ผลของ เอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย
ต่อตัวรับ กาบา เอ ในเซลล์ประสาทที่แยกได้ทันทีจากฮิปโปแคมปัสของหนูแรท

เรือเอก สุเทพ จันทร์เทศ

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EFFECT OF N-(2-PROPYLPENTANOYL) UREA ON $GABA_A$ RECEPTOR IN ACUTELY DISSOCIATED RAT HIPPOCAMPAL NEURONES

Lieutenant Suthep Jenthet

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การวิจัยนี้มีจุดมุ่งหมายที่จะศึกษาฤทธิ์ของ เอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย และ กรดวาลโปรอิก ซึ่งเป็นสารต้นแบบ ที่มีต่อตัวรับชนิด กาบา เอ และไกลซีน ในเซลล์ประสาทที่แยกได้ทันทีจากฮิปโปแคมปัสของหนูแรท ทำการศึกษาโดยการวัดกระแสทั้งหมดที่ไหลผ่านเยื่อหุ้มเซลล์ของเซลล์ประสาท

จากการศึกษาพบว่า เอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย ในขนาดความเข้มข้นตั้งแต่ 1-300 ไมโครโมลาร์ ไม่มีผลโดยตรงในการที่จะทำให้เกิดกระแสไหลผ่านเข้าเซลล์ประสาทปิรามิดที่แยกได้ทันทีจากฮิปโปแคมปัสของหนูแรท ในขณะที่เอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย สามารถออกฤทธิ์เพิ่มกระแสกาบา เอ ตามความเข้มข้นที่เพิ่มขึ้นเช่นเดียวกับเพนโตบาบิทอลและไดอะซีแพม เมื่อเปรียบเทียบกันพบว่า เอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย ต้องให้ในขนาดความเข้มข้นที่สูงกว่าเพื่อให้มีฤทธิ์ใกล้เคียงกับเพนโตบาบิทอลและไดอะซีแพมโดยยาทั้งสองชนิดมีฤทธิ์สูงสุดต่อตัวรับชนิดกาบา เอ ที่ความเข้มข้น 300 และ1 ไมโครโมลาร์ ตามลำดับ ส่วนกรดวาลโปรอิกซึ่งเป็นสารต้นแบบไม่มีผลกระตุ้นให้เกิดกระแสโดยตรง แต่ในระดับความเข้มข้นสูง (30-5,000 ไมโครโมลาร์) กรดวาลโปรอิกสามารถเพิ่มกระแสกาบา เอ และมีฤทธิ์สูงสุดที่ระดับความเข้มข้น 4,000 ไมโครโมลาร์ นอกจากนี้ยังพบว่าเอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรียและกรดวาลโปรอิกไม่มีผลต่อตัวรับชนิดไกลซีน

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ความสามารถในการเพิ่มกระแสที่ไหลผ่านตัวรับกาบา เอ ของเอ็น-(ทู-โพรพิลเพนทาโนอิล) ยูเรีย อาจเป็นกลไกอย่างหนึ่งในการต้านการชัก การศึกษาฤทธิ์ของสารนี้ต่อตัวรับชนิดอื่นๆที่เกี่ยวข้องกับภาวะชักเป็นสิ่งที่น่าสนใจจะศึกษาต่อไป

สหสาขาวิชาสรีรวิทยา ลายมือชื่อนิสิต.....

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ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: $GABA_A$ RECEPTOR / HIPPOCAMPUS / N-(2-PROPYLPENTANOYL) UREA / PATCH CLAMP

SUTHEP JENTHET: EFFECT OF N-(2-PROPYLPENTANOYL) UREA ON $GABA_A$ RECEPTOR IN ACUTELY DISSOCIATED RAT HIPPOCAMPAL NEURONES. THESIS ADVISOR : ASST. PROF. THONGCHAI SOOKSAWATE, Ph.D. 112 pp. ISBN 974-17-1826-8.

The present study was aimed to investigate the effects of n-(2-propylpentanoyl) urea or valproyl urea (VPU) and valproic acid (VPA), as a reference drug, on $GABA_A$ and glycine receptors in acutely dissociated rat hippocampal neurones using the whole-cell application of the patch clamp techniques.

Concentration range of 1-300 μM, VPU did not directly induce inward currents in acutely dissociated rat hippocampal pyramidal neurones. The $GABA_A$ currents could be enhanced by VPU, in a concentration-dependent manner, as well as pentobarbital sodium (PB) and diazepam (DZP). The GABA potentiating effect of VPU required higher concentration to reach the same potentiation effect in comparison to PB and DZP which had maximal potentiation effects at 300 μM and 1 μM, respectively. Valproic acid (VPA), a reference drug, did not directly elicite inward currents. However, at high concentration (30-5,000 μM), VPA could potentiate the $GABA_A$ currents with maximal potentiation effect at 4,000 μM. VPU and VPA did not affect the glycine currents.

Flumazenil, a benzodiazepine antagonist, could not inhibit the potentiation of the $GABA_A$ currents by VPU. However, VPU could inhibit the inward currents induced by PB. Moreover, coapplication of VPU with PB increased the potentiation of the $GABA_A$ currents by each of these drugs applied with GABA. These results show that the effect of VPU on the $GABA_A$ receptor may have some interaction directly or indirectly with the barbiturate site(s) on the $GABA_A$ receptor channel. However, the clear mechanism(s) of the interaction need further investigation.

The potentiation of the $GABA_A$ currents by VPU may, at least in part, contributes to its mechanism of anticonvulsant action. The effects on other receptors involved in convulsion would also be interesting to investigate.

Inter-Departmental Program in Physiology Student's signature.....
Field of study Physiology Advisor's signature.....
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List of Abbreviations

α	=	alpha
β	=	beta
γ	=	gamma
δ	=	delta
ε	=	epsilon
θ	=	theta
π	=	pi
ρ	=	rho
Ω	=	omega
μM	=	micromolar
μm	=	micrometre
pA	=	picoampare
%	=	percent
^{0}C	=	degree celcius
AEDs	=	antiepileptic drug
ATP	=	adenosine 5′-triphosphate
BMC	=	bicuculline methochloride
BZ	=	benzodiazepine
Ca^{++}	=	calcium ion
Cl^{-}	=	chloride ion
CNS	=	central nervous system
CSF	=	cerebrospinal fluid
DMSO	=	dimethyl sulfoxide
DZP	=	diazepam
ED_{50}	=	median effective dose
EEG	=	electroencephalogram
et al.	=	et alii (and other)

etc.	=	et cetera (and so on)
FMZ	=	flumazenil
g	=	gram
GABA	=	gamma-aminobutyric acid
GABA-T	=	gamma-aminobutyric acid transaminase
GAD	=	L-glutamate decarboxylase
GHB	=	γ-hydroxybutyric acid
GLY	=	Glycine
GOT	=	glutamic-oxaloacetic transaminase
G-proteins	=	guanine nucleotide binding proteins
5-HT	=	5-hydroxytryptamine (serotonin)
ILAE	=	International League Against Epilepsy
IP_3	=	inositol-1, 4, 5,-triphosphate
K^+	=	potassium ion
LD_{50}	=	medial lethal dose
MES	=	maximal electroshock seizure
Mg^{++}	=	magnesium ion
min	=	minute
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
ms	=	millisecond
mV	=	millivolt
Na^+	=	sodium ion
NMDA	=	*N*-methyl-D-aspartate
PB	=	pentobarbital sodium
PDS	=	paroxysmal depolarizing shift
PSS	=	physiological salt solution
PTX	=	picrotoxinin

PTZ	=	pentylenetetrazole
sec	=	second
S.E.M.	=	standard error of the mean
SGOT	=	glutamic oxaloacetic transaminase
SGPT	=	glutamic pyruvic tranferase
SHMT	=	serine hydroxymethyl transferase
SRF	=	sustained repetitive firing
SSADH	=	succinic semialdehyde dehydrogenase
STR	=	strychnine
TBPS	=	*t* -butyl-bicyclophosphorothionate
TM	=	transmembrane domain
VPA	=	valproic acid
VPU	=	n-(2-propylpentanoyl)urea
v/v	=	volume by volume
w/v	=	weight by volume
Zn^{++}	=	zinc ion

CHAPTER I

INTRODUCTION

1. Epilepsy

1.1 Definition and epidemiology

The word epilepsy is derived from the Greek words meaning "to be seized" or "to be attacked". This terminology derived from the even older notion that all diseases represented attacks by the gods or evil spirits, considered to be "the sacred disease" (Adams et al., 1997; Trescher and Lesser, 2000).

Epilepsy was known to the ancient Babylonians and was described by Hippocrates, who considered it as disease of the brain that must be treated by diet and drugs (Engel and Pedley, 1997). In 1890, John Hughlings Jackson concisely defined epilepsy as "……an occassional excessive and disordered discharge of gray matter" (Fukuzako and Izumi, 1991; McNamara, 2001).

Epilepsy is not a disease but a syndrome of many different cerebral disorders characterized by recurrent seizures, the clinical manifestation of an abnormal discharge of a set of neurones in the brain, due to excessive fluctuations in cerebral electrochemical balance (Avoli, 1997; Foldvary and Wyllie, 2000; Gilroy, 2000; Hopkins and Shorvon, 1995).

Epilepsy afflicts at least 2.5 million people in the United States and about 0.5% of people worldwide (Löscher, 2002; McNamara, 2001; Ure and Perassolo, 2000). The incidence of seizures is highest in the first year of life, especially in the first month. Rates are low during most of adulthood, and there is a secondary rise after age 60 years. Male tends to predominate in both prevalence and incidence (Honavar and Meldrum, 1997; Trescher and Lesser, 2000).

1.2 Classification of epilepsy

The classification of epilepsy is complex and can be based on the etiology, pathology, age of onset, clinical seizure, electroencephalogram (EEG) findings and prognosis. From the mid-19th century, epilepsies have been classified into two major groups. Patients with epilepsy in the absence of any history of prior neurologic insults are usually categorized as "primary or idiopathic epilepsy". If historical information identified a reasonable predisposing factor, the epilepsy is termed "secondary or symptomatic epilepsy" (Engel and Pedley, 1997; Foldvary and Wyllie, 2000).

A revised classification of individual seizure types was accepted in 1981 by the General Assembly of the International League Against Epilepsy (ILAE) and had been widely used in the management of epilepsy (Commission on Classification and Terminology of the ILAE, 1981). However, this does not accurately reflect the complicated epileptic syndrome which is characterized by a cluster of signs and symptoms customarily, occuring together. These include such item as type of seizure, etiology, anatomy, precipitating factors, age of onset, chronicity, diurnal and circadian cycling and sometimes prognosis (Dreifuss, 1997; Fukuzako and Izumi, 1991).

The 1981 classification of epileptic seizures is considered pragmatic for clinical use and serves as a useful tool for guiding decisions about when to treat epilepsy and how to choose among the antiepileptic drugs (Fukuzako and Izumi, 1991; Rall and Schleifer, 1990). For propose of drug treatment, it is more useful to classify patients according to the types of seizures they experience. A simplified form of the proposal from the Commission on Classification and Terminology of the ILAE (1981), based on the clinical manifestrations of the attacks and the pattern of the EEG, is presented in term of seizure types and characteristics in table 1.1 (Dreifuss, 1997).

