belong to one of the other neuronal types described in the glomerular layer, namely, external tufted cells, short-axon (SA) cells, or astrocytes. In fact, even if external tufted cells may sometimes be mistaken for PG cells at DIC microscopy, the error of identification is immediately revealed by the measure of the membrane capacity and by the excitability profile, as explained above and shown in Fig. 1. Some of the classification criteria adopted (location around the glomeruli, membrane capacity, and single action potential) cannot entirely rule out the possibility that some of the cells studied in the rat were SA cells. However, it should be observed that SA cells were rare enough to escape the careful investigation of Golgi (1874), Ramon y Cajal (1890), and Blanes (1898) in the same preparation used for most of these experiments. Furthermore, in the frog where, as we have shown, GABAergic synapses are present on PG cells, SA cells are not present. Finally, SAs are slightly larger than PG cells (Getchell and Shepherd 1975; Pinching and Powell 1971a) and could belong to a relatively well-identifiable small group of cells averaging 12–13 pF that are not included in this study.

From our experiment it cannot be decided with certainty if these GABAergic terminals originate from other interneurons in the glomerular layer or from centrifugal fibers, but it should be observed that, although there is a good histological evidence sustaining the first hypothesis, there are no findings...
neous GABAergic input would also extend the potential during prolonged depolarization. Furthermore, spontaneous activity of the existence of GABAergic synapses and driven in each experimental condition. Recordings in symmetrical chloride, \( E_{\text{Cl}} = -70 \text{ mV} \).

Several hypotheses can be advanced to attribute a physiological meaning to the existence of GABAergic synapses among interneurons in the glomerular layer. Assuming that these synapses are inhibitory, the most obvious hypothesis is that this arrangement is designed to trim the PG cell excitability. In fact, ultrastructural studies from serial slices have clearly established that axons originating from PG cells impinge onto the proximal dendrites, soma, and initial segment of other PG and SA cells (Pinching and Powell 1971b; White 1973) and that these synaptic contacts had the typical hallmarks of inhibitory synapses (flattened vesicles and symmetrical thickening). On the other hand, GABA was found to be involved in centrifugal innervation of the OB both in the frog (Kratskin et al. 1992) and in mammals (Zaborszky et al. 1986). However, although there is good anatomic and functional evidence that GABAergic centrifugal fibers terminate in the granule cell layer (Kratskin et al. 1992; Kunze et al. 1992), the centrifugal projections to the glomerular layer appear to be mostly cholinergic (Le Jeune and Jourdan 1993; Macrides and Davis 1983; Pinching and Powell 1972) and serotonergic (McLean and Shipley 1987); to our knowledge there is no evidence to date of GABAergic centrifugal fibers impinging onto interneurons in the glomerular layer of rat or frog (for a recent review see also Duchamp-Viret and Duchamp 1997).

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FIG. 5. Single-channel properties of \( \gamma \)-aminobutyric acid (GABA) receptor channels. A: strips of single-channel recordings at 0 mV and filtered at 1 kHz; outside-out patches. Single channel activity was started by applying 10 \( \mu \text{M} \) GABA. Holding potential \( +100 \text{ mV} \); \( E_{\text{Cl}} = -70 \text{ mV} \). B: amplitude of single-channel currents as a function of membrane potential. Each point represents the mean of the Gaussian describing the current amplitude. C: normalized open time distribution of GABA-activated, single-channel currents. Bin size 1 ms, 257 openings.

FIG. 6. Evoked synaptic activity recorded in rat PG cells under voltage-clamp conditions following stimulation in the glomerular layer. Paired pulses, 0.5-ms duration and separated by an interval of 2 s, were delivered in each experimental condition. Recordings in symmetrical chloride, \( E_{\text{Cl}} = -70 \text{ mV} \).
citability profiles because of distinct composition in voltage-dependent channels (Puopolo and Belluzzi 1998), and, probably, different intracellular concentration of chloride ions (Siklós et al. 1995), with the obvious consequence that this could have on GABAergic signals. We conclude that an essential prerequisite for any reasonable guess about the role of PG cells is a much better understanding of their different types, properties, and synaptic connections, for which a considerable effort must still be spent.

We are grateful to P. Duchamp-Viret, A. Duchamp, and I. Kratskin for useful comments on this manuscript. This laboratory work was carried out with a personal grant to Prof. Virgilio Peri.

Address for reprint requests: O. Belluzzi, Dipartimento di Scienze Bio-mediche e Terapie Avanzate-Sezione di Fisiologia Umana, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy.

Received 8 January 1998; accepted in final form 25 March 1998.

REFERENCES


