MODULATION OF NICKEL-INDUCED BURSTING WITH 4-AMINOPYRIDINE IN LEECH RETZIUS NERVE CELLS

DHRUBA PATHAK1,2, S. LOPICIC2, MARIJA BRATIC-STANOJEVIC2, D. PAVLOVIC3, P. R. ANDJUS1 and V. NEDELJKOV2

1 Department of Physiology and Biochemistry, School of Biology, University of Belgrade, 11000 Belgrade, Serbia
2 Institute for Pathological Physiology, School of Medicine, University of Belgrade, 11000 Belgrade, Serbia
3 Ernst Moritz Arndt University, Greifswald, Germany

Abstract - Paroxysmal depolarization shift has been identified as a characteristic feature of the cellular basis of epilepsy. On Na+ dependent bursting, 1 mmol/l 4-aminoopyridine (4-AP) produced a two-phase effect - a significant depolarization accompanied by an increase in the frequency of bursting, followed by repolarization along with a diminished frequency of bursting. Neither 1 μmol/l apamin nor 150 nmol/l charybdotoxin (ChTX) elicited any significant effect on either bursting or standard conditions. Our results suggest that 4-AP affects the bursting indirectly by altering the excitability of the cell. The lack of effects of apamin and ChTX is probably due to channel insensitivity to these blockers in leech.

Key words: Paroxysmal depolarization shift (PDS), Na+ dependent bursting, 4-AP, Apamin, ChTX,

INTRODUCTION:

At the cellular, level epilepsy is described by the spontaneous and synchronous depolarization of a network of neurons within the epileptic focus. Characteristic of the epileptiform activity of an individual neuron is the paroxysmal depolarization shift (PDS) (Gorji and Speckmann, 2009), to the extent that a PDS is considered a diagnostic cellular expression of epileptic activity (Üre and Altrup, 2006). Although some investigators regard any depolarization which is abrupt, substantial, and has an all or nothing character to be an epileptic PDS (Prince and Wong, 1981), a stricter definition of a PDS is a steep plateau of depolarization superimposed by action potentials, followed by a steep repolarization (Altrup, 2004). However, it is not clear whether PDSs are made up of synaptic potentials depending on neural interactions within a network, or whether they are generated by endogenous membrane processes that are mainly governed by membrane channels of the single neuron (Timofeev et al., 2004, Heilman and Quattrochi, 2004).

Although PDSs and their causes are diverse, two key underlying theories have been proposed for their generation. The first theory, “calcium-dependent PDS”, explains the generation of PDSs through synaptic and calcium dependent processes. According to this theory the initial change is a synaptic activation of ionotropic glutamate receptors that eventually results in a prolonged increase of intracellular Ca2+ concentration, thereby triggering Ca2+-dependent processes that lead to and sustain the bursting (Segal, 1981; Xin et al., 2005).

The second theory is the “sodium-dependent PDS”, which is Ca2+- and synapse-independent. Na+-dependent PDSs have been shown to exist in a variety of species, both in vitro and in vivo. Removal of the avian Edinger Westphal nucleus leads to extracellular calcium increase and frequency of action potential calcium increase and frequency of action potential discharges of repetitively firing cells...
(Fujii, 1992). Furthermore, low and zero Ca\(^{2+}\) solutions cause the development of spontaneous, rhythmic and synchronous bursting discharges in rat hippocampal slices (Wang et al. 2004) and supraoptic nucleus neurons (Li and Hatton, 1996). Finally, low calcium levels can cause, or at least contribute to, myoclonic seizures, infantile spasms, and seizures concurrent with Down syndrome (Thiel, 2006).

It has been shown in leech that superfusion of the Retzius neurons with blockers of voltage–dependent Ca\(^{2+}\) currents like Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), La\(^{3+}\), and Zn\(^{2+}\), induces rhythmic epileptiform bursting activity. This activity is eliminated in Na\(^{+}\)-free solutions (Angstadt et al. 1998; Pathak et al. 2009). In addition, these neuronal cells exhibit bursting behavior both in isolation and as an assembly, in which case the bursting occurs synchronously (Angstadt and Friesen, 1991; Ghosh et al. 2009). It is presumed that the block of voltage-gated Ca\(^{2+}\) channels prevents the activation of Ca\(^{2+}\)-activated K\(^{+}\) channels. Reduction of outward K\(^{+}\) currents unmasks the persistent inward Na\(^{+}\) current which leads to bursting (Angstadt et al. 1998; Angstadt, 1999).

