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Blockade of Intrinsic Oscillatory Activity of Cerebellar Purkinje Cells by Apamin and Nickel

Whasook Seo[1], Jean C. Strahlendorf[2], and Howard K. Strahlendorf[3]

[1]Department of Nursing, College of Medicine Inha university, Incheon 402–751, Korea; Departments of [2]Physiology and [3]Pharmacology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, U.S.A.

Intracellular recordings of oscillatory firing (bursting activity) were obtained from Purkinje cells (PCs) in rat cerebellar slices. Apamin inhibited post-burst hyperpolarizations (PBHs) progressively and finally terminated oscillatory firing activity of PCs. Apamin did not affect the amplitude or duration of the after-hyperpolarization (AHP) between spikes within the burst. In the voltage clamp mode, apamin shifted the whole-cell, quasi-steady state I/V relationship in an inward direction and abolished the zero slope resistance (ZSR) region by blocking outward current. Nickel (Ni^{2+}) terminated oscillatory activity and also abolished the ZSR region. However, Ni^{2+} did not have progressive blocking action on the post-burst hyperpolarization before it blocked oscillatory activity. Ni^{2+} blocked an inward current at potentials positive to approximately −65 mV, which was responsible for the ZSR region and outward current at more negative potentials. These data indicated that oscillatory activity of PCs is sustained by a balance between a slow Ni^{2+}-sensitive inward current and an apamin-sensitive outward current in the region of ZSR of the whole-cell I/V curve.

Key Words: Purkinje cell (PC); Oscillatory firing; Nickel-sensitive inward current; Apamin-sensitive outward current; Zero slope resistance (ZSR) region

INTRODUCTION

Llinas (1988) proposed that oscillatory activity potentially affects overall brain function by establishing global frames of reference on which extrinsic environmental information is imposed. At the cellular level, it also has been suggested that oscillatory firing activity in general stabilizes synaptic input and localizes excitability by regulating calcium entry (Greenberg et al, 1986; Llinas, 1988). In PCs, oscillatory firing properties potentially play a role in the formation of stable network for the cell growth and synaptogenesis since immature cerebellar cultures have activity patterns with a marked absence of high-frequency somatic spike bursts (Kapoor et al, 1988)

Corresponding to: Whasook Seo, Department of Nursing, College of Medicine Inha University, Yong-Hyun Dong, Nam-Gu, Incheon 402-751, Korea

Oscillatory firing activity in PCs can be produced by direct stimulation or can occur spontaneously even in the absence of extracellular Na^+ (Kapoor et al, 1988; Llinas & Sugimori, 1980a; Llinas & Sugimori, 1980b). General consensus holds there is no unique single mechanism for sustaining rhythmic bursting activity in single neurons (Harris-Warrick & Flamm, 1987). Rather a number of different ionic mechanisms could produce variants in oscillatory behavior (Gorman et al, 1987). In PCs, mechanisms underlying generation of oscillatory activity are poorly delineated but appear to include a voltage-dependent Ca^{2+} conductance change followed by a Ca^{2+}-dependent conductance (Hounsgaard & Midtgaard, 1988).

One of the most significant membrane properties of neurons displaying oscillatory activity is the presence of a negative slope resistance (NSR) region in the whole-cell, steady-state I/V relationship which is responsible for inward currents (I_{NSR}) at membrane

potentials more negative than approximately −60 mV (Benson & Adams, 1987). In Aplysia neuron R15, it has been shown that Ca^{2+}, Na^{+}, or both, are responsible for I_{NSR} (Adams & Benson, 1985; Gorman et al, 1987). Meech and Standen (1975) also suggested that the N-shaped region of the I/V relationship is partly determined by $I_{K(Ca)}$. A zero slope resistance region (ZSR) region precedes the NSR region of a whole-cell, steady-state I/V relationship and is that portion in which the net current through the variable conductance pathway is close to zero, representing a balanced flow of inward and outward current at this voltage region. In our previous report (Chang et al, 1993), we showed that a ZSR region of the whole-cell, quasi-steady state I/V relationship from PCs was obtained in all neurons generating bursting activity without exception. We also reported that oscillatory activity associated with the presence of the ZSR region was sustained in the presence of TTX or QX-314 (blockers of sodium channels) and cesium (a blocker of hyperpolarization-activated inward current, I_h) although the pattern of the oscillatory activity was altered by application of those blockers.

