ผลของกรดวาลโปรอิลไฮดรอกซามิก ต่อเซลล์ประสาทเปอร์กินเจในเปลือกสมองน้อยในหนูแรท

นางสาว สลิล มิ่งมาลัยรักษ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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EFFECT OF VALPROYL HYDROXAMIC ACID ON CEREBELLAR PURKINJE NEURONES IN RATS

Miss Salin Mingmalairak

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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จุฬาลงกรณ์มหาวิทยาลัย

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การวิจัยนี้มีจุดมุ่งหมายที่จะศึกษาผลของกรดวาลโปรอิลไฮดรอกซามิก ซึ่งเป็นอนุพันธ์ของกรดวาลโปรอิก โดยทำการศึกษาเปรียบเทียบกับกรดวาลโปรอิกต่อเซลล์ประสาทเปอร์กินเจในเปลือกสมองน้อยในหนูแรท โดยใช้เทคนิคไมโครไอออนโตฟอเรซีสในการผลักสารออกจากปลายอิเล็คโตรด

จากการวิจัยนี้พบว่ากรดวาลโปรอิลไฮดรอกซามิกออกฤทธิ์ลดอัตราการปลดปล่อยกระแสประสาทของเซลล์ประสาทเปอร์กินเจในเปลือกสมองน้อยในหนูแรทได้เช่นเดียวกับกรดวาลโปรอิก ซึ่งเมื่อศึกษาร่วมกับสารสื่อประสาทชนิดยับยั้ง ได้แก่ กรดแกมมาอะมิโนบิวไทลิกและไกลซีน และสารสื่อประสาทชนิดกระตุ้น ได้แก่ กลูตาเมตและแอสปาเตต พบว่ากรดวาลโปรอิลไฮดรอกซามิกมีผลเพิ่มการออกฤทธิ์ของสารสื่อประสาทชนิดยับยั้ง ได้แก่ กรดแกมมาอะมิโนบิวไทลิก ได้เช่นเดียวกับกรดวาลโปรอิก จากผลที่เหมือนกันระหว่างกรดวาลโปรอิลไฮดรอกซามิกกับกรดวาลโปรอิก แสดงว่าสารทั้งสองตัวอาจจะมีกลไกการออกฤทธิ์เหมือนกัน เมื่อศึกษาร่วมกับสารไบคูคูลลินและสารสตริกนินซึ่งเป็นสารที่ต้านฤทธิ์แบบแข่งขันกับกรดแกมมาอะมิโนบิวไทลิกและไกลซีนตามลำดับ เพื่อศึกษาผลของกรดวาลโปรอิลไฮดรอกซามิกและกรดวาลโปรอิกต่อตัวรับกรดแกมมาอะมิโนบิวไทลิกชนิดเอและตัวรับไกลซีน พบว่าสารทั้งสองชนิดสามารถต้านฤทธิ์ของกรดวาลโปรอิลไฮดรอกซามิกได้เช่นเดียวกับที่สามารถต้านฤทธิ์ของกรดแกมมาอะมิโนบิวไทลิกและไกลซีนได้ตามลำดับ ซึ่งแตกต่างจากกรดวาลโปรอิกที่สารไบคูคูลลินและสารสตริกนินไม่สามารถต้านฤทธิ์ของกรดวาลโปรอิกได้

จากการทดลองที่ได้ดำเนินมาก่อนพบว่ากรดวาลโปรอิลไฮดรอกซามิกสามารถป้องกันการชักในหนูถีบจักรที่ถูกเหนี่ยวนำให้เกิดการชักโดยการให้สารไบคูคูลลินและสารสตริกนินทางช่องท้องได้ โดยสารสตริกนินมีค่าขนาดของกรดวาลโปรอิลไฮดรอกซามิกที่ต้านการชักในสัตว์ทดลองจำนวนครึ่งหนึ่งเมื่อเหนี่ยวนำการชักด้วยสารสตริกนินค่อนข้างสูง และจากการศึกษาผลของกรดวาลโปรอิลไฮดรอกซามิกร่วมกับสารไบคูคูลลินและสารสตริกนิน พบว่าการลดอัตราการปลดปล่อยกระแสประสาทจากเซลล์ประสาทเปอร์กินเจของกรดวาลโปรอิลไฮดรอกซามิกนี้ถูกต้านฤทธิ์ได้ด้วยสารไบคูคูลลินและสารสตริกนิน ผลดังกล่าวจึงอาจสรุปได้ว่าการออกฤทธิ์ของกรดวาลโปรอิลไฮดรอกซามิกอาจมีความเกี่ยวข้องกับตัวรับกรดแกมมาอะมิโนบิวไทลิกชนิดเอและส่วนหนึ่งอาจมีความเกี่ยวข้องกับตัวรับไกลซีน

ภาควิชา สหสาขาวิชาสรีรวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา สรีรวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2545	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : CEREBELLAR PURKINJE CELL / VALPROYL HYDROXAMIC ACID / MICROIONTOPHORESIS / RATS

SALIN MINGMALAIRAK : EFFECT OF VALPROYL HYDROXAMIC ACID ON CEREBELLAR PURKINJE NEURONES IN RATS. (THESIS TITLE) THESIS ADVISOR : ASSOC. PROF. BOONYONG TANTISIRA, Ph.D., THESIS COADVISOR : ASSOC. PROF. MAYUREE TANTISIRA, Ph.D., 63 pp. ISBN 974-17-1963-9.

Effect of newly synthesized valproic analogues, valproyl hydroxamic acid (VHA) was investigated in comparing to valproic acid (VPA) on cerebellar Purkinje neurons by microiontophoretic techniques.

VHA and VPA depressed spontaneous firing of cerebellar Purkinje neurons. Furthermore, we examined the effect of microiontophoretically applied VHA in comparing to VPA on neuronal response to inhibitory amino acids neurotransmitters (GABA, glycine) and excitatory amino acids neurotransmitters (glutamate and aspartate). It was found that VHA enhanced the inhibition of GABA without showing any appreciable effect on the inhibition of glycine and the excitation of both glutamate and aspartate. The effects were similar to those of VPA which potentiated the GABA responses while not affecting the response of glycine, glutamate and aspartate. These results suggested that VHA exerted anticonvulsant activity by mechanism similar to those of VPA. The direct effect of VHA and VPA on $GABA_A$ or glycine receptor was further probed by their respective receptor antagonists (bicuculline and strychnine). In these studies, we found that bicuculline and strychnine antagonized the depressant effect of VHA, while bicuculline and strychnine had no effect on the depressant effect of VPA.

The effect of VHA was previously reported to be effective in bicuculline-induced convulsion and rather ineffective (high ED_{50} value) in strychnine–induced convulsion in whole animal model. Based on our finding that bicuculline and strychnine antagonized the depressant effect of VHA. It could be concluded hereby that $GABA_A$ receptor mediated inhibition seemed to be a principal anticonvulsant action of VHA whereas glycine receptor mediated inhibition could be part of its anticonvulsant action in concert with other undefined mechanism.

Department Inter-department of Physiology	Student's signature…….
Field of study Physiology	Advisor's signature…….
Academic year 2002	Co-advisor's signature……...

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List of Abbreviations

AMPM	=	alpha-amino-3-hydroxy-5-methyl-Isoxyzole-4-propionic acid
Asp-T	=	aspartate transaminase
Ca^{2+}	=	calcium ion
CNS	=	central nervous system
CSF	=	cerebrospinal fluid
°C	=	degree celseius
ED_{50}	=	median effective dose
EEG	=	electroencephalogram
FDA	=	Food and Drug Administration
g	=	gram
GABA	=	gamma-aminobutyric acid
GABA-T	=	gamma-aminobutyric acid transaminase
GAD	=	L-glutamate decarboxylase
ILAE	=	International League Against Epilepsy
i.p.	=	intraperitoneal
K^{+}	=	potassium ion
kg	=	kilogram
M	=	molar
mΩ	=	megaohm
μm	=	micrometer
MES	=	Maximal Electroshock Seizure
mM	=	millimolar
Mg^{2+}	=	magnesium ion
mg	=	milligram
min	=	minute
ml	=	milliliter

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mm	=	millimeter
nA	=	nanoampare
Na^+	=	sodium ion
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NMDA	=	N-methyl-D-aspartate
PDS	=	paroxysmal depolarization shift
PI	=	protective index
PTZ	=	Pentylenetetrazole
sec.	=	second
SHMT	=	serine hydroxymethyltransferase
SRF	=	sustained repetitive firing
SSADH	=	succinic semialdehyde dehydrogenase
TD_{50}	=	median toxic dose
μV	=	microvolt
VHA	=	valproyl hydroxamic acid
VPA	=	valproic acid
VPD	=	valpromide
Zn^{2+}	=	zinc ion
α	=	alpha
β	=	beta
γ	=	gamma
%	=	percent

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CHAPTER I

INTRODUCTION

Epilepsy

Epilepsy has been recognized for at least 2,400 years. It is derived from the Greek “epilepsia”, meaning “to come upon, to be grabbed hold of or thrown down, to attack, to seize hold of” (Graves and Garnett, 1999). The modern view of epilepsy began with John Hughlings Jackson’s work at Queen Square in London in 1861. Jackson recognized the existence of partial epileptic seizures, localized a site of origin to discrete areas of the cerebral cortex, and established a scientific approach to the study of epileptic phenomena (Engel and Pedley, 1998; Kandel, Schwartz and Jessel, 2000).

Epilepsy is a chronic and often progressive disorder characterized by the periodic and unpredictable occurrence of epileptic seizure which are caused by an abnormal and excessive synchronized discharge of a set of cerebral neurons. The clinical manifestations of epileptic seizures are sudden and transient and can include a wide variety of motor, psychic and sensory phenomena, with or without alteration in consciousness or awareness (Engel, 1995; Löscher, 1998; Shorvon, 2000).

Epidemiology

Epilepsy affects at least 50 million people worldwide. The incidence ranges between 50 and 120 cases per 100,000 persons per year and the point prevalence is about 5-10 cases per 1,000 persons. The cumulative incidence of epilepsy is about 3 and 5%. The highest incidence rates are observed in neonates and young children and then with a second peak in old age. In recent time the rate in children seem to be falling and the rate in the elderly rising because of cerebrovascular disease. The prevalence of epilepsy is relatively static after early childhood, but again

shows a tendency to rise in old age. The overall risk for epilepsy is slightly higher in male than in females (Hausen, 1998; Shorvon, 2000).

Classification of epileptic seizures

The International League Against Epilepsy (ILAE) introduced a classification scheme in 1969 and published a revised version in 1981 (Table 1). Epileptic seizures are divided into two major groups, partial seizures and generalized seizures, by clinical characteristics and electroencephalographic (EEG) features (McLachlan, 2000; Trescher and Lesser, 2000).

Generalized seizures are those that arise from large areas of cortex in both hemispheres and consciousness is always lost. Generalized seizures are subdivided into six categories. Partial seizures are those that arise in specific often small loci of cortex in one hemisphere. They are divided into simple partial seizures which occur without alteration of consciousness and complex partial seizures in which consciousness is impaired or lost. A secondarily generalized seizure is a seizure with a partial onset (the aura) which spreads to become a generalized attack. Simple partial seizures may spread to become complex partial seizures and either can spread to become secondarily generalized (Shorvon, 2000).

There are many reasons why classification is vital to the interest of the patients under treatment, as well as to the advancement of knowledge concerning the epilepsies. The accurate definition of the individual epileptic seizure is one of the principal factors on which the choice of antiepileptic drug in a particular instance is predicated. Classification is also essential to the compilation of accurate medical statistics. A further reason for accuracy in classification is that the success of medical research, particularly collaborative research, is entirely dependent on the ability to pool patient data (Dreifuss, 1998).

Etiology

Epileptic seizures are results of a shift in the normal balance of excitation and inhibition within the CNS. Given the numerous properties that control neuronal

Table 1. International Classification of Epileptic Seizures (Shorvon, 2000)

I. Partial (focal, local) seizures

- A. Simple partial seizures
  1. With motor symptoms
  2. With somatosensory or special sensory symptoms
  3. With autonomic symptoms or signs
  4. With psychic symptoms
- B. Complex partial seizures
  1. Simple partial onset followed by impairment of consciousness
     a. With simple partial features (as in A.1-4) followed by impaired consciousness
     b. With automatisms
  2. With impairment of consciousness at onset
     a. With impairment of consciousness only
     b. With automatisms
- C. Partial seizures evolving to secondarily generalized seizures

II. Generalized seizures (convulsive and non-convulsive)

- A. Absence seizures
  1. Typical absence
  2. Atypical absence
- B. Myoclonic seizures
- C. Clonic seizures
- D. Tonic seizures
- E. Tonic-clonic seizures
- F. Atonic seizures

III. Unclassified epileptic seizures

Modified from Commission on Classification and Terminology of the International League Against Epilepsy (1981).

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excitability, it is not surprising that there are many different ways to perturb this normal balance, and therefore many different causes of both epileptic seizures and epilepsy. Many causes of epileptic seizures and epilepsy are resulted from a dynamic interplay between genetic factors, endogenous factors, epileptogenic factors and precipitating factors (Engel, 1995; Lowenstein, 1998).

Epilepsy is currently considered to be genetically transmitted conditions (idiopathic or primary epilepsies) or consequences of specific cerebral disturbances (acquired or symtomatic or secondary epilepsies). Symtomatic or acquired epilepsies comprise about one-third of all new cases of epilepsy and a majority of those at older ages. They arise as sequelae from a spectrum of acute or chronic injury to the brain, including vascular insufficiency, infection, tumor, trauma and metabolic and drug-induced encephalopathies. Most patients who present with epileptic seizures do not have an identifiable cause and thus have idiopathic or primary epilepsies. The incidence of idiopathic or primary epilepsies is higher in children (Shorvon, 2000; Noebels, 2001).

Identification of the cause of epileptic seizures is of primary importance in the determination of subsequent management. In some cases if the cause can be identified, it can be corrected, and the patient will not require chronic antiepileptic drug therapy (Graves and Garnett, 1999).

Mechanisms of epileptic seizures

Epileptic seizures result from the synchronous interactions of large populations of neurons that intermittently discharge in abnormal patterns. Because of the large number of processes that regulate cortical excitability, it is unlikely that there is a single epileptogenic mechanism. Nonetheless, neurophysiologic studies in a variety of experimental preparations have shown that “epileptic” neurons share a number of properties. Although the total expression of these varies according to the particular model (Figure 1) (Pedley, 2000; McNamara, 2001).

