Kor J Physiol (1995) 29(1), 103-111

Induction of Oscillatory Firing Activity by TTX in Rat Cerebellar Purkinje Cells

Whasook Seo

Inha University, Department of Nursing, College of Medicine

=ABSTRACT=

Intracellular recordings were obtained from Purkinje cells in rat cerebellar slices maintained in vitro. Adding tetrodotoxin to the superfusion solution produced a typical pattern of repetitive burst firing consisting of a cluster of action potentials followed by a long hyperpolarization. TTX-induced oscillatory activity was not due to modulation of membrane potential although underlying mechanisms for maintenance of oscillatory activity were influenced by membrane voltage. The mechanism of TTX-induced oscillation was not related to the presence or amplitude of I_h and could still induce the oscillatory activity after blockade of I_h by cesium. The result from an experiment in which QX-314 was injected intracellularly strongly suggested that TTX-induced oscillatory firing activity was due to blockade of post-synaptic Na^+ currents intrinsic to PCs.

Key Words: Purkinje cell, Oscillatory firing activity, Sodium current, Tetrodotoxin

INTRODUCTION

Burst responses can be produced in cerebellar Purkinje cells (PCs) by direct stimulation or spontaneously in the absence of extracellular Na^+ (Llinas and Nicholson, 1971; Llinas and Sugimori, 1980a and 1980b; Kapoor et al, 1988). The mechanism underlying the generation of this response appears to be a voltage-dependent Ca^{2+} conductance change followed by a Ca^{2+}-dependent conductance (Llinas and Sugimori, 1980a and 1980b). However, the ionic conductances involved in the oscillatory activity are yet to be fully determined.

The oscillatory activity in CNS has been proposed to have diverse functional roles such as establishing a frame of reference (Llinas, 1988), controlling calcium concentration (Greengard, 1987; Llinas, 1988), and regulating gene expression and development (Llinas, 1988). Kapoor et al (1988) suggested that this activity in PCs may related to cell growth and synaptic formation since immature cerebellar cultures have patterns with a marked absence of high-frequency somatic spike bursts. Autorhythmic electrical properties, therefore, may form the basis for an intrinsic functional context of PCs.

Tetrodotoxin (TTX) is commonly used to block the sodium channel specifically in order to study other membrane conductances in many cell types (Llinas and Sugimori, 1980a and 1980b; Aubry et al, 1991). TTX has been accepted also as a synaptic blocking agent since it suppresses the action potential-induced release of neurotransmitters. In the cerebellar slice preparation, unex-

pected induction of oscillatory firing behavior was observed after TTX application. The present study was to define the effect of TTX on cerebellar PCs in vitro.

METHODS

Experiments were performed in sagittal cerebellar slices (350 μm) of adult Sprague-Dawley rats (80~120 gm) prepared using a vibroslice (Campden Instruments). Slices were held at room temperature submerged in an oxygenated bath containing an artificial cerebrospinal fluid (ACSF, composition given below) and superfused with ACSF containing (in mM): NaCl 124, KCl 5, $MgSO_4$ 1.15, KH_2PO_4 1.25, $NaHCO_3$ 28, $CaCl_2$ 2.5, Glucose 10 (Wang et al, 1991), that was gassed with 95% O_2/5% CO_2 to a pH of 7.3 at 33°C. Intracellular recordings of PCs were made using micropipettes filled with 3 M KCl, having impedance of 50~100 MΩ. Only cells which had membrane potentials more negative than -50 mV and input resistances exceeding 10 MΩ were included in the present report. These values are typical of PCs recorded in this manner (Llinas and Sugimori, 1980a and 1980b; Crepel and Penit-Soria, 1986).

The neuron was current or voltage clamped with a bridge or switching clamp circuit (3~5 kHz, 50% duty cycle, Dagan 8100) (Wang et al, 1991). Voltage commands, data acquisition and analysis were performed with the aid of a computer running pClamp and Axotape software (Axon Instruments).