Table 1.1 International Classification of Epileptic Seizures (Modified from Dreifuss, 1997)

SEIZURE TYPE		CHARACTERISTICS
I. Partial seizures (focal, local seizures)	A. Simple partial seizures	Various manifestations, without impairment of consciousness, including convulsions confined to a single limb or muscle group (*Jacksonian motor epilepsy*), specific and localized sensory disturbances (*Jacksonian sensory epilepsy*), and other limited signs and symptoms depending upon the particular cortical area producing the abnormal discharge
	B. Complex partial seizures	Attacks of confused behavior, with impairment of consciousness, with a wide variety of clinical manifestations, associated with bizarre generalized EEG activity during the seizure but with evidence of anterior temporal lobe focal abnormalities even in the interseizure period in many cases
	C. Partial seizures secondarily generalized	
II. Generalized seizures (convulsive or nonconvulsive)	A.1. Absence seizures	Brief and abrupt loss of consciousness associated with high-voltage, bilaterally synchronous, 3-per-second spike-and-wave pattern in the EEG, usually with some symmetrical clonic motor activity varying from eyelid blinking to jerking of the entire body, sometimes with no motor activity.
	A.2. Atypical absence seizures	Attacks with slower onset and cessation than is usual for absence seizures, associated with a more heterogeneous EEG
	B. Myoclonic seizures	Isolated clonic jerks associated with brief bursts of multiple spikes in the EEG
	C. Clonic seizures	Rhythmic clonic contractions of all muscles, loss of consciousness, and marked autonomic manifestations
	D. Tonic seizures	Opisthotonus, loss of consciousness, and marked autonomic manifestations
	E. Tonic-clonic (*grand mal*) seizures	Major convulsions, usually s sequence of maximal tonic spasm of all body musculature followed by synchronous clonic jerking and a prolonged depression of all central functions
	F. Atonic seizures	Loss of postural tone, with sagging of the head or falling

A classification of epilepsies and epileptic syndromes has been published by the Commission on Classification and Terminology of the ILAE (1989). The classification stratifies epilepsies in four major classes on the basis of the presumed site of origin of the seizures (localization–related or generalizes) and the etiology of the seizures (idiopathic or cryptogenic or symptomatic). Within each class, further attempts are made to classify patients in more detailed subgroup with reference to selected variables such as age, family history, brain development, intellectual function, specific etiology, EEG findings and outcome (Rinaldi et al., 2000).

1.3 Etiology

Epilepsy has been considered a genetic disease for centuries. As early as 450 B.C. in his treatise, *The Sacred Disease*, Hippocrates concluded that epilepsy was inherited (Hauser et al., 1983). Numerous studies suggest that the genetic susceptibility to seizure is normally distributed in the general population and that there is a threshold above with the condition becomes clinically evident. Genetic factors appear to be most significant in patients with various primary epilepsies (Hopkins, 1993).

Seizures can occur in patients with almost any pathologic processes that affect the brain, such as perinatal injury, trauma, tumors, stroke, vascular disease and degenerative disorders (Hopkins, 1993). Several areas of the brain, especially the hippocampus, appear to be particularly vulnerable to the homeostasis alterations produced by recurrent and prolonged seizures. Cellular changes are also seen in the cerebellum and to a lesser extent in the cerebral hemispheres (Menkes, 1990).

From a neurophysiologic point of view, an epileptic seizure has been defined as an alteration of central nervous system (CNS) function resulting from spontaneous electrical discharge in a disease population of cortical gray matter or the brainstem (Menkes, 1990). Although a major seizure may involve nearly entire CNS, in its simplest form a focal seizure represents an abnormality of function in only a small portion of the brain, the epileptic focus that John Hughlings Jackson established this basic concept

many years ago. Epileptogenesis requires a set of epileptic neurones and circuity to permit multicellular synchronization (Menkes, 1990).

The transmembrane potentials of many neurones within an epileptic focus undergo a sudden, long-lasting depolarization coincident with the cortical paroxysm. This was named the "paroxysmal depolarizing shift" (PDS) by Matsumoto and Ajmone Marsan (Ayala et al., 1973). The synchronous depolarization of a large number of neurones in epileptic focus is associated with a burst of action potentials. The summation of these action potentials produces an "interictal spike" of EEG that can be recorded from the surface of the brain or from the skull. The PDS is usually followed by a hyperpolarization potential and neuronal inhibition that correspond to the slow wave seen on the EEG (Ayala et al., 1973; Menkes, 1990) (Figure 1.1).

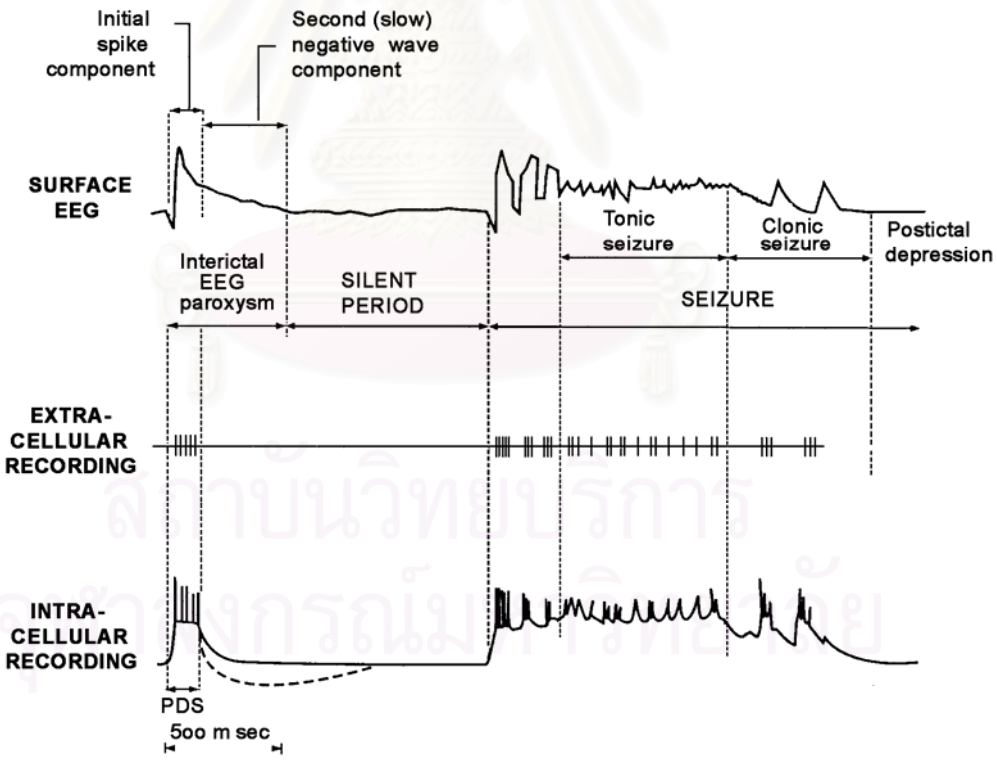

Figure 1.1 The schematic diagram of relations between cortical EEG, extracellular, and intracellular recordings in an epileptic focus (From McNamara, 2001).

2. Drugs for treatment of epilepsy

In order to have a complete control of epilepsy, the appropriate treatment must be selected on the basis of the type of epilepsy and the cause of seizures (Löscher, 1998; Mattson, 1995). The goal of antiepileptic drugs (AEDs) should be the restoration of a normal life through complete control of seizures, while minimizing side effects and without interfering with normal brain function (Brodie and Dichter, 1996; Löscher, 2002; Stringer, 1998). Monotherapy should be recommended in a newly diagnosed epileptic patient becaused polytherapy does not suppress seizures more effectively and because drug toxicity and interactions become increasingly common as the number of drugs administered increases (Rall and Schleifer, 1990; Stringer, 1998).

Treatment with an antiepileptic drug is usually begun when the patient has more than one unprovoked seizure within a year, whatever the type. The best drug for the particular type of seizure is selected and administered in a dose high enough to bring the plasma drug concentration into a therapeutic range without unacceptable side effects (Brodie and Dichter, 1996). The chioces of conventional and recently developed AEDs considered to be appropriate for each seizure type are giving in table 1.2

3. Mechanisms of action of antiepileptic drugs

At the cellular level, three basic mechanisms of antiepileptic drugs are recognized: (1) modulation of voltage-dependent ion channels (Na^{+}, Ca^{++}, K^{+}), (2) enhancement of γ–aminobutyric acid (GABA)-mediated inhibitory neurotransmission, and (3) attenuation of excitatory, particularly, glutamate-mediated neurotransmission (Kwan et al., 2001; Meldrum, 1996).

Table 1.2 Drugs for treatment of epileptic seizures (Modified from McNamara, 2001).

SEIZURES		ANTISEIZURE DRUGS	
		CONVENTIONAL	RECENT DEVELOPED
1. PARTIAL SEIZURES:	1.1 Simple partial seizure	Carbamazepine Phenytoin Valproate	Gabapentin, Zonisamide Lamotrigine, Topiramate Levetiracetam, Tiagabine
	1.2 Complex partial seizure	Carbamazepine Phenytoin Valproate	Gabapentin, Zonisamide Lamotrigine, Topiramate Levetiracetam, Tiagabine
	1.3 Partial with secondarily generalized tonic-clonic seizure	Carbamazepine Phenobarbital Phenytoin, Valproate	Gabapentin, Zonisamide Lamotrigine, Topiramate Levetiracetam, Tiagabine
2. GENERALIZED SEIZURES:	2.1 Absence seizure	Ethosuximide Valproate	Lamotrigine
	2.2 Myoclonic seizure	Valproate	Lamotrigine Topiramate
	2.3 Tonic–clonic seizure	Carbamazepine Phenobarbital Phenytoin Primidone Valproate	Lamotrigine Topiramate

3.1 Modulation of voltage-dependent ion channels

3.1.1 Na^+ channels

Electrophysiological analysis of individual neurone during a partial seizure demonstrates that the neurones undergo depolarization and fire action potentials at high frequencies. This pattern of neuronal firing is the characteristic of a seizure and is uncommon during physiological neuronal activity (McNamara, 2001). Inhibition of the high-frequency firing is thought to be mediated by reducing the ability of Na^+ channels to recover from inactivation, which little or no effect on low-frequency firing. Carbamazepine, lamotrigine, phenytoin and valproic acid exhibit anticonvulsant activity by this mechanism (Kwan, 2001; McNamara, 2001; Porter and Meldrum, 2001; Ragsdale and Avoli, 1998; Waterhouse and Delorenzo, 1996).

3.1.2 T-type Ca^{++} channel

In primary generalized seizures, the cortex becomes diffusely and synchronously involved in a discharge that appears as rhythmic spike and wave activity, usually at 3-per-second on the electroencephalogram. It is probable that the thalamic acts as a "pace-maker" and that thalamocortical relay neurones synchronized cortical discharges. The low-threshold T-type Ca^{++} channel is expressed predominately in the thalamocortical relay cortical neurones and inactive at the normal resting potential. When thalamic neurones exhibit tonic firing, the T-type Ca^{++} channel mediated a low threshold calcium spike and a burst of sodium-dependent spikes occurs. Blocking of the T-type Ca^{++} currents by ethosuximide, dimethadione and valproic acid are thought to explain the effect of these compounds in absence seizure (Kwan, 2001, McNamara, 2001; Porter and Meldrum, 2001; Waterhouse and Delorenzo, 1996).

3.1.3 K^+ channels

Direct activation of voltage-dependent K^+ channels hyper polarizes the neuronal membrane and limits action potential firing. Accordingly, K^+ channel activators have anticonvulsant effects in some experimental seizure models. Potentiation of voltage-sensitive K^+ channel currents may prove to be an important target for future AEDs

development. The novel antiepileptic agent retigabine, currently undergoing Phase II clinical trial, is believed to exert its effects, at lease in part, by activation of the KCNQ2/KCNQ3 K^+ channels (Kwan, 2001; Wickenden, 2002). Mutations in the KCNQ2/KCNQ3 K^+ channels have been reported in benign neonatal familial convulsions, a generalized epilepsy (Rogawski, 2000).

3.2 Enhancement of γ-aminobutyric acid-mediated inhibition

Enhancement of GABA-mediated inhibition can be produced in many different way, involving either direct action on the GABA receptor-chloride channel complex (as with benzodiazepine, barbiturate) or actions on the reuptake or metabolism of GABA (as with tiagabine and vigabatrin). This mechanism provides protection against generalized and focal seizures (Kwan, 2001; Porter and Meldrum, 2001; Waterhouse and Delorenzo, 1996).

3.3 Attenuation of glutamate-mediated excitation

Reduction of excitatory glutamatergic neurotransmission is potentially important: AMPA(α-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptor blockage probably contributes to the effect of phenobarbital and topiramate, NMDA (*N*-methyl-D-aspartate) receptor blockage probably contributes to the effect of felbamate (Dichter and Brodie, 1996; Porter and Meldrum, 2001).

4. Valproic acid

In 1882, Valproic acid (2-propylpentanoic acid, dipropylacetic acid; VPA) was first synthesized in the United State by Burton and used as an organic solvent. Its anticonvulsant activity was fortuitously discovered when it was used as a solvent in a drug screening program by Meunier and coworkers in 1963. After its first clinical trial in 1964, VPA was introduced into list of drugs for treatment of epilepsy. Unlike the other antiepileptic drugs, which are heterocyclic compounds containing nitrogen molecule,

VPA is a simple branched carboxylic acid and therefore radically different from the other antiepileptic drugs (Kupferberg, 1982; Löscher, 1999; Rogawski and Porter, 1990).