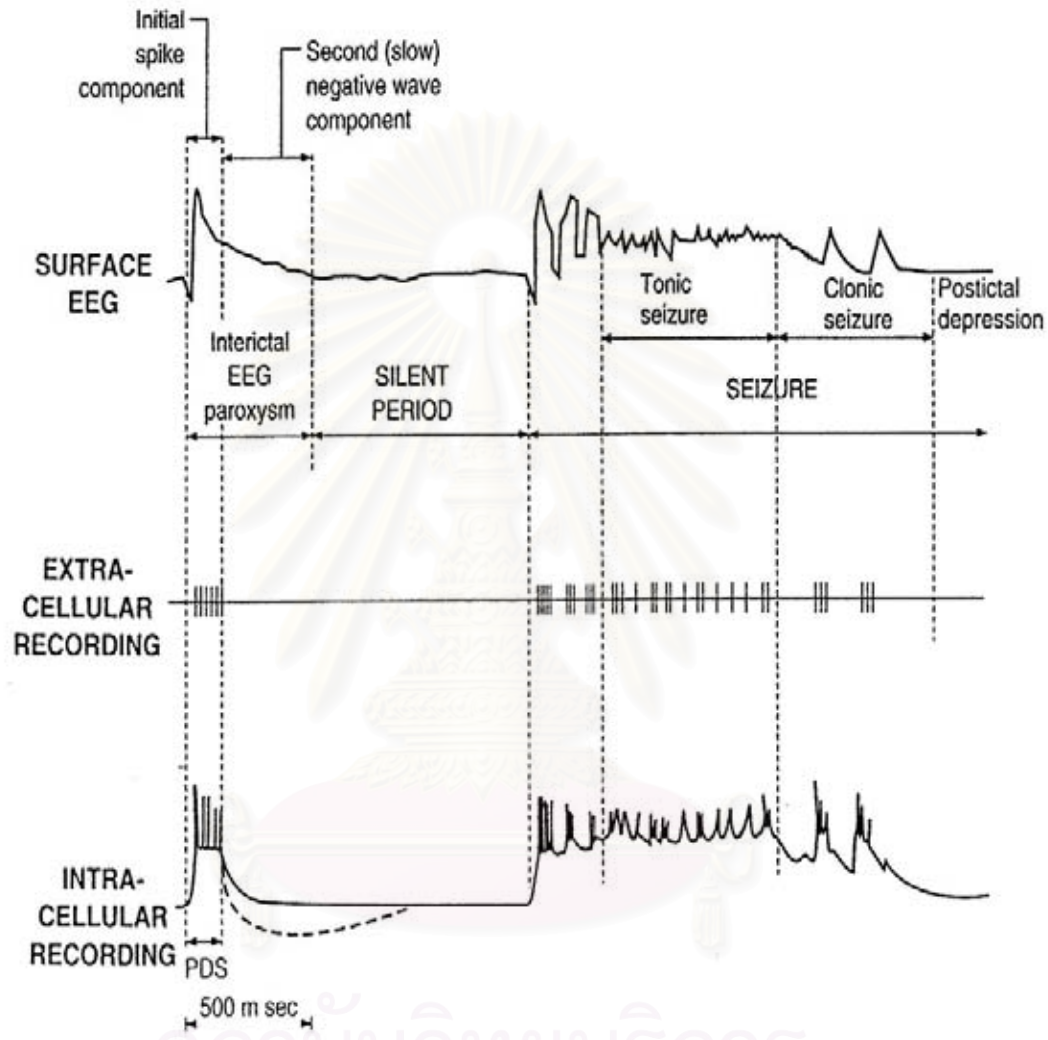

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Figure 1. Relations among cortical EEG, extracellular, and intracellular recordings in a seizure focus induced by local application of a convulsant agent to mammalian cortex (McNamara, 2001).

Intracellular recordings from neurons in an epileptogenic focus show recurring high-voltage, long-duration depolarizations with superimposed high-frequency bursts of action potentials. The extracellular current flow generated by these paroxysmal depolarizing shifts (PDSs) results in the interictal EEG spike or sharp wave, the characteristic epileptiform discharge that signifies susceptibility to epileptic seizures. PDS generation involves several mechanisms, including increased excitability resulting from changes in intrinsic voltage-dependent membrane currents, newly active excitatory circuits, and attenuation or loss of effective postsynaptic inhibition and other inhibitory processes, as well as increased effectiveness of excitatory synapses. In neurons showing "epileptic" patterns of behavior, ordinary synaptic inputs may elicit exaggerated or pathologically amplified responses (Ure and Perassolo, 2000; Pedley, 2000; McNamara, 2001).

Although it is not known in detail what causes the transition from an interictal to an ictal state, development of experimental epileptic seizures reflects decreasing effectiveness of inhibitory mechanisms accompanied by increasing evidence of excitation. PDSs become more frequent and involve ever larger numbers of neurons and more distant areas of cortex, a situation that results in progressive depolarization of neurons both within and outside the original focus. During frequent interictal epileptiform discharges and especially during seizures, extracellular potassium and intracellular calcium concentrations increase and contribute to the overall excitability of the epileptic neuronal aggregate. During the epileptic seizure itself, neurons are tonically depolarization and fire continuously in a sustained, high-frequency discharge (corresponding to the tonic phase of the seizure). The seizure ends as phasic repolarizations interrupt the continuous firing pattern (the correlate of the clonic phase) and gradually restore membrane potentials to normal or to a temporary hyperpolarized state (postictal depression) (Ure and Perassolo, 2000; Pedley, 2000; McNamara, 2001).

Amino acid neurotransmitters in epilepsy

For the majority of human epilepsy, the principal hypothesis currently concerned is a reduction of inhibitory synaptic activity or enhancement of excitatory synaptic activity. These defective synaptic actions might be expected to trigger a seizure. The neurotransmitter mediating the bulk of synaptic transmission in mammalian brain are amino acids, namely, γ-aminobutyric acid (GABA) and glutamate which are the principal inhibitory and excitatory neurotransmitters, respectively (Burnham, 1998; McNamara, 2001).

Inhibitory amino acid neurotransmitters

GABA is the principal inhibitory neurotransmitter and exerts a hyperpolarizing action in all forebrain neurons. Ever since the discovery of the powerful inhibitory actions of GABA in the brain by Eugene Roberts in 1950, it has been postulated that epileptic seizures would result if GABAergic function was impaired (Jarrott, 1999).

GABA is synthesized from glutamic acid by the enzyme glutamic acid decarboxylase (L-glutamate decarboxylase, GAD) and degraded to succinic semialdehyde by enzyme GABA transaminase (GABA-T), and then to succinic acid by succinic semialdehyde dehydrogenase (SSADH) (Cooper, Bloom and Roth, 1996; Macdonald, 1998).

Two types of GABA receptors have been characterized, termed $GABA_A$ and $GABA_B$ and distinguished on the basis of their pharmacologic characteristics (Trimble, Ring and Schmitz, 2000). $GABA_A$ are macromolecular proteins that contain specific binding sites at least for GABA, picrotoxin, barbiturates, benzodiazepines, and the anesthetic steroids and that form a chloride ion-selective channel. Activation of the $GABA_A$ receptor by GABA agonists results in the opening of the chloride channel. The ensuing influx of chloride anions inhibits the firing of the neurons by causing hyperpolarization. Benzodiazepines increase the frequency of channel opening without appreciably altering the channel conductance or duration of opening. Barbiturates, in contrast, slightly decrease the opening frenquency and prolong the duration of opening. Picrotoxin and pentylenetetrazol may block the

ionophore, while bicuculline is a $GABA_A$ competitive antagonist. $GABA_B$ are seven transmembrane proteins and are coupled to calcium or potassium ion channels via GTP binding proteins. $GABA_B$ are located on presynaptic terminals and on postsynaptic membranes. When activated by GABA presynaptically, $GABA_B$ reduce synaptic transmitter release by decreasing presynaptic calcium entry, and when activated postsynaptically, $GABA_B$ produce slow postsynaptic inhibition by increasing potassium conductance (Cooper, Bloom and Roth, 1996; Macdonald, 1998; Ure and Perassolo, 2000).

Glycine is an amino acid which has the simplest chemical structure found in substantial amounts in all mammalian body fluids and tissue proteins. It is believed to play a role as a neurotransmitter in the spinal cord and brain stem (Cooper, Bloom and Roth, 1996; Ure and Perassolo, 2000).

Glycine is synthesized from glucose via glycolytic pathway to produce 3-phosphoglycerate and 3-phosphoserine which is then converted to glycine by a reversible folate-dependent reaction catalysed by the enzyme serine hydroxymethyltransferase (SHMT). Glycine can also be formed from glyoxylate by transamination (Cooper, Bloom and Roth, 1996; Macdonald, 1998).

Glycine receptor can be classified into two types according to strychnine sensitivity. The strychnine-sensitive glycine receptor appears to exist in a macromolecular complex which consists of the glycine recognition site, chloride channel, and strychnine binding site (Browning, 1992). Activation of strychnine-sensitive glycine receptor, like the $GABA_A$ receptor, causes an increase in chloride conductance which usually results in hyperpolarization and inhibition of postsynaptic membrane (Cooper, Bloom and Roth, 1996). The strychnine-insensitive glycine receptors are linked to the NMDA receptor. This is a high affinity site that appears to increase the action of glutamate at its NMDA receptor. Although the specific glycine receptor antagonist strychnine produces convulsions in experimental animals and in man, an alteration of glycine inhibition has not been proposed as a basis for epilepsy in humans (Cooper, Bloom and Roth, 1996; Macdonald, 1998; Ure and Perassolo, 2000).

Excitatory amino acid neurotransmitters

Glutamate and aspartate are the major excitatory amino acid transmitters in the brain that are able to trigger epileptic seizures and provoke cell damage (Jarrott, 1999; Ure and Perassolo, 2000).

Glutamate is produced from α-ketoglutarate by glutamic acid dehydrogenase and from glutamine by glutaminase. In addition, transamination of α-ketoglutarate and oxaloacetate to glutamate and aspartate respectively can also be achieved with the help of the enzyme aspartate transaminase (Asp-T) (Cooper, Bloom and Roth, 1996; Dichter and Wilcox, 1998).

Glutamate receptor has been divided into at least four broad subclasses based upon receptor sensitivity to synthetic agonists and antagonists : the three ionotropic subtypes : NMDA, AMPA and kinate receptors and the metabotropic G-protein linked receptors (Jarrott, 1999; Ure and Perassolo, 2000). NMDA receptor has become a major focus of attention because of evidence suggesting that it may involve in a wide range of both neurophysiological and pathological processes such as memory acquisition, developmental plasticity, epilepsy, and the neurotoxic effects of brain ischemia (Cooper, Bloom and Roth, 1996). NMDA receptor regulates a channel permeable to Ca^{2+}, K^{+} and Na^{+}, and has several binding sites for glycine, Zn^{2+} and Mg^{2+}, which regulate the functioning of this channel in different ways (Dichter and Wilcox, 1998; Kandel, Schwartz and Jessel, 2000).

Treatment of epilepsy by antiepileptic drugs

The treatment of epilepsy has three main objectives : (1) to eliminate epileptic seizures or reduce their frequency to the maximum extent possible, (2) to avoid chronic drug-related adverse effects, and (3) to assist the patient in maintaining or restoring normal vocational and psychosocial adjustment (Pedley, 2000). Antiepileptic drug therapy is the mainstay for most patients with epilepsy. The choice of antiepileptic drug should be based on the seizure classification, the age and sex of the patient, concurrent medical conditions, potential adverse effects, and the pharmacokinetic features of the individual drugs (Craig, 1997; Lott and McAuley, 2001). Monotherapy is preferred to polytherapy with antiepileptic drugs because of the lower cost associated

Table 2. Drugs used in treating different types of epileptic seizures. The preferred agents are shown in italics (Dreifuss and Fountain, 1999)

Seizure type	Drugs
Partial seizures	
Simple partial seizures	*Carbamazepine*, valproic acid, phenytoin, vigabatrin[a], (gabapentin, lamotrigine, topiramate)
Complex partial seizure	*Carbamazepine*, phenytoin, valproic acid, vigabatrin[a], primidone, (gabapentin, lamotrigine, topiramate)
Partail seizures, secondarily generalized	*Carbamazepine*, valproic acid, phenytoin, phenobarbitone, (gabapentin, lamotrigine, topiramate)
Generalized seizures	
Absence seizures	*Ethosuximide*, *valproic acid*
Myoclonic seizures	Valproic acid[b], clonazepam, lamotrigine[b], felbamate, pyridixine
Atonic seizures	Valproic acid[b], lamotrigine[b], felbamate, corticosteroids
Tonic, clonic or tonic-clonic seizures	Valproic acid[b], carbamazepine, phenytoin, barbiturates

In parenthesis, Food and Drug Administration (FDA) approval restricted to add-on medication.

[a] Not yet available in U.S.A..

[b] Not yet FDA approved for this indication.

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with the medication and blood level monitoring, reduced potential for adverse reactions and undesirable drug interactions, and improved medication compliance with a simplified drug administration schedule. For patients in whom single drug therapy does not provide sufficient seizure control, polytherapy may be necessary to achieve the goals of treatment (Burnham, 1998; Lowenstein, 1998). Drugs used to treat epileptic seizures are listed in Table 2 and valproic acid has been widely used in the treatment of several types of epileptic seizures (McNamara, 2001).

Valproic acid

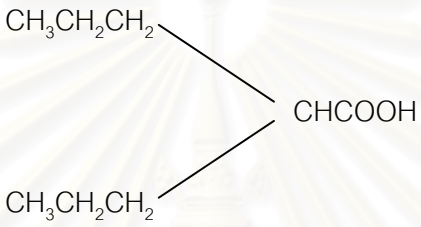

Figure 2. Structural formula of valproic acid (Löscher, 1999)

Valproic acid or valproate (2-propylpentanoic acid; VPA) (Figure 2) was first synthesized in 1882 by Burton and its anticonvulsant activity was fortuitously discovered by Pierre Eymard in 1962 in the laboratory of G. Carraz, which was published by Meunier et al. in 1963. It was approved for use as an anticonvulsant in the United States in 1978 after more than a decade of use in Europe. Structure of VPA is a simple branched-chain carboxylic acid, and differs from other antiepileptic drugs due to its lack of a nitrogen molecule or a heterocyclic moiety (Davis, Peters and McTavish, 1994; Löscher, 1999).