For all experiments, TTX (0.3 μM) was applied by microdrop onto the slice. Lidocaine-N-ethyl iodide quaternary salt (QX-314, 10 mM) was mixed with 3 M KCl electrode solution and injected intracellularly. Cesium (Cs^+, 5~10 mM) was dissolved in the superfusion medium.

The parameters used to define the characteristics of oscillatory activity are as follows: duration of burst (sec), duration of post-burst hyperpolarization (sec), amplitude of post-burst hyperpolarization (mV), number of spikes per burst, threshold of plateau potential (mV), amplitude of plateau potential (mV), threshold of burst (mV), threshold of spike (mV), maximum amplitude of spikes (mV), and maximum amplitude of afterhyperpolarization between spikes (mV). To compare characteristics of TTX-induced and QX-314-induced oscillatory activity (several sets of bursts were averaged), the unpaired student's t-test was used. However, when the sample did not support the normality assumption (tested by using Shapiro-Wilk's normality test), a nonparametic procedure, Wilcoxon's rank-sum test, was applied. To analyze the effect of TTX on the amplitude of I_h in voltage responses of membrane during hyperpolarizing current injections (0.2, 0.4, 0.6, 0.8, and 1.0 nA), the analysis of variance procedure was used. Averages are presented with standard error of the mean (mean $\pm$ S.E.M.).

RESULTS

Effect of TTX on PCs

Adding TTX (0.3 μM) on PCs which did not display oscillatory activity spontaneously produced the following responses (Fig. 1): (1) suppression of single spikes followed by the typical repetitive bursting mode of firing (Fig. 1A), (2) appearance of oscillatory bursting mode of firing in cells that were quiescent (Fig. 1B), and (3) no visible effect on quiescent neurons (Fig. 1C). Although the first two types of responses could be attributed to the application of TTX, the third was difficult to interpret in the absence of activity. TTX also applied on the spontaneous bursting PCs and it changed the pattern and frequency of oscillatory firing activity (Chang et al, 1993). Table 1 summarizes the results from 64 PCs before and after TTX application. The ability of TTX to

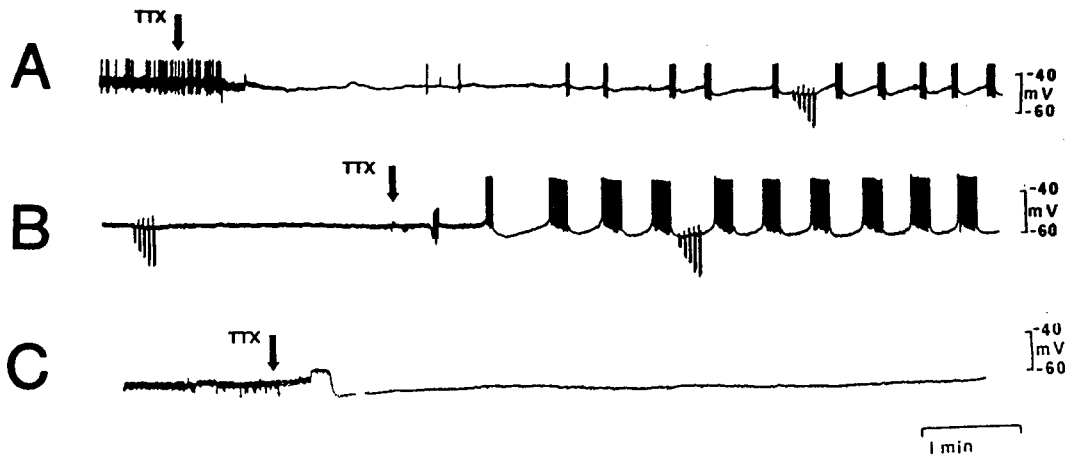

Fig. 1. Effects of TTX on the spontaneous display of oscillatory activity in PCs. A. In a cell displaying random spiking, application of TTX first stopped all activity and then initiated oscillatory activity. B. In a quiescent cell, TTX induced oscillatory activity. C. In a quiescent cell, TTX had no effect on firing activity. Downward deflections in panel A and B signify hyperpolarizing current pulses to test the slope resistance of the cell membrane.

