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Specific actions of cyanide on membrane potential and voltage-gated ion currents in rostral ventrolateral medulla neurons in rat brainstem slices

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Abstract

The present study examined specific effects of sodium cyanide (CN) on the membrane potential (MP), spontaneous discharge (SD) and voltage-gated ion current of the identified bulbospinal rostral ventrolateral medulla (RVLM) neuron in the rat pup brainstem slice. 125 μM CN rapidly depolarized MP in the RVLM neuron by 11.6 mV as well as enhanced the SD rate by 300%. In contrast, the same dose of CN immediately hyperpolarized unlabeled, non-RVLM neurons by 4.8 mV. 50 μM CN did not significantly affect voltage-gated Ca^{++} or A-type K^+ currents. The same concentration of CN, however, rapidly and reversibly suppressed voltage-gated Na^+ currents and sustained outward K^+ currents in the RVLM neuron by 22.5% and 23%, respectively. Tetraethylammonium could mimic the effect of CN on MP, SD and sustained K^+ current in the RVLM neuron. It is concluded that: (1) like that from the adult rat, the rat pup bulbospinal RVLM neuron can be selectively and rapidly excited by CN; (2) the hypoxia-sensitive, sustained outward K^+ channel may play an important role in the acute hypoxia-induced excitation of the RVLM neurons.

Keywords: Rostral ventrolateral medulla neurons; Hypoxia; Cyanide; Oxygen-sensitive ion channels; Patch-clamp; Brainstem slices; Pre-sympathoexcitation

The principal source of excitatory drives to pre-ganglionic sympathetic neurons in the spinal cord emanates from a few hundred pre-sympathoexcitatory bulbospinal vasomotor neurons in the brainstem rostral ventrolateral medulla (RVLM) area [1,3,16]. These neurons are a subset of the C1 cells that are adrenergic and monosynaptically innervate spinal sympathetic neurons [1,11,14,16]. They are spontaneously active and discharged with a cardiac rhythmicity, providing basic excitatory drive to maintain normal resting blood pressure [1,3,11,16,19]. The RVLM neuron itself also serves as an O_2-sensor in central nervous systems (CNS) [3,5,18]. It is rapidly (< 1 min) excited by hypoxic hypoxia and histotoxic hypoxia evoked by sodium cyanide (CN), resulting in a stereotyped autonomic response of sympathetic activation including hypertension, bradycardia, expiratory apnea and global elevations of resistance of cerebral blood flow without changes in cerebral metabolism [3,15,18–20]. Acute responses of the RVLM neuron to hypoxia or CN include a rapid enhanced pacemaker activity [5,18,19] and involvement of O_2-sensitive ion channels in plasma membrane. However, there has been conflicting evidence about the exact ionic mechanism underlying the cellular hypoxic activation of RVLM neurons [4,5,9,19]. In order to elucidate this ionic mechanism, we examined acute responses of membrane potential (MP) and spontaneous discharge (SD) in the identified rat bulbospinal RVLM neuron to histotoxic hypoxia evoked by CN and determined links between the O_2-sensitive ion channels in these neurons and hypoxia-induced excitation in the RVLM neuron. The present study utilized the rat pup RVLM neuron in brainstem slices for whole-cell patch-clamping, because: (1) only those RVLM neurons in brainstem slices, rather than in dissociated states, exhibit the pacemaker activity [9,11]; (2) the GΩ-seal method is not feasible for the adult RVLM neuron in slices [11,19].

Retrograde labeling: All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Weill Medical College of Cornell University. As

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described previously [11], rat pups (P4–10) were anesthetized by hypothermia with CO_2. Fluorescent rhodamine-labeled microbeads (Lumafluor) were injected bilaterally into thoracic T_2-T_4 spinal cord segments (0.5 μl/side) to retrogradely label the bulbospinal RVLM neurons. Two to 6 days later, the rat pups were anesthetized by hypothermia and sacrificed. The brainstem was removed and immersed into an ice-cold, sucrose-artificial cerebrospinal fluid (s-ACSF) composed of (in mM): 26 $NaHCO_3$, 1 NaH_2PO_4, 3 KCl, 5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose, 248 sucrose, (pH = 7.4) as equilibrated with 95% O_2 + 5% CO_2. The brainstem was then coronally sectioned with a Vibratome (Lancer 1000) at 150–200 μm in thickness. The sections were perfused in a recording chamber at room temperature with a lactic acid (l)-ACSF equilibrated with 95% O_2 + 5% CO_2 (in mM): 124 NaCl, 26 $NaHCO_3$, 5 KCl, 1 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 4.5 lactic acid (pH = 7.4). Most of C1-RVLM neurons are anatomically located in the rostral ventrolateral area in one slice immediately caudal to the facial nucleus [9,11]. Within this area, the labeled RVLM neurons were visualized under an upright, epi-fluorescent microscope (Nikon). In order to examine whether they are the C1-cells, contents of recorded neurons were usually aspirated into the electrode for the single-cell RT-PCR to detect the tyrosine hydroxylase (TH)-transcript [9].

