Ionic Basis of Tonic Firing in Spinal Substantia Gelatinosa Neurons of Rat

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INTRODUCTION

Dorsal horn of the spinal cord is the first relay station in processing the sensory input from primaryafferent terminals. Diversity of sensory modalities in dorsal horn is encoded by the type of peripheral afferent fiber, synaptic connectivity, and intrinsic firing properties of dorsal horn neurons (Brown 1981; Cervero 1987; Willis and Coggeshall 1991). Substantia gelatinosa (SG) is the dorsal horn region where most fine-calibre C-fibers terminate (LaMotte 1977; Light and Perl 1977; Rethelyi 1977; Sugita et al. 1986) and is therefore considered as an important element of nociceptive processing system. Several types of SG neurons are distinguished on the basis of their intrinsic firing properties (Grudt and Perl 2002; Lopez-Garcia and King 1994; Thomson et al. 1989; Yoshimura and Jessell 1989). Among them, tonically firing neurons (TFNs) were described as exhibiting little spiking frequency adaptation (Grudt and Perl 2002), suggesting that the intrinsic firing behavior might be mostly determined by the properties of ionic conductances present in the membrane. However, the knowledge about major types of ion channels specifically expressed in TFNs as well as their role in sustained firing is insufficient. Therefore we carried out this study and created a computer model to elucidate the ionic basis of tonic firing in SG neurons.

METHODS

Tight-seal recordings were done using 300-μm parasagittal slices prepared from the lumbar enlargement of the spinal cord of 2- to 5-wk-old rats (Bentley and Gent 1994; Cherry et al. 2000; Edwards et al. 1989). The animals were killed in accordance with the local guidelines. After the anesthesia by intraperitoneal injection of pentobarbital sodium (30 mg/kg) the vertebral column was quickly cut out and immersed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). The 5- to 7-mm-long segment of the lumbar enlargement was dissected and glued to the stage of the tissue slicer (Leica VT 1000S). Four parasagittal slices (2 from each half of the spinal cord) were prepared and incubated for 30–60 min in ACSF at 33°C. SG (laminas II) was identified as a translucent band in the dorsal horn.

ACSF contained (in mM) 115 NaCl, 5.6 KCl, 2 CaCl2, 1 MgCl2, 11 glucose, 1 NaH2PO4, and 25 NaHCO3 (pH 7.4 when bubbled with 95% O2-5% CO2). In some cases, 2 mM kynurenic acid was added to ACSF during preparation. Low-Ca2+-free ACSF (ACSF*) was obtained from ACSF by setting [Ca2+]o to 0.1 mM and [Mg2+]o to 0.5 mM. In some experiments K+ currents were recorded in Na+-free choline–Cl solution containing (in mM) 135 choline-Cl, 1.1 KCl, 0.1 CaCl2, 5 MgCl2, 11 glucose, and 10 HEPES. The pH value was adjusted to 7.4 by KOH (final [K+]o was 5.6 mM). Apamin and charybdootoxin were dissolved in ACSF with 0.05% BSA.

Standard pipette solution contained (in mM) 6 NaCl, 128 KCl, 2 MgCl2, 10 EGTA, and 10 HEPES. The solution with low Ca2+ buffering capacity contained (in mM) 6 NaCl, 145 KCl, 2 MgCl2, 1 EGTA, and 10 HEPES. The pH value in both solutions was adjusted to 7.3 with KOH (final [K+]o was 160.5 mM). Pipette solution for studying Na+ channels contained (in mM) 4 NaCl, 131 CsCl, 2 MgCl2, 10 EGTA, and 10 HEPES. The pH value was adjusted to 7.3 by CsOH (final [Cs+]o was 153 mM) and NaOH (final [Na+]o was 5.6). All chemicals were purchased from Sigma.

The patch pipettes were pulled from thick-walled borosilicate glass tubes (Modulohm, Denmark; 1.50 mm OD/0.86 mm ID) and had a resistance of 3–5 MΩ after fire-polishing. The EPC-9 amplifier (HEKA, Lambrecht, Germany) was used in all experiments. The effective corner frequency of the low-pass filter was 3 kHz. The frequency of digitization was 10 kHz. Transients and leakage currents were digitally subtracted using standard P/4 protocol. Offset potentials were nulled directly before formation of a seal. Liquid junction potentials were calculated and corrected for in all experiments. In

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neurons subjected to detailed analysis the series resistance measured in the whole cell mode was 6–20 MΩ and was compensated by ≥60%. Action potentials were recorded using the fast current-clamp mode of the EPC-9 amplifier. Input resistance (Rm) was measured in voltage-clamp mode using negative 10- to 40-mV pulses from a holding level of −80 mV. Only cells with a resting potential (Vr) negative to −60 mV were included into this study.

Ion channels were studied in two types of isolated structures: nucleated patches excised from somatic membrane (Sather et al. 1992) or entire somata (Safronov et al. 1997). Nucleated patches were usually obtained from deep neurons or larger superficial neurons and had diameters ranging from 5 to 8 μm. From the majority of superficial neurons, however, the entire soma with a diameter of 8–10 μm was isolated. We did not distinguish between those two structures and in the following text will refer them to as nucleated patches. For estimation of the current densities in isolated patches their mean diameter was assumed to be 7.5 μm.