Table 1. Result of TTX application on PCs

Type of spontaneous activity	Oscillation after TTX	No Oscillation after TTX	Total	Percentage (%)
Random spiking	9	26	35	54.7
Quiescent	8	12	20	31.3
Oscillation	9	0	9	14.1
Total	26	38	64	
Percentage (%)	40.6	59.4		

induce membrane oscillatory activity was not different (χ^2=1.2163, $0.2<p<0.3$) between quiescent and randomly spiking PCs. The effectiveness of TTX was evidenced by disappearance of Na^+ spikes (Fig. 2).

Membrane responses to constant hyperpolarizing current injection

The membrane of PCs hyperpolarized by approximately 4 to 5 mV immediately before oscillatory activity began after TTX application, irrespective of the ability of TTX to induce oscillatory activity. For this reason, constant hyperpolarizing current pulses were injected in cells that did not display spontaneous oscillatory activity to test whether the membrane potential could trigger oscillatory activity (Fig. 3A and 3B). The amount of injected current was varied (0.1 to 0.6 nA) to determine if there was a critical membrane potential for induction of this activity. Among 14 cells tested, whether cells had random spike activity (n=8) or were quiescent (n=6), none initiated oscillatory activity during or after constant hyperpolarizing current injection (membrane

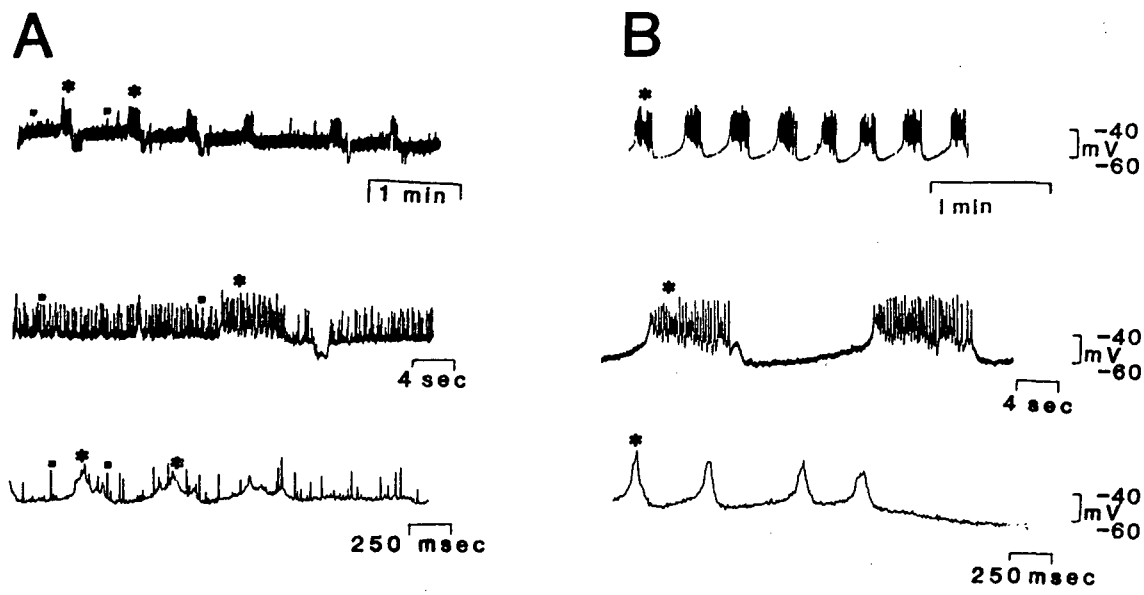

*Fig. 2. Blockade of Na^+-dependent spikes by TTX application. A. A cell's oscillatory activity was maintained by Ca^{2+}-dependent action potentials (indicated by *) and exhibited Na^+-dependent spikes (indicated by dot) superimposed upon the resting membrane potential (top trace). High-speed recording showed Ca^{2+}- and Na^+-dependent spikes in detail (bottom trace). B. After TTX application, the pattern of oscillatory activity changed (top trace) and Na^+-dependent action potentials disappeared (bottom trace) but oscillatory firing activity was maintained. Descending traces in each column are at successively faster sweep rates.*