Although initially identified on the basis of its ability to protect against pentylenetetrazole (PTZ) induced seizures. VPA was subsequently demonstrated to have broad spectrum of anticonvulsant activity in a wide variety of animal seizure models (Rogawski and Porter, 1990). VPA is active against tonic, clonic and tonic-clonic seizures induced by a variety of chemoconvulsants in addition to PTZ, including to bicuculline, picrotoxin, penicillin and strychnine. Furthermore, it is also active in the maximal electroshock test as well as in kindling model (Davis et al., 1994; Rowan, 1997).

4.1 Mechanisms of action of VPA

Despites an accumulating literature on the metabolic and neurophysiological effects of VPA, the basic mechanism of its action remains obscure and the anticonvulsant activity may relate to a combination of mechanisms in the central nervous system (Johannessen, 2000; Löscher, 1999; McNamara, 2001). Several hypotheses on quite different experimental studies exist for the mechanisms of action of VPA.

4.1.1 Effects on brain GABA level

The first hypothesis suggests that VPA increases GABA level in the brain. Administration of VPA to experimental animals causes an increase in whole brain and synaptosomal GABA (Godin et al., 1969; Löscher and Vetter, 1985; Simler et al., 1973). Plasma and cerebrospinal fluid levels of GABA also increase under long-term treatment of VPA in epileptic patients and healthy volunteers (Löscher, 1999; Löscher and Schmidt, 1980). VPA has been shown to inhibit enzymes involved in GABA degradation including GABA aminotransferase (Tunnicliff, 1991) succinic semialdehyde dehydrogenase and aldehyde reductase (van der Laan et al., 1979). In addition, VPA increases the activity of glutamic acid decarboxylase, a major enzyme responsible for GABA synthesis (Davis et al., 1994; Löscher, 1981; Phillips and Fowler, 1982).

4.1.2 VPA potentiates GABA action

Electrophysiological recording techniques have reported that VPA selectively enhanced neuronal responsed to exogenously applied GABA (Davis et al., 1994; Gent and Phillips, 1980; Johnston and Slater, 1982; Macdonald and Bergey, 1979; Rogawski and Porter, 1990). However, the concentrations required to potentiate GABA responses in the electrophysiology studies are far higher than the normal therapeutic levels, indicating that augmentation of GABA-mediated inhibition by the postsynaptic mechanism is unlikely to account for the anticonvulsant action of the drug under normal circumstances (Rogawski and Porter, 1990; Waterhouse and Delorenza, 1996).

4.1.3 Effects on neuronal membranes

VPA appears to reduce sustained repetitive firing through its influence on sodium and potassium conductances (Davis et al., 1994). When high concentrations of VPA were applied to isolated *Aplysia* neurones, an increase in membrane potassium conductance developed, leading to hyperpolarize neurones (Slater and Johnston, 1978). At concentrations equivalent to therapeutic cerebrospinal fluid level in human, VPA diminished sustained repetitive firing of sodium dependent action potentials in mouse spinal cord and cortical neurones in culture (McLean and Macdonald, 1986).

In cultured hippocampal neurones, VPA indeed strongly delayed the recovery from inactivation of sodium channels, which would be consistent with reduction of sodium conductance (Van den Berg et al., 1993). In hippocampal slices, VPA interfered with Ca^{++}-entry into nerve endings and increased potassium conductance, which would contribute to a decreased synaptic transmission (Franceschetti et al., 1986). VPA also produced small reduction of low-threshold T-type Ca^{++} current in ganglion neurones (Kelly et al., 1990).

4.1.3 Effects on other neurotransmitters

VPA reduced neurotransmission mediated by excitatory amino acid such as aspartic acid, glutamic acid and γ-hydroxybutyric acid (GHB), which produced absence-like seizures in animals (Chapman et al., 1982). In amygdaloid slices

VPA suppressed the response mediated by NMDA receptors (Gean, 1994). However, glycine, which is an inhibitory neurotransmitter in brainstem and spinal cord, is not altered by VPA (Löscher, 1999).

4.2 Side effects and toxicity of VPA

The incidence of toxicity associated with the clinical use of VPA is remarkably low compared with other AEDs. The most common adverse effects are gastrointestinal disturbance, weight gain, thrombocytopenia, pancreatitis, neurological effects such as tremor or sedative and transient hair loss (Davis et al., 1994; Greenwood, 2000; Löscher, 1999). However, the drug is associated with two severe, albeit rare toxic effects, fatal hepatotoxicity and teratogenicity (Brodie and Dichter, 1996; Davis et al., 1994; Löscher, 1999; Rowan, 1997).

Transient elevation of liver enzyme activity (alkaline phosphatase, glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT) have been observed in approximately 11% of patients receiving VPA (Jeavons, 1982). Fatal hepatotoxicity, occurs more frequently in patients <2 years old receiving polytherapy and does not appear to be related to the dose of VPA (Davis et al., 1994; Löscher, 1999). Studies in animals have shown that VPA exhibits teratogenic effects (Jeavons, 1982). In human, an estimated risk of 1 to 2% for neural tube defects, predominantly spina bifida aperta, with maternal use of VPA therapy has been reported (Bjerkedal et al., 1982; Davis et al., 1994; Dreifuss and Langer, 1988).

Despite the hepatotoxic and teratogenic effects of VPA in patients, this drug is considered a safe medication, provided certain precautions are dealt with, particularly in children <2 years of age and pregnant women (Löscher, 1999). Since VPA has two severe side effects, hepatotoxic and teratogenicity, and less potent than other three established antiepileptic drugs; phenobarbital, phenytoin and carbamazepine, there is a substantially need to develop new derivatives of VPA with higher potency but lower toxicity (Bialer, 1999, Bialer et al., 1994).

5. N-(2-Propylpentanoyl) Urea or Valproyl urea (VPU)

N-(2-propylpentanoyl) urea is a synthetic chemical which was synthesized by Boonardt Saisorn and co-worker (Saisorn et al., 1992) in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The structure of VPU modelled partially on barbiturate ring and VPA in the same molecule (Figure 1.2).

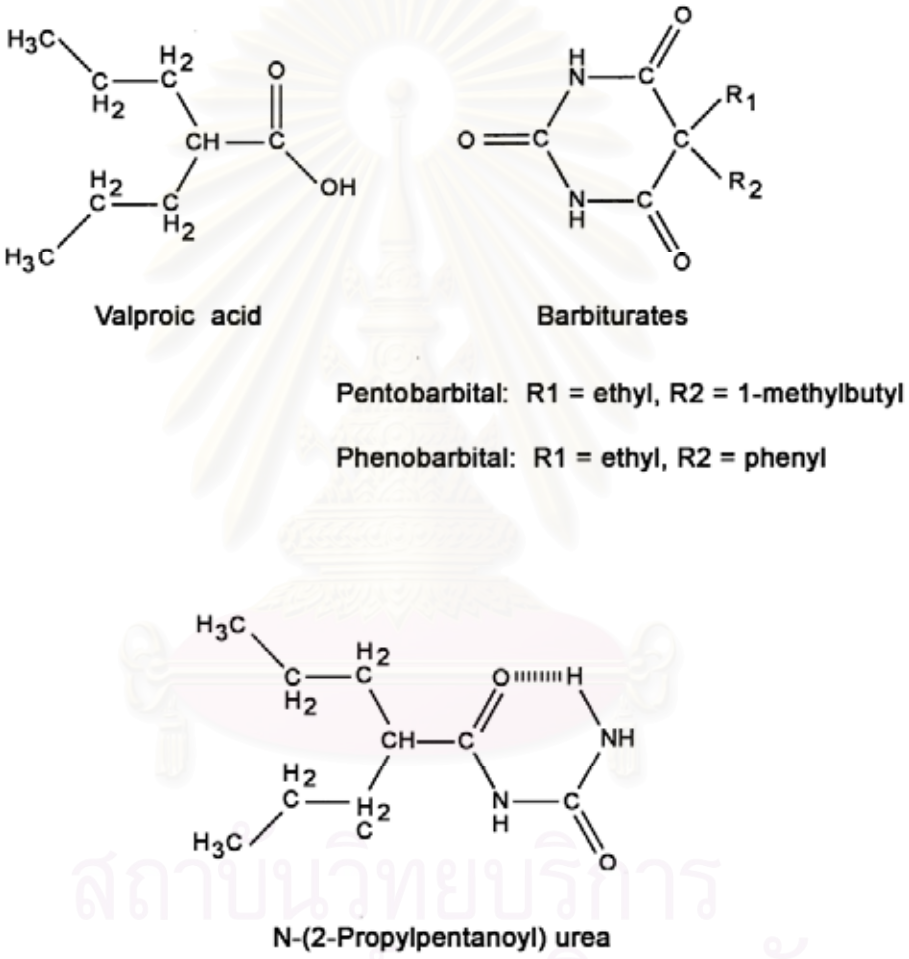

Figure 1.2 The structures of valproic acid, barbiturates and structure of n-(2-propylpen tanoyl) urea showing intramolecular hydrogen bonding (From Sooksawate, 1995).

In preliminary studies, intraperitoneally administered VPU demonstrated a higher protection than VPA in both the maximal electroshock seizure (MES) and the pentylenetetrazole (PTZ) tests. VPU weakly blocked the effect of bicuculline and was ineffective in strychnine test. Furthermore, VPU was also active orally demonstrating an ED_{50} approximately 6 times higher than its ED_{50} by the intraperitoneal route. Base on the relatively high medial lethal dose (LD_{50}), VPU possessed a greater margin of safety (LD_{50}/ED_{50}) than did VPA. VPU appeared to possess rather low side effects as predicted from its minor effects on rotorod test and locomotor activity test (Tantisira et al., 1997).

In brain microdialysis studies, VPU significantly decreased the levels of cortical excitatory (aspartate and glutamate) and inhibitory (GABA and glycine) amino acid neurotransmitters in dose dependent manner. The depression was greatest on glutamate and least on glycine (Sooksawate, 1995).

Developmental toxicity, regarding effects on axial rotation and embryonic growth, was lower in VPU-treated animals compared with those of VPA-treated (Meesomboon et al., 1997). Hepatotoxic effects were observed *in vivo* and *in vitro* only at large dose of VPU administration (Patchamart, 1996). Pharmacokinetic studies utilizing ^{14}C-VPU and autoradiographic technique demonstrated a rapid distribution characteristic of VPU into various organ tissues. In addition, *in vitro* studies using carboxylesterase from human liver and phenobarbital-treated mice liver shown that VPU was negligibly hydrolysed into VPA. Therefore, it was postulated that VPU *per se* and/or any metabolites other than VPA was responsible for the anticonvulsant activity (Kijsanayothin et al., 1997).

6. GABA and the $GABA_A$ receptor

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS (Hevers and Lüddens, 1998; Macdonald and Olsen, 1994; Tyndale et al.,1994). Estimations of the percentage of all central synapses that use GABA as their transmitter range from, depending on the brain region, 20-50% (Hevers and Lüddens, 1998;

Sieghart, 1995; Sieghart et al., 1999). The presence of a large amount of GABA in the brain was reported by Roberts and Frankel in 1950. Subsequently, GABA was proved to be an inhibitory neurotransmitter in mammalian brain by Roberts in 1974 (McGeer et al., 1988).

6.1 GABA synthesis and degradation

GABA is synthesized from glutamic acid by the enzyme glutamic acid decarboxylase (GAD) and degraded to succinic semialdehyde by enzyme GABA transaminase (GABA-T), and then to succinic acid by succinicsemialdehyde dehydrogenase (SSADH) (Cooper et al., 1996). GABA is packed into synaptic vesicles, where it is released in response to presynaptic calcium influx. GABA is taken up from the extracellular space by a specific GABA transporter in neuronal and glial membranes. In glia cells, GABA is degraded to succinic semialdehyde (SSA), wherease part of the GABA taken up into neurones can be reused for synaptic release (Figure 1.3) (Löscher, 1999).