The current–voltage (I–V) relationship for Na+ channels was fitted with equation: G/N[1 + exp(V50 – V/R)(V – Vrev)], where G0 is the maximum conductance, V50 is the potential of half-maximum channel activation, k is a steepness factor, and Vrev is the reversal potential. Vrev obtained by fitting for each I–V curve was 10–20 mV more negative than a theoretical V50 (+79 mV) due to appearance of outward Cs+ current through delayed-rectifier K+ channels incompletely blocked by 1 mM TEA. K+ conductances were calculated assuming Vrev equal to Vr of −84 mV, in agreement with the data shown in Fig. 4D. The activation and steady-state inactivation characteristics were fitted with Boltzmann function: 1/(1 + exp(V50 – V/R)). The time course of Na+ channel recovery from inactivation was fitted with a two-exponential function: 1 – A × exp(−t/τA) – (1 – A) × exp(−t/τA), where τA and τA are the fast and slow time constants and A is the relative amplitude of the fast component.

All numbers are given as mean ± SE. The values obtained by data fitting with a linear or nonlinear least-squares procedure are given as mean ± SE. In all figures the error bars are given when exceeding the symbol size. The present study is based on recordings from 266 SG neurons, 187 of which were classified as TFNs, and from 65 nucleated patches. All experiments were carried out between 22–24°C.

In some experiments, 0.5% biocytin (Sigma) was included in the pipette solution for later cell reconstruction. Following the recording session, the slices with biocytin-filled TFNs were transferred into a fixative containing 4% paraformaldehyde, 1.25% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) for 7–10 days. After resectioning at 60 μm, slices were treated according to the avidin-biotinylated horseradish peroxidase method (Extravidin, diluted 1:1000, Vector Labs, Burlingame, CA) and the reaction was completed with a diaminobenzidine (Sigma, St. Louis, MO) chromogen reaction. Sections were counterstained with toluidine blue, dehydrated, and mounted with DPX (Fluka, Buchs, Switzerland). The somata as well as the dendritic and axonal arbors of labeled neurons were reconstructed from serial sections using a camera lucida with a ×100 oil immersion objective.

Computer simulations were done using NEURON software (Hines 1993; Hines and Carnevale 1997) with an integration step of 50 μs. Model consisted of the axon initial segment (AIS) and soma connected to an equivalent dendrite. This allowed us to construct a model of TFN independent of its belonging to one of three morphological groups described below. Parameters of the equivalent dendrite were calculated using standard procedure (Rall 1959, 1969; Dodge and Cooley 1973) on the basis of our recordings from six TFNs with Rm = 1.7 ± 0.3 GΩ. Electrotonic length of the cylinder was calculated from the equation L = πr2/(τm/τl)1/2, where τm = 91 ± 10 ms (n = 6) and τl = 4.1 ± 0.3 ms (n = 6) were the two slowest membrane time constants obtained by exponential “peeling” from the averaged (500 episodes) low-amplitude (<2 mV) passive decay transients evoked in current-clamp mode by short (1 ms) hyperpolarizing current pulses (not shown). Calculated L value was 0.68, indicating a compact electrotonic structure of TFNs. Assuming a uniform membrane capacitance (Cm) of 1 μF/cm², the specific membrane resistivity, Rm = τm/Cm, was estimated to be 91 kΩ/cm². Using standard equations describing a passive cable, L = Dτm/Rm, the resistance of the equivalent dendrite, R, is an axial resistance, l is a length of the equivalent cylinder, and, assuming Rm of 80 Ohm (Barrett and Crill 1974; Thurbon et al., 1998) and Rm of 1.7 GΩ, one could estimate the dimensions of the equivalent dendrite, l = 1371 μm and δ = 1.4 μm. The soma was considered a 10-μm-long cylinder with a 10-μm diameter. AIS was 30 μm long and linearly tapered in diameter from 1.0 μm at base to 0.5 μm at its distal end (Gobel et al. 1980). The Cm, Rm, and Rl values in the soma and AIS were the same as in the dendrite. The soma, AIS, and equivalent dendrite consisted of 10, 30, and 50 compartments, respectively. Vr in the model was −70 mV.

The models of Na+ and K+ currents were developed on the basis of our recordings. Na+ current was described by a Hodgkin–Huxley style equation gNa h(V – Vrev), where gNa was the Na+ conductance, m and h were the variables of activation and inactivation, respectively, and Vrev was −60 mV. The steady-state activation variable (mst) and the time constant of activation (τm) were determined as mst = α(V)/mst and τm = 1/(αmst + αmst), where αmst = 0.182(3)/m + 33/[1 + exp(V − 33)/9] and αmst = −0.0124(3)/m + 33/[1 + exp(V − 33)/9] were reaction rates. The time constant of inactivation (τh) was determined as τh = 1/(αh + βh), where αh = −0.0018(3)/m + 82/[1 + exp(V − 82)/181] and βh = 0.061(V + 46)(1 − exp(−(V − 46)/3)) + 0.0166. The steady-state inactivation variable was given as h = 1/(1 + exp(V + 75)/9). Kdr current was described as gKdr h(V − Vr), where gKdr was the Kdr conductance, n was the variable of activation, and Vr was −84 mV. The parameters were n = αn/βn, τn = 1/(αn + βn), αn = 0.035(V + 15)/[1 − exp(−(V + 15)/9)], βn = 0.014exp(−(V − 12)/46), h = αh/αh + βh, τn = 1/(αn + βn), αh = 0.0038/[1 + 1/exp(V + 20/10)] + 1] and βh = 0.0083/[exp(V + 20)/10] + 1].

