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Research Report

Iberiotoxin-sensitive large conductance Ca^{2+}-dependent K^+ (BK) channels regulate the spike configuration in the burst firing of cerebellar Purkinje neurons

Hashem Haghdoost-Yazdi[a,*], *Mahyar Janahmadi*[b], *Gila Behzadi*[b]

[a]*Department of Physiology and Medical Physics, Faculty of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran*
[b]*Neuroscience Research Center and Department of Physiology, Faculty of Medicine, Shaheed Beheshti University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Cerebellar Purkinje cells (PCs) are the sole output neurons of the cerebellar cortex. Mature PCs discharge either tonically Na^+ spikes or bursts of Na^+ spikes ending to a Ca^{2+} spike. These cells express inactivating and non-inactivating large conductance Ca^{2+}-dependent K^+ (BK) channels in their soma and dendrites. Somatic intracellular recording of acutely prepared brain slices was performed to examine the role of BK channels-mediated current in the tonic and burst firing of PCs. Continuous injection of a negative DC current was used to both suppress the spontaneous activity and stabilize the resting membrane potential around −70 mV. Then, the short depolarizing current injection was used to evoke spike discharge. For establishing of the burst firing, 4-aminopyridine (4-AP) was bath applied to the bath solution. Blockade of BK channels with iberiotoxin (IbTx); a specific blocker of BK channels, did not affect the Na^+ spike configuration in the tonic firing but caused a remarkable change in the shape of Na^+ and Ca^{2+} spikes in 4-AP-induced burst. Our results showed that during the burst firing, strong activation of IbTx-sensitive BK channels enhances the amplitude of fast afterhyperpolarization while decreases the duration of both Na^+ and Ca^{2+} spikes. The current from these channels contributes to both the repolarizing of Na^+ spike in the burst and setting of the amplitude of post-pulse AHP that occurs immediately after a depolarizing pulse. These data reveal an important role of IbTx-sensitive BK current in regulating of the spike configuration during the burst firing of PCs.

1. Introduction

In many neurons, a rise in cytosolic concentration of calcium ions activates a number of potassium channels. These Ca^{2+}-activated K^+ channels participate in many of the electrophysiological activities in neurons such as shaping of the action potential, regulating of the firing frequency and synaptic transmission (Canepari and Ogden, 2006; Edgerton and Reinhart, 2003; Faber and Sah, 2002; Kim and Trussell, 2007; Petrik and Brenner, 2007; Sausbier et al., 2004; Shao et al., 1999; Womack and Khodakhah, 2004). Three broad families of Ca^{2+}-activated K^+ channels have been identified that based on the single channel conductance have been classified to large (BK), intermediate (IK) and small conductance (SK) channels (Sah and Faber, 2002). BK channels have single channel conductance 200–400 pS and are selectively blocked by scorpion toxin of iberiotoxin (IbTx) and mycotoxin of paxilline (Galvez et al., 1990). These channels comprise α pore forming and accessory

* *Corresponding author*. Fax: +98 281 3324970 1.
E-mail address: hhaghdoost@yahoo.com (H. Haghdoost-Yazdi).

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β subunits. So far, one α subunit has been identified, while three β subunits, β1, β2/3 and β4 have been cloned. Combination of the α and the β2/3 subunits, which occurs in the brain gives rise to channels that show rapid inactivation. On the other hand, co-assembly of the α and the β4 subunits gives rise to channels that do not show inactivation (Sah and Faber, 2002).

Cerebellar Purkinje cells (PCs) are the sole output neurons of the cerebellar cortex. Their firing pattern is largely controlled by intrinsic membrane properties and PCs show spontaneous spike discharge even after the synaptic inputs are blocked. A striking feature of PCs is their tendency to fire in closely spaced bursts. These bursts contain a varied number of the Na^+ spikes riding on a Ca^{2+} spike and often called Na^+–Ca^{2+} burst (Cavelier et al., 2002; Edgerton and Reinhart, 2003; McKay and Turner, 2004; Llinas and Sugimori, 1980a,b; Swensen and Bean, 2003; Womack and Khodakhah, 2002a,b). For firing of Na^+–Ca^{2+} burst, PCs require intact dendritic arborization. The dendrites generate Ca^{2+} spike, which is propagated to the soma and produces an intense depolarization that in turn, evokes a burst of Na^+ spikes. Clustering of Na^+ spikes into bursts of high-frequency discharge provides a powerful inhibitory control over the activity of cerebellar nuclear neurons (McKay and Turner, 2004; Stuart and Hausser, 1994; Telgkamp and Raman, 2002).