Because TTX-sensitive Na^{2+} current apparently was not an important contributor, and earlier investigations suggested an involvement of Ca^{2+} and Ca^{2+}-dependent conductances (Hounsgaard & Midtgaard, 1988), we hypothesized that the ZSR region in PCs may be mediated by coupled Ca^{2+} and Ca^{2+}-activated K^{+} conductances. Therefore, the purpose of present study was to evaluate the contribution of Ca^{2+} and Ca^{2+}-activated K^{+} conductances to the oscillatory cycle and the ZSR region using combined current-and voltage-clamp procedures. We used nickel, a blocker of Ca^{2+} conductance, and apamin, an antagonist of the low conductance Ca^{2+}-dependent K^{+} channel ($I_{s,K(Ca)}$), to determine the effect on oscillatory firing and correlated these effects to actions on the ZSR region of the whole-cell, quasi-steady state I/V relationship.

METHODS

Experiments were performed on parasagittal cerebellar slices (350 μm) of adult Sprague-Dawley rats (80~120gm). Slices were held submerged in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature and placed for recording in an interface type chamber that was constantly superfused with ACSF and gassed with 95% O_2/5% CO_2 to a pH 7.3 at 33°C. The ACSF consisted of (in mM): NaCl 124, KCl 5, $MgSO_4$ 1.15, KH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 2.5, and glucose 10 (Wang et al, 1992). All slices were allowed to recover for at least 1 hour before recording.

Intracellular recordings were obtained with micropipettes (50 to 100 MΩ) filled with 3M KCl. Only cells which had membrane potentials more negative than −50 mV and input resistance exceeding 10 MΩ were included in the present report. These values are typical of PCs recorded in this manner (Llinas & Sugimori, 1980a; Llinas & Sugimori, 1980b). PC were current or voltage clamped with a bridge or switching clamp circuit (3~5 KHz, 50% duty cycle, Dagan 8100). During voltage clamping procedures, the membrane potential was swept slowly (approximately 1 mV/sec) to allow voltage-and time-dependent currents to approach presumed quasi-steady state conditions.

To study Ca^{2+} and Ca^{2+}-dependent K^{+} currents active between bursts that presumably contribute to the interburst hyperpolarization-depolarization sequence, the voltage clamp was turned on only between bursts, and the PC was allowed to burst freely at the termination of the voltage clamp. The membrane could not be voltage-clamped at the potentials positive to approximately −45 mV since the clamp was unquenchable to prevent regenerative membrane currents in oscillating PCs. Therefore, the slow voltage ramp was delivered to a cell initially voltage clamped at close to its mean overall membrane potential (−50 to −65 mV), and the voltage command spanned −125 to −45 or −40 mV, depending on whether regenerative currents were elicited. The whole-cell, quasi-steady state I/V curve was obtained by plotting the measured current against the corresponding potential. Membrane potential was corrected for over- or under-shoot by measuring the actual voltage from the voltage monitor trace. Voltage commands, data acquisition and analysis were performed with the aid of a computer running pClamp and Axotape software (Axon Instruments). Data were stored on a pen recorder, videotape, and computer for further analysis.

The characteristics of oscillating activity were analyzed before and after any intervention in current clamp experiments. Parameters of oscillatory activity were as follows: amplitude of post-burst hyperpolarizations (PBHs), duration of after-hyperpolarization

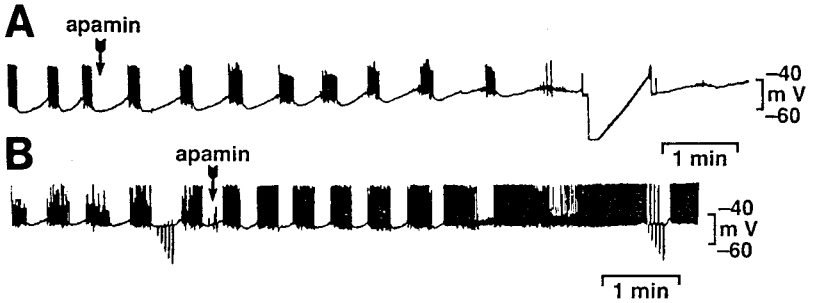

Fig. 1. The effect of apamin on oscillatory activity. A. After local application of apamin (100 nM), post-burst hyperpolarizations decreased progressively culminating in cessation of oscillating activity. Slow ramp was run in the middle of the sample voltage record. B. After local application of apamin (1 nM) in a different PC, the pattern of rhythmicity changed in a few minutes; oscillatory activity ceased, but spontaneous random spikes continued. Downward deflections signify hyperpolarizing current test pulses.