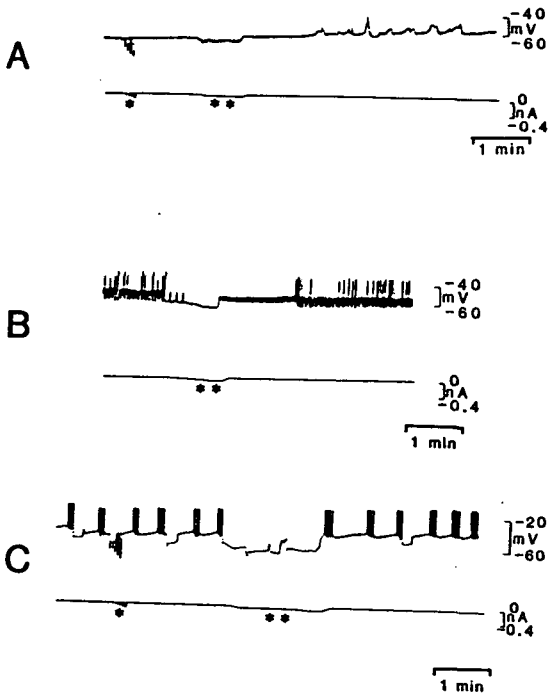

*Fig. 3. Membrane responses (upper traces) to constant hyperpolarizing current injection (lower traces) on a non- oscillating PC. A. Constant hyperpolarizing current injection (indicated by **) did not initiate oscillatory activity in a quiescent PC. Membranae potential was hyperpolarized to −65 mV by rapidly increasing the current to 0.1 nA for 50 seconds. Downward deflection signify hyperpolarizing test pulses (indicated by *) B. the same result is shown in a cell that did not display spontaneous oscillatory activity and had only random spiking activity. In this cell, the membrane potential was gradually hyperpolarized to −70 mV by slowly increasing the current to 0.2 nA over 1 minute. C. Membrane responses of an oscillatory neuron to constant hyperpolarizing current pulses. Injected hyperpolarizing current (**) suppressed bursting activity completely. Within 2 minutes the membrane hyperpolarized to −65 mV. Note that a shorter duration of hyperpolarization (*) did not stop this activity.*

Table 2. Analysis of variance for the amplitude of I_h before and after TTX application

Source	DF	MS	F Value	Pr<F
Model	6	160.88	48.49	0.0001
Error	113	3.32		
Corrected Total	119			
Group (Before vs After TTX)	1	2.92	0.88	0.35
Hyperpolarizing Currents	5	192.47	58.01	0.0001

DF=Degree of Freedom, MS=Mean Squares

hyperpolarization obtained by constant hyperpolarizing currents was −9.4±2.7 mV). In 4 cells that had a spontaneous oscillatory activity, complete suppression of the burst activity was obtained by constant hyperpolarizing current pulses (the membrane hyperpolarization achieved by constant hyperpolarizing current was −7.8±3.1 mV) (Fig. 3C).

TTX-induced oscillatory activity and I_h

Although it is generally considered that TTX does not affect I_h (Foehring and Waters, 1991), Hotson et al (1979) reported that I_h was blocked by TTX in hippocampal neurons. The result of this study showed that TTX did not affect the amplitude of I_h significantly (Table 2). The amplitude of underlying I_h was estimated by measuring the magnitude of the time- and voltage-dependent sags that could be seen in the voltage responses of the membrane to hyperpolarizing current pulses (0.2, 0.4, 0.6, 0.8, and 1.0 nA).