Electrophysiology: The patch pipette was pulled from borosilicate glass pipettes (Drummond Sci) on P80/PC puller (Sutter Instru.). For the current-clamp and Na^+ or K^+ current recording, the pipette solution was used (in mM): 114 K^+-gluconate, 17.5 KCl, 4 NaCl, 4 $MgCl_2$, 10 K^+-EGTA, Mg_2ATP, with pH = 7.25. For Ca^{++} current recording, K^+ in the pipette solution was replaced with equivalent Cs^+. The patch-clamp whole-cell current- and voltage-clamp [7] were employed using Axoclamp-2A

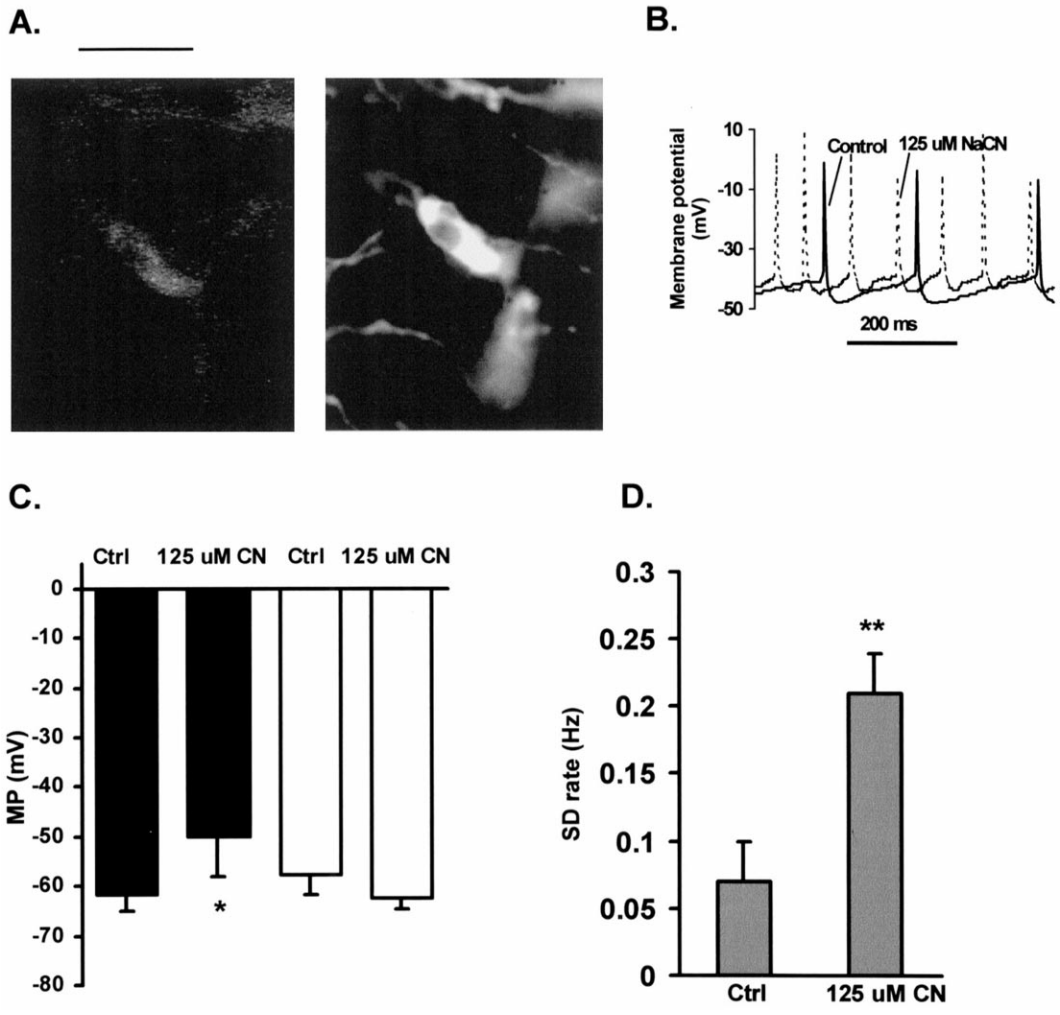

Fig. 1. (A) The C1-RVLM neuron marked by Cy3-conjugated, horse anti-mouse anti-TH antibody (right panel) was co-labeled by the retrogradely transported rhodamine microbeads (left panel). The bar indicates 20 μm. (B) Representative traces of MP and SD from a RVLM neuron are shown. The rate of SD of controls (solid line) was significantly enhanced by 125 μM CN (dotted line). (C) Amplitudes of MP in controls ($n = 6$) versus CN-treated groups amid RVLM (closed, $n = 4$) and unlabeled non-RVLM neurons (open, $n = 3$) are illustrated. (D) Changes in the SD rate of controls ($n = 6$) to presence of 125 μM CN ($n = 4$) were statistically compared. *$P < 0.05$; **$P < 0.01$.

and 200A, separately. pClamp 6.01 was used for data acquisition and analysis. Images of the labeled neurons were stored on 2.2 Workbench Image (Axon Instru.).

Immunostaining: The brainstem slice was fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.3). After washes with 0.5% bovine BSA, it was incubated with the primary mouse monoclonal antibody against TH (Chemicon, 1:10000) for 2 h and was then treated with Cy3-conjugated secondary antibody. The catecholaminergic C1-RVLM neuron was detected with its TH-immunoreactivity.