VPA is currently one of the major antiepileptic drugs with a broad spectrum of antiepileptic activity. It is useful both as monotherapy and as add-on therapy in generalized seizures (absence, tonic-clonic, myoclonic) and partial seizures (simple, complex, secondarily generalized) (Rowan, 1998). Clinical experience with VPA has continued to grow in recent years, including use of VPA for treatment of bipolar disorder and migraine prophylaxis (Johannessen, 2000; Porter and Meldrum, 2001).

Mechanisms of action

In spite of its wide use for many years, the mechanism of VPA action is still not fully understood. The action of VPA may therefore be exerted through several different mechanisms (McNamara, 2001).

Effects on the GABA system

It seems generally accepted that impairment of GABAergic inhibitory neurotransmission can lead to convulsions, whereas potentiation of GABAergic transmission result in anticonvulsant effects (Löscher, 1989a). There have been many reports suggesting that the mechanism of action of VPA involves a facilitation of GABAergic inhibition by increasing GABA levels in the brain or by potentiating the GABA receptors but, these hypotheses have been seriously challenged (Fromm, 1992).

The mechanism by which VPA increased GABA levels is not well understood. VPA has been shown to inhibit GABA degradative enzymes, GABA-transaminase (GABA-T) (Godin et al., 1969; Löscher, 1981a,b; Löscher, 1993) and succinic semialdehyde dehydrogenase (SSADH) (Whittle and Turner, 1978; Van der Laan, De Boer and Bruinvels, 1979). In addition, VPA can stimulate the activity of the GABA synthetic enzyme, glutamic acid decarboxylase (GAD) (Taberner, Charington and Unwin, 1980; Löscher, 1981a,b; Phillips and Fowler, 1982; Löscher, 1989b). However, there have been several other more significant objections to the increased GABA level hypothesis for VPA. Firstly, substantive elevation of brain GABA occurs only at relatively high doses of VPA. Secondly, anticonvulsant effects are observed before GABA brain levels are elevated (Shen and Levy, 1999; Porter and Meldrum, 2001).

Postsynaptic actions of VPA on GABA inhibition have also been studied. Macdonald and Bergey (1979) were the first to describe that VPA potentiates postsynaptic GABA responses *in vitro*, using microiontophoretic application for their experiments. However, the local (extracellular) concentration of VPA was unknown. Subsequent *in vitro* studies showed that increased postsynaptic GABA responses are only obtained with very high VPA concentrations (Harrison and Simmonds, 1982). There

is only one report which demonstrated a GABA potentiation at therapeutically relevant concentrations of VPA *in vitro* (Olpe et al., 1988).

Effects on amino acid neurotransmitters other than GABA

Glutamate concentrations in regional brain homogenates or in extracellular fluid obtained by microdialysis from hippocampus or substantia nigra were not significantly altered by systemic administration of VPA (Farrant and Webster, 1989; Biggs et al., 1992; Rowley, Marsden and Martin, 1995). However, Dixon and Hokin (1997) recently reported that VPA stimulates glutamate release in mouse cerebral cortex slices at therapeutic concentrations. This effect was discussed of being involved in the antimanic action of VPA. Some study showed that VPA suppresses glutamate response and, much more potently, NMDA-evoked transient depolarization in rat neocortex (Zeise, Kasparaow and Zieglgansberger, 1991). In amygdaloid slices VPA in a dose related manner suppressed the response mediated by NMDA receptors (Gean, 1994).

With respect to aspartate, VPA has been shown to reduce the concentration and release of the excitatory amino acid aspartate in rat and mouse brain (Chapman et al., 1982; Crowder and Bradford, 1987).

Furthermore, some reports found that VPA elevated concentrations of glycine in brain tissue (Similae et al., 1979).

Effects on neuronal membranes

At low concentrations (6-200 μM/L), VPA has been shown to diminish sustained repetitive firing (SRF) of sodium-dependent action potentials in mouse spinal cord and cortical neurons in culture, similar to the effects of phenytoin and carbamazepine (McLean and Macdonald, 1986). In electrophysiological study using cultured rat hippocampal neurons, VPA (1 mM) indeed strongly delayed the recovery from inactivation of sodium channels, which would be consistent with reduction of sodium conductance (Van den Berg, Kok and Voskuyl, 1993). This last finding was not confirmed by Albus and Williamson (1998) who did not find any changes in the refractory period and recovery from inactivation of sodium channels in rat hippocampal slices with VPA (1 mM). They conclude that at least in the hippocampal slice the drug's principal anticonvulsant effect can not be explained by its action on voltage-dependent sodium channels.

Effect on potassium homeostasis in the neurons have been observed. At high concentrations VPA (5-30 mM/L) hyperpolarizes the cell directly by increasing the K–conductance through a blockage of voltage sensitive Na-influx in *Aplysia* neurons, and a direct increase in K-conductance at high concentrations was shown (Slater and Johnston, 1978). Recent experiments using various potassium channel subtypes from vertebrate brain expressed in oocytes of Xenopus laevis have substantiated that the effects of VPA on potassium currents are too small to be of significance in its mechanism of anticonvulsant action (Roderfeld et al., 1994).

With regard to calcium channels, the antiabsence drugs ethosuximide and dimethadione have been shown to block use-dependent activation of T-type Ca^{2+} channels in thalamic neurons, which have been implicated in the generation of spike-wave activity associated with absence epilepsy. However, VPA (up to 1 mM) did not affect this T current in thalamic neurons, although VPA is as effective as ethosuximide to block absence seizures (Coulter, Huguenard and Prince, 1989). In contrast to thalamic neurons, VPA (100-1000 μM) was shown to block low threshold T calcium channels in peripheral ganglion neurons (Kelly, Gross and Macdonald, 1990).

Valproic acid has emerged as a first-line drug in the treatment of epilepsy. The incidence of toxicity associated with the clinical use of VPA is remarkably low compared with other antiepileptic drugs. The most common adverse effects are gastrointestinal disturbances, weight gain, neurological effects and transient hair loss. However, the drug is associated with two rare toxic effects, idiosyncratic fatal hepatotoxicity and teratogenicity (Davis, Perters and McTavish, 1994; Shorvon, 2000). Comparative analysis of the anticonvulsant potency and safety margin, utilizing the classical rodent models for anticonvulsant screening, shows that VPA is less potent than the other three major antiepileptics; phenobarbital, phenytoin and carbamazepine (Bialer et al., 1994). Consequently, there is substantially need to develop new derivatives of VPA with higher potency but lower toxicity.

Valpromide (VPD) is the primary amide of VPA. Studies in mice and rats showed that VPD is a non-teratogenic entity which is more potent than VPA. However, the advantages of VPD over VPA in rodents have no clinical implications, as in humans

VPD serves as a prodrug to VPA. Therefore, there is a need to develop stable VPD analogues. In the current study, several novel derivatives of VPD were synthesized. Valproyl hydroxamic acid (VHA) is one of the novel derivatives (Levi, Yagen and Bialer, 1997).

Valproyl hydroxamic acid

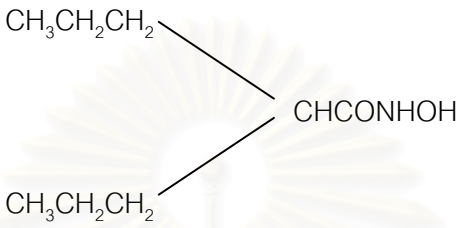

Figure 3. Structural formula of valproyl hydroxamic acid (Levi, Yagen and Bialer, 1997)

In previous studies by Levi, Yagen and Bialer (1997), valproyl hydroxamic acid (VHA) (Figure 3) is a novel derivative of valpromide (VPD) which showed better anticonvulsant activity than VPA, in maximal electroshock (MES) test, because of their greater intrinsic activity but not due to better pharmacokinetic characteristics. Pharmacokinetic analysis showed that VHA had the high clearance value. Its mean clearance was 4 and 8 times larger than of VPA and VPD respectively. The volume of distribution of VHA was similar to that of VPA and VPD. Consequently, VHA had the shortest half-life. In addition, it has been shown that unlike VPD, VHA was not biotransformed *in vivo* to VPA.

In Thailand, VHA was synthesized by Assistant Professor Chamnan Patarapanich et al. in 1996. The structure of VHA consists of two parts, one is the branched chain and the other is the structural changes of the substituents attached to the nitrogen of the amide moiety by a hydroxyl. The chemical structure of VHA was designed in expectation for a higher potency and less toxicity than VPA (Thongsathean, 1999).

Anticonvulsant activity of VHA was previously reported by Thongsathean (1999). VHA demonstrated a higher protection than VPA in maximal electroshock (MES)

test and bicuculline-induced convulsion but was as effective as VPA in pentylenetetrazole (PTZ) tests. However, VHA weakly blocked the effect of strychnine while VPA was ineffective. VHA and VPA exhibited the protective index ($PI=TD_{50}/ED_{50}$) of about 1-2 in both MES and PTZ tests. Though VHA is an active anticonvulsant molecule its anticonvulsant activity seemed to be short-life.

In brain microdialysis studies in freely moving rats, VHA was found to exert significant by positive effect on the level of cortical glycine whereas an increment of GABA was observed only in rats receiving high dose of VHA (Wanasuntronwong, 2001).

Purkinje cell of the cerebellar cortex

Because Purkinje cell has been the target of extensive electrophysiological investigation both in mammals as well as in other vertebrates, it is ideally suited for the study of the complex electrophysiological properties (Gould et al., 1995). Thus, it was used as a target neuron for elucidating the mechanism of action of VHA.

The cerebellar cortex is the superficial cell containing region of the cerebellum. It is organized into three layers and is composed of five principal neuronal cell types (Figure 4). The deepest cortical layer is the granular cell layer; the most superficial is the molecular layer. The Purkinje cell layer lies between them. The granular cell layer is composed primarily of densely packed small neurons called granule cell and a few larger Golgi cells. The Purkinje cell layer consists of a single row of the large ovoid cell bodies of Purkinje cells. These cells, which are the largest neurons in the brain, are the focal point of the cerebellar circuitry. The molecular layer consists primarily of granule cell axons and Purkinje and Golgi cell dendrites, along with a few scattered stellate and basket cells (Fredericks, 1996; Bear, Connors and Paradiso, 2001).

The basic neuronal connections of the cerebellar cortex are summarized in Figure 4 and 5. In brief, there are two main sources of input to the cerebellar cortex :

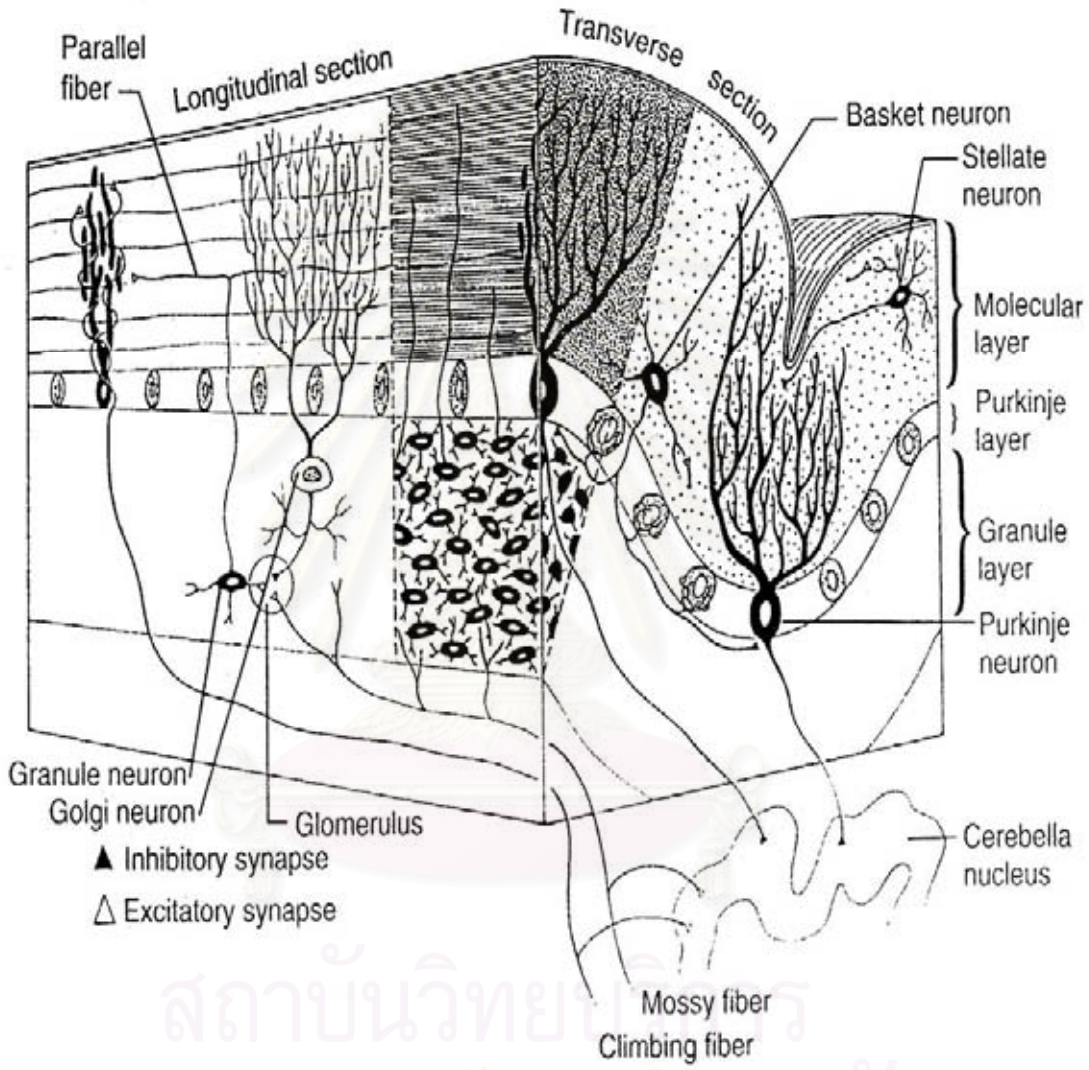

Figure 4. Functional histology of cerebellar cortex in a folium sectioned in the transverse and horizontal planes. Black synapses inhibitory; white synapses excitatory (Young and Young, 1997).

