Regulation of Granule Cell Excitability by a Low-Threshold Calcium Spike in Turtle Olfactory Bulb

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Pinato, Giulietta and Jens Midtgaard. Regulation of granule cell excitability by a low-threshold calcium spike in turtle olfactory bulb. J Neurophysiol 90: 3341–3351, 2003. First published July 16, 2003; 10.1152/jn.00560.2003. Granule cells excitability in the turtle olfactory bulb was analyzed using whole cell recordings in current- and voltage-clamp mode. Low-threshold spikes (LTSs) were evoked at potentials that are subthreshold for Na spikes in normal medium. The LTSs were evoked from rest, but hyperpolarization of the cell usually increased their amplitude so that they more easily boosted Na spike initiation. The LTS persisted in the presence of TTX but was antagonized by blockers of T-type calcium channels. The voltage dependence, kinetics, and inactivation properties of the LTS were characteristic of a low-threshold calcium spike. The threshold of the LTS was slightly above the resting potential but well below the Na spike threshold, and the LTS was often evoked in isolation in normal medium. Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) had only minimal effects on the LTS but revealed the presence of a high-threshold Ca\(^{2+}\) spike (HTS), which was antagonized by Cd\(^{2+}\). The LTS displayed paired-pulse attenuation, with a timescale for recovery from inactivation of about 2 s at resting membrane potential. The LTS strongly boosted Na spike initiation; with repetitive stimulation, the long recovery of the LTS governed Na spike initiation. Thus the olfactory granule cells possess an LTS, with intrinsic kinetics that contribute to sub- and suprathreshold responses on a timescale of seconds. This adds a new mechanism to the early processing of olfactory input.

INTRODUCTION

The olfactory bulb is the first central relay for olfactory input, where the first-order sensory neurons contact the second-order sensory neurons, the mitral and tufted cells (Fig. 1A; Shepherd and Greer 1998). Granule cells are the most numerous nerve cell type in the olfactory bulb, their main output being inhibitory dendrodendritic connections to the mitral/tufted cell secondary dendrites (Shepherd and Greer 1998), although a subpopulation of granule cells has recently been suggested to be excitatory (Didier et al. 2001). Granule cell-mediated inhibition modulates somatodendritic spike initiation in mitral cells (Chen et al. 1997) as well as the lateral propagation of spikes in the mitral cell secondary dendrites (Lowe 2002; Xiong and Chen 2002). The dendritic release of transmitter from granule cells involves calcium influx through N-methyl-D-aspartate (NMDA) receptors and voltage-gated calcium channels (Chen et al. 2000; Isaacson 2001; Jahr and Nicoll 1982). The neural representation of odorants includes temporal as well as spatial distribution of neural activity (Perez-Orive et al. 2002; Spors and Grinvald 2002), and granule cells are centrally placed in the olfactory circuitry to provide an early contribution to the time course and distribution of olfactory responses. It is therefore of interest to analyze the electrophysiological properties that shape the firing pattern of these cells, so as to gain an insight into the mechanisms contributing to spatial and temporal distribution of mitral/tufted cell activity.

Low-threshold spikes (LTSs) due to T-type calcium channels (Carbone and Lux 1984) are initiated around or below the resting potential of the cell, subthreshold for Na spikes (Huguenard 1996; Jahnsen and Llinàs 1984; Llinàs and Yarom 1981), and an LTS is a feature of neurons in many brain regions including the inferior olive (Llinàs and Yarom 1981), hippocampus (Christie et al. 1995), thalamus (Jahnsen and Llinàs 1984), and hypothalamus (Niespodziany et al. 1999), where it is responsible for driving Na spike firing (Kim and McCormick 1998; Sherman 2001).

In this paper, we show the existence of a LTS due to T-type calcium channels in olfactory bulb granule cells. The LTS contributes to subthreshold depolarizing responses and boosts Na spike firing, and may thus contribute to early sensory processing in the olfactory system.

Parts of these findings have been presented in abstract form (Pinato and Midtgaard 2001).

METHODS

Preparation

Experiments were performed in adult freshwater turtles (Pseudemys scripta elegans), a preparation that offers some advantages since experiments in vitro can be performed at physiological temperature in adult animals (Mori et al. 1981). Animals were anesthetized by intramuscular injection of pentobarbitone (100 mg/kg) and decapitated. The surgical procedures comply with the Danish legislation and are approved by the controlling body under the Danish Ministry of Justice. The brain was removed and placed in oxygenated Ringer solution. The olfactory bulb was isolated, and each hemisphere was hemisected (Mori et al. 1981) using fine scissors. One-half a hemisphere of the OB was glued to the bottom of the recording chamber (volume approximately 1 ml) and continuously superfused with oxygenated Ringer solution at an exchange rate of 1–2 ml/min. Both dissection and experiments were done at room temperature (22–24°C). Some experiments were performed in slices of 300–600 μm thickness, cut on a Vibratome (DSK).