(AHPs) between spikes, and amplitude of AHPs between spikes. Because all PCs studied were firing in an oscillatory fashion they had no true resting Vm; therefore no attempt was made to study effects of apamin or Ni^{2+} on this parameter. For voltage clamp studies, changes in the ZSR region of the whole-cell I/V relationship were examined.

For all experiments, tetrodotoxin (TTX, 0.3 μM, from Sigma) and apamin (1 to 500 nM, from Sigma) were applied by microdrop onto slice. $NiCl_2$ (Ni^{2+}, 100 to 300 μM, from Sigma) was added to the standard ACSF and superfused on to the slice. To compare characteristics of oscillatory activity (several bursts were averaged) before and after any intervention, the unpaired Student's t-test was used. However, when the samples did not support the normality assumption (tested by using Shapiro-Wilk's normality test), a nonparametric procedure (Wilcoxon's rank-sum test) was also applied. A p value of less than 0.05 was set for statistical significance. Error limits are given as standard error of the mean (mean$\pm$S. E.M.).

RESULTS

Blockade of oscillatory activity by apamin

Apamin, which blocks a small conductance $I_{K(Ca)}$ (often referred to as SK in invertebrates) irreversibly, was applied locally (1$\sim$500 nM) to 10 PCs which displayed oscillatory firing activity in the presence of TTX. Apamin decreased the amplitude of PBHs progressively which led to cessation of oscillatory activity in 7 of these 10 neurons (Fig. 1). Of PCs that ceased oscillatory activity after apamin application, some PCs were converted to random, spontaneous single-spike firing (Fig. 1B) and others stopped firing entirely (Fig. 1A). The amplitude of PBHs before apamin application averaged 3.3$\pm$0.2 mV (n=4) and became approximately zero after local application of apamin. Because the duration of the PBH is related

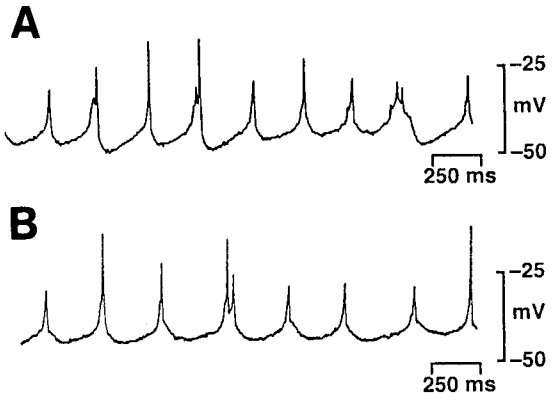

Fig. 2. Comparison of electrical activity before and after apamin application (1 nM) in the same cell as shown in Fig. 1B. A. Before apamin application. B. After apamin application the amplitude and duration of AHPs and the amplitude of action potentials did not change significantly.

Table 1. Effect of apamin on membrane characteristics of oscillating PCs. Data were obtained by measurement of 5 separate bursting sets in each of 4 PCs before and after apamin application

Characteristics of Burst	Mean±S.E.M. Before	After	Percentage Change	p value
Amplitude of PBH between bursts(mV)	3.3±0.2	0.0	100	<0.05
Duration of AHP between spikes(msec)	302.3±207.4	328.5±218.0	9	0.68
Amplitude of AHP between spikes(mV)	7.7±1.7	7.6±1.5	2	0.63
Maximum amplitude of spike (mV)	15.9±7.9	16.5±9.1	4	0.99