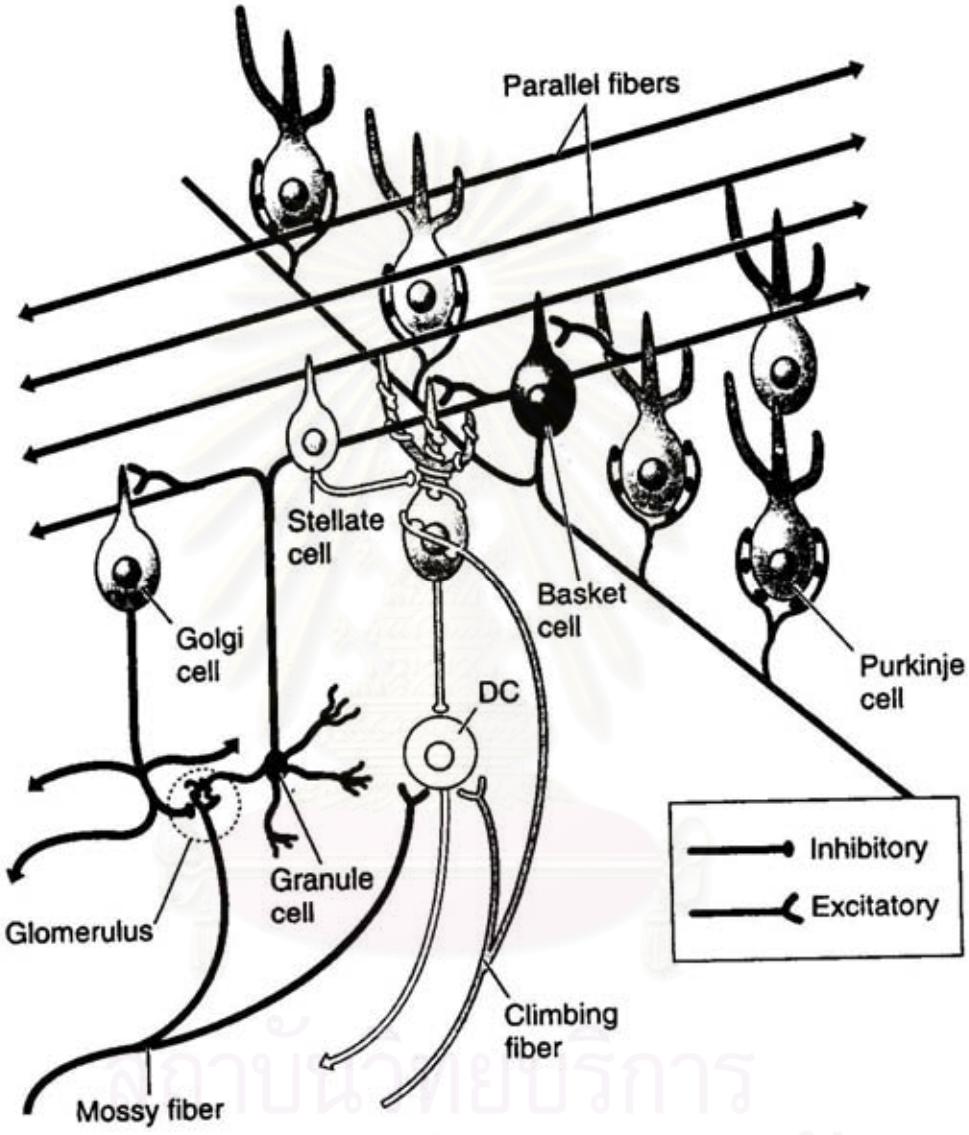

Figure 5. The synaptic relation among cerebellar cortical cells. DC, deep cerebellar nuclei (Kingsley, 2000).

climbing fibers and mossy fibers. The Climbing fibers inputs exert a strong excitatory amino effect on single Purkinje cell, whereas another source of excitation on this cell comes from mossy fibers input mediated through granule cells which originate parallel fibers whose endings form synapses on Purkinje cell dendrites. The basket and stellate cells are also excited by granule cells via the parallel fibers and their outputs inhibit Purkinje cell discharge. The Purkinje cell makes inhibitory synapses on the deep cerebellar nuclei, which are the major output cells of the cerebellum. The Golgi cells receive excitatory synaptic input from parallel fibers and cause inhibition to granule cells (Young and Young, 1997).

GABA is known to be the major inhibitory neurotransmitter in the cerebellum. There is convincing evidence that GABA is released by Purkinje cell. Additionally, this neurotransmitter is also released from terminals of Golgi cells, basket cells, and possibly from some stellate cells. Several kinds of evidence point to glutamate as the excitatory neurotransmitter released by granule cell whose parallel fibers terminated on Purkinje cell dendrites. In consideration of aspartate, neurochemical evidences make it likely that it is released by climbing fibers (Gould et al., 1991; Kingsley, 2000).

Electrophysiological analyses of individual neurons during a partial seizure demonstrate that the neuron undergo depolarization and fire action potentials at high frequencies. This pattern of neuronal firing is characteristic of a seizure and is uncommon during physiological neuronal activity. Thus, selective inhibition of this pattern of firing would be expected to reduce seizure (McNamara, 2001). From previous studies of VPA, it was found to exert anticonvulsant activity by decreasing high-frequency repetitive firing (McLean and Macdonald, 1986).

Thus, the technique of extracellular recording in conjunction with microiontophoresis was used in the present study to investigate:

1. The effects of VHA in comparing to VPA on spontaneous activity of cerebellar Purkinje cells.

2. Interaction of VHA and VPA on microiontophoretically applied γ-aminobutyric acid (GABA), glycine (Gly), glutamate (Glu), aspartate (Asp), bicuculline methochloride (BMC) and strychnine sulphate (STRY) on cerebellar Purkinje cells.

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CHAPTER II

MATERIALS AND METHODS

Experimental animal and anesthesia

The experiments were performed on male Wistar albino rats weighing 250-300 g. They were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. After arrival in the animal house, the rats were kept under controlled environmental condition (ambient temperature 25±2°C, humidity 60%-70%±10%, 12-h light/dark cycle) and were allowed free access to both standard laboratory chow (C.P.082 mice food, Thailand) and tap water. All animals were adapted to laboratory and housing conditions for at least one week before being used in the experiments. The experimental protocols used in this study were approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. All efforts were made to minimize pain or discomfort of animals used.

All electrophysiological investigations were conducted on rats anesthetized with urethane (ethyl carbamate) 1.6 g/kg i.p..

Chemicals

Sodium chloride (NaCl; APS Finechem, Australia)

Sodium hydroxide (NaOH; Riedel-de Haën, Germany)

Hydrochloric acid (HCl; Merck, Germany)

Urethane (ethyl carbamate) (Sigma, U.S.A.)

γ-Aminobutyric acid (GABA; Sigma, U.S.A.)

Glycine (Gly; Sigma, U.S.A.)

L-Glutamic acid (Glu; Sigma, U.S.A.)

L-Aspartic acid (Asp; Sigma, U.S.A.)

Bicuculline methochloride (BMC; Sigma, U.S.A.)

Strychnine sulphate (STRY; Macfarlan Smith)

2-Propylpentanoic acid (valproic acid (sodium salt)) (VPA; Sigma, U.S.A.)

Valproyl hydroxamic acid (VHA)*

*VHA was synthesized by Assist. Prof. Chamnan Patarapanich and coworkers at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Experimental instruments

5-barrelled microelectrode

Vertical microelectrode puller (Electronic Unit, Department of Physiology, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand)

Ωmega-Tip Z (OMEGAZ-A, World precision instruments, Inc., U.S.A.)

Stereotaxic apparatus (Narishige, Japan)

Micromanipulator (Narishige, Japan)

High input impedance probe (JZ 802J, Nihon Kohden, Japan)

Microelectrode amplifier (MEZ-8201, Nihon Koden, Japan)

Oscilloscope (Dual-beam memory oscilloscope VC-10, Nihon Kohden, Japan)

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Spike processor (Electronic Unit, Department of Physiology, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand)

Microiontophoretic apparatus (IP-2, Medical System Corp., U.S.A.)

Macintosh computer (LC630, Apple computer, Inc., U.S.A.)

Analog-to-Digital Instruments (MacLab/4, ADInstruments, Australia)

Software programs; Chart V.3.4.2 (ADInstruments, Australia)

Experimental methods

In this study 5-barrelled microelectrodes were used for recording extracellularly the spontaneous discharge of Purkinje cells and for applying chemical substances at the site of recording by microiontophoresis.

1. Stereotaxic techniques

The so-called stereotaxic technique was introduced for the first time by Clarke and Horsley in 1906, after which it has been considered essential for achieving precise placement of electrode or cannulae in many experiment manipulation (i.e. electrophysiological recording, lesioning, chemical injection and stimulation) of the central nervous system. In principle, electrode or cannulae are introduced into specific area by referring to a three dimensional system of co-ordinates determined by external land marks on the skull or by other reference point from pre-existing atlas of the rat brain (Pellegrino, Pellegrino and Cushman, 1979).

The animal was mounted on a stereotaxic apparatus (Narishige, Japan). The animal's head was rigidly secured in a conventional stereotaxic head holder. The upper jaw bar and two ear bar were aligned in the same horizontal plane.

2. Surgical procedure

After fixing the animal in the stereotaxic apparatus, a midline incision was made in the scalp from the level of fronto-nasal suture to the neck. The scalp was

reflected to expose the skull, and attached muscle was scraped away. The musculature covering the nape was cleared to expose the cisterna magna. To minimize cerebral edema and pulsation, a small incision was made through the cistern to provide a drainage for cerebrospinal fluid. The dorso-caudal surface of the cerebellum was exposed by craniotomy and the dura matter removed under microscopic control to expose the brain. Before insertion of the microelectrodes, a small patch was then made in the pia matter to facilitate the insertion of glass microelectrodes. This step was considered necessary since advancing a microelectrode through an intact pia matter tended to cause blockage. Care was taken from this stage to prevent the exposed tissue from drying by irrigation the surface with mixture of liquid paraffin and vaseline. After insertion of the microelectrodes, the exposed brain surface was covered with layer of 4% agar in saline to minimize pulsation which might interfere with electrophysiological observation.

3. Microiontophoresis

Microiontophoresis is a method of providing a controlled application of chemical substance from fine microelectrode by passing of electrical current. The term is sometimes interchangable with microelectrophoresis, but in a more strict sense the term microiontophoresis should be applied only when describing the movement of ions by current flow. Thus, in microelectrophoresis, microiontophoresis occurs which usually is associated with electro-osmosis.

Ejection of substances into the vicinity of neurons was achieved by passing current with the same polarity as that of the active ions species through the microelectrode barrel. For a strongly ionized ion, the ejection depends mostly on iontophoretic migration, while in case of poorly ionized substance the release has to be dependent on electro-osmosis (Curtis, 1964; Kelly, Simmonds and Straughan, 1975). This process could be enhanced by dissolving the particular substance in 165 mM NaCl. When being released, the expelled sodium ions were believed to be accompanied with water and dissolved solute (i.e. drug) molecules. Substances released by this process are shown in Table 3, using 165 mM NaCl solution as vehicle.

Table 3. Substances used in microiontophoretic study

Substance	Abbreviation	Conc. (M)	pH
γ-Aminobutyric acid	GABA	0.2	3.5
Glycine	Gly	0.5	3.5
L-Glutamic acid	Glu	0.5	7.5
L-Aspartate	Asp	0.5	7.5
Bicuculline methochloride	BMC	0.005*	3.5
Strychnine sulphate	STRY	0.005*	7
Valproic acid (sodium salt)	VPA	0.017	7.5
Valproyl hydroxamic acid	VHA	0.025	3.5

*prepared in 165 mM sodium chloride solution

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For microiontophoresis, one outer barrel of each microelectrode contained saline for automatic current balancing, and a current pump with programmable time was used for the precise control of currents and time of drug applications. Retaining currents sufficient to prevent drug leakage (usually 10-30 nA) were used for all drug applications. The net current at the microelectrode tip was continuously monitored and balanced with the aid of an iontophoresis pump (Microiontophoretic apparatus (IP-2, Medical System, U.S.A.)) (Jhamandas and Harris, 1992).

3.1. Microelectrode

In this study the microelectrode assembly consists of 5-barrels. This microelectrode was used to record and to apply drugs by microiontophoresis. It was fabricated in laboratory from borosilicated glass tubings (external diameter 1.55±0.05 mm., internal diameter 1.15±0.05 mm.). Each tube must be throughly cleaned. This is conveniently achieved by first soaking in cleansing solution for 12 hours and then throughly rinsing in tap water followed by a final rinse in distilled water. After that they are dried by being heated. These tubes are held together in a symmetrical array, either by fusion, by glue (epoxy resin) or by short length of shrink tubing (hishrink). The upper parts of the outer tubes in the array are bent outwards to obtain a good separation between the tops of the barrels. The microelectrode was pulled on a vertical type microelectrode puller (Vertical microelectrode puller (Electronic Unit, Department of physiology, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand)) and the tip was broken back to 6-9 μm under microscopic control. Each barrel of the microelectrode contained a fine glass fiber along its length to facilitate the filling of the drug solution to the tip by capillary attraction. Thereafter, the glass is stored in a dust-free environment.

3.2. Preparation of drug solutions

Drug solutions were prepared in clean detergent-free glasswares, and with particle free distilled water or normal saline. The necessary pH of a given solution was adjusted with HCl or NaOH for optimum ionization. Table 3 gives the details of concentration and pH of the solutions used in these investigations. Drug solutions are usually kept refrigerated until being used.

3.3. Filling the microelectrodes

Filling of solutions into each barrel of the microelectrodes was achieved by inserting a long small injection needle into each barrel as near to the tip as possible. Drug solution was injected from a small syringe at a slow rate to prevent formation of air bolus trapped in the barrel, which may cause insufficient filling. When glass tubings with glass fiber rib were used, the capillary attraction created by the strand of glass fiber was usually effective for drawing the solution up to the tip.