To assess the possibility that cells displaying TTX-induced oscillatory activity might contain a large I_h conductance, two sets of experiments were completed with the following results: First, the magnitude of I_h did not differ between cells in which TTX initiated oscillatory activity and those in which it did not (n=10) (Fig. 4). These neurons were selected by having membrane potentials

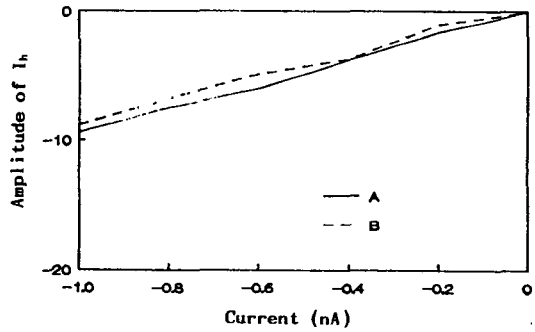

Fig. 4. Comparison of the amplitudes of I_h between PCs in which TTX initiated oscillatory activity (line A) and other PCs in which it did not (line B). The cells selected had membrane potentials between −60 to −70 mV.

between −60 to −70 mV because I_h should be activated in that voltage range. Second, TTX could induce membrane oscillatory activity after blockade of I_h by Cs^+ (n=4) (Fig. 5). Cs^+ (5 mM) blocked most of the voltage- and time-dependent sags that represented I_h as shown in Figure 5, therefore, the mechanism of TTX-induced oscillatory activity was not related to the presence or amplitude of I_h initially present in those neurons.

Comparison of TTX- and QX-314-induced oscillatory activity

QX-314, a lidocaine derivative that selectively blocks postsynaptic Na^+ channels, was introduced

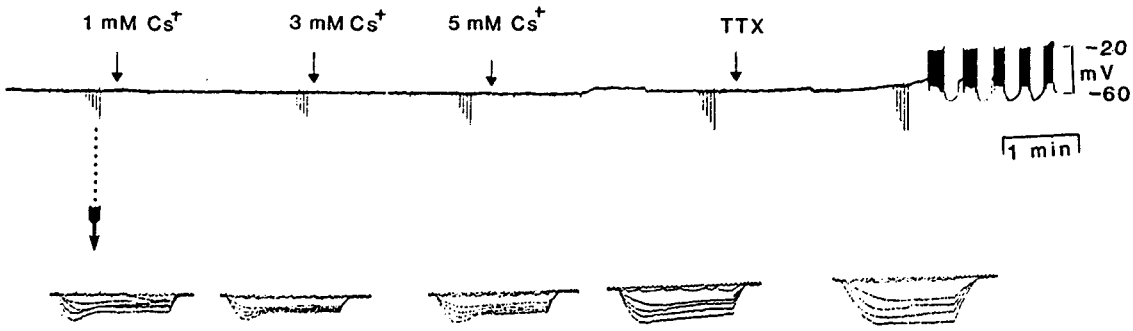

Fig. 5. The effect of TTX on oscillatory activity after blockade of I_h. TTX produced membrane oscillatory activity after I_h was blocked by bath application of cesium (top trace). The downward deflections in the top trace signify hyperpolarizing current pulses to measure slope resistance and amplitude of I_h (bottom trace). The amplitude of I_h decreased progressively after application of higher concentrations of cesium (bottom traces). Note that by the fourth test for the presence of I_h, it was blocked approximately 80% by cesium and by the fifth test, it was virtually eliminated at that time oscillatory activity begins.