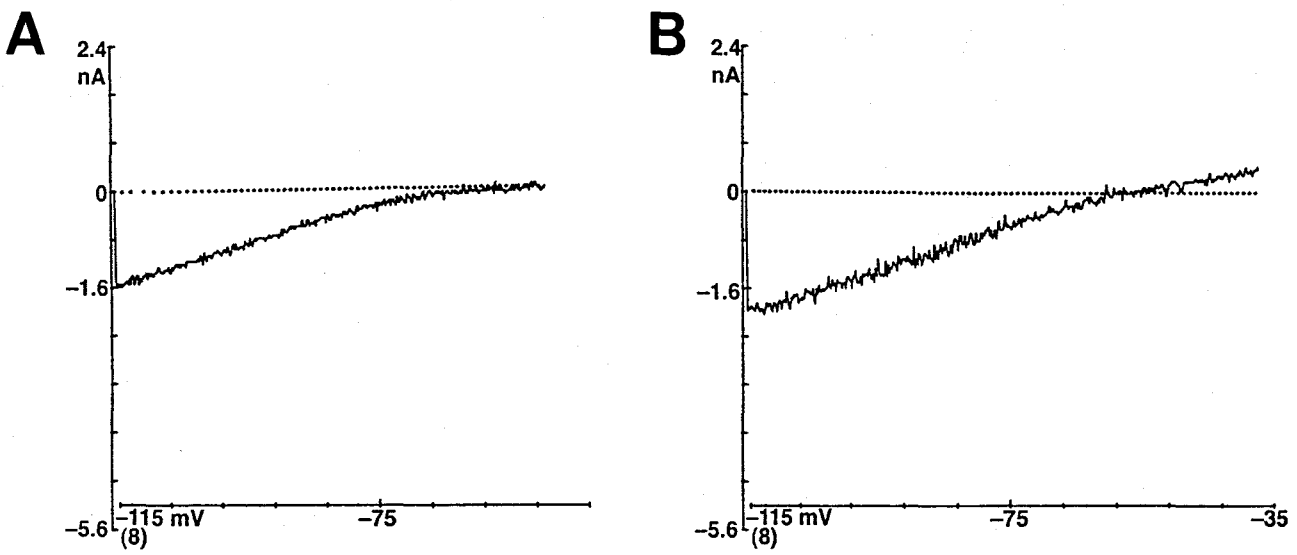

Fig. 3. The effect of apamin (100 nM) in the quasi-steady state I/V curve. A. Before apamin application, the I/V curve showed that the ZSR region started at −60 mV (holding potential was −55 mV). The voltage ramp was terminated at −45 mV because regenerative currents appeared at more depolarized potentials. B. The steady-state I/V curve after applying apamin was nearly linear and crossed the zero current axis at approximately −55 mV. The apamin-sensitive outward current was present constantly at membrane potentials more negative than −60 mV. Regenerative currents were blocked by apamin at potentials depolarized to −35 mV.

to its magnitude, variable effects of apamin on PBH durations were seen depending on whether apamin caused the PC to cease firing entirely (apparent prolonged PBH) or whether the PC assumed a steady random firing pattern (apparent shortened PBH). Apamin did not have any significant effect on the duration and amplitude of AHPs between spikes (Fig. 2 and Table 1). Apamin also did not affect significantly the maximum amplitude of spikes. On occasion, apamin appeared to decrease the maximum amplitude of AHP achieved between spikes and slow the trajectory of its onset (Fig. 2). However, these were transient effects succeeded by a return to near control amplitude of the AHP between spikes before oscillatory activity stopped. Collectively these results imply that an apamin-sensitive current helps sustain bursting activity by contributing to PBHs, but this conductance does not significantly shape interspike

AHPs within bursts.

Effect of apamin on the whole-cell, quasi-steady state I/V relationship of PCs

In normal ACSF, the quasi-steady state I/V curve exhibited an inward rectification and a ZSR region that began at approximately −60mV. Local application of concentrations of apamin identical to those employed in current-clamp experiments resulted in the elimination of the ZSR region and straightening of the whole-cell I/V in the potential range from −65 to −40 mV and (Fig. 3; n=3). This effect was associated with termination of oscillatory activity in the current-clamp mode. In addition, the I/V curve was shifted in the net inward direction with very little change in slope conductance, indicating that apamin blocked outward current at all membrane potentials from the ZSR and more negative in a voltage-independent manner (Fig. 3B). Static holding currents during voltage clamping at −50 to −60 mV were not altered markedly by apamin.