3.4. Microelectrode properties

After filling with drug solution, the microelectrode was then subjected to electrical tests. The most convenient and useful assessment was measurement the DC resistance of each barrel in the microelectrode assembly by Ωmega-Tip Z (OMEGAZ-A, World precision instruments, Inc., U.S.A.). In practice, the usual resistance range from 4 M NaCl filled barrel of a 5-barrelled microelectrode was 4-8 megaohm (mΩ), 8-12 mΩ for 2 M NaCl and 15-150 mΩ for barrel containing drug solutions.

3.5. Electrophysiological technique and processing of electrophysiological data

3.5.1. Recording microelectrodes

Extracellular unit activity was recorded through the center barrel, which contained 4 M NaCl. The other barrels were used for microiontophoresis application of drugs and balancing current.

3.5.2. Amplification and display of unit activity

To minimize electromagnetic and electrostatic interferences, successful recording was performed on the animal housed in earth screen cage (Faraday cage). Figure 6 shows detail of experimental arrangement used in all electrophysiological investigation in this study. Two stages of amplification were employed. Unit activity was delivered as single ended input into a high input impedance probe (JZ-802J, Nihon Kohden, Japan) which functioned as a unity gain buffer amplifier. The probe situated close to the recording microelectrode to minimize the “stray” capacitive effect. Output from the high input impedance probe was fed into a microelectrode amplifier (MEZ-8021, Nihon Kohden, Japan) (usuallyx500) which had

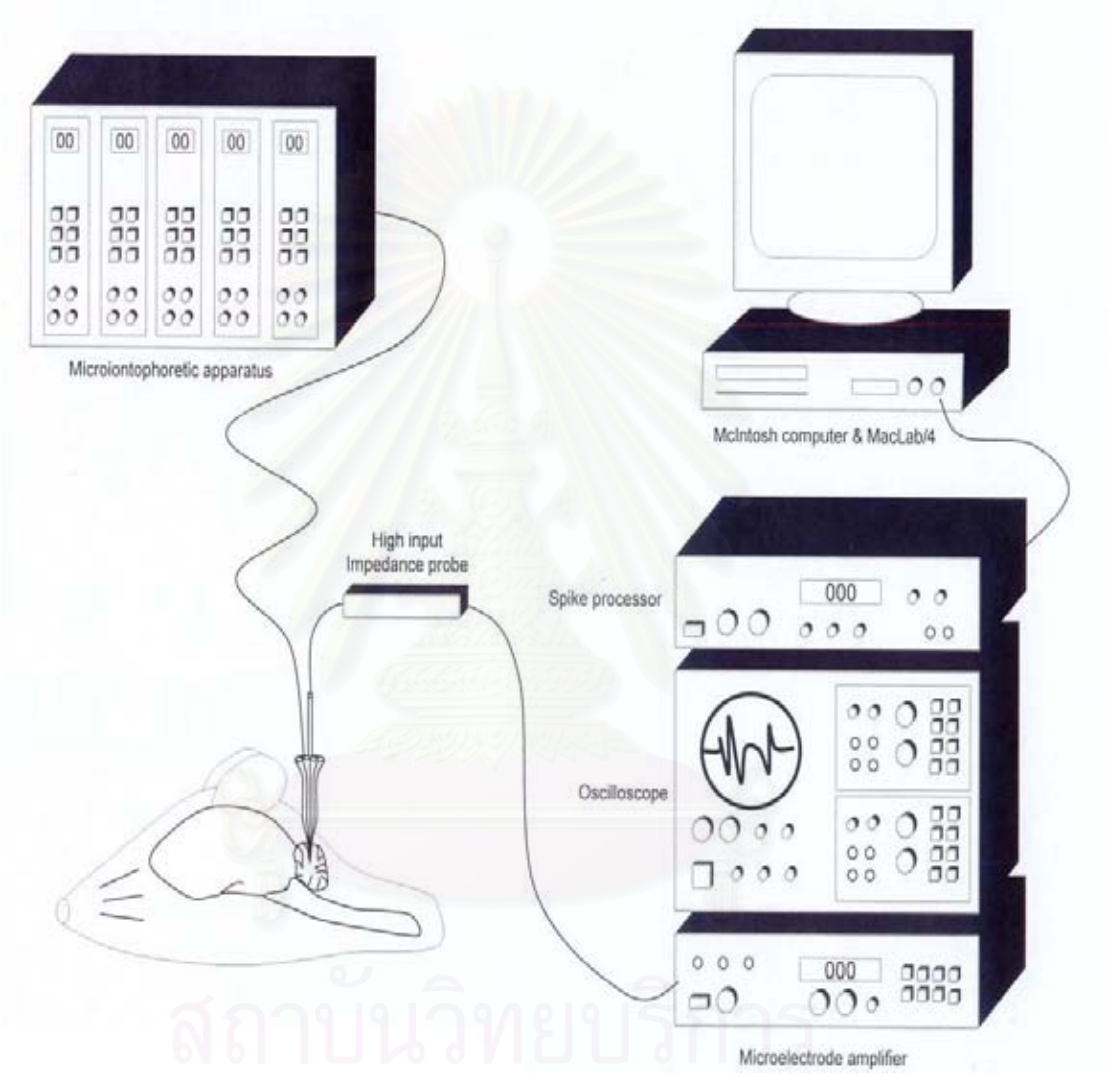

Figure 6. Diagram representation of experimental arrangement routinely employed in experiments involving electrophysiological investigation, microiontophoresis.

filter system to minimize both low frequency interference associated with line frequency, movement from animal pulses and breathing and high frequency interference from radio-transmission and switching artifact. The final output from the amplifier was displayed on a digital memory oscilloscope (Dual-beam memory oscilloscope VC-10, Nihon Kohden, Japan).

3.5.3. Processing of spike data

In order to obtain signals which were compatible with the input stage of the instruments, the amplified spike potentials were converted into corresponding pulses of standard amplitude and duration. This was achieved firstly by feeding the amplified spike in to a pulse height selector, which by adjusting a variable gate, would reject undesirable signals (i.e. noise) which had amplitudes below or above a set level. This method together with manoeuvering the microelectrode position in relation to a particular neuron enabled a single unit spike activity to be isolated. The selected amplified spikes were then used for triggering standard pulses (one pulse per spike).

The standard pulses were fed into a digital rate meter and epoch counter (Spike processor (Electronic Unit, Department of Physiology, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand)), the output from epoch counter was record on the McIntosh computer (LC 630, Apple computer, Inc., U.S.A.) emulated as a pen recorder with a digital-to-analog converter (Analog-to-Digital Instruments (MacLab/4, ADInstruments, Australia)) and software programs (chart V.3.4.2 (ADInstruments, Australia)), to obtain the neuronal firing rate and the counts displayed as peristimulus-time histogram of number of pulse over each consecutive epoch of 0.5 sec. The record was subsequently converted to neuronal firing rate of spikes per second for data presentation. The neuronal activities before, during and after microiontophoretic application of various substances were compared and analyzed.

Evaluation of microiontophoretic responses

The most conventional and most popular practice of data evaluation in a microiontophoretic experiment is by visual inspection of the firing rate records obtained

from a rate meter readout. Basal firing rates before drug application are compared with those obtained during and following microiontophoretic injection.

One essential feature of pharmacological study is to extract meaningful interpretation from the dose-response relationship of the model under study. Microiontophoretic study also, more or less, requires this general practice. However, it has always been difficult to perform full quantitative analysis of dose-response data in microiontophoretic experiments, as this has been known to be time consuming. Further, in microiontophoretic experiments performed *in vivo*, difficulties involve reproducibility of the response to a given dose of drug (in term of ejecting current in nA) are always experienced. These difficulties may be attributed to many variable factors involved, such as spatial change between the neuron and the microelectrode tip and variation in microelectrode properties.

When considering microiontophoretic results, it is of help to keep in mind that microiontophoretic application is, in nature, a continuous infusion of drug into microenvironment of the neuron tested. During a period of application at a given amount of current, drug concentration builds up over time at the rate depending on two contributing factors; first, the amount of injecting current, and second, the rate of removal of the drug by diffusion, metabolism and uptake process. It can therefore be assumed that the drug concentration surrounding the cell will eventually become constant at the balance point of the two factors (Kelly, Simmonds and Straughan, 1975; Tongroach, 1988).

The criteria used to identify Purkinje cells in cerebellum

Some preliminary comment is in order about the criteria used to identify the activity of Purkinje cells. On passing the microelectrode into the cerebellar cortex, the microelectrode encoutered an increased level of maintained multiunit activity. Of the unit that could be isolated in or near the Purkinje layer, most generated two distinctly different kinds of spike (Figure 7).

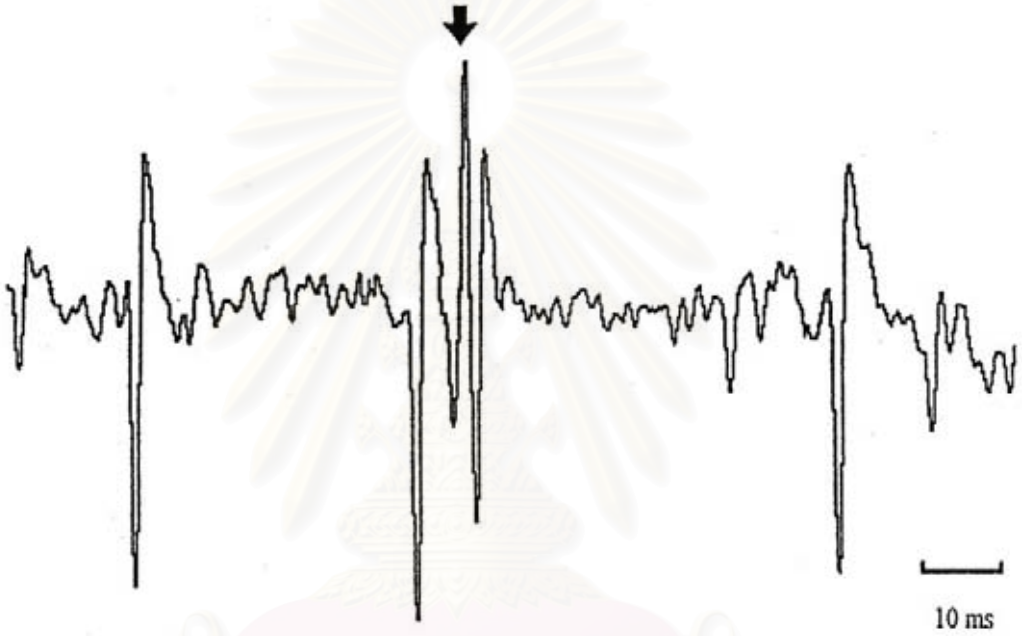

Figure 7. Maintained discharge of Purkinje cell, recorded extracellulary; The two types of spontaneous Purkinje cell discharges, namely the simple spike related to mossy fiber activation, and the complex spike (black arrow) related to climbing fiber activation. The trace shows their different pattern of discharge and shape; positive is up (Khongsombat, 1997).

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A simple spike socalled because of its relatively simple wave-form was first recorded as an initially negative potential that commonly became positive-negative on further advance of the microelectrode and fired (without indications of injury) at maintained frequencies about 20-150 spikes/sec. Discharge of this simple was frequently interrupted by another waveform that was more complex in shape (Figure 7, black arrow). The “complex” spike occurred in a sporadic pattern at a frequency about 1-4 spike/sec, and was followed by a brief silent period. Units whose activity was recorded extracellulary in the cerebral cortex and which generated these two different spike shapes are subsequently referred to as “Purkinje cells” (Eccles, 1973; Szentagothai and Arbib, 1974).

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CHAPTER III

RESULTS

Extracellular recording in urethane-anesthetized rats was obtained from 149 cerebellar cortical neurons which were identified as Purkinje cells (Table 4). Table 5 shows summary of the effects of VHA and VPA on cerebellar Purkinje neurons.

Responses of neurons to microiontophoretically applied substances were expressed as changes in basal firing rate. A requirement of at least 20% change in firing rate from control was considered a necessary criterion to support the hypothesis of a substance-induced change in neuronal response.

Effects of VHA and VPA on spontaneous firing rate

The effects of microiontophoretic application of VHA (5-100 nA; positive current, 77 neurons) and VPA (5-90 nA; negative current, 72 neurons) were consistent depression of the spontaneous discharge in a dose-dependence manner (Table 4). The responses were rapid in onset, and immediate recovery was observed upon cessation of VHA and VPA ejection.

Figure 8 and 9 showed the development of inhibition of spontaneous neuronal firing of the Purkinje cells during application of VHA 10, 20, 30, 40, 50 nA and VPA 10, 20, 30, 40, 50, 60, 70, 80, 90 nA.

Effects of VHA and VPA on neuronal responses to amino acids neurotransmitter (γ-aminobutyric acid (GABA), glycine, glutamate and aspartate)

Effects of VHA and VPA on the responses of GABA and glycine

Pulsatile cationic microiontophoretic applications (10 sec duration and at 10 sec regular intervals) of GABA (1-15 nA) and glycine (1-100 nA) produced consistent depression on activity of all Purkinje cells tested. Continuous microiontophoretic application of VHA (10-50 nA) were superimposed on the depressant pulses, the

Table 4. Summary of microiontophoretic study of VHA and VPA on cortical neurons of the cerebellum.

Action	VHA		VPA	
	No. of neurons		No. of neurons	
	tested	response	tested	response
1. Depressant action	77	77	72	72
2. Enhancement on the effect of inhibitory amino acid neurotransmitters				
GABA	12	12	9	9
Glycine	12	2	9	0
Inhibition on the effect of inhibitory amino acid neurotransmitters				
GABA	12	0	9	0
Glycine	12	1	9	0
3. Enhancement on the effect of excitatory amino acid neurotransmitters				
Glutamate	10	0	13	0
Aspartate	10	2	13	0
Inhibition on the effect of excitatory amino acid neurotransmitters				
Glutamate	10	3	13	0
Aspartate	10	3	13	0
4. Antagonistic effect on bicuculline methochloride	6	4	5	0
5. Antagonistic effect on strychnine sulphate	4	3	3	0

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Table 5. Summary of the effects of VHA and VPA on cerebellar Purkinje neurons.