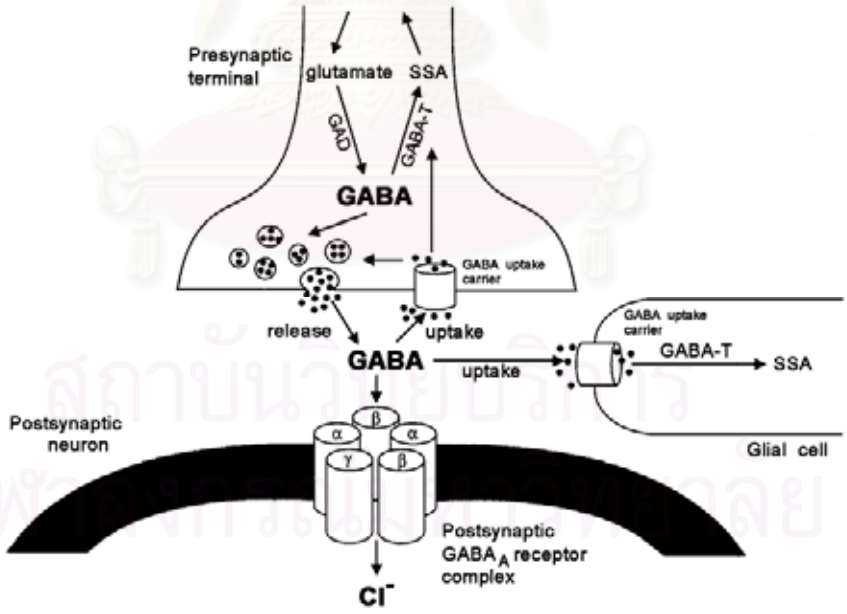

Figure 1.3 Schematic illustration of GABAergic inhibitory synapse in the brain with pre and postsynaptic processes involved in GABAergic transmission (From Löscher, 1999).

6.2 Classification of the GABA receptor

The actions of GABA are mediated by binding of GABA to two different receptor classes: $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) receptors (Barnard et al., 1998). The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily, that also includes the nicotinic acetylcholine, glycine, and $5\text{-}HT_3$ receptors. The activation of $GABA_A$ receptor is followed by the fast gating or opening of an integral chloride ion channel which results, in general, in the hyperpolarization of the neurones (Sieghart, 1995; Tyndale et al., 1994). The activation of $GABA_B$ receptor, as a member of the G-protein-linked superfamily, decrease Ca^{++} and increase K^+ conductance in neuronal membranes. The effect on Ca^{++} conductance appears to be primary associated with presynaptic P/Q and N-type Ca^{++} currents and modulation of K^+ conductance appears to be linked primarily with postsynaptic $GABA_B$ sites and with perhaps multiple types of K^+ channels. Decrease Ca^{++} conductance influence transmitter release and increase K^+ conductance associated neuronal hyperpolarization (Bowery, 2000; Bowery and Enna, 2000; Bowery et al., 2002).

6.3 Molecular structure of the $GABA_A$ receptor

The molecular structure of the $GABA_A$ receptor is believed to be a pentamer of subunits that assemble to form a functional $GABA_A$ receptor-ion channel (Figure 1.4) (Nayeem et al., 1994; Sieghart et al., 1999; Tretter et al., 1997). So far, a total of 20 $GABA_A$ receptor subunits belongings to several subunit classes ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, $\rho_{1\text{-}3}$, π, ε, δ, and θ) have been cloned from mammals (Barnard et al., 1998). The deduced amino acid sequence of $GABA_A$ receptor subunit show significant sequence identity about 20-30% with other ligand-gated ion channel (Mehta and Ticku, 1999; Whiting et al., 1995). Amino acid sequence identity among isoforms of the same class is about 70%, whereas the identity among classes is about 30% (Mehta and Ticku, 1999; Sieghart, 1995).

Each subunit of the $GABA_A$ receptor consists of four putative transmembrane hydrophobic segment (TM1-TM4). All $GABA_A$ receptor subunits consist of a large N-terminal extracellular domain and a large intracellular loop between TM3 and TM4 (Figure 1.4) (Macdonald and Olsen, 1994; Sieghart et al., 1999; Smith and Olsen, 1995). The large hydrophilic N-terminal part contains several glycosylation sites and a loop formed by two conserved cysteines and is thought to contain the GABA recognition site. The large cytoplasmic loop contained possible phosphorylation sites. The TM2 may contribute to the formation of a hydrophilic lining of the channel pore (Macdonald and Olsen, 1994).

The native $GABA_A$ receptors may have the stoichiometry two α, one β, and two γ, -subunits ($2\alpha\beta2\gamma$) or two α, two β, and one γ subunits ($2\alpha2\beta\gamma$) (with the γ subunits in some cases being replaced by δ or by ε) (Barnard et al., 1998; Mehta and Ticku, 1999). As shown in table 1.3 the largest population (43%) of the $GABA_A$ receptor subtypes in rat brain is the $\alpha_1\beta_2\gamma_2$ subtype. Two other major populations are the $\alpha_2\beta_{2/3}\gamma_2$ (18%) and $\alpha_3\beta_n\gamma_{2/3}$ (17%) subtypes (McKernan and Whiting, 1996).

6.4 Pharmacology of the $GABA_A$ receptor

The $GABA_A$ receptor is a macromolecular protein that contains specific binding sites. A part from the natural transmitter, GABA, there are several compounds that have been utilized as agonists at the $GABA_A$ receptor, including muscimol, isoguvacine, piperidine-4-sulphonic acid. Bicuculline acts as a competitive antagonists. Picrotoxinin, pentylenetetrazole, and *t*-butyl bicyclophosphorothionate (TBPS) antagonize GABA in a noncompetitive manner (Krogsgaard-Larsen et al., 1997; Sieghart, 1995). Additional, activation of the $GABA_A$ receptor can be positively modulated by a wide range of pharmacologically and clinically important drugs acting at distinct sites. These include benzodiazepines, barbiturates, neuroactive steroids, Zn^{++}, ethanol, propofol, loreclezole, inhalation anesthetics etc. (Sieghart, 1995).

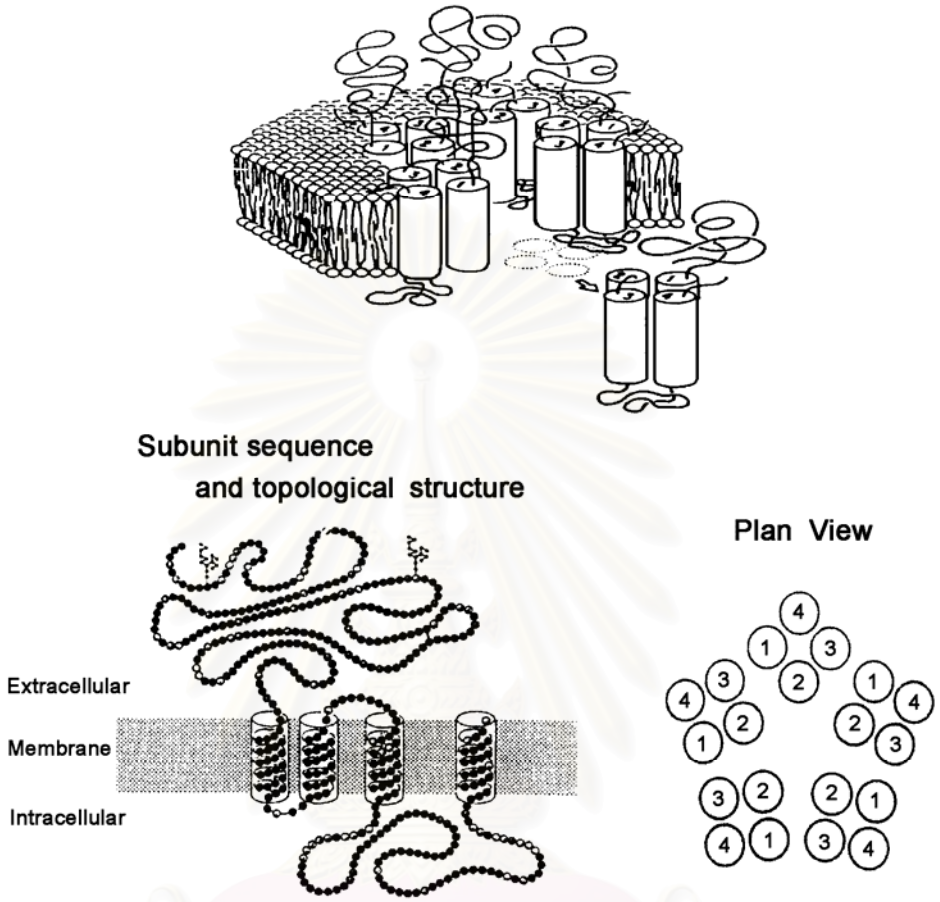

Figure 1.4 Model of the $GABA_A$ receptor. This receptor believed to be a pentamer of subunits that assemble to form a functional $GABA_A$ receptor-ion channel. Each subunit consists of four putative transmembrane domains numbered 1-4, a large extracellular domain at the N-terminal half of the polypeptide and a large cytoplasmic domain between TM3 and TM4. The TM2 may contribute to the formation of a hydrophilic lining of the channel pore (Modified from Macdonald and Olsen, 1994).

Table 1.3 Distribution of the major $GABA_A$-receptor subtypes in the rat brain (From McKernan and Whiting, 1996).

Subtypes	Relative abundance in rat brain (%)	Location and putative function
$\alpha_1\beta_2\gamma_2$	43	Present in most brain areas. Localised to interneurones in hippocampus and cortex, and cerebellar Purkinje cells
$\alpha_2\beta_{2/3}\gamma_2$	18	Present on spinal cord motor neurones and hippocampal pyramidal cells
$\alpha_3\beta_n\gamma_{2/3}$	17	Present on cholinergic and monoaminergic neurones where they regulate acetylcholine and monoamine turnover
$\alpha_2\beta_n\gamma_1$	8	Present on Bergmann glia, nuclei of the limbic systems, and in pancreas
$\alpha_5\beta_3\gamma_{2/3}$	4	Predominantly present on hippocampal pyramidal cells
$\alpha_6\beta_n\gamma_2$	2	Present on cerebellar granule cells
$\alpha_6\beta_n\delta$	2	Present on cerebellar granule cells
$\alpha_4\beta_n\delta$	3	Present in thalamus and hippocampal dentate gyrus
Other minor subtypes	3	Present throughout the brain

Location and function are listed where these have been investigated, and are not comprehensive. Other minor subtypes include $\alpha_1\alpha_6\beta_n\gamma_2$, $\alpha_1\alpha_3\beta_n\gamma_2$, $\alpha_2\alpha_3\beta_n\gamma_2$ and $\alpha_5\beta_n\gamma_2\delta$ subtypes and are represented together as a small population.

6.4.1 The GABA binding sites on the $GABA_A$ receptor

GABA binds to the $GABA_A$ receptor allowing a passive flow of negatively charge chloride ion down their electrochemical gradient into neurones. Activation of the adult CNS $GABA_A$ receptor almost always causes membrane hyperpolarization, results in a reduced excitability of the neurones (Tyndale et al., 1994). Nevertheless, in neonatal or some neurones, GABA demonstrated excitatory actions (Chen et al., 1996; Sieghart, 1995; Tyndale et al., 1994). Quantitative analysis of electro physiological experiment suggests that at lease two GABA molecules must bind to the receptor for full receptor activation (Sakmann et al., 1983). The GABA binding domain comprises of α and β subunit interface in the putative N-terminal extracellular domains (Smith and Olsen, 1995; Upton and Blackburn, 1997).

6.4.2 The interaction of picrotoxinin on the $GABA_A$ receptor

Picrotoxinin is a CNS convulsant that antagonize the $GABA_A$ receptor (Hevers and Lüddens, 1998; Sieghart, 1995). Picrotoxinin, active ingredient of picrotoxin, isolated from a poisonous species of the moonseed family (White et al., 1985). This nonnitrogenous compound exerts a noncompetitive inhibition via a site distinct from that for GABA, benzodiazepines, barbiturates, or steroid-binding sites (Hevers and Lüddens, 1998; Macdonald and Olsen, 1994). Since, the mechanism of picrotoxinin blockade of the GABA-induced chloride ionophore requires channel opening, the picrotoxinin binding site should be located within the channel (Sieghart, 1995). All regions or residues identified that affect picrotoxinin binding site are in the M2 domain of the $GABA_A$ subunits (Chang and Weiss, 2000).