BK channels are expressed in the soma and dendrites of PCs and participate in many aspects of PCs electrophysiological activity (Edgerton and Reinhart, 2003; McKay and Turner, 2004; Womack and Khodakhah, 2003, 2004). Single-channel analysis from inside-out patches has demonstrated that both inactivating and non-inactivating BK channels are expressed in the rat PCs (Widmer et al., 2003). However, function of these

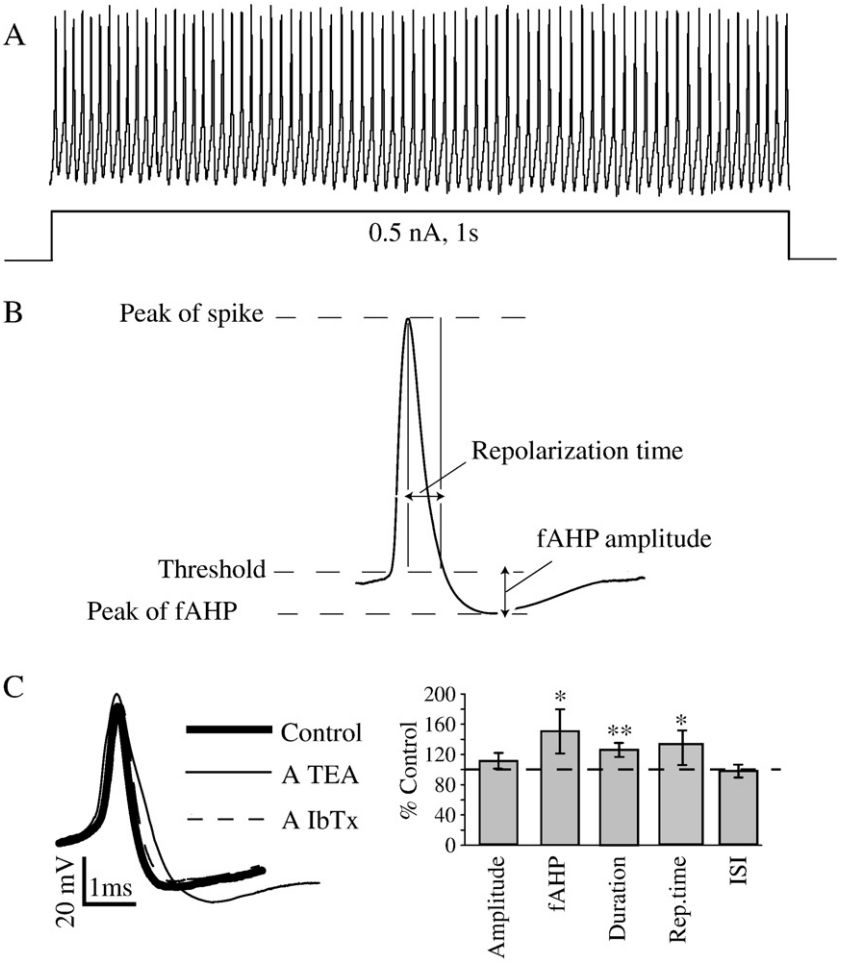

Fig. 1 – IbTx-sensitive large conductance Ca^{++}-activated K^+ (BK) channels have no contribution in the shaping of spike in the tonic discharge of cerebellar Purkinje cells (PCs). (A) Represents the tonic discharge of PCs. After injection of a steady and negative DC current, many of PCs discharged repetitive Na^+ spike in response to depolarizing current injection, which is considered as tonic discharge. (B) Displays protocols for measurement of the various Na^+ spike parameters. (C) Left panel represents superimposed Na^+ spikes recorded in control condition and after superfusion either TEA (1 mM, $n=13$), a non-specific blocker of BK channels or IbTx (100 nM, $n=7$), a specific blocker of BK channels. Plot in right shows percentage change of various parameters of the tonically fired Na^+ spike after application of TEA, normalized to control values (100%, dotted line). TEA had significant effect on the mean half-width, fast AHP (fAHP) and repolarization of Na^+ spike while IbTx had no effect on the configuration of spike. A: after; fAHP: fast afterhyperpolarization; ISI: Na^+ spikes interval; Rep: repolarization. Asterisks indicate significance of test vs. control recordings; $^*P<0.05$; $^{**}P<0.01$; Student's paired t-test.