Action	Agents	
	VHA	VPA
Depressant Action	+	+
Enhancement of GABA	+	+
Antagonism of GABA	-	-
Enhancement of glycine	-	-
Antagonism of glycine	-	-
Enhancement of glutamate	-	-
Antagonism of glutamate	-	-
Enhancement of aspartate	-	-
Antagonism of aspartate	-	-
Antagonism of Bicuculline Methochloride	+	-
Antagonism of Strychnine Sulphate	+	-

The effects of VHA and VPA on responses of Purkinje cells are represented as : +, positive effect ; -, no effect.

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inhibitory response to GABA was selectively augmented in 12 neurons. While augmentation of GABA responses was a consistent finding, the effect of VHA on responses to glycine was less consistent. VHA did not influence the inhibition produced by glycine in 9 out of 12 neurons. In 2 neurons, VHA augmented glycine responses, whereas in 1 neuron, VHA antagonized the glycine-induced inhibition. VPA (8-70 nA, 7 neurons), administered continuously, enhanced the inhibition produced by GABA but had no effect on response to glycine (Table 4).

Examples of these results were shown in Figure 10 and 11. The result of the interaction of VHA (20 nA) with GABA (5 nA) and glycine (20 nA) was illustrated in Figure 10. The depressant action of GABA was enhanced by VHA, while the depressant action of glycine remained unaffected. Figure 11 presented the effect of VPA (70 nA) over the response of GABA (3 nA) and glycine (100 nA). Similar to VHA, VPA selectively potentiates inhibitory action of GABA without having any appreciable effects on inhibitory action of glycine. Recovery of the potentiation to the control level of response was observed after cessation of VHA and VPA administration.

Effects of VHA and VPA on the responses of glutamate and aspartate

Pulsatile anionic microiontophoretic applications (10 sec duration and 10 sec regular intervals) of glutamate (1-50 nA) and aspartate (1-50 nA) produced consistent excitation on activity of all Purkinje cells tested. Continuous microiontophoretic application of VHA (10-40 nA) were superimposed on the excitant pulses, the excitation caused by glutamate remained unaltered in 7 out of 10 neurons and was antagonized in 3 out of 10 neurons. VHA did not change aspartate response in 5 out of 10 neurons, while in the remaining experiments VHA either reduced (3 out of 10 neurons), or enhanced (2 of 10 neurons) aspartate responses. While the excitation in response to glutamate and aspartate were unaffected by VPA (15-50 nA, 13 neurons) (Table 4).

As shown in Figure 12, effect of continuous microiontophoretic application of VHA (30 nA) on excitant action of microiontophoretic application of glutamate (1 nA) and aspartate (1 nA) on Purkinje cell was observed. When effects of

VHA were superimposed on the excitant pulses of glutamate or aspartate, the excitation of neuron remained unaffected. Similar result produced by interaction of VPA (35 nA) with glutamate (1 nA) and aspartate (1 nA) was shown in Figure 13.

Effect of GABA antagonist, bicuculline methochloride, on VHA and VPA

The convulsant alkaloid bicuculline, a competitive GABA receptor antagonist, was selected as a tool to investigate the possibility of GABA receptor involvement in the substance effects. The methochloride derivative of the alkaloid was chosen because it was more resistant to hydrolysis than the bicucuclline base under physiological condition (Hill, Simmonds and Straughn, 1974 ; Olsen et al., 1975).

Effect of bicuculline methochloride on the depressant effect of VHA

As shown in Figure 14, neuronal activity of Purkinje cells (6 neurons) were consistently depressed by pulsatile microiontophoretic application (10 sec duration and 10 sec regular intervals) of GABA (1-5 nA) and VHA (20-40 nA). Following continuous superimposition of bicuculline methochloride (BMC; 10-30 nA, positive current) on the depressant pulses, BMC antagonized the depression effect of GABA in all tested neurons and VHA in 4 neurons (Table 4).

Effect of bicuculline methochloride on the depressant effect of VPA

The pulsatile microiontophoretic application (10 sec duration and 10 sec regular intervals) of GABA (3-20 nA) and VPA (30-50 nA) produced consistent depression of Purkinje cells tested (5 neurons). When continuous microiontophoretic application of BMC (5-20 nA) were superimposed on the depressant pulse, BMC reversed the depression effect of GABA. While inhibition produced by VPA were not antagonized (Table 4). One of which was illustrated in Figure 15.

Effect of glycine antagonist, strychnine sulphate, on VHA and VPA

The convulsant alkaloid strychnine, a competitive antagonist of glycine, an amino acid presumable released as an inhibitory neurotransmitter on spinal

motorneurons, was selected as a tool to investigate the possibility of glycine involvement in the substance effects (Curtis, Duggan and Johnston, 1971).

Effect of strychnine sulphate on the depressant effect of VHA

Pulsatile microiontophoretic application (10 sec duration and 10 sec regular intervals) of glycine (20-40 nA) and VHA (20-50 nA) produced consistent depression of Purkinje cells tested (4 neurons). When continuous microiontophoretic application of strychnine sulphate (STRY; 10-20 nA, positive current) were superimposed on the depressant pulses, STRY antagonized the depression effect of glycine in all tested neurons and VHA in 3 neurons (Table 4). Example of these results was shown in Figure 16.

Effect of strychnine sulphate on the depressant effect of VPA

In Purkinje cells (3 neurons) which were consistently depressed by pulsatile microiontophoretic application (10 sec duration and 10 sec regular intervals) of glycine (6-30 nA) and VPA (20-40 nA). Following superimposition of STRY (20-30 nA) on the depressant pulses, STRY antagonized only the depression effect of glycine, while depression of neuronal activity caused by VPA remained unaffected (Table 4). Figure 17 showed the example for this result.

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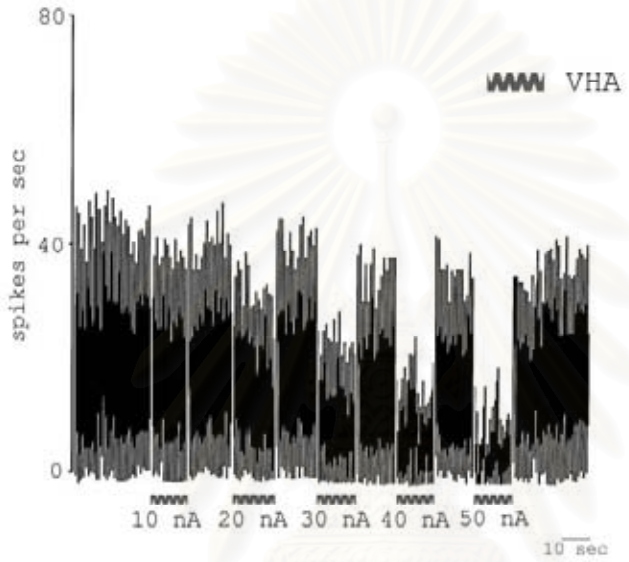

Figure 8. Effect of microiontophoretic application of VHA on spontaneous firing rate of Purkinje cells. VHA produced depression on neuronal firing of Purkinje cells.

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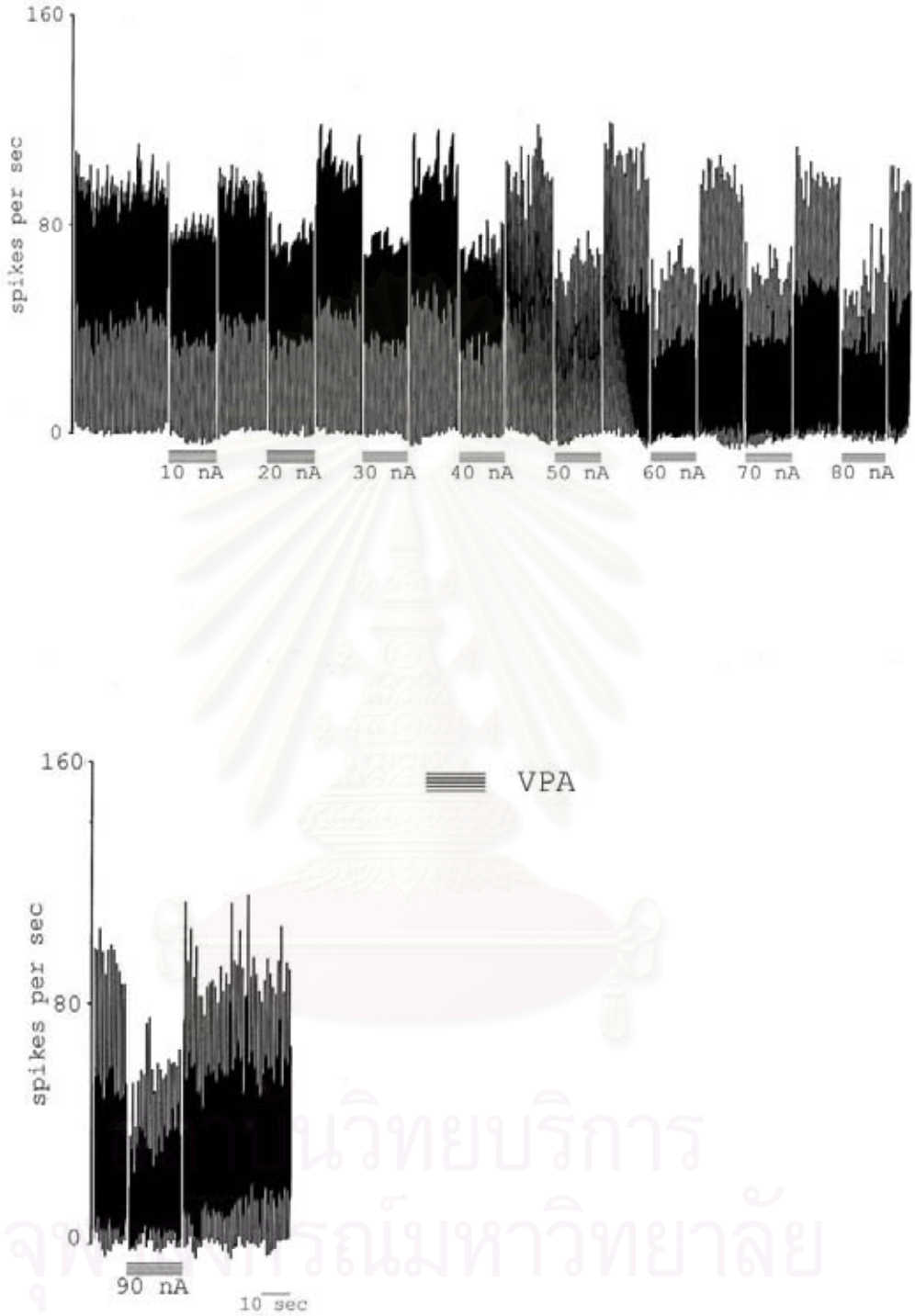

Figure 9. Effect of microiontophoretic application of VPA on spontaneous firing rate of Purkinje cells. VPA produced depression on neuronal firing of Purkinje cells.

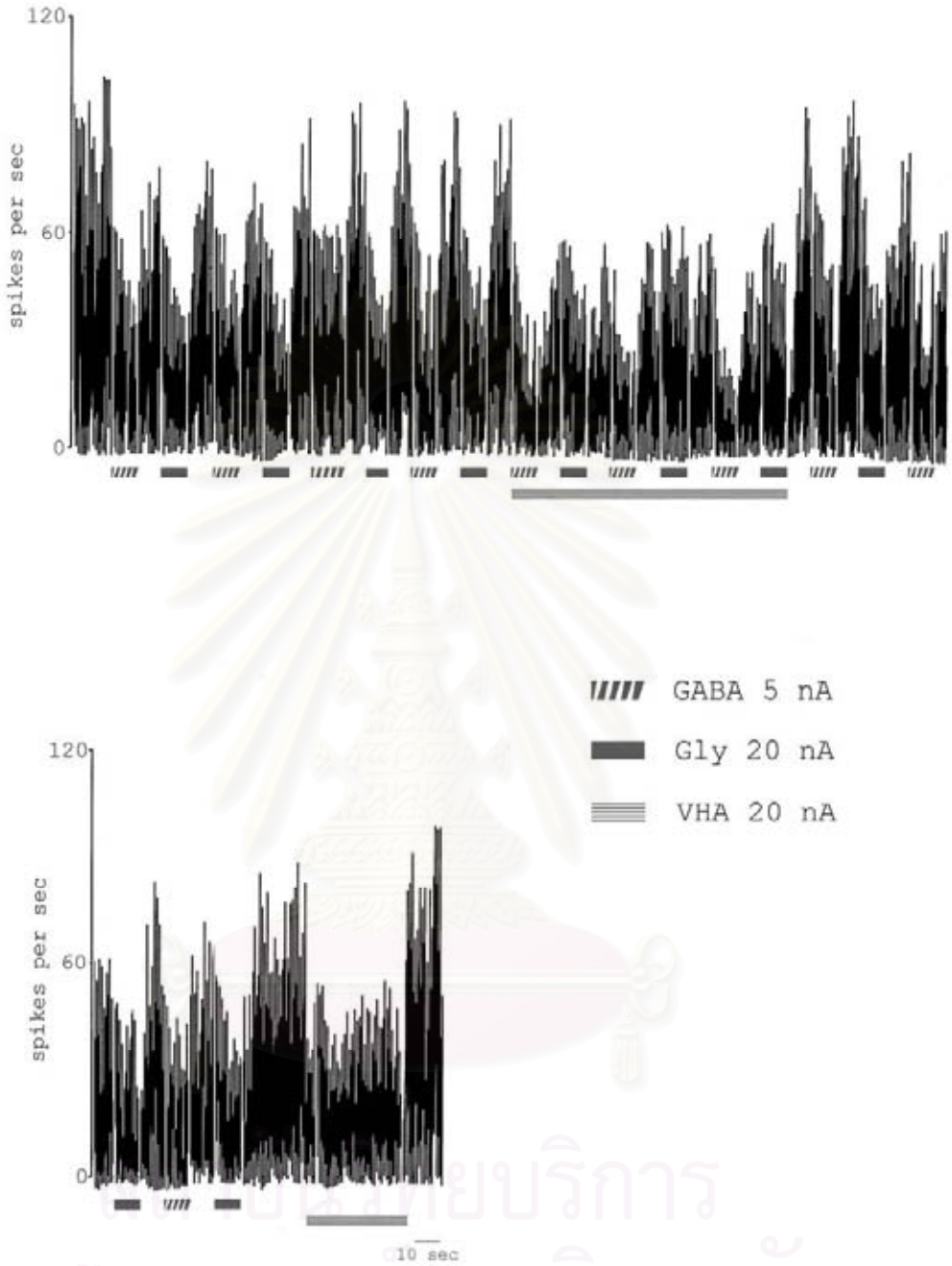

Figure 10. Effect of continuous microiontophoretic application of VHA (20 nA) on depressant actions of microiontophoretic application of γ-aminobutyric acid (GABA; 5 nA) and glycine (Gly; 20 nA) on neuronal firing of Purkinje cell. The depressant action of GABA was enhanced by VHA, while the depressant action of glycine remained unaffected.