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Solutions and drugs

The recording chamber was perfused by a gravity feeding system, and the Ringer solution had the following composition (in mM): 120 NaCl, 15 NaHCO₃, 5 KCl, 2 MgCl₂, 3 CaCl₂, and 20 glucose, pH 7.6, oxygenated with 98% O₂-2% CO₂. TTX was obtained from Alomone Labs (Jerusalem, Israel); amidrol, α-2-amino-3-phosphopentanoic acid (AP-5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and ZD7288 were obtained from Tocris (Bristol, UK); SR-95531 was obtained from RBI (Natick, MA); and mibefradil was kindly provided by Roche Pharma (Basel, Switzerland). CdCl₂, NiCl₂, and tetraethylammonium (TEA) were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole cell recordings were obtained from granule cells and mitral cells in the olfactory bulb. Recordings were performed both in voltage- and current-clamp mode. In some experiments, potassium-Lucifer yellow-CH (5 mM) was included in the electrode solution for anatomical identification of the cells. Cells were accepted for study if they displayed a stable resting potential, when no bias current was passed through the recording electrode, and fired repetitive Na spikes on depolarization. Two hundred cells were included in this study. In some experiments, AP-5 (30 μM), CNQX (50 μM), and SR-95531 (10 μM) were added to the medium to reduce the background synaptic activity. The data presented here were not corrected for the liquid junction potential between electrode and bath. Recording microelectrodes (thick-walled glass capillaries, 10–30 MΩ) were filled with the following solution (in mM): 127 KCH₃SO₄, 1.53 Mg-glucuronate, 3.7 MgCl₂, 1 glucose, 5 HEPES, 5 Na-HEPES, and 2 Na₂-ATP, adjusted to pH 7.5 with KOH.

Conventional techniques for obtaining “blind” whole cell recordings were used. The data were amplified (Axoclamp 2B, Axon Instruments), digitized at 20 kHz (Digidata, Axon Instruments), and analyzed using pClamp8 software (Axon Instruments). In some of the experiments, the cells were clamped at the potentials evoked by synaptic excitation: excitatory postsynaptic potentials (EPSPs) were recorded in granule cells in current clamp by stimulating the lateral olfactory tract in the presence of SR95531 (10 μM). The EPSPs were then used as voltage-clamp commands in other cells; the voltage traces shown in the figures are the ones actually recorded during the voltage-clamp experiment.

RESULTS

Most experiments were performed in one-half hemispheres of the olfactory bulb (Mori et al. 1981) to preserve the integrity of the cells and the network. For morphological identification of granule cells and subsequent correlation with the electrophysiology, the cells were stained with Lucifer yellow during recording (Fig. 1B). As shown in other species (Hall and Delaney 2002; Mori and Kishi 1982; Price and Powell 1970; Schneider and Macrides 1978; Shepherd and Greer 1998; Wells and Kauer 1994), olfactory bulb granule cells usually possess a number of basal dendrites, confined to the vicinity of the cell body, and several thin (approximately 1–2 μm), long (up to about 600 μm) dendrites projecting to the external plexiform layer, where they make contacts with mitral/tufted cell secondary dendrites. The identification of granule cells (see METHODS) was aided by electrophysiological criteria. As previously reported (Hall and Delaney 2002; Wells and Kauer 1994), granule cells fired a short burst of Na spikes when depolarized from a positive resting potential, with a gradual attenuation of spike amplitude often terminating in a plateau-like response (Fig. 2A). In contrast, mitral cells were often spontaneously active at low frequencies and produced robust firing throughout a depolarizing pulse with no inactivating plateau (data not shown; Hall and Delaney 2002; Mori et al. 1981; Wells and Kauer 1994). Granule cells had an input resistance of 1.28 ± 0.4 (SD) GΩ (range: 0.5–1.9 GΩ, n = 20), time constant of 46.94 ± 17.2 ms (range: 23–69 ms), resting potential in normal medium of −62.35 ± 8.7 mV (range: −44 to −75 mV), and Na-spike threshold of −28.14 ± 1.25 mV (range: −24 to −30 mV). Mitral cell input resistance was around 0.84 ± 0.18 GΩ (range: 0.6–1.1 GΩ, n = 8), resting potentials in normal medium was −60.8 ± 9.5 mV (range: −48 to −75 mV), and Na-spike threshold was −42.10 ± 3.83 mV (range: −38 to −48 mV).