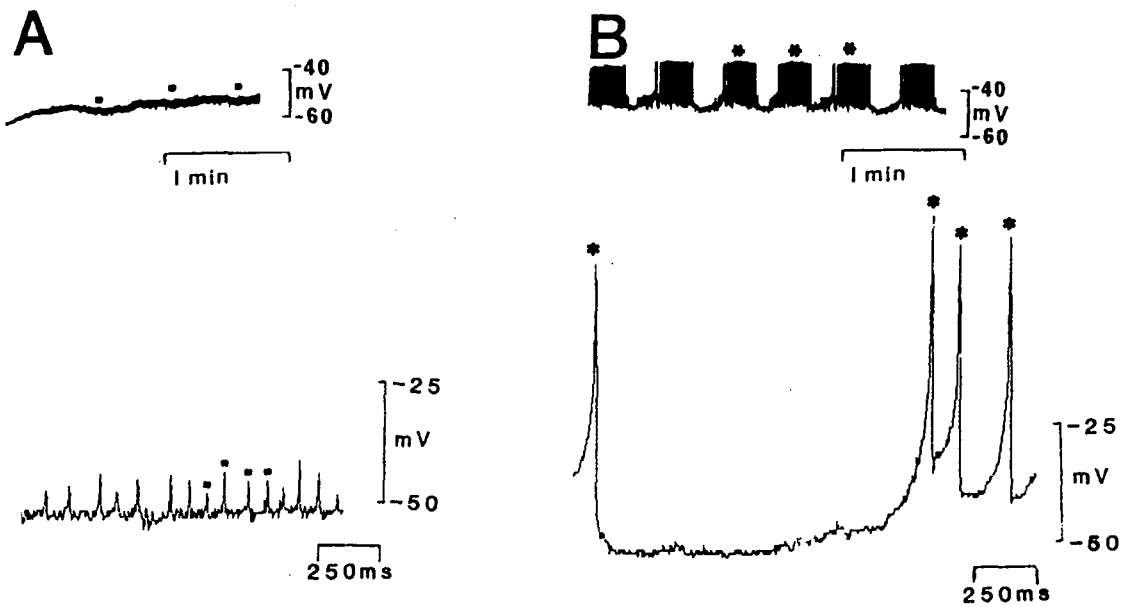

*Fig. 6. Blockade of Na^+-dependent spikes by QX-314 application. A. Intracellular activity of a cell immediately after impalement with a QX-314-filled electrode shows Na^+-dependent spikes (indicated by dots). Bottom traces in A and B are fast speed and high resolution recordings of the same cell. B. In a continuous recording from the same cell as in panel A, oscillatory activity comprising Ca^{2+}-dependent action potentials (indicated by *) is initiated after 5 minutes of impalement of QX-314-filled electrode, but Na^+-dependent spikes are blocked.*

Table 3. Comparison of parameters in sets of burst between TTX- and QX-314-induced oscillations. Data were obtained from 4 different bursting sets in each of 7 PCs in each groups (*: p value < 0.05)

Parameters of burst	Mean±S.D.		p value
	QX-314	TTX	
Duration of burst (sec)	9.0±2.5	10.4±4.0	0.7015
Duration of post-burst hyperpolarization (sec)	13.3±5.2	14.4±4.7	0.9999
Amplitude of post-burst hyperpolarization (mV)	6.9±1.8	5.1±1.9	0.1413
Number of spikes per burst	77.8±31.7	47.6±14.6	0.0736
Threshold of plateau potential (mV)	−55.5±3.0	−51.3±8.6	0.5510
Amplitude of plateau potential (mV)	6.1±2.3	8.4±2.8	0.3711
Threshold of burst (mV)	−50.7±4.5	−43.6±5.2	0.0383*
Threshold of spike (mV)	−52.5±3.9	−46.6±5.5	0.0550
Maximum amplitude of spikes (mV)	24.3±14.8	32.3±9.7	0.2496
Maximum amplitude of afterhyperpolarization between spikes (mV)	1.6±1.5	4.1±0.6	0.0024*

directly into cell to ascertain that TTX induced oscillatory activity by blocking Na^+ current. In this study, QX-314 was introduced by passive diffusion from the recording pipette. Because oscillatory activity is extremely voltage dependent, depolarizing pulses to inject QX-314 were not used. Cells initiated oscillatory activity approximately 10 minutes after impalement by a QX-314-filled electrode (n=13). The effectiveness of QX-314 in blocking Na^+ conductances was determined by the disappearance of fast, apparently Na^+-dependent spikes that could be observed on the oscilloscope (Fig. 6). To ascertain that both TTX and QX-314 induced oscillatory activity by same mechanism, the firing pattern was compared between TTX- and QX-314- induced oscillatory activity and the results were as follows. QX-314-induced oscillatory activity did not differ from TTX-induced oscillatory activity in most characteristics of bursting except that QX-314-induced oscillatory firing carried a smaller amplitude of afterhyperpolarization (AHP) between spikes and had a more hyperpolarized threshold for bursts ($p<0.05$) (Table 3).