Kdr current density in the soma was adjusted in agreement with our present measurements. Those in the dendrite and AIS were determined by simulating the experiments from Wolff et al. (1998), which showed that the ratio of somatic/dendritic/AIS components of Kdr current recorded in voltage-clamp mode with electrode placed on the soma was 15/47/38. These conditions were satisfied if gKdr was 34, 4.3 and 76 mmho/cm² for the dendrite, soma, and AIS, respectively. Inactivating Na+ channels were only inserted in the soma and AIS (Safronov 1999). On the basis of our present results gKdr was set to 8 mmho/cm² for the soma. In AIS gKdr was set to 1800 mmho/cm² to reach the maximum velocity of spike depolarization of 229 V/s (mean value for 70 TFNs). At a Vr of −70 mV, the density of available Na+ channels with a conductance of 11.6 pS (Safronov et al. 1997) was 0.55 μm−2 in the soma and 124 μm−2 in AIS. Since the Kdr current in TFNs was small and almost completely inactivated at Vr, it was not included into the model.

RESULTS

TFNs were identified in SG as neurons able to support sustained firing during 500-ms depolarization evoked by an injection of inward current. Of a total of 266 SG neurons tested, 187 (70%) were TFNs with Vr of −67 ± 1 mV (n = 117) and Rm of 1.14 ± 0.08 GΩ (n = 117). In all TFNs the firing frequency progressively increased with stimulation intensity (Fig. 1A). Frequency-current (f–I) curves for the first interspike interval (instantaneous) and the last few intervals (steady-state) as well as the instantaneous firing frequency as a function of time (f–I plot) are shown in Fig. 1B. The steady-state f–I characteristic was nonlinear with an initial slope of 0.61 ± 0.08 Hz/pA (n = 8).
Ca\(^{2+}\)-dependent conductances

Blockers of Ca\(^{2+}\) and K\(_{CA}\) channels were tested to study the role of Ca\(^{2+}\)-dependent conductances. In these experiments intracellular solution containing 1 mM EGTA was used. After equimolar substitution of 2 mM Ca\(^{2+}\) in ACSF by inorganic Ca\(^{2+}\) channel blockers Co\(^{2+}\) (n = 8) or Mn\(^{2+}\) (n = 5), several modifications of firing pattern were observed. At low stimulation intensities, the firing frequencies increased (Fig. 1, A and B, shown for Co\(^{2+}\)), so that the initial slope of the steady-state f-I curve reached 1.14 ± 0.12 Hz/pA (n = 8). At strong stimulation, however, the steady-state frequency was similar to control value, but pronounced spike attenuation within the train appeared in Co\(^{2+}\) or Mn\(^{2+}\). Thus the block of Ca\(^{2+}\) influx into the neuron led to a left-shift in the f-I characteristics and a reduction of firing stability. The effects of Co\(^{2+}\) or Mn\(^{2+}\) on TFN firing resulted from a reduction of a slow afterhyperpolarization (Fig. 1C). Similar effects were also seen in ACSF after addition of 500 nM apamin (Fig. 1D; n = 5) but not 100 nM charybdotoxin (Fig. 1E; n = 4), indicating the involvement of small conductance apamin-sensitive, rather than big conductance K\(_{CA}\) channels. It could be therefore concluded that 1) Ca\(^{2+}\)-dependent conductances are involved in regulation of firing frequency in TFNs, but 2) the basic pattern of tonic firing is generated by voltage-gated Na\(^{+}\) and K\(^{+}\) channels.

The following study of Na\(^{+}\) and K\(^{+}\) channels was carried out in ACSF* (0.1 mM Ca\(^{2+}\)–5 mM Mg\(^{2+}\)) with 10 mM EGTA in pipette solution to minimize the contribution of Ca\(^{2+}\)-dependent conductances. Under these conditions, TFNs showed sustained firing, but addition of 2 mM Co\(^{2+}\) did not shorten intervals between spikes (Fig. 1F, n = 5). Further substitution of internal EGTA with fast Ca\(^{2+}\) chelator BAPTA (10 mM) did not change the pattern recorded in ACSF* (n = 5; not shown). Thus a combination of external ACSF* and internal 10 mM EGTA, used in all of the following experiments, was adequate for minimizing Ca\(^{2+}\)-dependent conductances.

An appearance of tonic firing did not depend on V\(_{th}\) in the range between −80 and −60 mV (Fig. 1G). Hyper- or depolarization of TFN by injection of sustained current did not prevent generation of tonic firing evoked by depolarizing cur-