Data analysis: Data were expressed as mean ± SEM. Statistical comparisons between controls and drug-treated neurons were made using the Student's *t*-test. Sample number for controls versus drug-treated neurons was 3–6.

It is established that the bulbospinal RVLM neuron can be labeled by retrogradely transported fluorescent dye that is injected into the IML region of thoracic T_2 toT_4 spinal cord segments [11]. The RVLM neurons in brainstem slices were well labeled with rhodamine microbeads 2–4 days after the injection (Fig. 1A). Using a combination of the post-recording TH-staining (Fig. 1A) and single-cell RT-PCR (data not shown) methods, our results show that approximately 35% of the total labeled neurons in the RVLM area were catecholaminergic, similar to other reports [9,11].

As illustrated in Figs. 1B and 3A, the labeled RVLM neurons exhibited the pacemaker activity that is only observed when recorded on brainstem slices [9,11]. Like

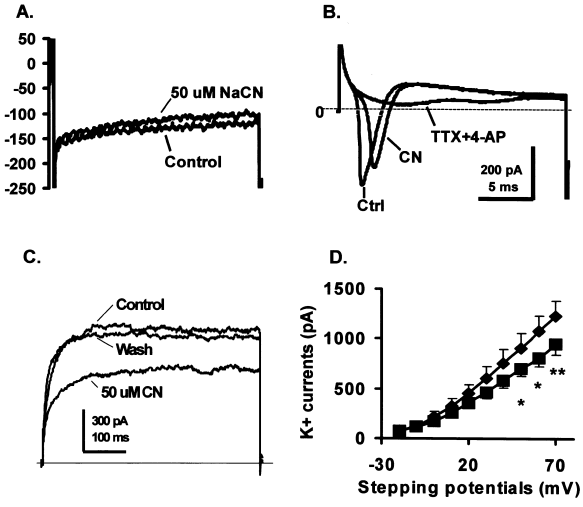

Fig. 2. (A) 50 μM CN exerted little influence on the voltage-gated Ca^{++} current in the RVLM neuron elicited from −80 mV to +10 mV. (B) 50 μM CN, however, partially inhibited the voltage-gated Na^+ current in the RVLM neuron elicited from −90 mV to −10 mV. Be noted that this Na^+ current was fully blocked by 10 μM TTX, along with 10 mM 4-AP blocking the A-type transient K^+ current. (C) 50 μM CN reversibly inhibited the voltage-gated, sustained outward K^+ current in the RVLM neuron elicited from −30 mV to +70 mV. (D) I-V curve of this $K^+_{(out)}$ current is shown. The $K^+_{(out)}$ current in controls (◆) and the presence of 50 μM CN (■) is plotted against each 10 mV step potential ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$).