DISCUSSION

TTX selectively blocks voltage-dependent sodium channels and is thought to have no other effects (Aubry et al, 1991; Hille, 1992). For this reason, TTX is widely used to block sodium-dependent action potentials in excitable cells. When TTX was used as a synaptic blocker, the unexpected phenomenon of TTX-induced oscillatory firing activity was observed. This study was to define the effect of TTX on the induction of oscillatory activity observed in cerebellar PCs.

Immediately before TTX-elicited oscillatory activity began, the membrane hyperpolarized by approximately 4 to 5 mV. However, injection of hyperpolarizing currents without TTX did not trigger oscillatory activity in quiescent and randomly spiking PCs. On the contrary, a complete suppression of the burst activity was obtained by constant hyperpolarizing current pulses in PCs which displayed spontaneous oscillatory activity. These results indicate that the underlying mech-

anisms for maintaining oscillatory activity are influenced by membrane voltage. However, the mechanism of TTX induced oscillatory activity in PCs is not related to modulation of membrane potential. The same result was shown in the experiment reported by Aubry et al (1991) while the present experiments were in progress.

Hotson et al (1979) reported that I_h was blocked by TTX in hippocampal neurons, although it is generally considered that TTX does not affect I_h (Foehring and Waters, 1991). We have already shown that I_h was an important determinant for the pattern of oscillatory activity by modulating the amplitude and duration of interburst hyperpolarization in the previous report (Chang et al, 1993). Results from experiments to assess whether TTX initiated oscillatory activity by affecting I_h showed that TTX did not block I_h. Also the magnitude of I_h recorded in cells in which TTX initiated oscillatory activity and those in which it did not were not different. Moreover, TTX could induce membrane oscillatory activity after I_h had been blocked with cesium. Therefore, the mechanism of TTX-induced oscillation is not related to the presence or amplitude of I_h originally observed and I_h is not necessary for the induction and maintenance of oscillatory activity.

The experiments with QX-314 showed the same results as those with TTX. Both TTX and QX-314 blocked Na^+ current and induced oscillatory activity. The patterns of the oscillatory activity induced by these two compounds were similar in most characteristics with two exceptions: the amplitude of afterhyperpolarization (AHP) between spikes, and the threshold potential for burst. The amplitude of AHP between spikes was smaller, and the threshold potential of bursts was more hyperpolarized in QX-314-induced oscillating PCs ($P<0.05$). A small effect of QX-314 on the $I_{K(Ca)}$ and G-protein gated potassium channels has been showed by Andrade (1991) and Oda et al (1992). Oda et al (1992) suggested that the blockade of $I_{K(Ca)}$ by QX-314 was relatively small compared with the blockade of the Na^+ channel. QX-314 also has been found to block some G-protein-gated potassium channels but not those responsible for the maintenance of the resting membrane potential nor those underlying slow after- hyperpolarization in hippocampal neurons (Andrade, 1991). The smaller AHP in QX-314-induced oscillatory activity, therefore, may be explained by effects of QX-314 on $I_{K(Ca)}$ or, which is less likely, G-protein-gated potassium channels.

It has been known that QX-314 blocks both fast, Na^+-dependent action potential and the voltage-dependent, non-inactivating Na^+ conductance. Because of its permanent cationic charges, QX-314 has the advantage that it can be applied intracellularly without appreciable diffusion through membranes to adjacent neural or synaptic elements (Cornnors and Prince, 1982). In this study, both TTX (applied onto the slice) and QX-314 (applied into the cell directly) could cause oscillatory activity. It appears that the mechanism of the TTX-induced oscillatory activity is related to the blockade of the post-synaptic Na^+ channel. Aubry et al (1991) also showed that TTX induced oscillatory activity in rat PCs; but bicuculline, a competitive antagonist at the GABA receptor, produced only an increase in the firing and did not trigger bursting activity. This result further indicated that the oscillatory activity elicited by TTX was not due to the blockade of presynaptic Na^+-dependent input to PCs.

Aubry et al (1991) proposed that the mechanism of TTX-induced oscillatory activity might be related to a Na^+/Ca^{2+} exchanger. They hypothesized that if TTX reduces Na^+ influx, cytoplasmic $[Na^+]$ should be decreased below normal by this activity. The increased driving force for sodium would increase the activity of a Na^+/Ca^{2+} exchanger, thus reducing intracellular free $[Ca^{2+}]_i$.