Some of the recordings were performed following the addition of CNQX (50 μM), AP-5 (30 μM), and SR95531 (10 μM) to block excitatory and inhibitory synaptic background activ-
oxaline-2,3-dione (CNQX), and SR95531 were added to the medium in B to block spontaneous synaptic events. Three different cells are shown in the LTS. D - 2-Amino-5-phosphonopentanoic acid (AP-5), 6-cyano-7-nitroquinolin-2-ones (indicated by the arrow in B). In the presence of a negative holding current, the cell responded with an LTS followed by a Na spike. TTX (10–30 pA current pulse, evoking an LTS followed by a Na spike. Due to trial-to-trial fluctuations, Na spikes were not evoked in every sweep; 2 sweeps are superimposed to show how the occurrence of a Na spike and the associated afterhyperpolarization rapidly terminates the underlying LTS. C: the cell, slightly hyperpolarized (−10–pA bias current), generated an LTS followed by a Na spike. Due to trial-to-trial fluctuations, Na spikes were not evoked in every sweep; 2 sweeps are superimposed to show how the occurrence of a Na spike and the associated afterhyperpolarization rapidly terminates the underlying LTS.

The LTS was evoked from the resting membrane potential in 64% of the cells tested, which corresponded to the percentage of the cells with a resting membrane potential more negative than −55 mV. The remaining cells showed an LTS when depolarized from potentials less than −55 mV. Studies of LTSs in different brain regions indicate that hyperpolarization of the cells was often an essential requirement for the activation of these spikes (Jahnsen and Llinás 1984; Llinás and Yarom 1981); in our experiments, hyperpolarization of the cell usually resulted in an increased LTS amplitude, and on the other hand, depolarization of the cell above rest prevented LTSs generation. For a given level of depolarization, the effect of prestimulus membrane potential or the depolarizing current step was varied (Fig. 3A). An incremental series of pulses was delivered to the cell from rest in TTX (Fig. 3Aa). For weak currents, the cells showed a passive response, while larger pulses evoked graded LTSs. Using a prestimulus potential and a current pulse strength ensuring maximal activation of the LTS, the influence of the speed of depolarization was analyzed by changing the rate of rise of the depolarizing current pulse (Fig. 3Ac). The results show that the LTS activates over a range of slopes with a similar threshold, suggesting that even slowly changing synaptic depolarizations may activate the LTS (Kawai and Miyachi 2001; McQuiston and Katz 2001; Sherman 2001), although maximal amplitude was reached for slopes over 40 pA/s (Fig. 3ae).

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**Pharmacological characterization of the LTS**

Three subtypes of T-type calcium channels, which are responsible for the low-threshold calcium spikes, have been characterized recently (Cribbs et al. 1998; Lee et al. 1999a; Perez-Reyes et al. 1998). Olfactory bulb granule cells express the mRNA for all three subtypes (Talley et al. 1999). These channels differ in terms of kinetics and pharmacological profile, albeit with an overlap between subtypes (Klockner et al.
In particular, the pharmacology of these channels is complex (Huguenard 1996; Lacinova et al. 2000a,b; Sidach and Mintz 2002, Todorovic and Lingle 1998), with heterogeneity in the pharmacological profile in different kinds of cells. Relatively selective blockers of T-type channels are mibefradil (Lacinova et al. 2000a; McDonough and Bean 1998; Michels et al. 2002), NiCl$_2$ (Huguenard 1996; Lee et al. 1999b; Todorovic and Lingle 1998), and amiloride (Lacinova et al. 2000a; Williams et al. 1999). Mibefradil (20 μM) was the most effective way to antagonize the LTS in olfactory bulb granule cells, reducing to 89.2% of control. The low concentration reduced the LTS by 49.2% (n = 4, P < 0.001), while at higher concentration, the amplitude was reduced 79.3% (n = 16, P < 0.001; Fig. 3Bb). Amiloride (≤150 μM), a blocker of T-type channels in certain cells (Gillesen and Alzheimer 1997; Lacinova et al. 2000a; Zhuravleva et al. 2001), did not have significant effect in reducing LTS amplitude (100.5 ± 2.5% of control, n = 4, P > 0.5).