Blockade of oscillatory activity by Ni^{2+}

A Ni^{2+}-containing solution (100 to 300 μM) was superfused onto 6 oscillating PCs in the presence of TTX. Ni^{2+} initially decreased the amplitude of Ca^{2+}-dependent spikes and eventually eliminated all Ca^{2+}-dependent regenerative oscillatory activity (Fig. 4). Furthermore, rhythmic bursting was converted to nonrhythmic bursts or prolonged depolarizing plateaus supporting single spikes that often spontaneously hyperpolarized to a quiescent state (Fig. 4). Unlike apamin, Ni^{2+} did not have any progressive effects on PBHs before it blocked oscillatory activity. Changes in Vm probably were not responsible for stopping oscillatory activity, as the overall Vm after Ni^{2+} ap-

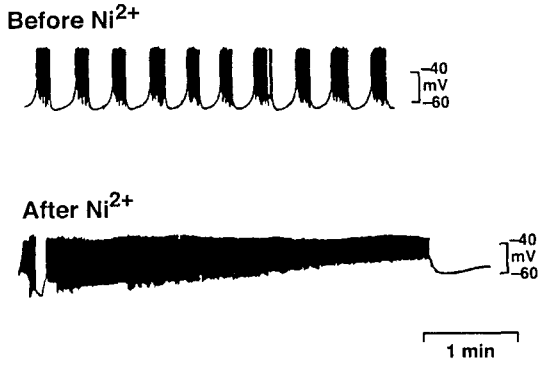

Fig. 4. The effect of Ni^{2+} on the oscillatory activity of PC. After superfusion of Ni^{2+}-containing solution (TTX present), the amplitude of Ca^{2+}-dependent action potentials decreased; finally, the cell became quiescent at a Vm between −50 and −40 mV.

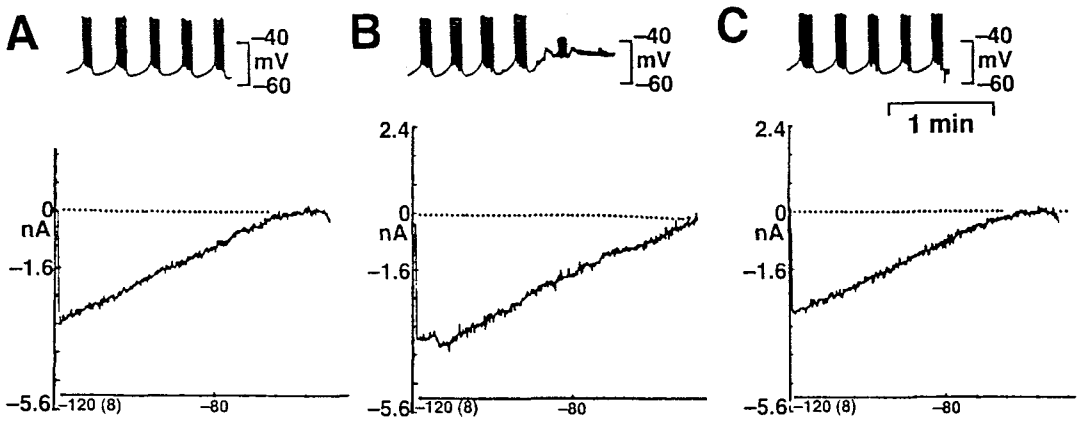

Fig. 5. The effect of Ni^{2+} on the quasi-steady state I/V curve of an oscillating PC. A. The I/V curve (bottom trace) of the oscillating neuron (top trace) before superfusion with the Ni^{2+}-containing solution shows the ZSR/NSR region starting at −60 mV. B. Ni^{2+} produced a decrease in the amplitude of Ca^{2+} spikes before it stopped oscillatory activity (top trace) and eliminated the ZSR/NSR region of the whole-cell I/V relationship (bottom trace). C. After a recovery period of 30 minutes, the I/V curve shows the ZSR/NSR region again (bottom trace) and oscillatory activity has resumed (top trace). The holding potential was −55 mV.

plication remained essentially unchanged and in the potential range (−40 to −65 mV) that readily supports oscillatory firing of PCs. To investigate further the action of Ni^{2+} voltage clamp experiments were conducted.

Effect of Ni^{2+} on the quasi-steady state, whole-cell I/V relationship of PCs

A quasi-steady state I/V curve was obtained before and after superfusion with Ni^{2+}-containing solution (100 to 300 μM). Under control conditions the quasi-steady state I/V curve exhibited typical inward rectification and a ZSR/NSR that began at approximately −60 mV (Fig. 5A). Superfusion of Ni^{2+}-containing ACSF resulted in the reversible elimination of the ZSR/NSR region; this effect was associated with termination of oscillatory activity in the current-clamp mode (Fig. 5B and C; n=4). Close inspection of I/V curves over the potential range of the ZSR revealed the Ni^{2+}-sensitive current to be inward. This was reinforced in that the static holding current underwent a 0.5 nA outward shift after Ni^{2+} application. The I/V curve was shifted in a net inward direction by Ni^{2+} at potentials negative to the ZSR (Fig. 5A and B) indicating that Ni^{2+} blocked outward current in this potential range.