6.4.3 The interaction of bicuculline on the $GABA_A$ receptor

The $GABA_A$ receptor currents are selective competitively antagonized by the plant convulsant bicuculline. Bicuculline reduces the $GABA_A$ currents by decreasing open frequency and mean duration. Bicuculline produced a competitive antagonism of the $GABA_A$ currents by competing with GABA for binding to the receptor (Macdonald and Olsen, 1994).

6.4.4 The interaction of barbiturates on the $GABA_A$ receptor

Depressant barbiturates, such as pentobarbital (PB) and phenobarbital, enhanced the action of GABA at the $GABA_A$ receptor by increasing the average channel open duration but did not alter receptor conductance or opening frequency (Mehta and Ticku, 1999; Sieghart, 1995; Study and Barker, 1981). At higher concentrations (>30 μM) barbiturates are able to direct open the $GABA_A$ receptor in the absence of GABA (Franks and Lieb, 1994; Mehta and Ticku, 1999; Sieghart, 1995). Barbiturates at concentrations exceeding millimolar values block the GABA receptor, leading to a reduced peak response (Peters et al., 1988; Rho et al., 1996). Based on the results from mutagenesis experiments, it has been proposed that the pentobarbital binding site is located within the second transmembrane regions of the receptor (Kardos, 1999). In addition, whole-cell currents directly activated by PB were blocked by the $GABA_A$ receptor antagonists, bicuculline and picrotoxinin (Rho et al., 1996).

6.4.5 The interaction of benzodiazepines on the $GABA_A$ receptor

Benzodiazepines (BZ) were introduced first into clinical practice in early 1960, and recognized to act by potentiating the inhibition action of GABA in the brain. The therapeutic applications of BZ as anxiolytic, anticonvulsant, sedative-hypnotic and muscle relaxant. Electrophysiological experiments have indicated that BZ enhance the action of GABA at the $GABA_A$ receptor by increasing the frequency of Cl^- channel opening. BZ are inactive at the $GABA_A$ receptor in the absence of GABA (Hevers and Lüddens, 1998; Sieghart, 1995; Smith and Olsen, 1995; Study and Barker, 1981).

The BZ site has the unique property of mediating two opposite effects, namely positive modulation (facilitation of GABA action by agonists) and negative modulation (depression of GABA action by inverse agonists). A spectrum of ligands has been display intrinsic activities ranging from full agonists to full inverse agonists. The BZ site is considered to be located at the interface between the α and γ_2 subunit (Möhler et al., 2002; Möhler et al., 2000). The types of α subunit are restricted to

α_1, α_2, α_3, and α_5, providing a heterogeneity in ligand affinities. In addition, site-direct mutagenesis has identified the amino acid residues histidine 101, tyrosine 159, glycine 200, threonine 206 and tyrosine 209 of the rat α_1 subunit, as well as phenylalanine 77 and methionine 130 of the γ_2 subunit as critical for ligand binding to benzodiazepine site (Figure 1.5) (Möhler et al., 2000).

The effect of BZ could be blocked by flumazenil, a prototypical specific BZ site antagonist. It has high affinity for BZ binding site throughout the brain. As an antagonist, flumazenil generally has no physiological effect on its own (Upton and Blackburn, 1997).

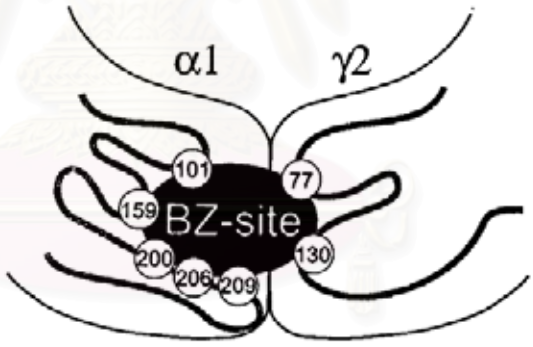

Figure 1.5 Amino acid constituents of the benzodiazepine site. The positions refer to the rat α_1 and γ_2–subunit sequences (From Möhler et al., 2000).

7. Glycine and the glycine receptor

Structurally, glycine is the simplest amino acid in all mammalial body fluids and tissue proteins in substantial amounts. It is recognized as one of the major inhibitory neurotransmitters in the vertebrate CNS (Cooper et al., 1996).

Glycine is synthesized in nerve tissue from glucose via the glycolytic pathway to produce 3-phosphoglycerate and 3-phosphoserine which is then converted to glycine by a reversible folate-dependent reaction catalyzed by the enzyme serine hydroxymethyltransferase (SHMT). Glycine can also be formed from glyoxylate by transamination with glutamate (Cooper et al., 1996). *In vitro* autoradiography study suggests that glycine occurs at high level in the spinal cord and medulla, lower in midbrain, hypothalamus and thalamus. Within the spinal cord glycine are present in the gray matter, but not the white columms (Rajendra et al., 1997).

Glycine exerts its action by binding to the glycine receptor in the postsynaptic neuronal membrane. Current findings suggest that at least three molecules of glycine are required to activate the glycine receptor (Olsen and DeLorey, 1999). Glycine receptor is also a member of the ligand-gated ion channel superfamily. Activation of the glycine receptor, like the $GABA_A$, caused an increase in chloride conductance which results in hyperpolarization and inhibition of postsynaptic neurones. This effect can be antagonized by strychnine (Kuhse et al., 1995; Rajendra et al., 1997). Immunological and molecular cloning studies have revealed that the glycine receptor is widely distributed throughout the mammalian CNS (Betz, 1991). The other site for glycine action, also call strychnine-insensitive glycine receptor, linked to the NMDA excitatory amino acid receptor (For review see Danysz and Parsons, 1998). Nanomolar concentrations of glycine increase the frequency of NMDA receptor channel opening in a strychnine-insensitive manner. The main effect of glycine is to prevent desensitization of the NMDA receptor during prolonged exposure to agonists by an acceleration of the recovery from its desensitized state (Cooper et al., 1996).

8. Patch-clamp techniques

The patch-clamp techniques is an electrophysiological method that allows the recording of macroscopic whole-cell or microscopic single channel currents flowing across biological membranes through ion channels (Penner, 1995). Patch-clamp techniques was developed in 1976 by Erwin Neher and Bert Sakmann to record current flow from single ion channels from membrane of denervated frog muscle fibres (Neher and Sakmann, 1976). The main targets of patch-clamp investigations are membrane-contained ion channels, including voltage-dependent ion channels (e.g., Na^+, K^+, Ca^{++}, Cl^- channels), receptor-activated channels (e.g., neurotransmitters, hormones, exogenous chemical mediator), and second messenger activated channels (e.g., $[Ca^{++}]_I$, cAMP, cGMP, IP_3, G-proteins or kinase) (Penner, 1995; Pun and Lecar, 1998).

8.1 Patch-clamp configuration

The basic patch-clamp configurations are cell-attached recording, inside-out recording, outside-out recording and whole-cell recording (Hamill et al., 1981; Liem et al., 1995; Penner, 1995). Several modifications of basic patch-clamp configurations have been developed in order to overcome some of their limitations or to perform certain experiments, these special techniques are perforated patch recording, double patch recording, loose patch recording, giant patch recording, detector patch recording etc. (Penner, 1995). The schematic representation of basic patch-clamp configuration was shown in figure 1.6

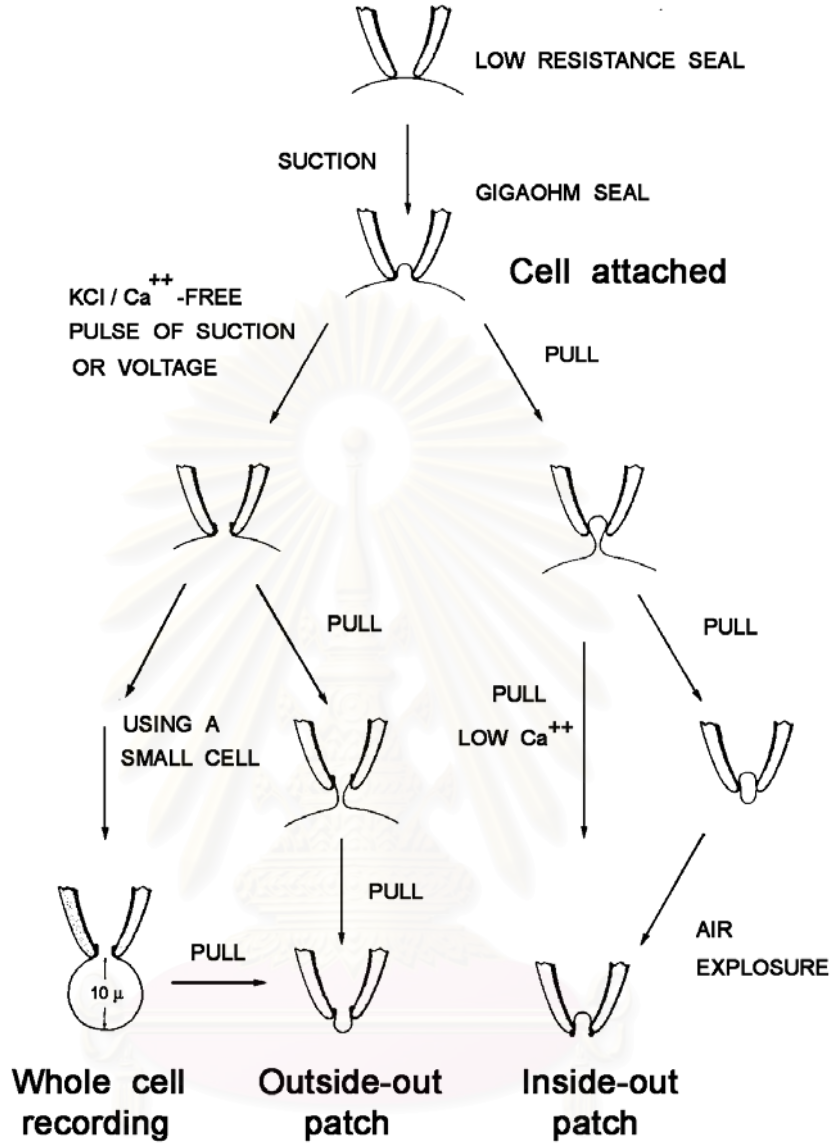

Figure 1.6 Schematic representation of basic patch-clamp configurations. A: low resistance seal when pipette touch cell. B: gigaseal formed by gentle suction, allowing cell-attached patch recording. C: whole-cell configuration created by ruptured the cell membrane patch with suction or a voltage pulse. D: outside-out patch formed by pulling from a whole-cell configuration. E: vesicle formation by pulling pipette from cell-attached configuration if calcium is present in the bath. F: inside-out excised patch obtained by briefly exposing vesicle to air or bath solution in the absent of calcium (From Hamill et al., 1981).

8.2 Whole-cell patch clamp

The whole-cell patch clamp is employed to study the function of the entire population of ionic channels on a cell membrane (Hamill et al., 1981; Penner, 1995). This configuration is particularly well suited for studying the effect of pharmacological events that interact directly with the membrane receptor channel. The whole-cell configuration is also suitable to measure exocytotic activity of secretory cells by measuring cell membrane capacitance (Liem et al., 1995; Penner, 1995).

The whole-cell patch clamp technique does offer several distinct adventage. This method is less traumatic to the cell than direct impalement with microelectrode. High-resistance seals minimize leakage currents, making low-noise recording. The internal milieu of the cell also can be altered via the pipette solution, even during the course of an experiment (Liem et al., 1995).

8.3 Formation of a gigaseal

The process of seal formation is monitored by observing the pipette currents on an oscilloscope. A conventional pulse amplitude can be obtained from electronic stimulator or pulse generator. Before the pipette is inserted into the bath, the current trace should be flat except for very small capacitive transients caused by the stray capacitance of the pipette and holder. When the pipette enter the bath, the pulses will cause current flow through the pipette (Figure 1.7).