Although the presumed mechanism of Na\(^{+}\)-dependent PDSs involves Ca\(^{2+}\)-activated K\(^{+}\) channels, the functional relevance of potassium dynamics in these oscillatory patterns in the absence of synaptic transmission is still poorly understood. Therefore, we have examined the acute effects of various K\(^{+}\) channel blockers (4-AP, apamin, ChTX). The experiments were performed using the established model of Ni\(^{2+}\)-induced bursting in the Retzius nerve cells of the leech (Pathak et al. 2009). The aim was to evaluate the contribution of specific K\(^{+}\) channel populations to the regulation of rhythmic oscillatory activity on our model.

**MATERIALS AND METHODS**

The experiments were performed at room temperature (22–25°C) on Retzius nerve cells of the isolated segmental ganglia of the leech *Haemopis sanguisuga*. The method of dissection has been previously described (Beleslin, 1971) and complies with institutional research council guidelines.

Dissected segments of 3 ganglia were immediately transferred to a 2.5 ml plastic chamber with leech Ringer solution (for composition see solutions) and fixed by means of fine steel clips. The plastic chamber was then placed in a grounded Faraday’s cage mounted on a fixed table in a manner that prevents vibrations. The cells were identified and penetrated using a micromanipulator under a stereomicroscope inside the cage. Retzius neurons were identified based on the position within the ganglion, the size and the bioelectrical properties of the cells.

Prior to the experiments, the chamber was flushed with fresh Ringer solution; microelectrode dipped into the solution and allowed 20–30 min for equilibration. For long-term experiments substances were applied by continuous flushing of the chamber and for short-term experiments by the dripping of the substance directly above the cell. While dripping, the utmost care was taken to avoid any mechanical vibrations (James et al. 1980).

**Electrical methods (electrophysiological recordings)**

The membrane potential was recorded using standard single-barrel glass microelectrodes. Micropipettes were pulled from the thick wall capillaries with internal filament (O.D. 1.5 mm, I.D. 0.6 mm, World Precision Instruments) on a vertical puller (PE-6, Narishige, Japan) and then filled with 3 mmol/l KCl shortly after pulling. The tip diameter of the electrodes was less than 1 μm, the tip potentials were less than 5 mV, and the microelectrode resistance was 20–25 MΩ in the standard Ringer solution (standard Ri).

The recordings were amplified using a high input impedance amplifier (model 1090, Winston Electronics, USA). Microelectrodes were connected to the amplifier via an Ag-AgCl wire. The ground electrode was an Ag-AgCl pallet in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3
mmol/l KCl 3% agar bridge. The recordings were displayed on a two-channel oscilloscope (HM 205-3, Hameg, Germany) and permanently recorded on a pen recorder (L 7025 II, Linseis, Germany) and a thermal printer (HM8148-2, Hameg, Germany).

For measurements of the input resistance of the directly polarized membrane, the high input impedance bridge amplifier (model 1090, bridge unit BR1, Winston Electronics, USA) was used to inject a current through the recording microelectrode. Rectangular hyperpolarizing pulses (0.3-1.0 nA, 500ms duration, applied at a frequency of 0.1-0.2 Hz) were delivered using a S48 dual output square-pulse stimulator and an SIU 5 stimulus isolation unit (both Grass Instruments, USA). The amplitude of the recorded voltage produced by these pulses was used as a measure of the input membrane resistance (IMR).

Solutions

The standard Ri used in these experiments had the following composition (in mmol/l): NaCl 115.5, KCl 4, CaCl₂ 2, NaH₂PO₄ 0.3, Na₂HPO₄ 1.2 (pH = 7.2). In the Ni²⁺-containing solutions (Ni-Ringer) 3 mmol/l NiCl₂ was added. 4-aminopyridine (4-AP, Sigma, St. Louis, USA) containing solutions were made in the following series of concentrations (in mmol/l): 0.01, 0.03, 0.1, 0.3 and 1 mmol/l of 4-AP in standard Ri solution. Apamin (Sigma-Aldrich, Steinheim, Germany) was applied in a final concentration of 1 μmol/l dissolved in Ringer solution just prior to application. Charybdotoxin (ChTX, RBI, USA) was dissolved in standard Ri and applied as a fresh solution in a final concentration of 150 nmol/l.