FIG. 1. Effects of Ca\(^{2+}\) and V\(_{th}\) on firing pattern. A: tonic firing evoked in control [artificial cerebrospinal fluid (ACSF), 2 mM Ca\(^{2+}\)] and after an equimolar substitution of Co\(^{2+}\) for Ca\(^{2+}\). Dashed lines indicate 0 mV. Depolarizing current pulses of 10, 30, and 130 pA were applied for 0.5 s. B: plots of the instantaneous and the steady-state firing frequency characteristics as functions of injected current (f-I) and the instantaneous firing frequency at 30 pA stimulation as function of time (f-t). Effects of 2 mM Co\(^{2+}\) (C), 500 nM apamin (D), and 100 nM charybdotoxin (E) on interspike potentials during sustained firing. Each superimposed pair of traces was recorded at the same stimulation intensity. F: tonic firing in ACSF* (0.1 Ca\(^{2+}\)–5 Mg\(^{2+}\)) before and after addition of 2 mM Co\(^{2+}\). Current protocol is the same for both traces. G: trains of action potentials evoked in a tonically firing neuron (TFN) at varying V\(_{th}\), which was set to −70, −80, and −60 mV by injecting a steady-state in- or outward current (<15 pA) through the recording pipette.
rent pulses \((n = 25)\). Therefore in current-clamp experiments all neurons were uniformly kept at \(-70 \text{ mV}\) by injecting the holding current, which did not exceed 45 pA.

**Na**\(^+\) channels

Na\(^+\) channels were studied in nucleated patches using pipette solution in which K\(^+\) was substituted with Cs\(^+\). Since neurons could not keep \(V_R\) and tonic firing without internal K\(^+\), their characterization was done during the first 10–15 s after membrane was broken, before pipette Cs\(^+\) substituted intracellular K\(^+\) (Fig. 2A). For Na\(^+\) current recording in patches, 1 mM TEA was added to ACSF\(^*\) to reduce outward K\(^+\) current. Na\(^+\) channels began to activate at \(-50 \text{ mV}\) and had fast opening kinetics (Fig. 2, B and D). Their activation characteristic fitted with Boltzmann equation had \(V_{50} = -35.7 \pm 0.6 \text{ mV}\) and \(k = 7.5 \pm 0.5 \text{ mV}\) (Fig. 2C, \(n = 6\)). The steady-state inactivation of Na\(^+\) channels, studied with 50-ms conditioning prepulses, revealed a half-maximum inactivation at \(-75.5 \pm 0.1 \text{ mV}\) and \(k = -9.1 \pm 0.1 \text{ mV}\) (Fig. 2C, \(n = 5\)).

The kinetics of Na\(^+\) channel inactivation was studied by fitting the current decay. Whenever possible, a monoeponential function was used. In cases where two-exponential fitting was needed, the time constant of the dominating fast component was considered as the inactivation time constant \(\tau_in\) of Na\(^+\) current. The \(\tau_{in}\) changed from 6.1 ± 2.1 ms at \(-50 \text{ mV}\) to 0.39 ± 0.04 ms at +10 mV (Fig. 2D, \(n = 6\)). The time course of Na\(^+\) channel recovery from inactivation at potentials close to \(V_R\) was studied using a standard two-pulse protocol (Fig. 3A). The membrane was held at \(-80 \text{ mV}\) and two 25-ms voltage pulses to \(-30 \text{ mV}\) with varying intervals were applied. Recovery of channels from inactivation followed a double-exponential time course. The time constants, fast and slow, were \(\tau_F = 21.8 \pm 1.7 \text{ ms (63\%)}\) and \(\tau_S = 793 \pm 92 \text{ ms (37\%)}\), respectively (Fig. 3B, \(n = 7\)).

Recovery from inactivation seemed to be too slow to explain firing frequencies about 100 Hz (10-ms spike intervals) observed in experiments from Fig. 1A. To understand the reason for this apparent discrepancy we analyzed a degree of Na\(^+\) channels.

**FIG. 2.** Activation and inactivation of Na\(^+\) channels. A: identification of a TFN in current-clamp mode a few seconds after the membrane rupture with a pipette containing Cs\(^+\) solution. B: Na\(^+\) currents activated in a nucleated patch by depolarization to \(-50, -40, -30, \) and \(-20 \text{ mV}\) following a 50-ms prepulse to \(-120 \text{ mV}\), holding potential \(-80 \text{ mV}\). Current-voltage (I-V) curve is shown for the same neuron. C: steady-state activation and inactivation characteristics of Na\(^+\) channels. Fitting parameters are given in the text. D: the rise time of a half-maximum current \(\tau_{in\text{(A)}}\) and inactivation time constants \(\tau_{in\text{(B)}}\) as a function of membrane potential \((n = 6)\).

**FIG. 3.** Kinetics of Na\(^+\) channel recovery from inactivation. A: Na\(^+\) currents activated by pairs of 25-ms pulses from \(-80\) to \(-30 \text{ mV}\) with varying intervals (4, 8, 20, and 60 ms). B: time course of Na\(^+\) channel recovery from inactivation at \(-80 \text{ mV}\) \((n = 7)\). The data are fitted using a double-exponential function. C: estimation of Na\(^+\) channel inactivation during sustained firing. Train of action potentials recorded in the presence of 2 mM Co\(^{2+}\) (taken from Fig. 1A) was differentiated to give a rate of membrane polarization as derivative dV/dt. **Derivatives of the first and last spikes are given below at expanded time scale.**
channel inactivation during a high-frequency firing in the presence of 2 mM Co<sup>2+</sup>. The top trace from Fig. 1A (right) was differentiated (Fig. 3C). The inward current charging the membrane is proportional to the depolarization rate according to an equation \( I = C(dV/dt) \), where \( C \) is a membrane capacitance. Each positive peak of the differentiated trace reflected maximum Na<sup>+</sup> current depolarizing the neuron during the corresponding spike. Within the train the depolarization rate was reduced from 290 V/s at the first spike to 58 V/s at the last one. In five neurons recorded either in the presence of 2 mM Co<sup>2+</sup> or in ACSF<sup>*</sup>, the amplitude of Na<sup>+</sup> current contributing to the last spike in a train was 0.22 ± 0.04 of the first spike. This is in good agreement with a value of 0.24 predicted from a half-maximum current (\( I_{V_{1/2}} \)). At potentials positive to 80 mV, the \( I_{V_{1/2}} \) was 79.8 ± 12.5 ms at −10 mV to 59.0 ± 3.2 ms at +60 mV (Fig. 4C, \( n = 9 \)). \( V_{REV} \) for K<sub>DR</sub> conductance was estimated from tail currents evoked by voltage return from +40 mV to different levels (Fig. 4D). The tail current changed almost linearly with a voltage and reversed its polarity near to \( V_C \) of −84 mV (\( n = 5 \)).