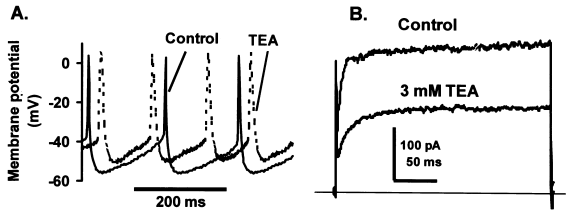

Fig. 3. TEA mimics the effect of CN on MP, SD and sustained outward K^+ currents in the RVLM neuron. (A) Representative traces of MP from a RVLM neuron are shown. Both MP and SD were affected by 3 mM TEA (dotted line) in the manner similar to that by CN. (B) 3 mM TEA significantly inhibited the voltage-gated, sustained $K^+_{(out)}$ current elicited from −30 mV to +50 mV ($n = 3$).

hypoxic hypoxia ($5\%O_2 + 95\%N_2$) (data not shown), CN rapidly and significantly evoked a bursting firing of SD of the RVLM neuron (Fig. 1B,D), a stereotyped response similar to that recorded from adult rats [19]. In addition, the same dose of CN significantly depolarized MP by decreasing it from −61.6 ± 3.6 mV to −50 ± 8.2 mV (Fig. 1B,C). Three unlabeled neurons picked up from non-RVLM regions displayed no SD, with an average MP of −57.7 ± 4.0 mV ($n = 6$). In contrast to the labeled RVLM neurons, CN (125 μM) immediately hyperpolarized MP in these unlabeled non-RVLM neurons to −62.5 ± 2.2 mV, yet could not evoke any SD (Fig. 1C).

In order to determine the role of voltage-gated Ca^{++} channels in hypoxia- and CN-induced excitation of the RVLM neurons, effects of CN on the voltage-gated Ca^{++} currents was examined first. As shown in Fig. 2A, 2 mM Ca^{++} -mediated Ca^{++} channel currents were elicited from −80 mV to 10 mV. 50 μM CN exerted little influence on this Ca^{++} current (Table 1).

Effects of CN on Na^+ and K^+ currents of the RVLM neurons were then examined. A TTX-sensitive, voltage-gated Na^+ current was elicited from −90 mV to −10 mV (Fig. 2B). 50 μM CN significantly inhibited this Na^+ current (Table 1, Fig. 2B).

A sustained outward K^+ ($K^+_{(out)}$) current in the RVLM neurons [16] was elicited from −30 mV. The I-V relationship curve of it reveals that the K^+ channel starts to activate at holding potentials ≥ −30 mV (Fig. 2D). 50 μM CN reversibly and significantly inhibited this K^+ current induced by step potentials from +50 mV to +70 mV (Table 1, Fig. 2C). This inhibition appears to be voltage-dependent, namely, the more positive depolarizing steps were applied, the more significant suppression of the current exhibited. At +60 mV, for instance, 50 μM CN suppressed this $K^+_{(out)}$ current from 1075.5 ± 154.3 pA of controls to 804.5 ± 79.1 pA ($P < 0.05$). At +70 mV, moreover, 50 μM CN inhibited the $K^+_{(out)}$ current more significantly, from 1230 ± 148.4 pA of controls to 947 ± 109.8 pA ($n = 4$, $P < 0.01$) (Fig. 2D). In contrast, 50 μM CN did not significantly affect a 4-AP-sensitive A-type K^+ current

Table 1
Effects of 50 μM CN on the voltage-gated ion channel currents in the RVLM neurons[a]

Ion currents (pA)	Ca^{++}	Na^{+}	$K^{+}_{(out)}$	A-type K^{+}
Control	− 172.6 ± 39.3	− 387.1 ± 61.1	1230 ± 148.4	665.4 ± 86.9
CN-treated	− 163.9 ± 25.8	− 300.1 ± 50.6*	947 ± 109.8**	687.5 ± 61.6
$n=$	4	4	5	3

[a] Mean ± SEM; *$P < 0.05$; **$P < 0.01$.

in the RVLM neuron elicited from −90 mV to +30 mV (Table 1).

Finally, we examined if tetraethylammonium (TEA), a typical K^{+} channel blocker, could mimic the CN-evoked actions on MP and SD in RVLM neurons. Like CN, 3 mM TEA significantly decreased MP from -52.6 ± 2.1 mV of controls to -37.3 ± 1.9 mV ($n = 3$, $P < 0.05$) (Fig. 3A). Additionally, it almost doubled SD rate from 0.04 ± 0.003 HZ of controls to 0.076 ± 0.005 Hz ($n = 3$, $P < 0.05$). Moreover, 3 mM TEA significantly inhibited the sustained $K^{+}_{(out)}$ current in the RVLM neuron, Amplitudes of this $K^{+}_{(out)}$ current elicited at step potential +50 mV decreased from 863.8 ± 120.9 pA of controls to 546.7 ± 121.2 pA in the presence of 3 mM TEA ($P < 0.05$, $n = 3$) (Fig. 3B).