DISCUSSION

Under voltage-clamp conditions, oscillating neurons display a ZSR/NSR region in the steady-state, whole-cell I/V relationship. The ZSR/NSR region can be accounted for mainly by the interaction between inward and outward currents. In the absence of sodium conductance, as in the present study (TTX present), a coupled calcium/potassium activation system can produce a variety of effects, especially on rhythmic activity, since regenerative calcium conductances are opposed by nonregenerative outward potassium current. Therefore, balance of inward and outward currents establishes a flat plateau (the ZSR region) of a few seconds in duration within a narrow range of Vms that gives way to the NSR region as a slow inward current ensues.

Our results show that Ni^{2+} and apamin, agents with distinctly different target conductances, both stopped oscillatory firing of PCs and appeared to affect the whole-cell, quasi-steady state I/V relationship similarly. Both agents abolished the ZSR/NSR region and caused an inward shift of the I/V curve at more negative Vms, but by apparent different mechanisms. Abolishing the ZSR region and I_{NSR} is critical to block oscillatory firing of neurons. Either decreased inward or outward current leads to an imbalance of currents around the ZSR region and prevents the plateau region and I_{NSR}. The I/V curve then crosses the zero current axis with positive slope and abolishes oscillatory activity (Benson & Adams, 1987).

Apamin, an $I_{K(Ca)}$ blocker (Blatz & Magleby, 1986; Cook, 1988; Kawai & Watanabe, 1986; Kukuljan et al, 1992), inhibited PBHs progressively and finally terminated oscillatory activity. Similar results on PBHs were reported by Kukuljan et al, (1992) who showed that in pituitary gonadotrophs blockade of an apamin-sensitive $I_{K(Ca)}$ diminished the amplitude of oscillations or completely abolished them. Apamin-sensitive current is apparently not responsible for AHPs between spikes as no differences in this parameter were seen in any of the PCs examined. Voltage-clamp analysis of PCs revealed block of outward current in the ZSR region by apamin, strongly suggesting that a Ca^{2+}-dependent K^+ conductance contributes to the PBHs and the ZSR region of the I/V curve. In PCs, a Ca^{2+}-dependent K^+ current is known to be active at Vms in the range of the ZSR/NSR region and contributes to inward rectification in this potential range (Crepel & Penit-Soria, 1986) and thus is the most likely target of apamin. Block of a sustained outward conductance in this range of Vms would be expected to produce an inward shift in the static holding current; an action not seen with apamin probably because the apamin-sensitive conductance is electrotonically distant on the dendrites. The cause of the inward current shift seen at more negative potentials is at present unclear, however $I_{K(Ca)}$ could be active at all potentials positive to the K^+ reversal potential since this conductance displays little voltage dependency (Hille, 1992). Alternatively, apamin may have non-selectively affected leak conductances (Reinhardt et al, 1984; Zemkova et al, 1988).

Oscillatory activity in PCs also is apparently Ca^{2+}-dependent, since rhythmic burst firing could be induced and maintained in the presence of TTX or QX-314, blockers of voltage-activated Na^+ current (Chang et al, 1993). Moreover, rhythmic bursting in PCs in the presence of TTX was completely abol-

ished by Ni^{2+}, a commonly utilized antagonist of Ca^{2+} channels with some selectively for the T-type channel (Kaneda et al, 1990; Regan, 1991). Ni^{2+} initially decreased the amplitude of Ca^{2+}-dependent spikes; at the same time, rhythmic single spikes developed into nonrhythmic bursts or prolonged depolarizing plateaus. In voltage clamp mode, application of Ni^{2+} eliminated the ZSR/NSR region by blocking an inward current at these Vms. Although Ni^{2+} might also indirectly affect the outward $I_{K(Ca)}$ current (see below), the I/V relationship showed that the main current affected by Ni^{2+} in the ZSR/NSR potential range was the net inward current. Static holding current also underwent an outward shift after Ni^{2+} application, indicating block of an inward current electrotonically close to the recording electrode. Furthermore, it has been suggested that three Ca^{2+} ions are necessary for the activation of each $I_{K(Ca)}$ channel while only one Ca^{2+} ion is necessary to pass through each inward current channel (Kramer & Zucker, 1985). Therefore, the inward current can be expected to be more sensitive to Ca^{2+} blockade than is $I_{K(Ca)}$.