Tail currents were also used to measure the closing rate of K<sub>DR</sub> channel at −60 mV. They were recorded after short and long depolarizing pulses to +60 mV (Fig. 4E). The short pulse of 3–5 ms was adjusted to terminate at a peak of the current giving the tails corresponding to the total (noninactivated) K<sub>DR</sub> current. This tail current decayed monoexponentially with a time constant of 5.9 ± 1.3 ms (\( n = 6 \)). Similar measurement was also done after a 200-ms pulse, for a partially inactivated K<sub>DR</sub> current. The tail currents became smaller but decayed monoexponentially with a similar time constant of 5.3 ± 1.1 ms. The observed fast closing rate of K<sub>DR</sub> channel correlated well with high frequencies of tonic firing seen in current-clamp experiments.

In 1 mM TEA, K<sub>DR</sub> current was blocked to 20.3 ± 1.5% (Fig. 4F, \( n = 4 \)). The kinetics of control and remaining currents were very similar, which was better seen when the traces were normalized and superimposed (Fig. 4F, bottom). In 10 mM TEA, the current was reduced to 8.4 ± 1.3% (\( n = 5 \)) but the current kinetics remained unchanged (not shown).

**Delayed-rectifier K<sup>+</sup> (K<sub>DR</sub>) channels**

The major voltage-gated K<sup>+</sup> current found in TFNs was a slowly inactivating K<sub>DR</sub> current (Fig. 4A). Patch was held at −80 mV and depolarizing voltage pulses were applied after a 150-ms prepulse to −60 mV inactivating transient A-type K<sup>+</sup> (K<sub>A</sub>) currents. The threshold of K<sub>DR</sub> current was −40 mV and conductance reached saturation at +20 mV (Fig. 4B). The fitting of the activation characteristic with Boltzmann equation gave \( V_{50} = -19.8 \pm 0.4 \) mV and \( k = 9.9 \pm 0.4 \) mV (\( n = 13 \)). To describe the activation kinetics we measured the rise time of a half-maximum current (\( \tau_{0.5} \)). At potentials positive to +30 mV, the \( \tau_{0.5} \) became shorter than 1 ms (Fig. 4B, \( n = 9 \)). Thus gating kinetics of K<sub>DR</sub> current was sufficiently fast for its involvement in spike repolarization.

Starting from −20 mV, a partial inactivation of K<sub>DR</sub> current with a monoexponential time course developed. The \( \tau_{in} \) was weakly voltage dependent, changing from 79.8 ± 12.5 ms at −10 mV to 59.0 ± 3.2 ms at +60 mV (Fig. 4C, \( n = 9 \)). \( V_{REV} \) for K<sub>DR</sub> conductance was estimated from tail currents evoked by voltage return from +40 mV to different levels (Fig. 4D). The tail current changed almost linearly with a voltage and reversed its polarity near to \( V_C \) of −84 mV (\( n = 5 \)).

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**FIG. 4.** K<sub>DR</sub> current in nucleated patches. A: K<sub>DR</sub> currents activated by 200-ms depolarizing pulses (from −40 mV to +20 mV in 10-mV steps) following a 150-ms prepulse to −60 mV. Holding potential, −80 mV. Decay of the current at +20 mV is fitted with a monoexponential function. B: steady-state activation curve and the rise time of a half-maximum current (\( \tau_{0.5} \)). C: \( \tau_{0.5} \) at different potentials. D: measurements of \( V_{REV} \) for K<sub>DR</sub> current. Tail currents at potentials between −40 and −90 mV in 10-mV steps. \( I-V \) relationship for these tail currents is shown below. E: closing rate of K<sub>DR</sub> current. Tail currents recorded at −60 mV after a short (3 ms) and long (200 ms) depolarization pulses to +60 mV (prepulse: 150 ms, −60 mV; holding potential, −80 mV). The tail currents are shown below enlarged. Superimposed dashed lines are monoexponential fittings. F: K<sub>DR</sub> current in control solution and in the presence of 1 mM tetraethylammonium (TEA). Both traces were normalized and are shown superimposed below. The control trace is given by white color. Pulse protocol: holding potential, −80 mV; prepulse, −60 mV; depolarizing pulse, +60 mV, 200 ms.
K_A current

In somatic patches from TFNs, a transient K_A current was much smaller than K_DR current. K_A current could not be separated using a standard procedure with two prepulses (to −120 and −60 mV), since the difference trace was always dominated by the slowly inactivating K_DR current, the amplitude of which also depended on the prepulse. Therefore we recorded K_A currents elicited by a voltage step from −120 to −60 mV before and after addition of 10 mM TEA (Fig. 5A). In control solution no fast inactivating component could be revealed and a monoexponential fitting with a slow inactivation characteristic adequately described inactivation. In 10 mM TEA, K_DR current decreased (Fig. 5A, left) and the fast inactivating component could be resolved in the remaining current (right, the same trace is shown amplified). A double-exponential fitting revealed the fast component of inactivation, τ_A = 6.6 ± 0.8 ms at +60 mV (n = 8).