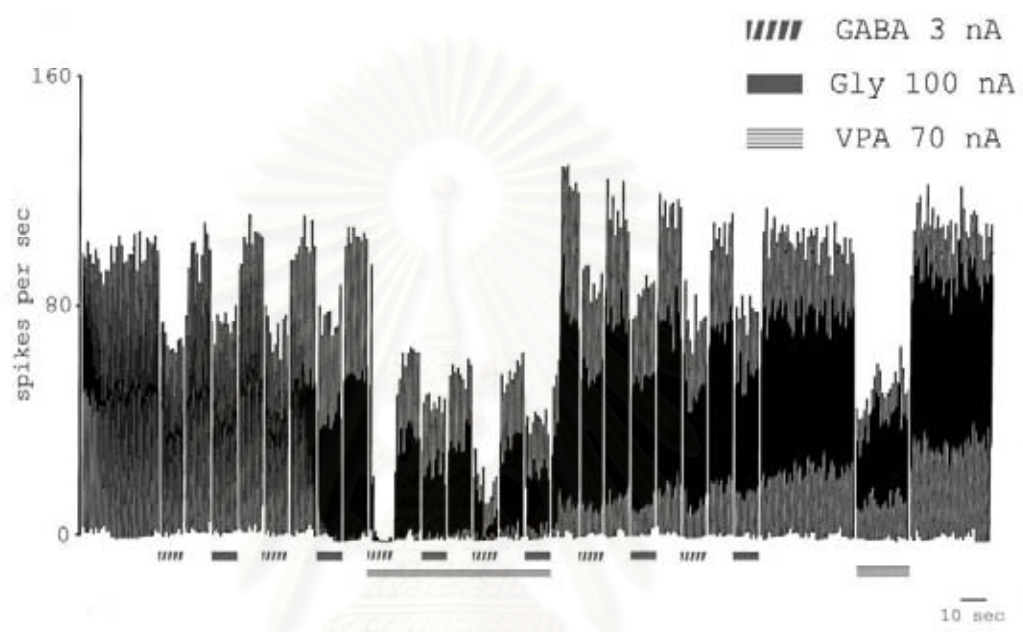

Figure 11. Effect of continuous microiontophoretic application of VPA (70 NA) on depressant actions of microiontophoretic application of γ-aminobutyric acid (GABA; 3 nA) and glycine (Gly; 100 nA) on neuronal firing of Purkinje cell. The depressant action of GABA was enhanced by VPA, while the depressant action of glycine remained unaffected.

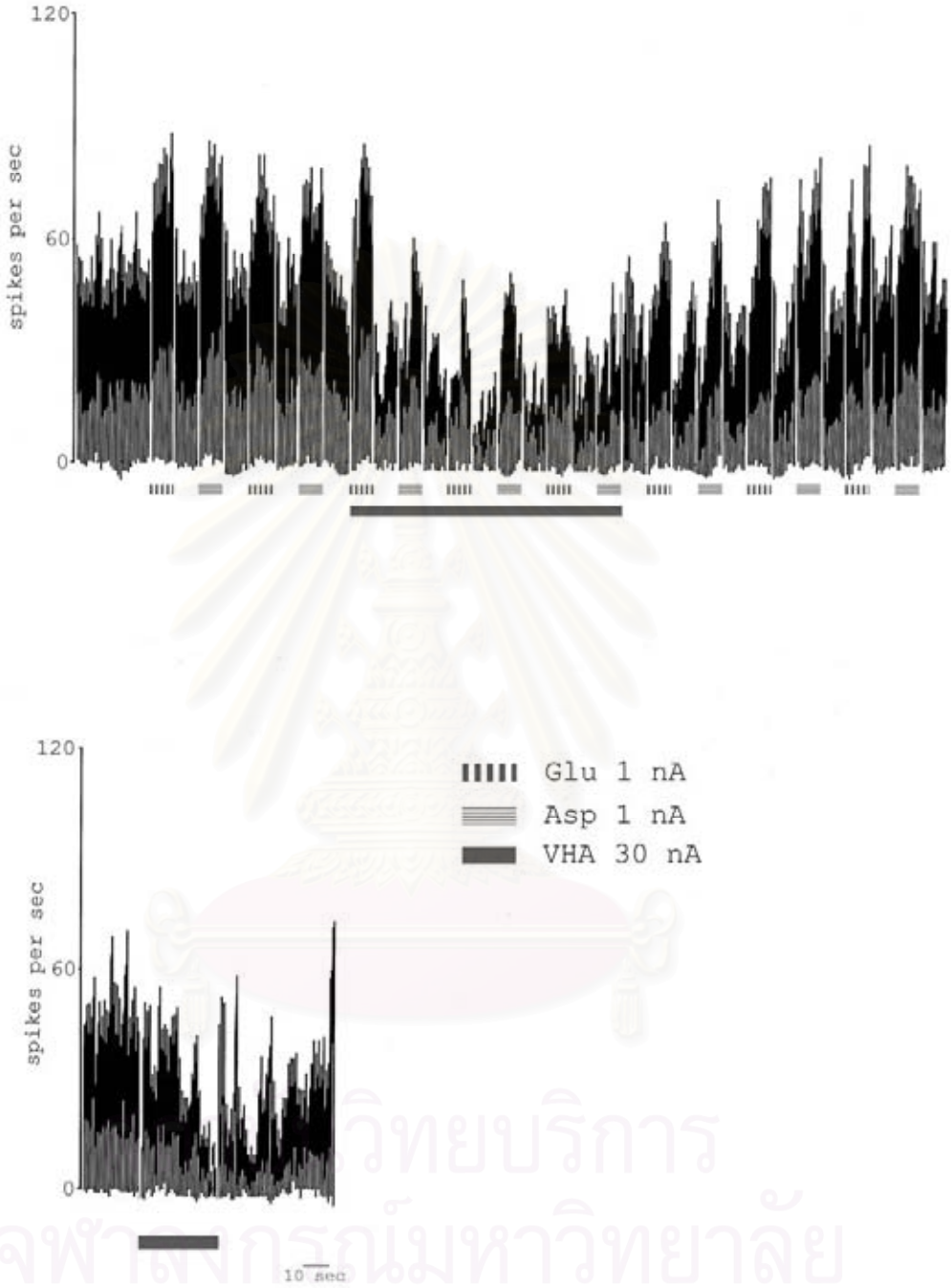

Figure 12. Effect of continuous microiontophoretic application of VHA (30 nA) on excitant actions of microiontophoretic application of glutamate (Glu; 1 nA) and aspartate (Asp; 1 nA) on neuronal firing of Purkinje cell. VHA produced no alteration on the neuronal response to glutamate and aspartate.

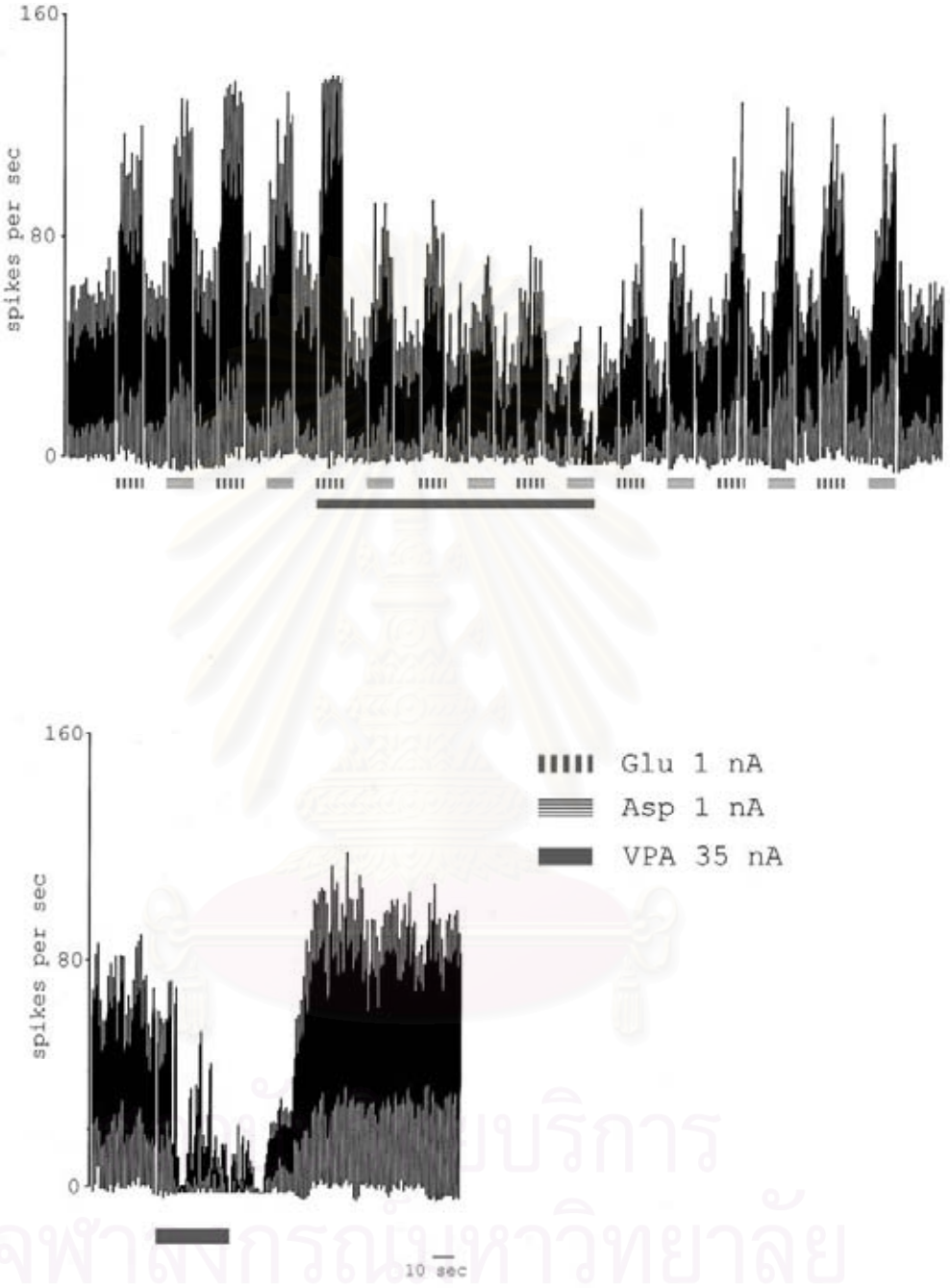

Figure 13. Effect of continuous microiontophoretic application of VPA (35 nA) on excitant actions of microiontophoretic application of glutamate (Glu; 1 nA) and aspartate (Asp; 1 nA) on neuronal firing of Purkinje cell. VPA produced no alteration on the neuronal response to glutamate and aspartate.

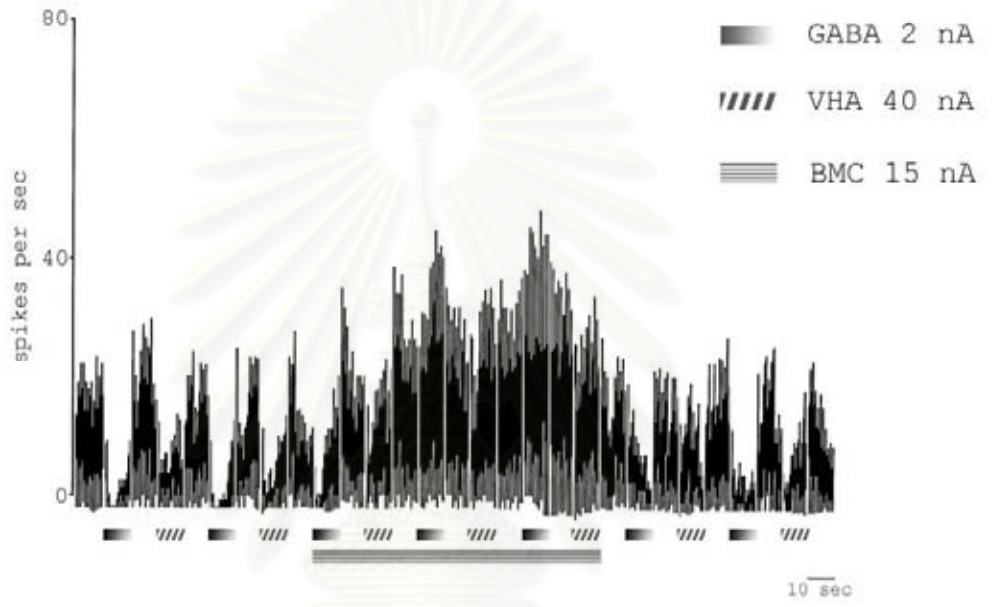

Figure 14. Effect of continuous microiontophoretic application of bicuculline methochloride (BMC; 15 nA) superimposed on the response of Purkinje cell to microiontophoretic application of GABA (2 nA) and VHA (40 nA). Application of BMC selectively and reversibly antagonized the depressant effects of GABA and VHA.