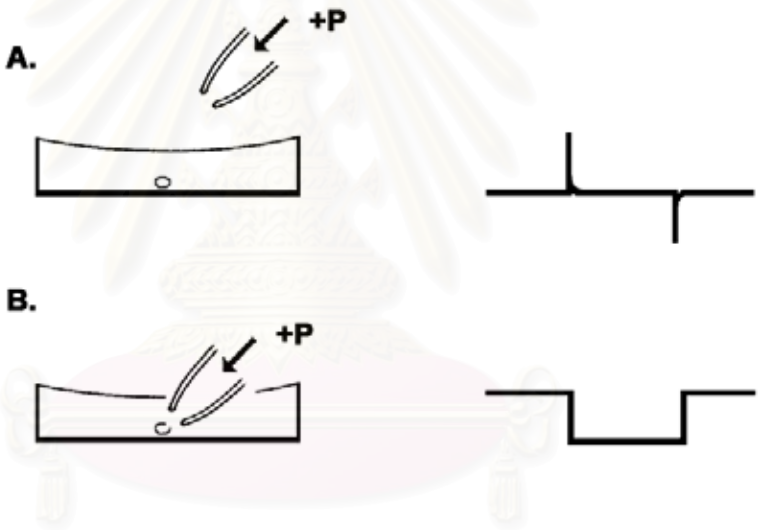

Figure 1.7 Pipette current recorded as the response to a low voltage pulse. When the pipette is out of the bath solution (A): the current consists of a very fast transient, as a result of the capacity component of the pipette. When it is introduced to the solution (B): a resistive component appears, from which it is possible to measure the pipette resistance (Modified from Moran, 1996).

To perform a gigaseal, the pipette is pushed against a cell, the current will become slightly small that reflecting an increase in resistance. Application of gentle suction should increase the resistance further and result in the formation of a gigaseal, which is characterized by the current trace becoming essentially flat again (Figure 1.8).

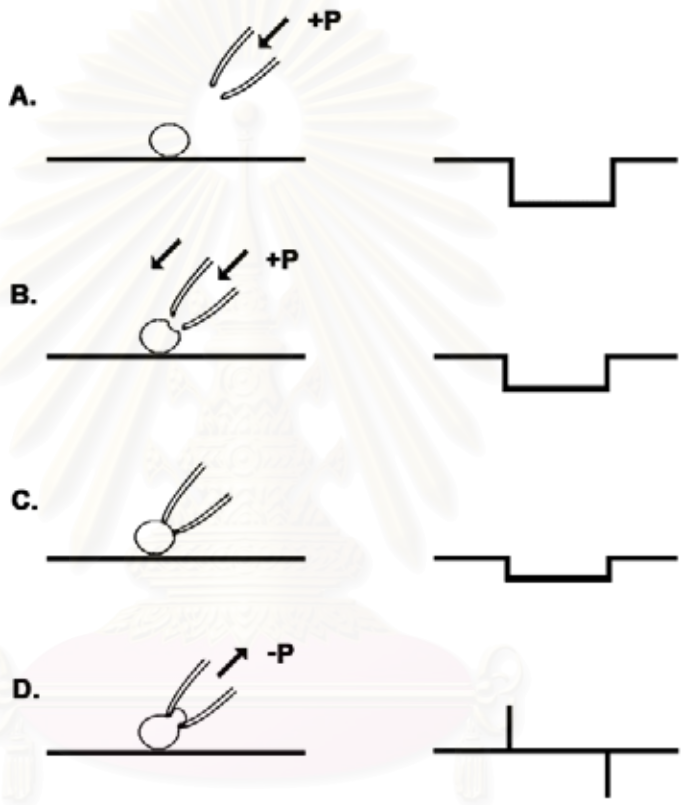

Figure 1.8 Making a gigaseal. (A): The pipette is near to the cell, and the resistive current observed as a response of a small voltage pulse corresponds to the pipette resistance. (B): The pipette is advanced to the cell, maintaining the positive pressure inside. Diminishing the access to the pipette tip is reflected as a reduction of the resistive component on the current. (C): When the pipette pressure is released, a further approach of the pipette to the cell in observed as an additional reduction of the resistive current. (D): Finally, a small negative pressure is applied to the pipette, and a *gigaseal* is formed (Modified from Moran, 1996).

8.4 Formation of whole-cell application

After a gigaseal is formed, the patch membrane can be broken by additional negative pressure or applying a voltage pulse of large amplitude and very short duration (1.3 volts DC, 0.5 ms) to induce membrane breakdown (“Zapping”). Electrical access to the cell interior is indicated by a sudden increase in the capacitive transients and decrease of the resistance (Figure 1.9) (Marty and Neher, 1995).

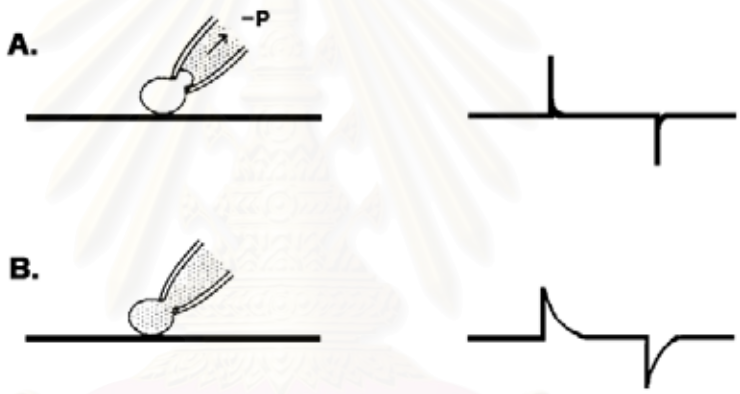

Figure 1.9 Formation of the whole-cell configuration. After obtaining the cell attached configuration (A): additional negative pressure or zapping, the membrane under the pipette tip can be broken, and a direct access of the pipette to the cell interior is obtained. (B): A significant increase of the measured capacity and decrease of the resistance (Modified from Moran, 1996).

9. Aims and objectives

The evidences presented above gave encouragement to extensive study on its precise mechanism of action of VPU, espectially modulation of GABA on the $GABA_A$ receptor. Thus, the present study was aimed to determined:

1. Effects of VPU on the $GABA_A$ currents in acutely dissociated rat hippocampal neurones.

2. A possible site(s) of action of VPU on the $GABA_A$ receptor.

CHAPTER II

MATERIALS AND METHODS

1. Experimental animals

All experiments were performed on male Wistar rats aged 21-27 days. They were obtained from National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom, Thailand.

The experimental animals were housed in animal care facilities at the Faculty of Pharmaceutical Sciences, Chulalongkorn University and maintained on 12:12 light-dark cycle at controlled temperature (25^0C). They were allowed free access to both food (C.P. Mice Feed) and water.

The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. Experimental instruments

1. Analog Digital Instruments (MacLab™/4, ADInstruments, Australia)

2. Automatic micropipette (Pipet-Lite™, U.S.A.)

3. Automatic controlled temperature (Model-7305, Polyscience, U.S.A.)

4. Automatic mixer (Vertex, U.S.A.)

5. Electronic stimulator (Model SEN 3201, Nihon Kohden, Japan)

6. Faraday cage

7. Inverted research microscope (Model IMT-2, Olympus, Japan)

8. Joystick manipulator (Model MN-151, Narishige, Japan)

9. Macintosh® Computer (Model LC 630, Apple Computer, Inc., U.S.A.) with software programs; Chart™ V 3.4 for data recording system (MacLab™, ADInstruments, Australia)

10. 0.22 μm membrane filter (Millipore, U.K.)

11. Oscilloscope (Model 420, Gould, U.K.)

12. Patch clamp amplifier (Model Axopatch 200 B, Axon Instruments, U.S.A.)

13. pH meter (Beckman, U.K.)

14. Thin wall borosilicate glass capillaries without filament (GC 150T-10, Clark Electromedical Instruments, U.K.)

15. Three dimensional hydraulic micromanipulator (Model MO-203, Narishige, Japan)

16. Vacuum pump (Model A-3S, Tokyo Rikakikai, Japan)

17. Vibroslice (Model 752 M, Campden Instruments, U.K.)

18. Vertical microelectrode puller (Model PP-83, Narishige, Japan)

3. Drugs and chemicals

1. N-(2-propylpentanoyl) urea (VPU was synthesized by Assistant Professor Dr. Chamnan Patarapanich and co-workers at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

2. Adenosine 5′-triphosphate (ATP; Sigma, U.S.A.)

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

4. Calcium chloride-2-hydrate ($CaCl_2$; Riedel-de Haën, Germany)

5. Cesium chloride (CsCl; Sigma, U.S.A.)

6. D(+)-Glucose monohydrate ($C_6H_{12}O_6$; Sigma, U.S.A.)

7. Diazepam (DZP; Sigma, U.S.A.)

8. Dimethyl sulfoxide (DMSO; Sigma, U.S.A.)

9. Ethylene Glycol-bis (β-aminoethyl Ether)-N,N,N′,N′,-Tetraacetic Acid (EGTA; Sigma, U.S.A.)

10. Flumazenil (FMZ; Sigma, U.S.A.)

11. Glycine (GLY; Sigma, U.S.A.)

12. γ -Amino-n-butyric acid (GABA; Sigma, U.S.A.)

13. Magnesium chloride-6-hydrate ($MgCl_2$; Riedel-de Haën, Germany)

14. N-(2-Hydroxyethyl) piperazine–N′-(2-ethanesulfonicacid)(HEPES; Sigma, U.S.A.)

15. Pentobarbital sodium (PB; Sigma, U.S.A.)

16. Picrotoxinin (PTX; Sigma, U.S.A.)

17. Potassium chloride (KCl; Riedel-de Haën, Germany)

18. Protease type X (Thermolysin; Sigma, U.S.A.)

19. Protease type XIV (Pronase; Sigma, U.S.A.)

20. Sodium chloride (NaCl; APS Finechem, Australia)

21. Strychnine (STR; Macfarlan Smith, U.K.)

22. Tris-base (Sigma, U.S.A.)

23. Valproic acid (VPA; Sigma, U.S.A.)

3.1 Drug and chemical preparation

All drugs were prepared as stock solutions, kept frozen in small microtubes, and dilute to appropriate concentrations in physiological salt solution (PSS) daily before use:

Bicuculline methochloride was dissolved in dimethyl sulfoxide (DMSO) at a concentration 5 mM. Diazepam was dissolved in DMSO at a concentration 1 mM. Flumazenil was dissolved in DMSO at a concentration 15 mM. GABA was dissolved in PSS at a concentration 0.1-1,000 mM. Glycine was dissolved in PSS at a concentration 1-3,000 mM. N-(2-propylpentanoyl) urea was dissolved in DMSO at a concentration 300 mM. Pentobarbital sodium was dissolved in DMSO at a concentration 30 mM. Picrotoxinin was dissolved in DMSO at a concentration 10 mM. Strychnine sulfate was dissolved in PSS at a concentration 20 mM. Valproic acid was dissolved in DMSO at a concentration 5,000 mM, the pH of the VPA-containing PSS became acidic and had to be adjusted to pH 7.4 with Tris-base.

The final concentration of DMSO was less than 0.1%(v/v) throughout and it was confirmed that this concentration did not alter or evoke any current in separate control experiments (see Appendix 1).

All drugs and chemicals were purchased from Sigma (U.S.A.) with some exceptions. N-(2-propylpentanoyl) urea was synthesized by Assistant Professor Dr. Chamnan Patarapanich and co-workers at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. $CaCl_2$, $MgCl_2$ and KCl were purchased from Riedel-de Haën (Germany). NaCl was purchased from APS Finechem (Australia).

3.2 Drug application

Rapid drug applications were performed with the "U-tube" method (Fenwick et al., 1982; Sooksawate and Simmonds, 1998). A U-shaped glass capillary of diameter ~150 μm with a small circular hole (~50 μm) at the tip was placed about 300 μm from the neurone to be recorded. One end (influx arm) of the U-tube was connected to a reservoir of external solution or drug solution held about 18 cm above the level of the U-tube tip. The other end (efflux arm) was connected to a vacuum pump (A-3S; Tokyo Rikakikai, Japan) which sucked solution from the reservoir through the U-tube and also drew external solution into the tube through the hole at its tip.

To reverse the flow through the hole of the U-tube, the line to the vacuum pump was occluded for 1-2 sec to allow gravity to drive the solution from the reservoir and superfuse the recorded neurone. Reopening of the valve caused to be resuctioned the solution surrounding a neurone within 50 ms.