Data analysis

All results are expressed as means ± S.E.M, with n indicating the number of trials. Comparison between the mean values was made using a two-tailed paired Student’s t-test; p values of less than 0.05 were considered significant.

RESULTS

Effect of 4-AP on Ni²⁺-induced bursting

The resting membrane potential (RMP) of the Retzius neurons was −50.2±2.7 mV (n= 7 cells). Spontaneous action potentials were generated at a low frequency (<1 Hz). In our previous paper (Pathak et al. 2009), we reported that the addition of 3 mmol/l Ni²⁺ to the standard Ri was able to induce depolarization of the membrane potential followed by spontaneous bursting activity, with the salient feature of rapid depolarizations to a plateau level during which bursts of action potential occurred in the Retzius neuron. Beyond the significance of using Ni²⁺ in an animal model, it has been additionally implicated for deposition in plants as well (Vukojević et al. 2009). This served as a foundation for further experiments in the present study (Fig. 1A). As usual, it took 2-7 min for the transformation of spontaneous action potential generation to bursting. Subsequent stabilization of bursting could be observed, allowing us to mark it as a final level as depicted before the application of 4-AP (Fig. 1D, phase I). In this Ni²⁺-induced stabilized bursting condition, the effects of 1 mmol/l 4-AP in Ni-Ringer were tested to examine the changes in bursting parameters, such as frequency, duration and amplitude of plateaus and number of spikes per plateau.

Two major effects were observed on bursting. First, the stabilized bursting level was depolarized by 14.9±0.7 mV (Fig. 1B) and the frequency of bursting was increased from 4.9 ± 0.5 min⁻¹ to 9.5±1.1 min⁻¹ (Fig. 1D, phase II; paired t-test, p<0.01). It is noteworthy to mention that during this depolarization the amplitude of the plateaus decreased and there was a marked increase in the number of action potentials per plateau. This period of depolarization lasted for 141±30 s.

Following this phase of depolarization, the membrane potential started to repolarize (Fig. 1D, phase III). During this phase of repolarization the number of plateaus began to subside and in most cases bursting was eventually abolished. After
washout with Ni -Ringer the membrane potential
fully recovered in all seven cells, and bursting activity
resumed at 4.7±0.4 min⁻¹ (Fig. 1D, phase IV).

**Effect of 4-AP on membrane potential in standard physiological saline**

The effects of 4-AP on the resting membrane potential of the Retzius cells of the leech were examined in order to elucidate the mechanism of dual 4-AP effect on Ni²⁺-induced bursting.

Fig. 2A-E illustrates experiments where five concentrations of 4-AP were applied in standard Ri solution. For each 4-AP concentration (0.01 mmol/l, 0.03 mmol/l, 0.1 mmol/l, 0.3 mmol/l, 1 mmol/l), the membrane potential sweeps were recorded and comparisons were made with their respective resting membrane potential recordings in standard physiological saline (Table 1, Fig. 3).

As shown in Fig. 2, in standard Ri the 4-AP effect also had two phases. The frequency of firing initially increased dramatically in all cases and membrane potential depolarized significantly in concentrations of 4-AP higher than 0.03 mmol/l. The second phase was characterized by a reduction of firing frequency, as well as by repolarization towards the resting level. These effects were concentration-dependent since all effects became more pronounced as the concentration of 4-AP increased (Fig. 3).