channels in the different firing modes of PCs has been poorly identified. This is partly, due to diversity in the structure and function of BK channels. The other reasons that may contribute to this issue are complex firing pattern and postnatal developmental changes in the firing mode. It has been demonstrated that PCs from rat pups fire just in the tonic mode while mature PCs show tonic, burst and silence modes in their firing (Cingolani et al., 2002; Hockberger et al., 1989; Mckay and Turner, 2005; Womack and Khodakhah, 2002a,b).

Therefore, the aim of the present study was to investigate the role of BK channels-mediated current on the PCs firing properties specifically the function of these channels in the burst firing. As it was described in a previous paper (Yazdi et al., 2007) the application of 4-aminopyridine (4-AP), a potent blocker of the fast inactivating K^+ current, on the cerebellar brain slices establishes the Na^+–Ca^{2+} burst discharge in PCs. The configuration of the 4-AP-induced burst is very similar to the configuration of spontaneously fired burst but for examining of the burst firing, the 4-AP-induced burst has some advantages. 4-AP-induced burst is more stable and shows little changes during long-lasting recording in compared to the spontaneous burst. By evaluating of the tonic firing and 4-AP-induced burst in the presence of BK channel blockers, we found that BK channels do not participate in the shaping of Na^+ spike in the tonic firing but make remarkable contribution in regulation of the spike configuration during the burst firing.

2. Results

In the presence of the synaptic blockers, most of the recorded PCs showed periods of the spontaneous activity separating by quiescent periods. Concurrent with these active and quiescent periods, the resting membrane potential (RMP) experienced oscillations and it was generally more negative in quiescent periods (−62.9 ± 0.8, n = 60). Because change in the RMP influences