Both CN and hypoxic hypoxia can induce a similar excitation of the RVLM neuron in vivo and in vitro [4,18,19], indicating a similar mechanism involved [12,13]. Using CN as the histotoxic agent quickly evoking hypoxia [9,10,18,19], we sought to determine if CN could reveal any specific feature of O_2-sensing in the identified rat pup RVLM neurons. Our results show that RVLM neurons from 5–16 day old rat pups displays the acutely hypoxic response similar to that from adult rats [18,19], strongly suggesting that they have already expressed the related ionic O_2-sensitive mechanism [7,17].

The present data also confirms that the hypoxia-induced excitation in the rat pup RVLM neuron is rapid and selective. This rapid property distinguishes itself from 'pathological hypoxia' that results in responses to prolonged energy deprivation (> minutes). Thus, it suggests that the CN-induced excitation of the RVLM neuron may result from direct O_2-sensors located around the plasma membrane coupled with final effectors. The effect of CN on RVLM neurons is also selective. The immediate action by CN on the labeled RVLM neuron was depolarization while, in contrast, its action on the unlabeled non-RVLM neuron was hyperpolarization, suggesting that the RVLM neuron possesses an O_2-sensing property that is distinct from that in other neurons [5,6].

The voltage-gated ion current in the RVLM neuron have been previously characterized [5,9,11]. However, there have been conflicting results about its ionic mechanism underlying the hypoxic activation [4,9,19]. Different roles of Ca^{++} and K^{+} currents in unidentified RVLM neurons were described [4,19]. In addition, Kawai et al. [9] reported that 1 mM CN unselectively increased all voltage-gated ion currents in dissociated RVLM as well as non-RVLM neurons. One major reason for the different results between Kawai's and our studies might be the much higher (1 mM) dose of CN applied by the former. We have recently examined effects by different doses of CN on the K^{+} currents in the isolated RVLM neuron, showing that like those obtained from the slice experiment, the sustained K^{+} current was inhibited by 30–50 μM CN. The high (0.5–1 mM) dose of CN, however, could result in immediate and irreversible pathological changes in the RVLM neuron (data not shown). Moreover, the fact that the O_2-sensitive actions of RVLM neurons were mimicked by TEA strongly reveals a key role of the sustained K^{+} channel in the hypoxic pre-sympathoexcitation. Thus, it appears likely that the acute suppression of sustained $K^{+}_{(out)}$ currents by both CN and TEA results in a bursting of spontaneous activity and initiation of the pre-sympathetic excitatory response in the RVLM neuron. The identity of this CN-sensitive, sustained K^{+} channel remains unclear, however. It has been assumed that Kv3 or Ca^{++}-activated potassium channels may play an important role in the spontaneous firing in the CNS neuron [2,6,17]. However, possible roles of ATP-sensitive K^{+} channels in the RVLM neuron excitation [4] may be excluded, since this channel should open when CN is present [2]. The CN- and TEA-sensitive K^{+} channels are also found in other chemoreceptor cells including type I glomus carotid and neuroepithelial body cells [8,10,12,20] as well as neocortical neurons [6]. In terms of the CN-induced partial block of Na^{+} channels in the RVLM neuron, it may be linked with an O_2-sensitive protective mechanism that restricts Na^{+} entry to the neuron and exerts prevention of cell death [6].

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[1] Brown, D.L. and Guyenet, P.G., Cardiovascular neurons of brain stem with projections to spinal cord, Am. J. Physiol., 247 (1984) R1009–R1016.
[2] Coetzee, W.A., Amarillo, Y., Chiu, J., Chow, A., Lau, D., McCormack, T., Moreno, H., Nadal, M.S., Ozaita, A., Pountney, D., Saganich, M., Vega-Saenz de Miera, E. and Rudy, B., Molecular diversity of K^{+} channels, Ann. N. Y. Acad. Sci., 868 (1999) 233–285.
[3] Dampney, R.A., Kumada, M. and Reis, D.J., Central neural mechanisms of the cerebral ischemic response: characterization, effect of brainstem and cranial nerve transections, and simulation by electrical stimulation of restricted