Control of calcium spike excitability by potassium currents

Potassium currents contribute to the control of excitability in olfactory granule cells dendrites (Schoppa and Westbrook 1999). Therefore the effects of potassium channel blockers on the LTS were tested. Both 4-AP and TEA had minimal effects on the latency, threshold, and amplitude of the LTS in the concentrations used here; however, a slight prolongation of the LTS was observed (255 ± 23.8 ms vs. 218.7 ± 36.6 ms in control; n = 4; P < 0.01; Fig. 4, A and C). When the cell was depolarized from a more positive prestimulus membrane potential than required for evoking the LTS in isolation (Fig. 4, B and D), a large amplitude spike was elicited at a high-threshold (−21 ± 7.7 mV, n = 4). In some cases, this high-threshold spike (HTS) occurred on top of the LTS, making the separation of the two spikes difficult. The HTS in TEA was characterized by a relatively long duration (159.4 ± 51.4 ms, n = 5; Figs. 4B...
same range of membrane potential as the Na spike (Fig. 4 E and D) and Ba conductances. 

that the LTS and the HTS are controlled differently by potassium currents (Gasparini and DiFrancesco 1997; Williams et al. 1997). The second was a barium-sensitive current suggesting the presence of I_{KiR} (Fig. 6Aa, barium-conc. 1 mM, ▲). In current-clamp recordings, this was evident as a characteristic “sag” during hyperpolarizing pulses (Fig. 6B, a and b). Compared with the heart and thalamic relay neurons, where the time course of activation has been found to be around 1 s (Robinson and Siegelbaum 2003), granule cell inward rectifier current was fast (approximately 50 ms, Fig. 6, Ab, Ba, and Bb); similar kinetics have been reported in hippocampal CA1 neurons (Robinson and Siegelbaum 2003). Olfactory granule cells showed little evidence of anomalous rectification above approximately −80 mV (Fig. 6Aa), and the anomalous rectification did not overlap with the LTS (threshold approximately −55 mV; Fig. 6Ba). The time course and the voltage range of the anomalous rectification in granule cells makes it less likely that this phenomenon could interact with the LTS to generate bursting activity.

When the cell was stimulated repetitively, the LTS showed

and 5, A and B), while the full-amplitude HTS after prolonged exposure to 4-AP (Fig. 4E, top) was characterized by a relatively short duration (47.8 ± 4.43 ms, n = 5). The results show that the LTS and the HTS are controlled differently by potassium conductances.

Ranges for activation of LTS and HTS are shown in Figs. 4, D and E, and 5, B and C. In particular, the HTS falls within the same range of membrane potential as the Na spike (Fig. 4E).

To characterize the HTS further, NiCl_2 and CdCl_2, which are known blockers of low- and high-threshold voltage-activated calcium channels, respectively (Fox et al. 1987), were added to the bath (Fig. 5). NiCl_2 (100 μM) did not significantly affect the HTS amplitude (99.7 ± 2.2%, of control, n = 4, P > 0.5, Fig. 5A), while the HTS was reduced by 98.6 ± 1.7% (n = 13, P < 0.001) by 100 μM CdCl_2 (Fig. 5B); in contrast, the LTS was not significantly affected by CdCl_2 (85 ± 2.8% of control, n = 4, P > 0.5; Fig. 5C).

In several brain regions, an anomalous rectification coexists with T-type calcium currents and together they constitute the mechanism underlying pacemaker activity and oscillations (McCormick and Pape 1990; Robinson and Siegelbaum 2003).

Turtle olfactory granule cells displayed an anomalous rectification activated by hyperpolarization below −80 mV (−80.6 ± 4.1 mV, n = 9, Fig. 6Aa, □) and blocked by CsCl (2 mM, Fig. 6Aa, *). This resulted from the superposition of two components. The first was a barium insensitive current blocked by ZD7288 (100 μM, Fig. 6Aa, ●), a blocker of I_h currents (Gasparini and DiFrancesco 1997; Williams et al. 1997). The second was a barium-sensitive current suggesting the presence of I_{KiR} (Fig. 6Aa, barium-conc. 1 mM, ▲). In current-clamp recordings, this was evident as a characteristic “sag” during hyperpolarizing pulses (Fig. 6B, a and b). Compared with the heart and thalamic relay neurons, where the time course of activation has been found to be around 1 s (Robinson and Siegelbaum 2003), granule cell inward rectifier current was fast (approximately 50 ms, Fig. 6, Ab, Ba, and Bb); similar kinetics have been reported in hippocampal CA1 neurons (Robinson and Siegelbaum 2003). Olfactory granule cells showed little evidence of anomalous rectification above approximately −80 mV (Fig. 6Aa), and the anomalous rectification did not overlap with the LTS (threshold approximately −55 mV; Fig. 6Ba). The time course and the voltage range of the anomalous rectification in granule cells makes it less likely that this phenomenon could interact with the LTS to generate bursting activity.