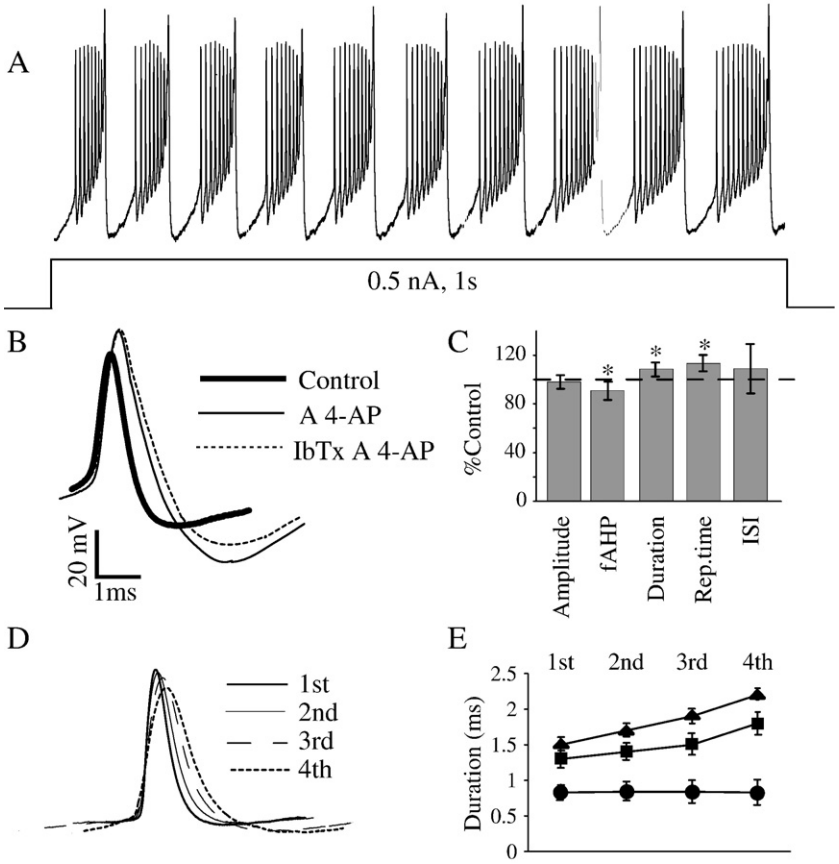

Fig. 2 – The effects of IbTx (100 nM) on Na^+ spike of 4-aminopyridine (4-AP)-induced burst. 4-AP (2 mM) was bath applied to the bath solution after the stabilizing of the resting membrane potential. IbTx was superfused several minutes after the establishment of Na^+–Ca^{2+} burst discharge. (A) Represents the Na^+–Ca^{2+} bursts evoked by injection of a depolarizing current and after application of 4-AP. (B) Superimposed representative of Na^+ spikes recorded in control condition, after application of 4-AP and following the superfusion of IbTx. 4-AP dramatically broadened Na^+ spike and augmented its amplitude (n = 22). Superfusion of IbTx after 4-AP further broadened this spike, reduced the amplitude of fAHP and slowed the repolarization of Na^+ spike. (C) Plot of percentage change of various parameters of Na^+ spike of 4-AP-induced burst after application of IbTx, normalized to control value (100%, dotted line, n = 11). (D) Represents spike broadening (1st to 4th) of Na^+ spikes in a 4-AP-induced burst. (E) Plot represents half-widths of Na^+ spikes (1st to 4th) in the tonic discharge (circles, n = 6), 4-AP-induced burst (rectangles, n = 11) and following superfusion of IbTx (triangles, n = 11). A: after; fAHP: fast afterhyperpolarization; ISI: Na^+ spikes interval; Rep: repolarization. Asterisks indicate significance of test vs. control recordings; *P < 0.05; Student's paired t-test.

the behavior of the ion channels especially voltage-sensitive ones, a negative DC current was continuously injected to both suppress the spontaneous activity and stabilize RMP around −70 mV. Depending on the input resistance and RMP, the magnitude of this DC current varied among neurons (−1.3± 0.9 nA), but it remained unchanged during the entire experiment in each neuron. After stabilizing of RMP, short depolarizing current injection was used to evoke spike discharge. In response to this depolarizing pulse, PCs discharged either slow rising depolarizations or repetitive fast spikes. It has been described that slow rising depolarization is sensitive to cadmium (blocker of voltage-gated Ca^{2+} channels) and is referred as Ca^{2+} spike. Also, fast spike is sensitive to tetradotoxin (TTX, specific blocker of voltage-gated Na^+ channels) and is referred as Na^+ spike and repetitive firing of Na^+ spike is considered as tonic firing. (Cavelier et al., 2002; Llinas and Sugimori, 1980a,b; McKay and Turner, 2004; Womack and Khodakhah, 2002a,b, 2003; Yazdi et al., 2007).

2.1. *The role of BK channels*

To test the role of BK channels on the firing properties of PCs, we examined the effect of BK channel blockers on both the tonic firing and 4-AP-induced burst. We first studied the effect of TEA (1 mM), a potent but non-specific blocker of BK channels on Na^+ spike in the tonic firing (Fig. 1). Superfusion of this agent resulted in a dramatic broadening in the half-width of spike by 23% and enhanced the amplitude of fast afterhyperpolarization (fAHP) up to 50%. The repolarization time, an interval time from peak of the spike to the threshold voltage in the repolarization phase, was also prolonged 33%. Firing frequency and height of the spike did not change significantly. However, in addition to BK channels, TEA also blocks other K^+ channels such as Kv3 and delayed rectifier channels. Therefore, in the next step we examined the effect of IbTx; a selective blocker of BK channels on the tonic firing. Application of this toxin (100 nM) had no affect on the configuration of Na^+ spike and failed to change the duration and the fAHP of spike. These results indicated that IbTx-sensitive BK channels have no contribution in shaping of the spike in the tonic discharge of PCs.

On the other hand, IbTx had marked effects on the spike configuration in 4-AP-induced burst (Fig. 2). To establish the burst firing, 4-AP was bath applied to the bath solution several minutes after the stabilizing of the RMP. Within 5 min after application of 4-AP (2 mM), PCs discharged the Na^+–Ca^{2+} burst. When it was compared to Na^+ spike discharged before application of 4-AP, Na^+ spike of this burst was bigger in amplitude and wider in duration. IbTx was superfused 10 min after 4-AP, when the burst firing was stabilized. Superfusion of IbTx increased further the duration of Na^+ spike up to 17% and decreased the amplitude of fAHP by 9%. Na^+ spikes interval (ISI) did not significantly change indicating that IbTx-sensitive BK channels have no contribution in regulation of the firing frequency during the burst.