A range of 0.1-1,000 μM GABA was superfused in the absence and presence of 100, 200 and 300 μM valproyl urea (VPU), 10 and 30 μM pentobarbital sodium (PB), 0.3 and 1 μM diazepam (DZP) and 1,000 and 3,000 μM valproic acid (VPA). A range of 1-3,000 μM glycine was superfused in the absence and presence of 300 μM VPU and 3,000 μM VPA. Also, a concentration range of 0.001-1,000 μM DZP, 1-1,000 μM PB, 1-5,000 μM VPA, 1-300 μM VPU with or without 3 μM GABA and 1-300 μM VPU, 1-5,000 μM VPA with or without 30 μM glycine was applied to a recorded neurones.

4. Experimental methods

Dissociation of hippocampal neurones

Hippocampal neurones were acutely dissociated from male Wistar rats aged 21-27 days as a method of Sooksawate and Simmonds (1998). Rats were killed by cervical dislocation and decapitated. The skull bones were removed with a rongeur and dissected dura. Brains were rapidly remove with a stainless steel spatula and placed into iced cold (~4^0C) physiological salt solution (PSS) for 3 min. The PSS containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, Glucose 11, HEPES 10, adjusted to pH 7.4 with Tris-base. The brain was removed and mounted, frontal side down, on the stage of a vibroslice (Campden Instruments, U.K.) using a thin film of α–cyanoacrylate glue. The slicing chamber was then immediately filled with iced-cold PSS. The brain was sliced into 400 μm thick sections in coronal plane. The slices containing the hippocampus were transfered to oxygen-saturated PSS at temperature 25^0C.

After 30 min preincubation in oxygen-saturated PSS, the slices were enzymatically treated in PSS containing 0.03-0.04% (w/v) protease type XIV (pronase; Sigma, U.S.A.) followed by 0.03-0.04% (w/v) protease type X (thermolysin; Sigma, U.S.A.) for 20 min each at 31^0C and continuously oxygenated throughout the procedure. After the enzymatic treatment, the sliced were washed three times with oxygen-saturated PSS.

The hippocampal regions were carefully dissected out with fine forceps. Mild trituration of the slices through fire-polished glass pipettes of progressively smaller tip diameter (2, 1, and 0.5 mm, respectively) served to dissociate the neurones from surrounding tissue. The neurones suspension was allowed to stand for 10 min before the supernatant containing dissociated neurones was taken off, leaving only 0.3-0.5 ml containing big lumps. The neurones were suitable for electrophysiological recording after being allowed to adhere to the base of recording chamber for 30-40 min.

5. Electrophysiological measurements

The effects of N-(2-propylpentanoyl) urea on $GABA_A$ and glycine receptors were investigated in acutely dissociated rat hippocampal neurones using the whole-cell application of the patch clamp technique (Hamill et al., 1981). Whole-cell membrane currents were recorded from the somata of the neurones under visual control with inverted microscope (IMT-2; Olympus, Japan). The holding potential was –20 mV in all experiments (Sooksawate and Simmonds, 1998).

Patch pipette was made from borosilicate glass capillaries without filament (1.5 mm O.D. and 1.17 mm I.D., Clark Electromedical Instruments, U.K.). The pipette was pulled by a two stages vertical microelectrode puller (PP-83; Narishige, Japan). In the first pull, the capillary was thined over a length of 7-10 mm to obtain a minimum diameter of 200 μm. The capillary was then recentered with respect to the heating coil and in the second pull the thin part was broken, producing two pipettes. The patch pipette was mounted on a suction pipette holder, which connected to amplifier head stage which was mounted on hydraulic micromanipulator (Model MO-203; Narishige, Japan). This, in turn, was mounted onto coarse manipulator (Model MM-33; Narishige, Japan).

The patch-pipette was filled with the intrapipette solution containing (mM): CsCl 140, $MgCl_2$ 4, Na_2ATP 4, EGTA 11, $CaCl_2$ 1, and HEPES 10, adjusted to pH 7.2 with Tris-base and filtered through 0.22 μm Millipore filter (Millipore, U.K.). The resistance between the patch-pipette and a reference electrode in the external solution (PSS) ranged from 3-5 MΩ.

Membrane current was measured with a patch clamp amplifier (Axopatch 200B; Axon Instruments, U.S.A.) and was monitored simultaneously on an oscilloscope (Model 420; Gould, U.K.). Currents from patch clamp amplifier were changed from analog to digital by MacLab™ (ADInstruments, Australia), and recorded by Macintosh computer using Chart™ V 3.4 (ADInstruments, Australia). During recording, neurone was superfused

with oxygen-saturated PSS at a rate of 2.5-3.0 ml/min. All experiments were performed at air conditioned room temperature (approximately 25^0C).

6. Data analysis

Experimental values are presented as mean±standard error of the mean (S.E.M.). To combine data from different neurones, the GABA and glycine responses on each neurone were expressed as percentages of the maximal response, or responses to 3 μM GABA or 30 μM glycine of the same neurone.

For concentration-response analysis, data were plotted using GraphPad PRISM™ Version 3.0 (GraphPad Software, U.S.A.) and fitted with a logistic equation in the form:

$$I = I_{min} + (I_{max} - I_{min}) / (1+(EC_{50} / [X])^H)$$

Where

I is the $GABA_A$ or glycine currents.

I_{min} and I_{max} are the minimal and the maximal currents recorded in a given neurone, respectively.

EC_{50} is the concentration of GABA or glycine eliciting 50% of the maximal currents.

$[X]$ is the GABA or glycine concentration.

H is the Hill coefficient.

Significance was tested by Student's *t*-test or one-way analysis of varient (ANOVA) followed by Dunnett's test where applicable. *P* value of less than 0.05 was considered to be significant.

CHAPTER III

RESULTS

1. The viability and morphological appearance of acutely dissociated rat hippocampal neurones.

After dissociation, some of hippocampal neurones were viable. Most of the dissociated hippocampal pyramidal neurones still had a typical morphological shape. Less than 10% of the neurones were still viable after plating into a recording chamber for 4 hrs. The photomicrograph of a representative hippocampal pyramidal neurone acutely dissociated from 21-day-old male Wistar rat has shown in figure 3.1.

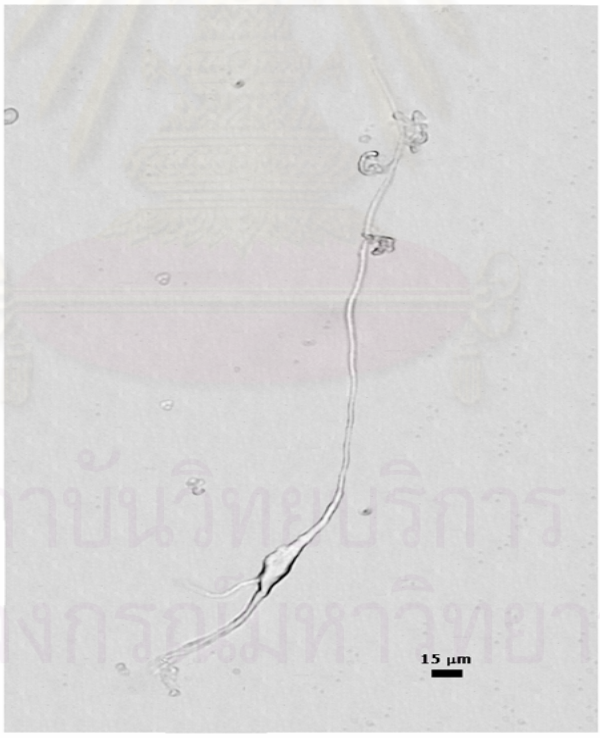

Figure 3.1 The photomicrograph of a representative hippocampal pyramidal neurone acutely dissociated from 21-day-old male Wistar rat by a method of Sooksawate and Simmonds (1998). Scale bar =15 µm.

2. Neuronal response to GABA.

The inward currents evoked by a rapid application of increasing concentrations of 0.3-1,000 μM GABA demonstrated dose-dependent manner. The desensitization characteristics of the current observed at high GABA concentration (10 μM GABA). The GABA concentration producing a maximal current amplitude was about 300 μM (Figure 3.2).

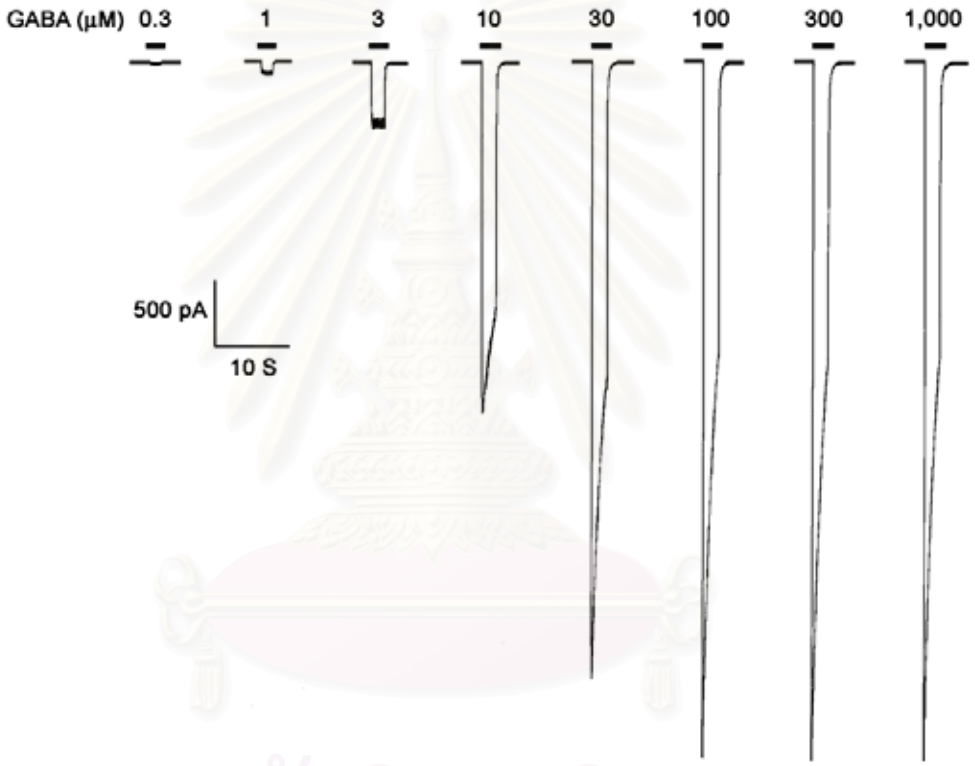

Figure 3.2 Representative current traces demonstrating whole-cell $GABA_A$ currents induced by increasing concentrations of 0.3-1,000 μM GABA to an acutely dissociated hippocampal pyramidal neurone from male Wistar rat aged 21-27 days. Drug applications were separated by at least 1-2 min interval and the duration was indicated by the solid line above the current traces. All record current traces are from the same neurone. Holding potential was –20 mV.

The EC_{50} values and Hill coefficient of the log.concentration-response relationship obtained from the control neurones were 8.29 $\pm$ 0.17 μM and 1.58 $\pm$ 0.04 respectively (n=16) (Figure 3.3).

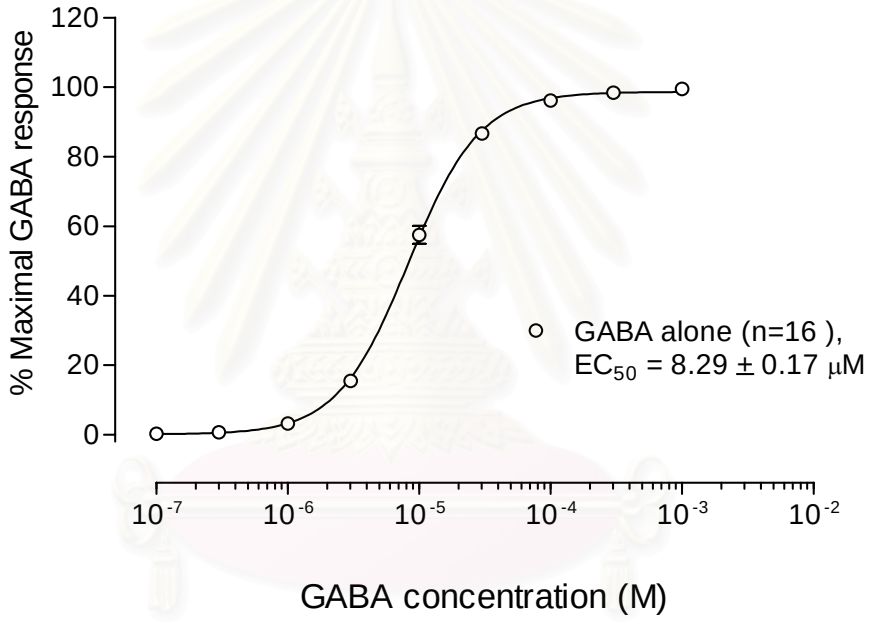

Figure 3.3 GABA log.concentration-response relationship in acutely dissociated rat hippocampal pyramidal neurones. Each point is the mean $\pm$ S.E.M. of the current response, expressed as percentage of the maximal response.