Because of its small amplitude in nucleated patches and lack of possibility of clear separation, some properties of K_A current were studied in a whole cell mode. K_A current was activated by pulses from −120 mV to −60 or −55 mV, voltages subthreshold for activation of Na^+ current. Since, in the majority of TFNs, K_A current was too small to be studied, we chose seven cells with the largest current ranging between 40 and 250 pA for analysis. The steady-state inactivation of K_A current with V_{50} = −96.3 ± 1.2 mV and k = −7.2 ± 0.8 mV (Fig. 5B, n = 5) showed that it was almost completely inactivated at V_R. The whole cell recordings were also used to study the sensitivity of K_A current to 10 mM TEA. For control of TEA diffusion into the slice we also monitored a reduction of K_DR current activated by a voltage step from −60 to −20 mV (Fig. 5C). K_A current was resistant to 10 mM TEA, the remaining part being 0.99 ± 0.17 (n = 5) of the control current.

Densities of Na^+ and K^+ currents

For estimation of the current densities the mean diameter of nucleated patch was assumed to be 7.5 μm giving a membrane area of 177 μm². The maximum amplitude of Na^+ current activated after a 50-ms prepulse to −120 mV was 208 ± 31 pA (n = 15), corresponding to a density of 1.18 pA/μm². The

FIG. 5. K_A currents in patches and TFNs. A: total K^+ current activated in a nucleated patch by a voltage step from −120 (150 ms) to +60 mV (200 ms) in the presence and absence of 10 mM TEA. Holding potential, −80 mV. The trace in TEA is shown at higher magnification (right). The amplitude of K_A current was obtained from a double-exponential fitting of the decay kinetics as the amplitude of the fast component (τ_A). B: steady-state inactivation. Whole cell K_A currents recorded at −60 mV after a 150 ms prepulse to varying potentials (−130, −110, −90 and −80 mV). Holding potential, −80 mV. Steady-state inactivation characteristic was fitted with Boltzmann equation. C: effects of 10 mM TEA on K_A and K_DR currents. Holding potential, −80 mV. K_A currents were activated by step from −120 to −60 mV, whereas K_DR currents by a voltage step from −60 to −20 mV. D: firing frequency at 10 pA stimulation is reduced in 1 mM TEA (left). Right, slowing down the spike repolarization in 1 mM TEA (stimulation, 190 pA).
amplitude of K\textsubscript{DR} current measured at a voltage step from −60 to +60 mV was 1049 ± 193 pA (n = 8), giving a mean current density of 5.9 pA μm\textsuperscript{-2}. It should be noted the K\textsubscript{DR} channels were inactivated to some degree at −60 mV. The total K\textsuperscript{+} current seen after a −120 mV prepulse was larger by a factor of 1.23 ± 0.05 (n = 11), but it represented a mixture of the K\textsubscript{DR} and K\textsubscript{A} currents that could not be clearly separated.

Based on our whole cell recordings, it could be assumed that K\textsubscript{A} currents in patches were not substantially reduced in 10 mM TEA. At the voltage step from −120 to +60 mV, the amplitude of K\textsubscript{A} current estimated by the fitting was 123.1 ± 34.6 pA (n = 8), giving a density of 0.7 pA μm\textsuperscript{-2}. It should be noted that the K\textsubscript{A} current formed only 8.6 ± 1.6% (n = 8) of a total (K\textsubscript{DR} + K\textsubscript{A}) current when recorded after a −120-mV prepulse and became negligible when depolarization was applied after prepulses close to the V\textsubscript{R} level.

**Role of K\textsubscript{DR} current in tonic firing**

An involvement of K\textsubscript{DR} channels in tonic firing was tested in current-clamp mode by comparing the patterns recorded in the presence and absence of 1 mM TEA, which partially blocked K\textsubscript{A}, but not K\textsubscript{DR}, current. The firing frequency and stability, at a given stimulation strength, were reduced in 1 mM TEA (Fig. 5D, n = 6). These effects resulted from slowing down the spike repolarization and disappearance of fast afterhyperpolarization (Fig. 5D, right). Thus a reduction of K\textsubscript{DR} rather than K\textsubscript{A} current was critical for generation of repetitive firing.