In hippocampal pyramidal neurons, Na^+ spikes during repetitive firing show spike broadening, which has been attributed to the fast inactivation of BK channels. We examined this phenomenon in both tonic and burst firing. In the tonic firing, Na^+ spikes did not show spike broadening

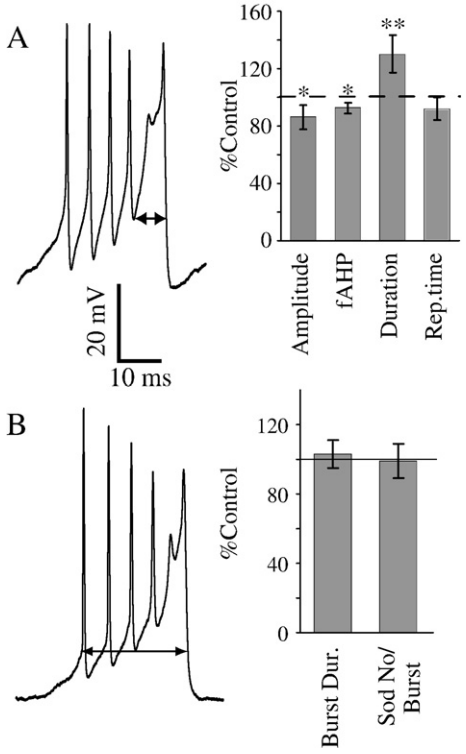

Fig. 3 – Displays the effects of IbTx (100 nM) on the configuration of Ca^{2+} spike and the burst parameters in 4-AP-induced burst. Left panels in A and B display protocols used for measurement of the Ca^{2+} spike duration and burst duration, respectively. Plots in A and B illustrate the effect of IbTx on the various parameters of Ca^{2+} spike and 4-AP-induced burst, respectively. Data was obtained from 11 neurons and has been normalized to control value (100%, dotted line). Dur: Duration; fAHP: fast afterhyperpolarization; No: Number; Rep: repolarization; Sod: Na^+ spikes. Asterisks indicate significance of test vs. control recordings; $^*P<0.05$; $^{**}P<0.01$; Student's paired t-test.

while it was clear during 4-AP-induced burst. In this burst, duration of the spikes was gradually increased from 1st spike to 4th spike and 4th spike was broader up to 40% relative to 1st spike. However, application of IbTx did not affect this broadening indicating that it is unlikely to be attributable to the inactivating BK channels (Figs. 2D and E).

Activation of IbTx-sensitive BK channels had pronounced effect on the configuration of Ca^{2+} spike in 4-AP-induced burst (Fig. 3A). Blockade of these channels decreased significantly the amplitude of both spike and fAHP by 7%. The duration of spike, an interval time from the peak of fAHP of latest Na^+ spike in the burst to falling phase of Ca^{2+} spike, was increased about 30%. Due to the decrease in the amplitude of spike, the repolarization time was decreased but the slope of repolarization did not change. IbTx had no affects on the burst parameters (configuration, duration and number of Na^+ spikes, Fig. 3B) and also did not change the firing pattern.

Beside of the burst and tonic firing, we also examined the role of BK channels in 2 electrical phenomenon of the PCs membrane. Like many of neurons, PCs show post-pulse AHP (PPAHP) that is a negative waveform in membrane potential