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FIG. 4. Control of calcium spike excitability by potassium currents. A: left: control with hyperpolarizing bias current (−5 pA). Right: Tetraethylammonium (TEA; 10 mM). B: left: control response from rest. Right: TEA (10 mM). C: left: control with hyperpolarizing bias current (−5 pA). Right: 4-aminopyridine (4-AP; 1 mM). D: left: control from rest. Right: 4-AP (1 mM). E: LTS (bottom) and full-amplitude high-threshold spike (HTS; top) in 1 mM 4-AP. Na spike in normal medium (inset, right). For comparison, the duration at threshold of the Na spike and the HTS were 3 and 15 ms, respectively. All experiments in TTX (1 μM). Note the long duration of the HTS reached after approximately 30 min in TEA (B); steady state for TEA effects were reached after about 20 min.

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FIG. 5. Pharmacological characterization of the HTS. A: left: HTS in TEA (10 mM). Right: further addition of NiCl_2 (100 μM). B: left: HTS in 10 mM. Right: further addition of CdCl_2 (100 μM). C: left: LTS in TEA (10 mM). Right: further addition of CdCl_2 (100 μM). All experiments in TTX (1 μM). TEA present for 30 min in A and for 8 min in B.
a refractory period that was defined as the minimal interval between stimulations able to evoke 100% of LTS amplitude at every pulse. The reduction of the LTS amplitude during repetitive stimulation was graded (Fig. 7A). The cell was stimulated at different intervals (200, 600, 800, and 2,100 ms) with pulses of a duration ensuring full activation and repolarization of the LTS (approximately 500–900 ms): at 200 ms, the LTS was completely inactivated during the second and subsequent pulses. By slightly increasing the stimulation interval (600 ms), the LTS gradually recovered, and at around an 800-ms stimulus interval, the response amplitude had recovered further, but showed a large pulse-to-pulse variation. Only at stimulus intervals longer than 2 s was the full amplitude of the LTS recovered, similar to the results of paired-pulse activation of the LTS (Hernandez-Cruz and Pape 1989; Klockner et al. 1999; Llinás and Yarom 1981).

The mean value of the inter-pulse interval for full recovery of the LTS was 2.8 ± 0.7 s (n = 20). The recovery curves of three cells are shown in Fig. 7B. Here, the normalized LTS amplitude for each cell versus interval of stimulation is plotted. The results also show that the effect of refractoriness is not all or none, but that the LTS amplitude recovered gradually with an increasing stimulus interval.

Prestimulus hyperpolarization of the resting potential resulted in an increased LTS amplitude (Fig. 3Ac); it was therefore possible that the inter-stimulus interval membrane potential influenced the refractory period. To test this possibility, pairs of pulses were delivered to the cell at intervals well below the refractory period (Fig. 8, same cell as Fig. 7A). The cell was held at a membrane potential that ensured maximal LTS amplitude at the first pulse. In subsequent trials, the inter-pulse membrane potential was varied, and the LTS gradually recovered with increasing hyperpolarization of the inter-pulse membrane potential (Fig. 8A). However, there appeared to be an absolute refractory period (Fig. 8B), below which the interpulse hyperpolarization had negligible effects on the recovery from inactivation. The value of the absolute refractory period was 315 ± 58 ms (n = 4). These results are consistent with studies of cloned T-type calcium channels (Klockner et al. 1999; Kozlov et al. 1999), suggesting that the kinetic properties of the T-type channels are responsible for LTS refractory period.