**Effect of apamin on Ni²⁺- induced bursting**

This set of experiments was carried out to check whether apamin, a blocker of SK potassium channels, affects the parameters of Ni²⁺-induced bursting. The average resting membrane potential in these experiments was -45.1 ± 1.3 mV. In the control solution (Ni Ringer) the frequency of
Fig. 2. Traces showing the effect of 4-AP in standard Ri, where frequency of firing has been increased. A. Upon application of 0.01 mmol/l 4-AP, RMP -41 mV remained unchanged. B. Example of a recording showing the effect of 0.03 mM 4-AP where RMP was depolarized from -62 mV to -60 mV by 2 mV. C. Example of a recording showing the effect of 0.1 mmol/l 4-AP where RMP was depolarized from -60 mV to -53 mV by 7 mV. D. Example of a recording showing the effect of 0.3 mmol/l 4-AP where RMP was depolarized from -38 mV to -25 mV by 13 mV. E. Example of a recording showing the effect of 1 mmol/l 4-AP where RMP was depolarized from -51 mV to -35 mV by 16 mV. PD, membrane potential.
plateaus, number of spikes per plateau, plateau duration and plateau amplitude were 6.4±0.9 min⁻¹, 15.1±1.6, 4.0±0.2 s and 11.3±1.6 mV, respectively. In the presence of 1 μmol/l apamin the respective values for the parameters were 6.0±0.7 min⁻¹, 15.1±2.0, 4.1±0.2 s and 9.6±1.2 mV (Fig. 4). The presence of apamin did not significantly change the parameters of bursting (n=7, p>0.05).

These results clearly show that apamin did not have any significant effect on Ni²⁺ induced bursting in leech. To further investigate this lack of effect, we performed the analysis of apamin in standard Ri.

Effect of apamin on membrane potential and input membrane resistance in standard Ri

In the standard Ri solution, the blocker of the Ca²⁺-activated K⁺ channels (apamin) did not affect the membrane potential, amplitude and frequency of spontaneous firing of the Retzius cell (1 μmol/l, n=4, p>0.05). The RMP in standard Ri had an average value of -49.6±1.6 mV and under apamin was -50.4±1.7 mV. Similarly, the amplitude of spontaneous firing in standard Ri and in the presence of apamin was 40.2±4.3 mV and 40.0±4.2 mV, respectively. Furthermore, the frequency of spontaneous firing in standard Ri and in the presence of apamin was 14.7±0.8 min⁻¹ and 14.3±0.8 min⁻¹, respectively.

Since apamin did not have any significant effect on the RMP, amplitude and frequency of spontaneous APs of the Retzius nerve cells, we conducted experiments to check for apamin-induced changes in input membrane resistance. The average RMP of the penetrated Retzius cells in this group of experiments was -45.8±1.4 mV. Upon application of 1 μmol/l apamin, the membrane potential on the average remained unchanged. On the basis of the amplitude of voltage responses to -0.4±0.0 nA hyperpolarizing pulses (duration 500 ms), we compared the mean input resistance of 3 cells in 1 μmol/l apamin vs. control (standard Ri). The choice of stimulus amplitude was based on the examination of minimum voltage response that served as representative. The mean input resistance in standard Ri was 31.3±6.2 MΩ, and in apamin it was 32.7±9.7 MΩ. The increase in input membrane resistance by 1.4±3.6 MΩ in 1 μmol/l apamin was statistically nonsignificant (p>0.05 n=3).

Table 1. Resting membrane potential values and the depolarization induced by administration of different concentrations of 4-AP in millimolar and submillimolar range in standard Ri. The drug was applied after three min when the spontaneous firing of Retzius cell was stable in Standard Ri (control).

<table>
<thead>
<tr>
<th>Conc.(mmol/l)</th>
<th>RMP (mV)</th>
<th>Effect of 4-AP</th>
<th>Depolarization of MP (mV)</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>-50.4±3.0</td>
<td>-49.3±3.2</td>
<td>1.1±0.3</td>
<td>5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.03</td>
<td>-50.3±3.7</td>
<td>-45.5±4.3</td>
<td>4.8±0.7</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>-49.9±2.0</td>
<td>-43.2±2.2</td>
<td>6.7±0.6</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.3</td>
<td>-51.2±2.9</td>
<td>-38.7±2.9</td>
<td>12.5±0.9</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>-47.8±2.4</td>
<td>-34.2±2.7</td>
<td>13.7±1.1</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The data are shown as mean value ± standard error. Conc. - Concentration, RMP - resting membrane potential, 4 - AP (4–Aminopyridine), MP - membrane potential, n - number of trials, p - p value for the paired t test.
number of action potentials. The number of action potentials in standard Ri was 3.7±0.3 and under apamin it was 3.7±0.3. No changes in evoked repetitive action potential activity was detected under 1 μmol/l apamin (n=3, p>0.05).