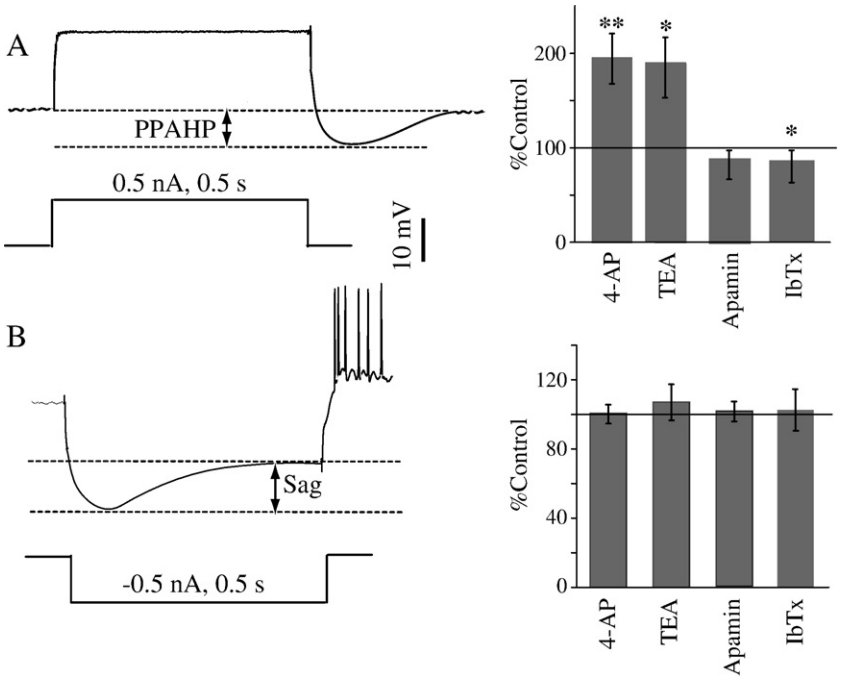

Fig. 4 – Represents the effects of 4-AP (2 mM), TEA (1 mM), IbTx (100 nM) and apamin (200 nM) on the post-pulse AHP (PPAHP) and the sag. Left panels in A and B display the PPAHP and the sag, respectively. Plots in A and B illustrate percentage change of the PPAHP and sag amplitudes after application of the different K^+ channel blockers, respectively. Data in plots have been normalized to control value (100%, dotted line). In both histograms *n* is for 4-AP = 20; for TEA = 13; for IbTx = 11 and for apamin = 13 neurons. Asterisks indicate significance of test vs. control recordings; *P < 0.05; **P < 0.01; Student's paired *t*-test.

occurring immediately after a depolarizing pulse (Fig. 4A). Also PCs show the "sag" that is another negative waveform in membrane potential appearing in the beginning of a hyperpolarizing pulse (Fig. 4B). Application of K^+ channel blockers changed the amplitude of PPAHP. 4-AP and TEA enhanced significantly the amplitude of PPAHP (98%, $n=20$ and 85%, $n=13$, respectively) and IbTx and apamin (200 nM) reduced it (33% and 18%, respectively), the effect of apamin was not significant though. On the other hand, these blockers had no significant effect on the sag ($n=8$ for each blocker) indicating that this phenomenon is created by the ionic currents that are resistant to these agents.

3. Discussion

To identify the role of BK channels in firing properties of PCs, we examined the effects of BK channel blockers on the evoked firing, which in comparison to spontaneous firing is more stable and shows little change in the long-lasting recording. We stabilized the RMP and in this way eliminated its oscillations mediating partly by variations in the I_h potassium current (Williams et al., 2002). The Na^+–Ca^{2+} burst discharge was induced by application of 4-AP that has no known effect on BK channels (Coetzee et al., 1999; Mathie et al., 1998). We found that IbTx-sensitive BK channels make contribution in the shaping of spike in the burst firing but not in the tonic firing. Our results showed in the burst firing, IbTx-sensitive current enhances the amplitude of fAHP, decreases the repolarization time and shortens the duration of both Na^+ and Ca^{2+} spikes. Also, this current enhances the amplitude of PPAHP but does not affect the sag.