Fig. 6. Inward rectification in granule cells. Aa: plot of the current responses recorded during voltage-clamp steps from a holding potential of −40 mV in control (●) and after subsequent addition of ZD 7288 (100 μM, ■), BaCl₂ (1 mM, ▲), and CsCl (2 mM, ▲). Hyperpolarizing steps ranged from −45 to −120 mV. Control recordings were done in presence of AP-5, CNQX, SR95531, TTX, CdCl₂, and NiCl₂. Currents were measured 500 ms after the start of voltage steps, at the steady state for the inward rectification. Ab: current traces from the same experiment shown in Aa (data indicated by the dashed line) in control and after subsequent addition of ZD 7288, BaCl₂, and CsCl. Currents were recorded during a voltage step of −115 mV from −40 mV. B: current-clamp measurements of voltage responses to hyperpolarizing current pulses from −50 (Ba) and −75 mV (Bb) prepulse potentials, corresponding, respectively, to +20- and 0-pA bias currents. Current steps decremented by 10 pA for each trial from +10 to −80 pA and −10 to −100 pA, respectively, in left and right. Voltage recordings show a small, fast “sag,” consistent with fast kinetics of the inward rectifier currents. A rebound LTS was evoked in Ba. No rebound depolarization was evoked when the cell was held at −75 mV (Bb).
spike excitability when the cell is relatively hyperpolarized. In this respect the inactivation properties of LTS become meaningful as a general feature of granule cell excitability. When Na spikes were driven by the LTS (Fig. 9Ba, n = 4), an increase in the rate of stimulation induced the cell to be completely silent after the first pulse (Fig. 9Bb), similar to the results in Fig. 7. Similarly, the inactivation of the LTS (Fig. 9Ca) could be reduced by hyperpolarizing the cell during the inter-pulse interval with a subsequent partial recovery of the LTS results accompanied by Na spiking (Fig. 9Cb). At more depolarized resting membrane potentials, where the LTS was not activated by depolarizing pulses, the intrinsic capability of the cell to produce Na spikes (Fig. 9Cc) was not influenced by the rate of stimulation. In particular, the onset of Na spike firing was minimally affected by the paired-pulse stimulation. In conclusion, the results demonstrate that the amplitude and refractoriness of the LTS could contribute to the Na spike excitability. The inactivation kinetics of the LTS made the cell very sensitive to the onset of a depolarizing event and to the stimulation frequency when activated from rest.

EPSPs evoked in granule cells by electrical stimulation in vitro (Schoppa and Westbrook 1999; Wells and Kauer 1994) and odor stimulation in vivo (Cang and Isaacson 2003; Luo and Katz 2001; Margrie and Schaefer 2003) readily traverse the voltage range from the resting membrane potential to the Na spike threshold, corresponding to the activation range of the LTS. This makes it likely that synaptic excitation may activate T-type calcium channels in olfactory granule cells.

To analyze this possibility, we first recorded EPSPs from granule cells in current clamp during the stimulation of the lateral olfactory tract. Cells were then voltage clamped using the recorded EPSP as a voltage-clamp command. In this way, it was possible to test whether T-type currents could be activated by synaptic excitation, without interference from other pre- and postsynaptic inward currents. First, a T-type calcium current was identified by clamping the cells using voltage steps. An inward current was evoked when the cell was brought at approximately −50 mV (Fig. 10Aa, left). This current was blocked by NiCl₂ (Fig. 10Aa, right). When the cell was clamped with an EPSP waveform (Fig. 10B, a–c), a similar, Ni-sensitive inward current (Fig. 10Bb), was evoked during the EPSP. Voltage steps evoking a T-type current preceding the EPSP within 500 ms prevented further activation of T-type currents (Fig. 10Bc), consistent with the refractory period of the LTS (Figs. 7–9). The EPSP recorded at the granule cell soma is most likely due to synaptic conductances activated along the proximal and distal dendrites (Price and Powell 1970); the data in Fig. 10 show that the resulting somatically recorded EPSP can evoke low-voltage-activated calcium currents.

**DISCUSSION**

Olfactory bulb granule cells possessed a LTS, characterized by its relatively slow onset, threshold at membrane potentials well below Na spike threshold and a refractory period of up to about 2 s. For moderate subthreshold depolarizations, the LTS contributed significantly to Na spike initiation. The slow recovery kinetics of the LTS made the cell particularly sensitive to depolarizing events occurring at low frequencies. An HTS was evoked in the presence of potassium channel blockers; this HTS was initiated around the peak of the LTS and overlapped in voltage range with the Na spike. By contrast, potassium...
channel blockers had only small effects on the LTS. Thus olfactory granule cells possess two distinct types of calcium spikes, controlled separately by intrinsic potassium currents. The LTS endows these cells with a mechanism for boosting subthreshold excitatory inputs and may add a timescale of seconds to the conversion of synaptic inputs to Na spike firing.

FIG. 8. Paired-pulse protocol revealed inter-stimulus membrane-potential dependence of the refractory period. A: paired-pulse protocol with a period of 700 ms, i.e., below the refractory period of the LTS. Stimulation parameters were tuned to evoke maximum LTS response at the 1st pulse (−15-pA bias current, 15-pA pulse amplitude, 500-ms duration). Inter-pulse hyperpolarization was gradually increased (−45, −55, −60 pA) until full recovery of the LTS. B: reduction of paired-pulse period under a given “absolute” value (300 ms) prevented the LTS from recovering fully when the cell was hyperpolarized (−60 pA) during the inter-pulse interval (−15-pA bias current, 15-pA pulse amplitude, 500-ms duration). All the experiments were performed in TTX, CNQX, AP-5, and SR95531.