**Morphology of TFNs**

Twelve biocytin-filled TFNs were reconstructed and six of them are shown in Fig. 6. On the basis of their dendritic arborization and appearance, TFNs could be divided into three major groups. The cells belonging to the first group (Fig. 6A, n = 5) had triangular/pyramidal soma with three to four main dendrites passing the border between lamina II (SG) and III. The axon, when reconstructed, stayed at the level of the cell body and was extensive in a rostrocaudal orientation. Neurons with a similar appearance were classified as stalked cells (Eckert et al. 2003; Gobel 1978; Todd 1988). The second group of neurons (Fig. 6B, n = 3) was characterized by smaller fusiform somata, dendrites bearing large beads, and axon branching around the cell body. The cells from the final group (Fig. 6C, n = 4) had rounded somata with multiple extensively branching dendrites either in a multipolar or a bipolar organization. The axons were recovered mainly in lamina II (SG). Neurons with similar features were referred to as islet cells (Eckert et al. 2003; Gobel 1978; Todd 1988). The neurons from all three groups had similar electrophysiological parameters: V\textsubscript{R} = −70 ± 2, −73 ± 3, and −71 ± 4 mV; R\textsubscript{m} = 1.25 ± 0.30, 1.16 ± 0.26, and 1.17 ± 0.15 GΩ; g\textsubscript{N} = 94.4 ± 9.3, 98.3 ± 23.4, and 102.7 ± 7.4 ms for the first (n = 5), second (n = 3), and third (n = 4) group TFNs, respectively.

**Computer simulation**

To test our assumption about the role of Na\textsuperscript{+} and K\textsubscript{DR} currents in the generation of tonic firing, we built a computer model of TFN. Inclusion of these conductances was sufficient to provide firing in a broad range of frequencies (Fig. 7A). The steady-state firing f–I characteristic of the model with the initial slope of 1.5 Hz/pA (Fig. 7B) was very similar to that recorded in TFNs. At high-frequency firing the degree of interspike recovery of Na\textsuperscript{+} channels from inactivation was about one-fifth of the resting level seen before the first spike (Fig. 7C), indicating that a high safety factor for the spike generation is important for the appearance of high-frequency firing.

To study the interplay between Na\textsuperscript{+} and K\textsubscript{DR} channels, we plotted the variables h (Na\textsuperscript{+}) and n\textsuperscript{4} (K\textsubscript{DR}) during the steady-state firing (Fig. 7D). At low frequencies (left), both parameters returned to their interspike level 10–20 ms after each spike and the delay to the next one was mostly determined by the passive membrane properties. At maximum frequencies, however, the next spike was generated just after the major part of K\textsubscript{DR} conductance, preventing membrane depolarization, was deactivated (right). The degree of Na\textsuperscript{+} channel recovery from inactivation, in turn, determined the spike overshoots, which were reduced to 0 mV at maximum frequencies. The kinetics of the Na\textsuperscript{+} channel activation was much faster (m\textsuperscript{3}, Fig. 7D), indicating that it did not limit the firing rate. Removal of Na\textsuperscript{+} channels from the soma reduced the single spike overshoot by <0.5 mV and did not affect the tonic firing.

**DISCUSSION**

Tight-seal recordings from TFNs in SG were performed to study ionic mechanisms of tonic firing. TFNs represented the most frequent SG cell type able to fire spikes during sustained depolarization without remarkable frequency adaptation and amplitude attenuation. Our data support most reports describing this abundant type of neuron in superficial dorsal horn (Grudt and Perl 2002; Lopez-Garcia and King 1994; Prescott and De Koninck 2002; Thomson et al. 1989), but conflict with Ruscheweyh and Sandkühler (2002) who have not found TFNs in rat lamina II (SG). According to cutaneous afferent input, most TFNs were shown to be either wide-dynamic-range or nociceptive-specific neurons (Lopez-Garcia and King 1994). The intrinsic tonic firing seems to be an important property of this class of neurons, allowing them to fire bursts of spikes in response to synaptic stimulation (Thomson et al. 1989).
The present study determined the role of major conductances in excitability of TFNs. Both Ca$^{2+}$ and K$_{CA}$ currents are involved in modulation of firing frequency. The effect is based on activation of apamin-sensitive K$_{CA}$ channels, regulating the slow component of afterhyperpolarization as was shown for several types of neurons (Barrett and Barrett 1976; Nishimura et al. 1989; Savic et al. 2001; Schwindt et al. 1988; Smith et al. 2002; Viana et al. 1993). Activation of K$_{CA}$ channels by entering Ca$^{2+}$ reduced the firing at a given depolarization strength and therefore modified the input–output characteristic of the neuron. Thus Ca$^{2+}$-dependent conductances play an important modulatory and stabilizing role in firing of SG neurons, but they are unlikely to be responsible for the appearance of the basic form of tonic firing. In similar manner, the variation of V_{th} does not change the pattern of tonic firing but regulates the strength of stimulation needed to evoke it. Therefore the voltage-gated Na$^+$ and K$^+$ channels are mostly responsible for the appearance of tonic firing.

The activation kinetics of Na$^+$ channels was sufficiently fast to provide spike depolarization. A low channel density found in the soma supports previous observations that the major part of Na$^+$ conductance necessary for the spike generation in spinal neurons is located in AIS (Alessandri-Haber et al. 1999; Safronov et al. 1999a,b). In addition, our simulations showed that removal of somatic channels did not change the spike overshoot. Rapid inactivation of Na$^+$ channels at positive potentials ($\tau_{in} < 0.4$ ms at +10 mV), in combination with fast K$_{DR}$ channel opening ($\tau_{0.5} < 0.9$ ms at +40 mV), is critical for the spike repolarization. Double-exponential recovery of Na$^+$ channels from inactivation was similar to that observed in rat motoneurons (Safronov and Vogel 1995) and hippocampal neurons (Martina and Jonas 1997). About one-fifth of the channels reprimed within the first 10 ms, which corresponds to interspike intervals at 100 Hz firing. However, our recordings and model showed that this amount of Na$^+$ channels is sufficient to support firing. Thus a high safety factor for the spike generation (see also Eckert 1978) plays a critical role in maintaining the tonic firing under conditions in which most Na$^+$ channels are inactivated.