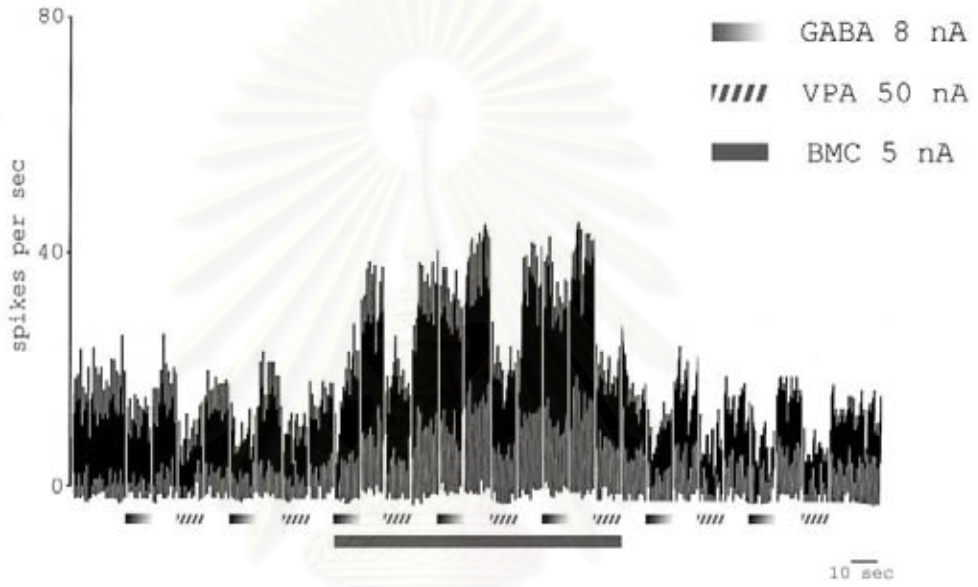

Figure 15. Effect of continuous microiontophoretic application of bicuculline methochloride (BMC; 5 nA) superimposed on the response of Purkinje cell to microiontophoretic application of GABA (8 nA) and VPA (50 nA). Application of BMC selectively and reversibly antagonized the depressant effect of GABA, while the depression of neuronal activity caused by VPA remained unaffected.

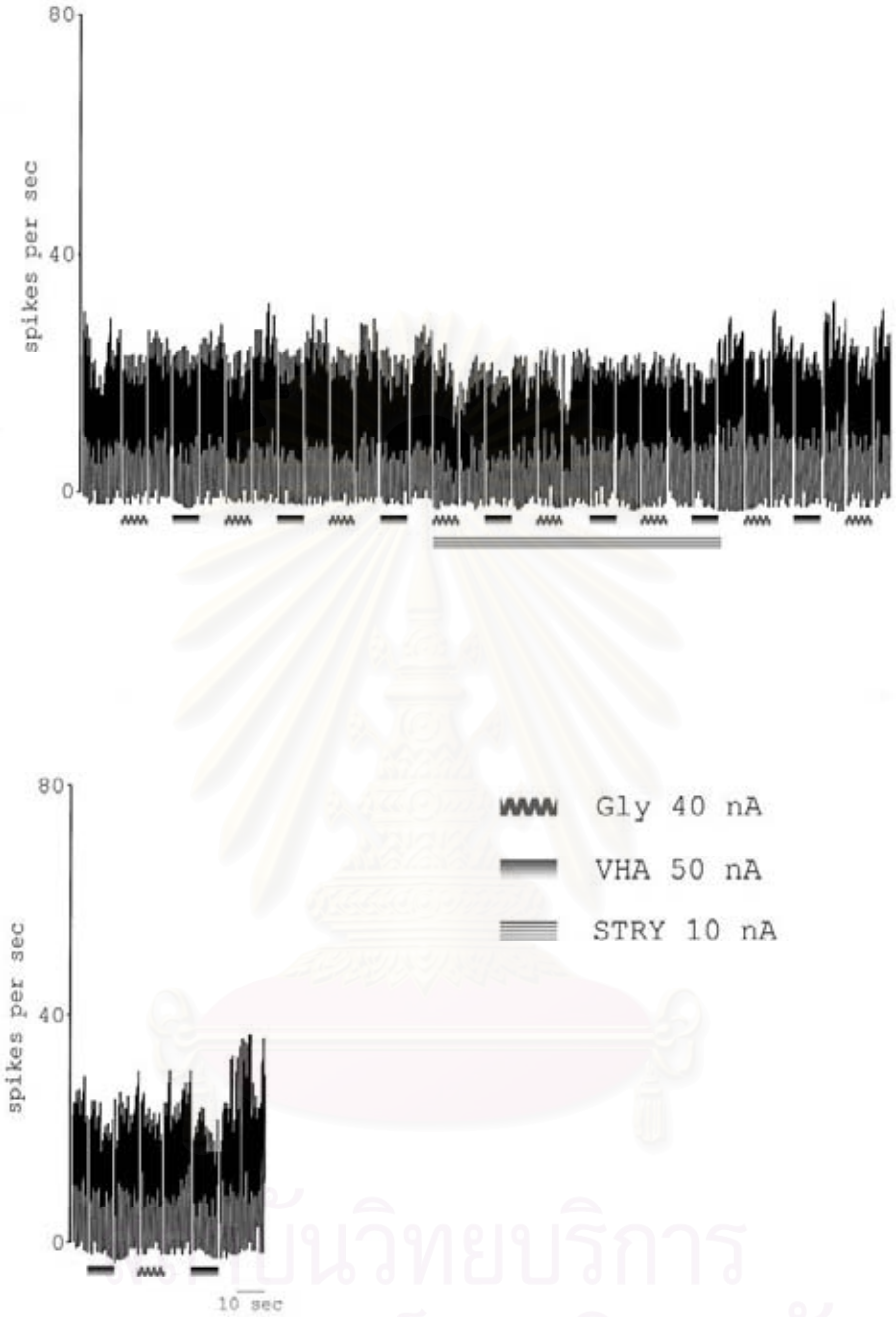

Figure 16. Effect of continuous microiontophoretic application of strychnine sulphate (STRY; 10 nA) superimposed on the response of Purkinje cell to microiontophoretic application of glycine (Gly; 40 nA) and VHA (50 nA). The depressant action of glycine and VHA were antagonized by STRY.

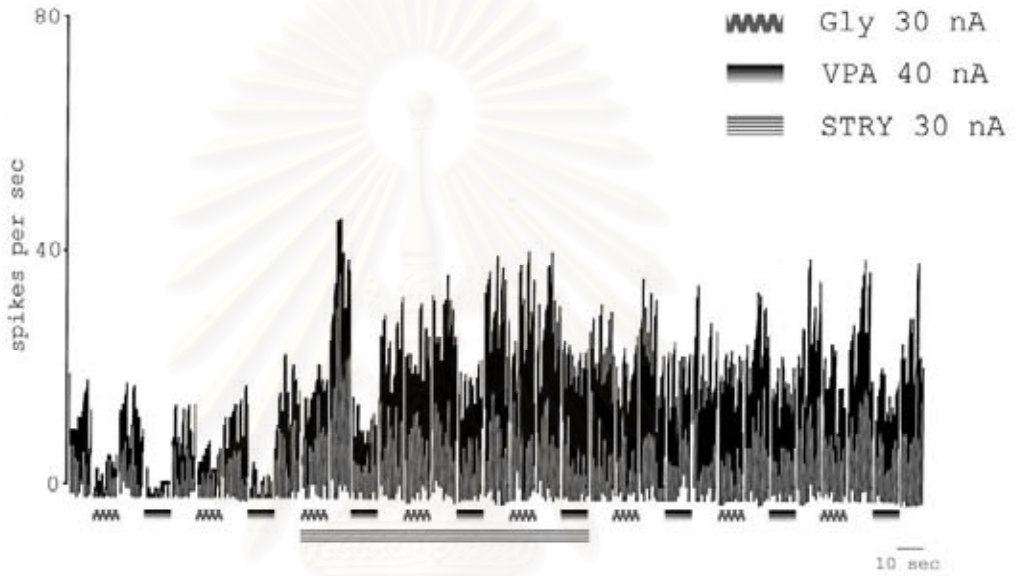

Figure 17. Effect of continuous microiontophoretic application of strychnine sulphate (STRY; 30 nA) superimposed on the response of Purkinje cell to microiontophoretic application of glycine (Gly; 30 nA) and VPA (40 nA). The depressant action of glycine was antagonized by STRY, while the depressant action of VPA remained unaffected.

CHAPTER IV

DISCUSSION AND CONCLUSION

Microiontophoretic technique is the technique for application of substances in minute quantity from multibarrel microelectrode into immediate extracellular environment of single neurons. This technique is method for proving action of putative synaptic transmitters and for investigating the mechanism of action of these neurotransmitters as well as substances which may interfere with their functions (Tongroach, 1988).

Previous work using microiontophoretic techniques has yielded conflicting results. Slater and Johnston (1978) demonstrated that VPA caused hyperpolarization of *Aplysia* neurons *in vitro* which was associated with an increase in potassium conductance. If VPA had caused hyperpolarization a decrease in firing rates should have ensued, but this was not observed in the rat medullary reticular formation when VPA was delivered by microiontophoresis (Gent and Phillips, 1980). Baldino and Geller (1981a) found that cerebral cortical neurons did not change their activity in response to microiontophoretically applied VPA. When VPA was microiontophoresed directly onto the neocortical neurons, there was either no effect or a slight increase in spontaneous firing rate (Schmutz, Olpe and Koella, 1979). Blume et al. (1979) reported that VPA increased firing rate of cerebral cortical and hippocampal neurons *in vivo*, findings essentially similar to those reported by several studies but in different regions (Kerwin, Olpe and Schmutz, 1980; Baldino and Geller, 1981b). In contrast are the results of Khongsombat (1997) who found that both neurons of cerebral cortex and cerebellar Purkinje neurons responded to microiontophoretically applied VPA with a decrease in firing rate. The present study also demonstrated inhibition of spike generation by cerebellar Purkinje neurons with VPA (Figure 9).

Similar disparities were reported on the effects of VPA on GABA inhibition but not on glycine inhibition. In rat cortical neurons, microiontophoretically

applied VPA was reported to augment the inhibitory effects of GABA with no affect on the inhibitory effects of glycine (Schmutz, Olpe and Koella, 1979; Kerwin, Olpe and Schmutz, 1980; Baldino and Geller, 1981a). Gent and Phillips (1980) found similar effects of VPA on GABA and glycine –induced inhibition in rat medullary reticular formation. Likewise, in this study, VPA enhanced the inhibition produced by GABA while not affecting the response of the neuron to glycine (Figure 11). However, a recent study showed that neuronal responses of cerebellar Purkinje neurons to GABA and glycine were not altered by VPA (Khongsombat, 1997). Additionally, Baldino and Geller (1981b) reported that VPA antagonized the inhibitory effects of GABA on hypothalamic neurons.

No effect of VPA on the excitation of both glutamate and aspartate was observed in this study (Figure 13). Similarly, Gent and Phillips (1980) found that the responses of medullary reticular formation neurons to glutamate were not affected by microiontophoresis of VPA. These results seem contradictory to decrement of both glutamate and aspartate excitation produced by VPA in rat cerebellar Purkinje neurons (Khongsombat, 1997).

The apparent disparity in these results may be due to such factors as differences in the types of neuron and species studied. However, indirect actions and/or interactions with anesthetic may also be contributing factors. Furthermore, the high-pH solution (8.0-9.0) used to dissolve VPA may have had local pH effects. Additionally, indirect effects of VPA on nearby neurons could also explain this disparity (Blume et al., 1979; Baldino and Geller, 1981a; Johnston, 1984).

In the present studies, VHA and VPA demonstrated their consistent depression of neuronal activity as indicated by decreasing spontaneous firing rates in a dose dependent manner on cerebellar Purkinje neurons (Figure 8 and 9).

Furthermore, in an attempt to rule out the possible mechanisms for the observed electrophysiological effects, we examined the effects of microiontophoretically applied VHA in comparing to VPA on neuronal response to inhibitory amino acids neurotransmitters (GABA and glycine) and excitatory amino acids neurotransmitters (glutamate and aspartate).

The results of VHA on GABA and glycine demonstrated the enhancement of GABA inhibition, while the inhibitory effects of glycine remained unaffected (Figure 10). In interaction with glutamate and aspartate, VHA had no effect on responses of the neurons to these excitatory amino acids neurotransmitters (Figure 12). The responses were similar to those of VPA which enhanced the inhibition of GABA without showing any appreciable effect on the inhibition of glycine and the excitation of both glutamate and aspartate. These results suggested that VHA exerted anticonvulsant actions by mechanism similar to those of VPA.

The direct effect of VHA on $GABA_A$ or glycine receptor was further probed by their respective receptor antagonists, bicuculline for the involvement of $GABA_A$ receptor and strychnine for the strychnine-sensitive glycine receptor. The data obtained from this study indicate that, bicuculline antagonized the inhibitory effects of VHA (Figure 14). These results did suggest role of $GABA_A$ receptor as a possible site of anticonvulsant action of VHA. Involvement of glycine receptor in the action of VHA was also included by the finding that strychnine antagonized the depressant effects of VHA on spontaneous firing activity of cerebellar Purkinje neurons (Figure 16); however, in this case changes in basal firing rate of at least 20% was difficult to observe responses to glycine, VHA and strychnine. Taken this result together with *in vivo* finding that the median effective dose (ED_{50}) of VHA for protection of mice against maximal electroshock (MES) test, pentylenetetrazole (PTZ) seizure test, bicuculline and strychnine -induced convulsion were 114, 97, 153 and 441 mg/kg B.W. respectively (Thongsathean, 1999). It is likely that though VHA may exert anticonvulsant actions by directed effect on $GABA_A$ and glycine receptor. However, directed effect of VHA on glycine receptor should be comparatively of minor importance in relation to other mechanism as indicated by rather high ED_{50} in strychnine-induced convulsive model.

In conclusion, the results from present study suggest that VHA produced inhibition by decreased firing rates of spontaneous firing of cerebellar Purkinje neurons. It exerted anticonvulsive action by potentiation of GABA responses. This action was similar to enhancement of GABA inhibition produced by VPA. From these results, VHA exerted anticonvulsive mode of action by mechanism similar to those of VPA.

The observation that bicuculline and strychnine antagonize the inhibitory effects of VHA, suggests that VHA may exert the inhibitory effect on the neurons by simply acting on the same receptor as $GABA_A$ and glycine respectively. However, based on the results that VHA did not potentiate the inhibitory effects of glycine and its ED_{50} in strychnine-induced convulsion was rather high. It is suggestive that direct effect of VHA on glycine receptor should not be a principal mechanism underlying the anticonvulsant actions observed.

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