FIG. 9. The LTS boosts Na spike firing for depolarizing events occurring at low frequencies. Aa: LTS boosted Na spikes at hyperpolarized resting potentials (control; left; 3 sweeps superimposed). NiCl₂ (100 μM) abolished the LTS and the accompanying Na spike. Ab: same cell as in Aa. At resting membrane potential, Na spike excitability was not reduced by NiCl₂. Aa and Ab: left, control; right, in NiCl₂. B: pulse trains of depolarizing current (0-pA bias current, 15-pA pulse amplitude, 1-s pulse duration) were delivered with periods above (7 s, Ba) and below (500 ms, Bb) the refractory period for the LTS. At high stimulus frequencies, the LTS inactivated and the associated Na spike firing was prevented. C: paired-pulse protocol was applied to granule cells below LTS refractory period (300 ms, 500-ms pulse duration), preventing Na spike generation at the 2nd pulse (Ca). Inter-pulse hyperpolarization (~30 pA) reduced LTS inactivation resulting in a Na spike with an increased latency (Cb). At depolarized prestimulus potentials, Na spikes were evoked by paired-pulse stimulation with little reduction of the early response to the 2nd stimulus (Cc). Experiments in B and C were performed in CNQX, AP-5, and SR95531.
The pharmacology of low-voltage-activated calcium currents is complex; studies of various T-type currents in different brain regions have revealed large variations in their sensitivity to different compounds, and differences have also been found regarding the kinetic properties (Huguenard 1996; Niespodziany et al. 1999).

Recently, three subunits of T-type calcium channels have been isolated: the α1G (Perez-Reyes et al. 1998), the α1H (Cribbs et al. 1998), and the α1I (Lee et al. 1999a). The localization, kinetics, and pharmacology of these isoforms have been characterized (Chemin et al. 2002; Klockner et al. 1999; Kozlov et al. 1999; Lacinova et al. 2000a; Lee et al. 1999b; Michels et al. 2002; Santi et al. 2002; Talley et al. 1999). The granule cell layer of the olfactory bulb is a very distinctive case in this respect; the brain region is where the highest level of expression of all three subunits has been found (Talley et al. 1999). In the present study, mibebradil and NiCl₂ effectively antagonized the LTS, while amiloride did not have significant effects. The sensitivity of the LTS to low concentrations of NiCl₂ (Fig. 3Bb) suggests that the T-type channels responsible share some similarities with the α1H T-channel isoform (Lee et al. 1999b). However, the insensitivity to amiloride makes this categorization less certain (Lacinova et al. 2000a; Zhuravleva et al. 2001), but this pharmacological profile is similar to one of the LTS found in hypothalamic neurons (Niespodziany et al. 1999).

**Role of potassium currents in the control of calcium spike excitability**

Potassium channels blockers had moderate effects on the LTS (Fig. 4) but revealed the presence of high-threshold calcium spikes initiated around the peak of the LTS. The results suggest that while I_A currents control Na spike firing and EPSP shape in granule cells (Schoppa and Westbrook 1999), the initiation of the LTS is primarily determined by the intrinsic properties of the T-type channels. The HTS, which overlaps with the Na spike voltage range (Fig. 4E), is controlled by both 4-AP and TEA-sensitive potassium currents. Although the subcellular localization of the HTS has not been determined, the presence of an HTS is consistent with results showing that high-threshold calcium channels contribute to dendritic transmitter release in granule cells (Isaacson 2001).

**Contribution of the LTS to granule cell excitability**

The LTS was evoked at membrane potentials well below the Na spike threshold (Fig. 2) and could be of considerable amplitude in this membrane potential region. When a Na spike was evoked by the LTS, the latency to Na spike initiation appeared determined mainly by the activation time-course of the LTS (Fig. 2). Repetitive stimulation resulted in diminished LTS amplitude and a concomitant slowing of the rising phase, accompanied by an increased latency to Na spike initiation (Fig. 9B). In terms of membrane potential threshold, Na spike initiation occurred at more negative (by about 15 mV) membrane potentials in mitral cells than in granule cells, and the more positive Na spike threshold in granule cells could allow the LTS an important role in boosting Na spiking.

The LTS showed a refractory period of about 2 s (Figs. 7–9). This could be shortened by inter-stimulus hyperpolarization (Figs. 8 and 9), and the data are consistent with the refractory period found as an intrinsic property of the T-type calcium channels (Hernandez-Cruz and Pape 1989; Klockner et al. 1999; Kozlov et al. 1999).