The reduced $[Ca^{2+}]_i$ might close calcium dependent K^+ channels which, in turn, might depolarize the membrane sufficiently to active Ca^{2+} conductances to produce bursting. This might explain the increased number of spikes during a bursting period if a certain increase in $[Ca^{2+}]_i$ is required to activate enough $I_{K(Ca)}$ to terminate the burst. However, it is not known whether or not Na^+/Ca^{2+} exchangers are present in PCs. Further studies using fluorescent dyes to track the changes in the various ions will be necessary to establish the presence of this exchanger in PCs. Moreover, the development of pharmacological compounds to approach the Na^+/Ca^{2+} exchanger specifically will also be necessary.

Another possibility, which we showed in a previous report (Chang et al, 1993), is that elimination of the I_{Na} pathway increases membrane resistance in the ZSR region. This would possibly allow any small current perturbation (i.e., spontaneous channel opening) to produce greater depolarization, thereby moving Vm into the ZSR/NSR region. When a sufficient number of action potentials occur in rapid succession, the accumulated $[Ca^{2+}]_i$ could activate enough $I_{K(Ca)}$ to prevent the next burst from firing.

REFERENCES

Andrade R (1991) Blockade of neurotransmitter-activated K^+ conductance by QX-314 in the rat hippocampus. *European J Pharmacology* **199**, 259-262

Aubry A, Batini C, Billard JM, Kado RT & Morian P (1991) Tetrodotoxin induced calcium spikes: in vitro and in vivo studies of normal and deafferented purkinje cells. *Experimental Brain Research* **84**, 297-302

Chang WS, Strahlendorf JC & Strahlendorf HK (1993) Ionic contributions to the oscillatory firing activity of rat Purkinje cells in vitro. *Brain Research* **614**, 335-341

Cornnors BW & Prince DA (1982) Effects of local anesthetic QX-314 on the membrane properties of hippocampal pyramidal neurons. *J Pharmacology and Experimental Therapeutics* **220**, 2, 476-481

Crepel F & Penit-Soria J (1986) Inward rectification and low threshold calcium conductance in rat cerebellar purkinje cells. An in vitro study. *J Physiology* **372**, 1-23

Foehring RC & Waters RS (1991) Contributions of low-threshold calcium current and anomalous rectifier (I_h) to slow depolarizations underlying burst firing in human neocortical neurons in vitro. *Neuroscience Letters* **124**, 17-21

Greengard P (1978) Phosphorylated proteins as physiological effectors. *Science* **199**, 13, 146-152

Hille B (1992) Ionic channels of excitable membrane (2nd ed.). Sinauer, Sunderland, Massachusetts

Hotson JR, Prince DA & Schwartzkroin (1979) Anomalous inward rectification in hippocampal neurons. *J Neurophysiology* **42**, 3, 889-895

Kapoor R, Jaeger CB & Llinas R (1988) Electrophysiology of the mammalian cerebellar cortex in organ culture. *Neuroscience* **26**, 2, 493-507

Llinas R & Nicholson C (1971) Electrophysiological properties of dendrites and somata in alligator purkinje cells. *J Neurophysiology* **34**, 534-551

Llinas R & Sugimori M (1980a) Electrophysiological properties of in vitro purkinje cell somata in mammalian cerebellar slices. *J Physiology* **305**, 171-195

Llinas R & Sugimori M (1980b) Electrophysiological properties of in vitro purkinje cell dendrites in mammalian cerebellar slices. *J Physiology* **305**, 197-213

Llinas R (1988) The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. *Science* **242**, 1654-1664

Oda M, Yoshida A & Ikemoto Y (1992) Blockade by local anesthetics of the single Ca^{2+}-activated K^+ channel in rat hippocampal neurons. *Br J Pharmacology* **105**, 63-70

Wang Y, Strahlendorf JC & Strahlendorf HK (1991) A transient voltage-dependent outward potassium current in mammalian cerebellar purkinje cells. *Brain Research* **567**, 153-158