Several authors have addressed the role of BK channels in regulation of the spike configuration in neurons (Edgerton and Reinhart, 2003; Faber and Sah, 2002; McKay and Turner, 2004; Shao et al., 1999; Womack and Khodakhah, 2002a,b, 2004). However, this role has been differently revealed in these studies and therefore, it is difficult to identify the precise function of this current. It has been reported that in PCs with the spontaneous tonic firing, blockade of BK channels with IbTx attenuates the amplitude of fAHP but has no affect on the both duration and repolarization of the spike (Womack and Khodakhah, 2002a,b). Some reports show IbTx also affects various burst parameters in PCs (Womack and Khodakhah, 2004). On the other hand, in the amygdalar and pyramidal neurons, application of paxilline (another specific BK channel blocker) broadened significantly the mean half-width of spike, slowed the spike repolarization and just slightly attenuated the fAHP (Faber and Sah, 2002; Shao et al., 1999). While these results describe a role for BK channels in the shaping of spike, McKay and Turner (2004) have demonstrated that application of IbTx has a much small effect on the Na^+ spike configuration in the burst firing of PCs. They have explained that under current clamp recording conditions, IbTx has no effect unless presented in combination with other BK channel blockers. In confirming this report, single channel studies have shown that there are 2 types of BK current in neurons; type I inactivating and type II non-inactivating currents. Type I is sensitive but type II is resistance to IbTx (Sah and Faber, 2002; Wang et al., 2008). Both of the currents are present in PCs but

the non-inactivating current is more dominant (Widmer et al., 2003). Our results obtained from the tonic firing are in agreement with later reports and indicate that IbTx-sensitive and inactivating BK current has no contribution in regulation of firing properties in PCs. But, we cannot entirely rule out the BK channels because TEA affected remarkably the shape of tonically fired Na^+ spike. However, because TEA also blocks Kv3 channels that make large contribution in the spike repolarization in PCs (McKay and Turner, 2004), it is difficult to consider a role for BK channels based on the results obtained with this blocker.

In contrast, our results from burst firing indicate that IbTx-sensitive current contributes significantly in regulation of the spike configuration. Obviously, in comparison to the tonic firing, the burst discharge elicits a different voltage trajectory for membrane potential that certainly affects voltage-sensitive ion channels. For activation, BK channels need both depolarization and influx of Ca^{2+} ions from high-threshold Ca^{2+} channels (Marrion and Tavalin, 1998). They are inactive in RMP and increase in depolarization of the membrane that increases their affinity to calcium and therefore their activity (Womack and Khodakhah, 2002a,b). BK channels comprise from α and 3 different β (β1, β2/3, β4) subunits. Expression of the brain-specific β4 subunits reduces BK channels opening at low Ca^{2+} but increases channel opening at high Ca^{2+} (Wang et al., 2008). This subunit is expressed in Purkinje neurons (Petrik and Brenner, 2007) describing why in our experiments the role of BK channels was revealed in the burst firing. But BK channels containing β4 subunit mediate the IbTx-insensitive current (Petrik and Brenner, 2007; Wang et al., 2008) and accordingly, IbTx must not affect the burst firing. Nevertheless, it has been suggested that alternative splicing in the RNA of the α subunit produces diversity in the physiology of BK channels. BK channels also appear to be regulated by cAMP and different protein kinases and finally there are other β subunits in PCs too (Petrik and Brenner, 2007; Widmer et al., 2003). Taken together, we propose that firing into burst provides conditions for high activation of the IbTx-sensitive BK channels.

There was clear spike broadening in Na^+ spike during 4-AP-induced burst. This phenomenon that was not observed in the tonic firing has been reported for some other cell types. In hippocampal CA1 pyramidal neurons this phenomenon has been attributed to rapid inactivation of BK channels during train of action potentials (Shao et al., 1999). In contrast, we found that in PCs, the spike broadening is unaffected after blockade of BK channels, indicating that it is likely to be caused by a different mechanism. Our results show IbTx-sensitive current makes contribution in PPAHP indicating that this current does not inactivate during depolarizing pulse. Depolarization pulse inactivates many of voltage-sensitive ion channels and in this way produces spike adaptation in some neurons (Faber and Sah, 2002). Thus, it seems that an IbTx-sensitive and non-inactivating BK current contributes in the PPAHP.

Similar to the findings from other authors (Edgerton and Reinhart, 2003; Mckay and Turner, 2004), blockade of BK channels reduced the amplitude of fAHP in Ca^{2+} spike and prolonged it in the burst. However, in contrast to our results, it has been reported that blockade of BK channels augments the height of Ca^{2+} spike. This effect can be attributed to facilitating in dendrosomatic propagation of Ca^{2+} spike (Cavelier et al., 2002; Rancz and Häusser, 2006). 4-AP also augments Ca^{2+} spike in the similar way (Cavelier et al., 2003; Seo et al., 1999) and thus possibly saturated dendrosomatic propagation so that IbTx could not increase further the amplitude of Ca^{2+} spike.