regions of medulla oblongata in rabbit, Circ. Res., 45 (1979) 48–62.
[4] Golanov, E.V. and Reis, D.J., A role for KATP + -channels in mediating the elevations of cerebral blood flow and arterial pressure by hypoxic stimulation of oxygen-sensitive neurons of rostral ventrolateral medulla, Brain Res., 827 (1999) 210–214.
[5] Guyenet, P.G., Neural structures that mediate sympathoexcitation during hypoxia, Respir. Physiol., 121 (2000) 147–162.
[6] Haddad, G.G., Oxygen sensing in neurons: direct and indirect modulation of ion channels, In J. Lopez-Barneo and E.K. Weir (Eds.), Oxygen Regulation of Ion Channels and Gene Expression, Futura Publishing Company, New York, NY, 1998, p. 285.
[7] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J., Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches, Pflugers Arch., 391 (1981) 85–100.
[8] Hatton, C., Carpenter, E., Pepper, D., Kumar, P. and Peers, C., Developmental changes in isolated rat type I carotid body cell K^+ currents and their modulation by hypoxia, J. Physiol. (Lond.), 501 (1997) 49–58.
[9] Kawai, Y., Qi, J., Comer, A.M., Gibbons, H., Win, J. and Lipski, J., Effects of cyanide and hypoxia on membrane currents in neurones acutely dissociated from the rostral ventrolateral medulla of the rat, Brain Res., 830 (1999) 246–257.
[10] Lahiri, S., Ehleben, W. and Acker, H., Chemoreceptor discharges and cytochrome redox changes of the rat carotid body: role of heme ligands, Proc. Natl. Acad. Sci. USA, 96 (1999) 9427–9432.
[11] Li, Y.W., Guyenet, P.G. and Bayliss, D.A., Voltage-dependent calcium currents in bulbospinal neurons of neonatal rat rostral ventrolateral medulla: modulation by alpha2-adrenergic receptors, J. Neurophysiol., 79 (1998) 583–594.
[12] Lopez-Barneo, J., Lopez-Lopez, J.R., Urena, J. and Gonzalez, C., Chemotransduction in the carotid body: K^+ current modulated by PO_2 in the type I chemoreceptor cells, Science, 241 (1988) 580–582.
[13] Lopez-Barneo, J., Pardal, R. and Ortega-Saenz, P., Cellular mechanisms of oxygen sensing, Annu. Rev. Physiol., 63 (2001) 259–287.
[14] Milner, T.A., Morrison, S.F., Abate, C. and Reis, D.J., Phenylethanolamine N-methyltransferase-containing terminals synapse directly on sympathetic preganglionic neurons in the rat, Brain Res., 448 (1988) 205–222.
[15] Reis, D.J., Golanov, E.V., Ruggiero, D.A. and Sun, M.K., Sympathoexcitatory neurons of the rostral ventrolateral medulla are oxygen sensors and essential elements in the tonic and reflex control of the systemic and cerebral circulation, J. Hypertens., 12 (1994) S159–S180.
[16] Ross, C.A., Ruggiero, D.A., Park, D.H., Joh, T.H., Sved, A.F., Fernandez-Paridal, J., Saavedra, J.M. and Reis, D.J., Tonic vasomotor control by the rostral ventrolateral medulla: effect of electrical and chemical stimulation of the area containing C1 adrenaline neurons on arterial pressure, heart rate, plasma catecholamines and vasopressin, J. Neurosci., 4 (1984) 474–494.
[17] Rudy, B., Chow, A., Lau, D., Amarillo, Y., Ozaita, A., Saganich, M., Moreno, H., Nadal, M.S., Hernandez-Pineda, R., Hernandez-Cruz, A., Erisir, A., Leonard, C. and Vega-Saenz de Miera, E., Contributions of Kv3 channels to neuronal excitability, Ann. N. Y. Acad. Sci., 868 (1999) 304–343.
[18] Sun, M.K., Jeske, I.T. and Reis, D.J., Cyanide excites medullary sympathoexcitatory neurons in rats, Am. J. Physiol., 262 (1992) R182–R189.
[19] Sun, M.K. and Reis, D.J., Hypoxia-activated Ca^{2+} currents in pacemaker neurons of rat rostral ventrolateral medulla in vitro, J. Physiol. (Lond.), 476 (1994) 101–116.
[20] Youngson, C., Nurse, C.A., Yeger, H. and Cutz, E., Oxygen sensing in airway chemoreceptors, Nature, 365 (1993) 153–155.