K$_{OR}$ channel underlies the major type of K$^+$ conductance in TFNs. Delayed activation and a high sensitivity to TEA allowed us to attribute it to a family of delayed-rectifier K$^+$

FIG. 7. Computer simulation of tonic firing. A: tonic firing evoked by 500-ms current pulses of +10, +30, and +120 pA. A negative (–10 pA) current pulse evoked a passive response. Dashed line indicates 0 mV. B: steady-state firing frequency (f-I) characteristic of the model neuron. C: changes in Na$^+$ channel inactivation variable h during tonic firing evoked by +120 pA current pulse. D: dynamics of n', h (Na$^+$), and m$^+$ during steady-state firing at low and high frequencies. All variables are shown for the middle point of AIS.

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channels. Its inactivation was by an order of magnitude slower than that of $K_A$ channels. Moreover, in contrast to the $K_A$ component, $K_{DR}$ current did not show inactivation kinetics at potentials below $-10$ mV. $K_{DR}$ current was proportionally reduced in 1 and 10 mM TEA, indicating homogeneity of the channel population with respect to its sensitivity to the blocker. The study of the tail currents at $-60$ mV did not provide any evidence for existence of a channel subpopulation, which was active at the beginning of the pulse but inactivated at the end of 200 ms depolarization. Thus, based on analysis of tail currents and sensitivity to TEA, which are used as standard tools for separating delayed-rectifier current components (Dubois 1981; Safronov et al. 1996; Stühmer et al. 1989), we assume that $K_{DR}$ current is carried through a one-channel type highly permeable for $K^-$. Somatic density of $K_{DR}$ current of 5.9 pA $\mu m^{-2}$ found here is much higher than one calculated from Wolff et al. (1998) for nonidentified dorsal horn neurons of 3- to 7-day-old rats (240 pA for the 10 $\mu m$ soma, corresponding to 0.76 pA $\mu m^{-2}$). This difference can be explained if it is assumed that the neuronal development during the first postnatal month is accompanied by somatic expression of $K_{DR}$ channels, in contrast to the $Na^+$ channels that are exclusively expressed in AIS (Safronov et al. 1999b).

The activation of $K_{DR}$ current was sufficiently rapid to provide membrane repolarization during an action potential. The channel closing with a time constant of $\leq 6$ ms, in turn, was important for sustained firing at high frequencies. An involvement of $K_{DR}$ channels in both processes was confirmed by a spike prolongation and reduction of firing frequency seen in 1 mM TEA (see also Olschewski et al. 2001).

$K_A$ current was very small in somatic patches and strong inactivation at $V_R$ would further reduce it, making its participation in tonic firing unlikely. It appears that lack of $K_A$ channels is critical for appearance of sustained firing, since a pronounced $K_A$ current in dorsal horn neurons was shown to result in delayed firing onset, irregular burst-like firing, or frequency adaptation (Grundt and Perl 2002; Ruscheweyh and Sandkuhler 2002; Yoshimura and Jessell 1989).

Our model has confirmed that a combination of $Na^+$ and $K_{DR}$ channels is sufficient for appearance of a basic pattern of tonic firing. The model could reproduce the firing in a broad range of frequencies. The maximum firing rates were determined by biophysical properties of the channels in such a way that at a given stimulus strength the closing of $K_{DR}$ channels determined the length of the interspike interval, whereas the recovery of $Na^+$ channels from inactivation determined the spike amplitude. Our model was not based on the specific anatomy of any particular type of neurons and, therefore, it can be useful for studying the mechanisms of firing adaptation or appearance of delayed-firing patterns in other types of SG neurons.

The population of TFNs was morphologically inhomogeneous and at least three groups of neurons were distinguished on the basis of their somatodendritic organization. Our results support the observations of others that tonic firing can be generated by several anatomical groups of SG neurons (Grundt and Perl 2002). Moreover, neurons belonging to one group could show firing patterns with differing degrees of adaptation (Grundt and Perl 2002), implying the absence of strict correlation between the firing pattern and cell morphology in SG. It can be therefore suggested that the balanced expression of ion channels described here is responsible for appearance of tonic firing in morphologically distinct types of SG neurons. We could not combine a nucleated patch recording with staining in the same neuron, because the isolation of the nucleus usually resulted in a deterioration of relatively small SG neuron and did not allow us to obtain a sufficiently good staining. Nevertheless, in more than 60 nucleated patches from TFNs, we recorded ion channels with similar properties, suggesting their presence in cells of all three subtypes.

In conclusion, a balanced system of ionic conductances underlies tonic firing in SG neurons. Voltage-gated $Na^+$ current in combination with a pronounced $K_{DR}$ but small $K_A$ currents generate a basic firing pattern, while $Ca^{2+}$-dependent conductances stabilize tonic firing, efficiently regulate discharge frequency, and modulate an input–output characteristic in a neuron.

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