All these results indicate that apamin does not affect the RMP and IMR of leech Retzius cells.

Effect of charybdotoxin on Ni2+- induced bursting

In view of the lack of effect of apamin described above, one might suspect that another population of Ca2+-dependent K+ channels (high conductance BK channels) may be active under our experimental conditions. Therefore, in this set of experiments we applied a specific BK channel blocker ChTX.

In order to check for the effects of ChTX, we utilized an experimental protocol, known as the drop application, at the concentration that would be expected to block BK channels (150 nmol/l).

The average resting membrane potential of the Retzius neurons in these experiments was -52.4±2.5 mV. In Ni-Ringer the frequency of plateaus, number of spikes per plateau, plateau duration and plateau amplitude were 1.6±0.2 min⁻¹, 3.8±0.4, 7.1±0.4 s and 13.2±0.8 mV, respectively, whereas in ChTX these parameters had the values of 2.4±0.4 min⁻¹, 5.0±0.6, 7.0±0.4 s and 13.7±0.9 mV (Fig. 5). The results show no significant effect of ChTX on Ni2+ - induced bursting in the leech (p>0.05, n=5).

Since ChTX did not have significant effects on bursting, we investigated the effects of the substance in standard Ri.

Effect of charybdotoxin on membrane potential and input membrane resistance in standard Ri

The RMP in standard Ri had an average value of -44.8±0.8 mV and under 150 nmol/l ChTX it was -43.5±1.0 mV. Similarly, the amplitude of spontaneous firing in standard Ri and in the presence of
ChTX was 40.2±1.5 mV and 33.0±3.85 mV, respectively. Furthermore, the frequency of spontaneous firing in standard Ri and in presence of ChTX was 7.2±1.2 min⁻¹ and 8.2±0.7 min⁻¹, respectively. Data from our experiments indicate that ChTX did not have any significant effect on the membrane potential (n=6), amplitude (n=4) and frequency of spontaneous firing (n=5) of the Retzius cell in standard Ri solution (p>0.05). As ChTX did not have any significant effect on the RMP, amplitude and frequency of spontaneous APs of the Retzius nerve cells, we conducted experiments to examine whether ChTX induces any changes in input membrane resistance. The average RMP of the Retzius cells in this group of experiments was -48.7±1.9 mV. Upon application of 150 nmol/l ChTX, the mean input resistance in standard Ri was 19.2±1.6 MΩ, while in ChTX it was 19.9±1.3 MΩ (n=4, p>0.05).

Injecting the constant depolarizing current pulse 0.7±0.1 nA to the control cells and treated cells for the same duration of 500 ms generated a number of action potentials. The number of action potentials in standard Ri was 6.3±1.8 and under ChTX it was 6.3±1.8. No changes in evoked repetitive action potential activity was detected under 150 nmol/l ChTX (n=3, p>0.05).

Together, these results indicate that ChTX does not affect the RMP and IMR of leech Retzius cells.

**DISCUSSION**

In a range of vertebrate and invertebrate preparations the initiation and synchronization of bursting can be achieved by lowering the extracellular Ca²⁺ concentration, and in the presence of Ca²⁺ channel blockers. Since in these conditions the chemical synaptic transmission is blocked, this type of bursting is often termed non-synaptic bursting. Although altered potassium dynamics play a major role in this type of neuronal activity (Dudek et al. 1998), the role of K⁺ channels is still incompletely understood. In this paper we have examined the effect of K⁺ channel blockers 4-AP, apamin and ChTX on Ni²⁺-induced bursting in leech Retzius nerve cells.

**The effect of 4-AP**

In leech Retzius cells 4-AP blocks the A-type potassium current (Stewart et al. 1989). In our experiments, when applied during established Ni²⁺ induced bursting, the effect of 4-AP had two phases.

In the first phase 4-AP caused a marked depolarization with an increase in frequency of bursting. In standard conditions, 4-AP led to a dose-dependent depolarization with an increase in the frequency of spontaneous action potential generation. Since the effects of 4-AP are very similar in standard Ri and during bursting, it seems that the changes in bursting parameters observed
during the first phase of 4-AP effect are superimposed on changes in membrane potential and the overall electrical activity of the cell. These effects are most probably the consequence of increased cell excitability following the block of $I_{K\lambda}$, rather than the effect of 4-AP on the mechanism of bursting generation. Furthermore, it has been recently reported that nitric oxide synthase inhibitors could play a useful role in the neurological diseases in which excitotoxic mechanisms play a role (Radenović et al. 2005).