A Ca^{2+} conductance in many neurons contributes to the slow inward current during the depolarizing phase of pacemaker activity. This inward current is activated by depolarization, first appears between -70 and -50 mV, and produces the characteristic NSR region of the steady-state I/V curve obtained from invertebrate and vertebrate neurons alike (Adams, 1985; Gorman et al, 1987; Schwindt & Crill, 1980). The plateau-generating Ca^{2+} conductance first reported by Llinas and Sugimori (1980a; 1980b) from current clamp experiments in guinea pig PCs has some of the requisite characteristics to initiate and sustain Ca^{2+}-dependent bursting. The plateau Ca^{2+} current activates approximately 10 mV positive to rest (-50 to -45 mV), is slowly or non-inactivating and has been suggested to be of the N or P type (Bertolino & Llinas, 1992; Llinas & Sugimori, 1980b). The T-type Ca^{2+} current (I_T) is another possible current that may depolarize the membrane sufficiently to trigger the plateau Ca^{2+} conductance (Crepel & Penit-Soria, 1986). Intrinsic oscillatory activity in thalamic and inferior olivary nucleus neurons can occur as a result of the activation of I_T, which produces an after-depolarization responsible for the initiation or maintenance of the oscillatory activity (Bertolino & Llinas, 1992). In PCs, T-current is present but rapidly inactivates (Regan, 1991); therefore, this characteristic does not support the idea that I_T is responsible for a NSR region or slowly inactivating inward current in the steady-state I/V relationship. However, an overlap of I_T activation and inactivation curves ("window" current) might contribute a steady influx of calcium over a fairly broad range of potentials (-60 to -40 mV) (Regan, 1991). Moreover, I_T may serve as a trigger for a non-inactivating I_{Ca} and is believed to be located primarily on the somatic membrane of PCs. This could account for the change in holding current after Ni^{2+} mentioned above. Hounsgaard and Prince (1992) reported a distinct form of T-current (I_{TS}) in rat thalamic reticular nucleus. I_{TS} is inactivated much more slowly than I_T and has the proposed function of initiating the generation of long-duration calcium-dependent spike bursts in theses neurons. However, further studies are required to clarify the presence of characteristics of I_{TS} in cerebellar PCs.

Because $I_{K(Ca)}$ is co-activated with I_{Ca}, its presence may have lessened the magnitude of inward current blocked by Ni^{2+}, but indirect removal of $I_{K(Ca)}$ would have enhanced the straightening of the ZSR region of the I/V relationship and acted in concert to stop oscillatory firing. Indeed, one of the first detectable actions of Ni^{2+} was to reduce the height of the Ca^{2+} action potentials, thereby limiting the amount of internal Ca^{2+} available to activate the K^+ conductance. Part of the overall inward shift of the whole cell I/V relationship at potentials between the ZSR region and more negative potentials conceivably resulted from this indirect action of Ni^{2+} on a K^+ conductance. It is reasonable to postulate that oscillatory activity in PCs is controlled by different systems which are linked, including inward and outward currents. Persistent inward current, carried primarily by Ca^{2+} ions through voltage-dependent channels seems to exhibit some steady-state activation at Vms within the ZSR/NSR range. This inward current provides the depolarization needed to bring the membrane potential to threshold for action potential initiation. During a burst of action potentials, a large influx of Ca^{2+} through voltage-dependent Ca^{2+} channels may inactivate high threshold Ca^{2+} channels, thereby limiting the depolarization, and activate K^+ channels, thus causing an opposing outward current. Hence, the burst period is terminated and the membrane starts to hyperpolarize, which removes inactivation of I_{Ca}. At more hyperpolarized potentials, inward flux of cations through I_h (I_q) channels (Chang

et al, 1993), counterbalances outward flux and results in membrane depolarization towards the threshold for activation of I_{Ca}. Thus, the linkage between inward and outward currents which accounts for the ZSR/NSR region of the whole cell I/V curve provides for the activation of I_{Ca}, the rise and decay of intracellular Ca^{2+}, and for the activation of $I_{K(Ca)}$ that is apparently crucial for maintaining oscillatory firing activity in cerebellar PCs.

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