In conclusion, our findings show that a non-inactivating and IbTx-sensitive BK current contributes in regulation of firing properties in PCs. This current has no contribution in the shaping of Na^+ spike in the tonic firing but plays an important role in the shaping of both Na^+ and Ca^{2+} spikes in the Na^+–Ca^{2+} burst discharge.

4. Experimental procedures

4.1. *Preparation of slices*

15- to 30-day-old male Sprague–Dawley rats were anesthetized by inhalation of ether and were then decapitated. The cerebellum was exposed by removing the occipital bone, quickly detached and immersed in ice-cold ACSF consisting of (in mM): 124 NaCl, 5 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2 and 10 glucose, bubbled continuously with a mixture of 95% oxygen and 5% carbon dioxide. Parasagittal slices (300 μm thick) were cut from the vermis using a vibroslicer (752 M, Campden Instruments Ltd, UK). They were allowed to recover in oxygenated ACSF at 36 °C for 1 h and were then kept at room temperature (1–7 h) before experiments were performed. These procedures were in accordance with the guidelines of the Institutional Animal Ethics Committee at Shaheed Beheshti Medical Sciences University.

4.2. *Electrophysiology*

Slices were mounted in a home-made submerged chamber on the stage of an upright microscope (Olympus; BX 51WI) and visualized using a 40× water immersion objective. They were continuously superfused using a peristaltic pump (Hugo Sachs Electronik, Ismatec, Germany) at a rate of 1–2 ml/min with the bubbled ACSF containing 1 mM kynurenic acid and 100 μM picrotoxin to block ionotropic glutamate (Stone, 1993) and GABA (Yoon et al., 1993) receptors, respectively, at room temperature (23–27 °C) and were held in place with a U-shaped platinum-frame nylon net. PCs were easily identified based on their location, size and shape. Intracellular recordings were made from Purkinje cells using thick-walled borosilicate micropipettes (WPI, 120F, with inner filament) pulled by a horizontal puller (APP-1, 52500, Stoelting, USA) and filled with 3 M potassium chloride (resistance 40–80 MΩ) that were connected to the head stage of an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA). The reference electrode in all experiments was a silver–silver chloride wire within an agar bridge (4% agar in ACSF). A piezoelectric stepping motor (Burleigh Instruments Ltd, Harpenden, Herts, UK) was used to advance the microelectrode through the slice in 2 μM steps.

4.3. *Data acquisition and analysis*

Membrane potentials were recorded using the active bridge mode of the amplifier. Traces were filtered at 30 kHz, digitized

on-line using a 16-bit A/D converter (ADInstrument Pty Ltd., Sydney, Australia) at 40 kHz and stored on an IBM-compatible PC for further analysis using Chart 5, Matlab, MiniAnalysis and Excel spreadsheet. For evaluating the role of BK channels-mediated current in firing properties and by continuous injection of a negative DC current through the recording micropipette, both the spontaneous activities were suppressed and resting membrane potential was stabilized around −70 mV. Then, depolarizing current injections were used to evoke spike discharge. For Na^+ spike, the threshold was defined as the potential at which the greatest change in slope of the membrane potential occurred, which was determined from the first derivative of the membrane waveform. The amplitude of the spike and repolarization time was measured relative to this threshold. For Ca^{2+} spike, the amplitude and repolarization time was measured from the peak of spike to the peak of the fast AHP. The Ca^{2+} duration in the burst was determined by measuring the interval time from the latest Na^+ spike and the falling phase of the Ca^{2+} spike in the burst. The burst duration was measured from onset of the first Na^+ spike to the falling phase of Ca^{2+}. All data are expressed as means ± SEM. Student's paired *t*-test was used for statistical evaluation and differences were considered significant if $P<0.05$.

4-amionpyridine (4-AP), tetraethylammonium (TEA), apamin and IbTx were obtained from Sigma. 4-AP was prepared as concentrated stock solution in distilled water and stored at −20 °C in single-use aliquots. TEA, IbTx and apamin were made up to a known concentration in ACSF containing synaptic blockers and applied to the slice by switching the perfusion inlet tube to a different reservoir. Kynurenic acid and picrotoxin were obtained from Fluka and Tocris, respectively.

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