In the second phase, the membrane potential repolarized toward the resting level, the bursting subsided, and was completely abolished in some cases. This effect of 4-AP could be due to the activation of $Ca^{2+}$-dependent $K^+$ channels. The $Ca^{2+}$-dependent $K^+$ channels have been shown to exist throughout the leech nervous system and represent a major $K^+$ current in Retzius neurons (Stewart et al. 1989). 4-AP can activate this channel by increasing the intracellular $Ca^{2+}$ concentration. 4-AP has been shown to increase the intracellular $Ca^{2+}$ concentration in leech glial cells by a process that does not involve the influx of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels, and is sustained in presence of nickel (Muller et al. 1999). 4-AP also raises intracellular $Ca^{2+}$ levels without the activation of voltage-gated $Ca^{2+}$ channels in rat cortical type I astrocytes, primary cortical neurons, and skeletal muscle cells (Grimaldi et al. 2001). This intracellular $Ca^{2+}$ rise could activate the CaK channels and cause the repolarization as well as diminution and cessation of bursting.

Furthermore, it has been established that glial cells (Muller and Schlue, 1997) as well as AP neurons (Pellegrini et al. 1989) in the leech nervous system posses $K^+$ channels that are activated by both depolarization and an increase in the intracellular $Ca^{2+}$ concentration, resembling mammalian BK (Kv1.1, maxi-K) channels. If the same were true for Retzius neurons, 4-AP-induced depolarization could further contribute to the activation of $Ca^{2+}$-dependant $K^+$ channels leading to the repolarization and diminution of bursting as seen in the second phase of the 4-AP effect.

Our results therefore indicate that 4-AP has no direct effect on the mechanism of $Ni^{2+}$-induced bursting. The significant effects recorded in our study are probably a result of multiple actions of 4-AP on the membrane potential and intracellular $Ca^{2+}$ level, rather than on the bursting mechanism itself.

In accordance with the apparent inability of 4-AP to directly affect bursting is the fact that 4-AP itself did not produce bursting when applied in standard Ri. Although 4-AP is used as a proconvulsant in some preparations, the lack of 4-AP-induced bursting on our model is not surprising since 4-AP induces synaptic bursting which is dependent on the activation of glutamate ionotropic receptors (Pena and Tapia, 2000), while on our model the bursting is
of a non-synaptic type, as mentioned above. It is also noteworthy that in other preparations where non-synaptic bursting occurs, such as the CA1 region of the hippocampus, 4-AP is also unable to induce bursting, at least during the ictal phase of epilepsy (Bruckner and Heinemann, 2000).

The effects of apamin and charybdotoxin

Ca²⁺-activated K⁺ channels are essential for the production of bursting activity in mammalian cortical neurons (Jin et al. 2000) and alterations in the genes associated with these channels can lead to generalized epilepsy with paroxysmal dyskinesias (Du et al. 2005). Having this in mind, we tested the effects of specific SK and BK channel blockers, apamin and ChTX, on Ni²⁺-induced bursting on our model.

In standard Ri neither apamin nor charybdotoxin induced any changes in the resting membrane potential or spontaneous activity of the Retzius neuron, which leads to the conclusion that the Ca²⁺-activated K⁺ channels present in Retzius cells are insensitive to these blockers. This conclusion is further substantiated by the absence of changes in input membrane resistance upon the application of apamin or charybdotoxin. The insensitivity of the channels is in agreement with other results of our model. Stewart et al. (1989) and Merz (1995) have shown that IKCa in Retzius cells is not blocked by high concentrations of either ChTX or apamin. Apamin and ChTX do not affect the membrane potential or the input resistance of neuropile glial cells of the leech (Muller et al. 1999), and specific Ca²⁺-activated K⁺ channel blockers also had no effect on the T neurons (Scuri et al. 2002) or S neurons (Burrell and Crisp, 2008) of the leech.

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