Only one Na spike was usually evoked by an LTS; this is in contrast to thalamic neurons where a burst of Na spikes often crowns each LTS (Sherman 2001), but similar to cells in the hypothalamus (Niespodziany et al. 1999), where the intrinsic membrane properties of different cell types seem to determine the number of Na spikes evoked by a single LTS. Consistent with this, the Na spike in granule cells was accompanied by a prominent spike afterhyperpolarization, which effectively truncated the LTS (Fig. 2B). In this way, the contribution of the LTS to membrane depolarization and thus the window for
temporal integration associated with the LTS was dependent in part on whether a Na spike occurred.

Other cell types endowed with an LTS may display prominent membrane potential-dependent oscillations, like thalamic neurons (Jahnsen and Llinàs 1984), inferior olivary neurons (Llinàs and Yarom 1981), and juxtaglomerular cells in the olfactory bulb (McQuiston and Katz 2001). However, this was not observed in the present experiments. An anomalous rectifier (Ih current) is often an integral part of such membrane potential oscillations (McCormick and Pape 1990). Olfactory granule cells showed anomalous rectification (Fig. 6); however, the activation kinetics of this current was relatively fast (approximately 50 ms), and the voltage range for activation did not overlap with the LTS. This may in part explain the lack of oscillatory behavior in granule cells.

This study has emphasized the role of the LTS in Na spike firing using presumably somatic recordings in current-clamp mode, to provide a first, basic characterization of the LTS' role in granule cell excitability. The function and localization of the different T-type calcium channel subtypes expressed in olfactory bulb granule cells may be analyzed further using single channel and whole cell voltage-clamp recordings. Furthermore, the electrophysiological properties of these cells may be rather complex: previous studies have suggested the presence of fast spikes in the very fine and long apical dendrites (Luo and Katz 2001; Mori and Takagi 1978), and the distribution of some ion channels over the somatodendritic membrane is not uniform (Schoppa and Westbrook 1999).

In this study, the LTS was evoked by current pulses to facilitate the electrophysiological characterization. However, results in other cell types show that EPSPs and inhibitory postsynaptic potentials (IPSPs) control the occurrence of the LTS (de la Pena and Geijo-Barrientos 2000; Kim and McCormick 1998; Magee and Johnston 1995; McQuiston and Katz 2001). The excitatory synaptic response evoked in granule cells in vitro (Schoppa and Westbrook 1999; Wells and Kauer 1994) and in vivo (Cang and Isaacson 2003; Luo and Katz 2001; Margrie and Schaefer 2003) readily evokes sodium spikes even from relatively negative membrane potentials and therefore spans the range for activation of the LTS subthreshold for the Na spike. Our results (Fig. 10) indicate that excitatory synaptic responses can activate T-type calcium currents in granule cells.

Functional significance

Granule cells constitute a huge population of neurons in the olfactory bulb modulating information transfer along mitral cell secondary dendrites as well as the axonal output of mitral/tufted cells (Cang and Isaacson 2003; Chen et al. 2000; Isaacson and Strowbridge 1998; Schoppa et al. 1998; Shepherd and Greer 1998). The present results report a cellular mechanism by which granule cells are able to boost sodium spike firing in response to a subthreshold depolarization. Even single spikes in neurons can contribute to information processing (Borst and Theunissen 1999) and subthreshold mechanisms that facilitate activation and synchronization of single spikes can be of importance, as reported for the insect olfactory system (Perez-Orive et al. 2002). In this respect, the LTS in olfactory bulb granule cells could play a role as a stimulus detector boosting neuron responses.

The responses of olfactory neurons are affected by behaviors such as the background respiratory rhythm and sniffing, and electrophysiological phenomena in the olfactory bulb cover a range of timescales, including field-potential oscillations around 15–20 Hz (Dorries and Kauer 2000; Lam et al. 2000; Schoppa and Westbrook 2002), intrinsic membrane oscillations in mitral cells around 10–40 Hz (Chen and Shepherd 1997; Desmaissons et al. 1999), and LTS-induced oscillations around 2 Hz in juxtaglomerular cells (McQuiston and Katz 2001). During odorant stimulation, glomeruli as well as individual mitral and granule cells display a variety of responses, often evolving in time and space with repeated stimulations on a relatively slow timescale (Cang and Isaacson 2003; Friedrich and Stopfer 2001; Luo and Katz 2001; Spors and Grinvald 2002) seemingly comparable to the kinetics of the LTS, in particular the relatively long refractory period. For instance, some granule cells are excited vigorously by the first odor pulse, while subsequent stimulations result in a diminishing response; conversely, some mitral cells show a similar evolution of an early inhibitory response over some seconds (Wells and Scott 1990).

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DISCLOSURES

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REFERENCES