2.1 Effects of bicuculline methochloride (BMC) and picrotoxinin (PTX) on the GABA-induced inward currents.

The inward currents induced by GABA could be antagonized by 5 μM bicuculline methochloride (BMC), a competitive antagonist of GABA at the $GABA_A$ receptor (n=9) (Figure 3.4). BMC shifted the GABA concentration-response curve to the rightward without change in maximal GABA response (Figure 3.5). BMC increased the GABA EC_{50} values to 29.98 $\pm$ 0.12 μM (Student's *t*-test, $P<0.001$, n=9), compared with 7.10 $\pm$ 0.16 μM (n=9) of control.

The inward currents induced by GABA could also be blocked by 10 μM picrotoxinin (PTX), a noncompetitive antagonist of GABA at the $GABA_A$ receptor (n=16) (Figure 3.6). However, PTX not only shifted the GABA concentration-response curve to the right but also reduced the maximal GABA response (Figure 3.7). PTX increased the GABA EC_{50} values to 11.78 $\pm$ 0.37 μM (Student's *t*-test, $P<0.001$, n=16), compared with 8.15 $\pm$ 0.12 μM (n=16) of control.

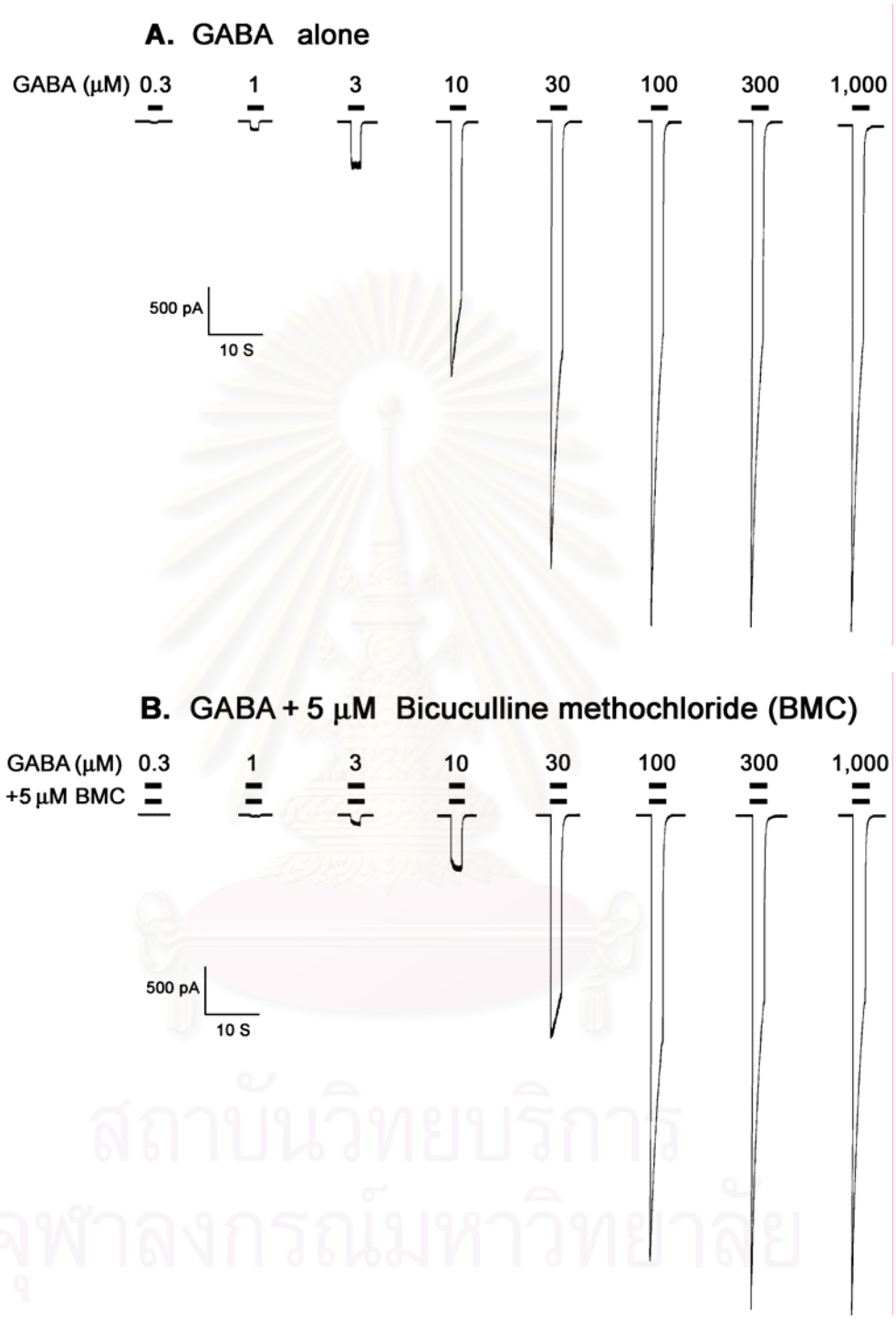

Figure 3.4 Representative current traces demonstrating the inhibition of the $GABA_A$ currents by 5 μM bicuculline methochloride (BMC).

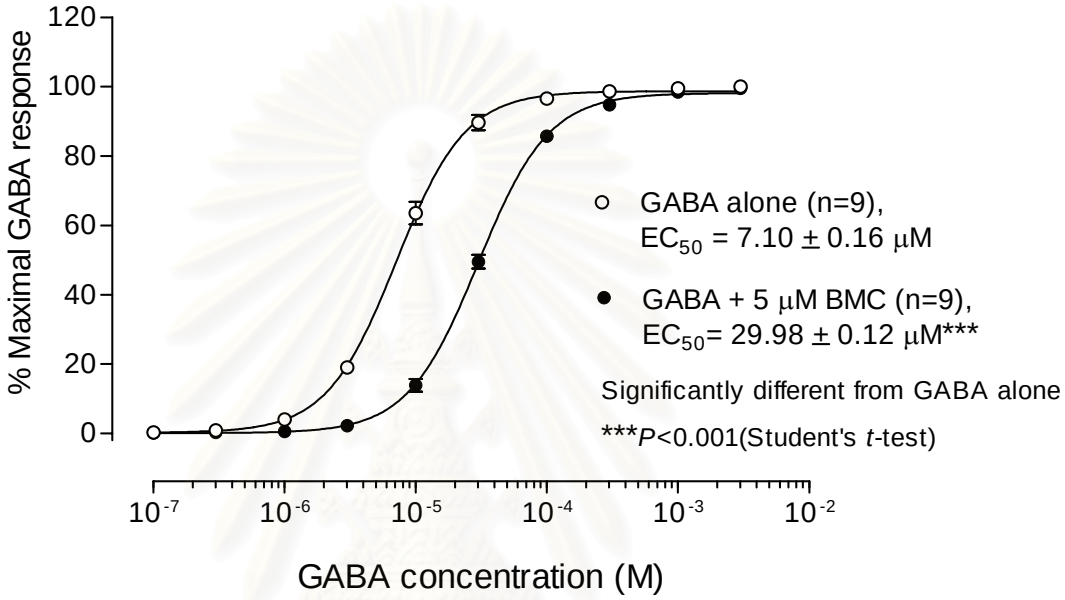

Figure 3.5 The GABA concentration-response curves demonstrating the inhibition of the $GABA_A$ currents by 5 μM bicuculline methochloride (BMC). BMC shifted the GABA concentration-response curve to the right without change in maximal GABA response.

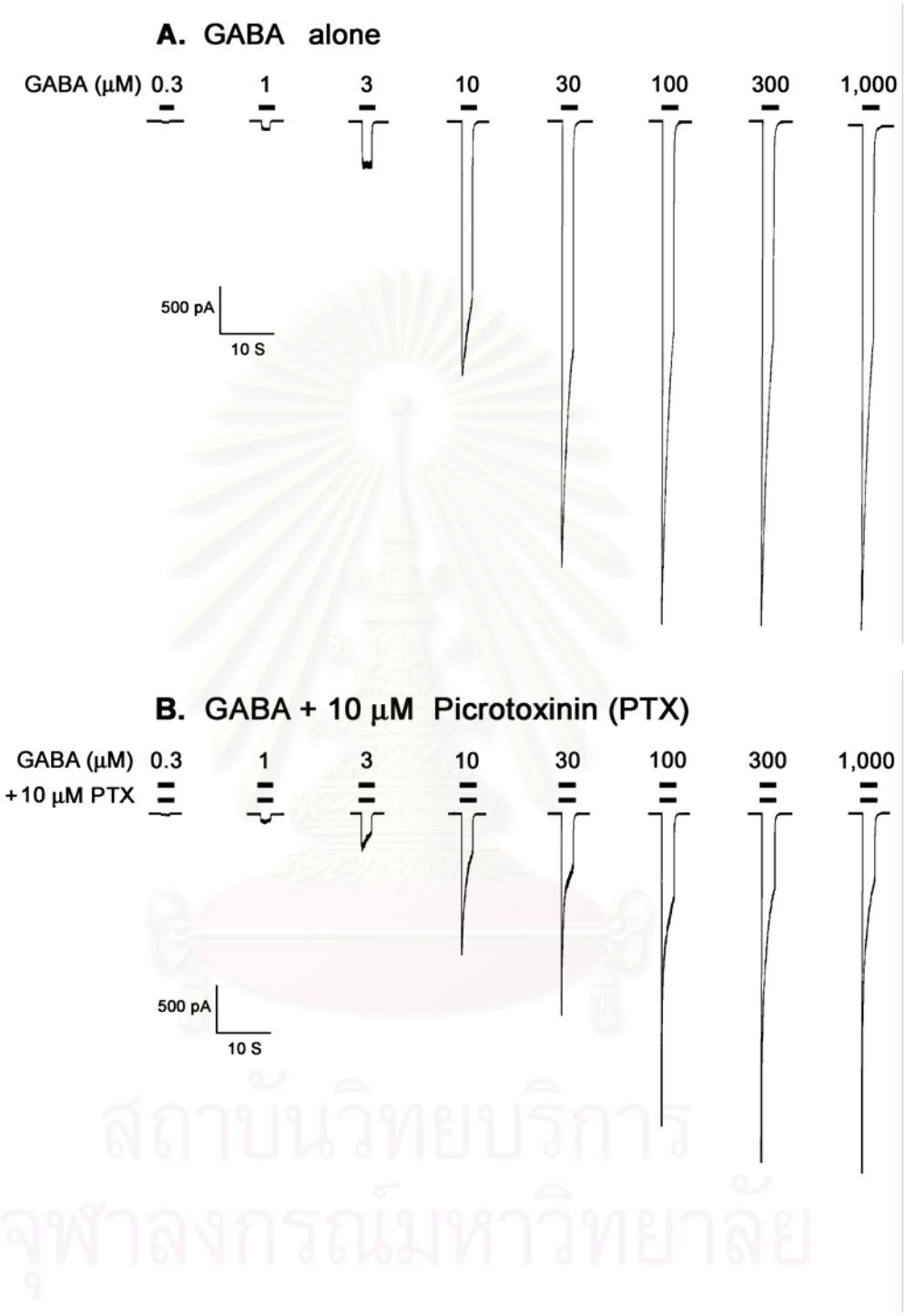

Figure 3.6 Representative current traces demonstrating the inhibition of the $GABA_A$ currents by 10 μM picrotoxinin (PTX).

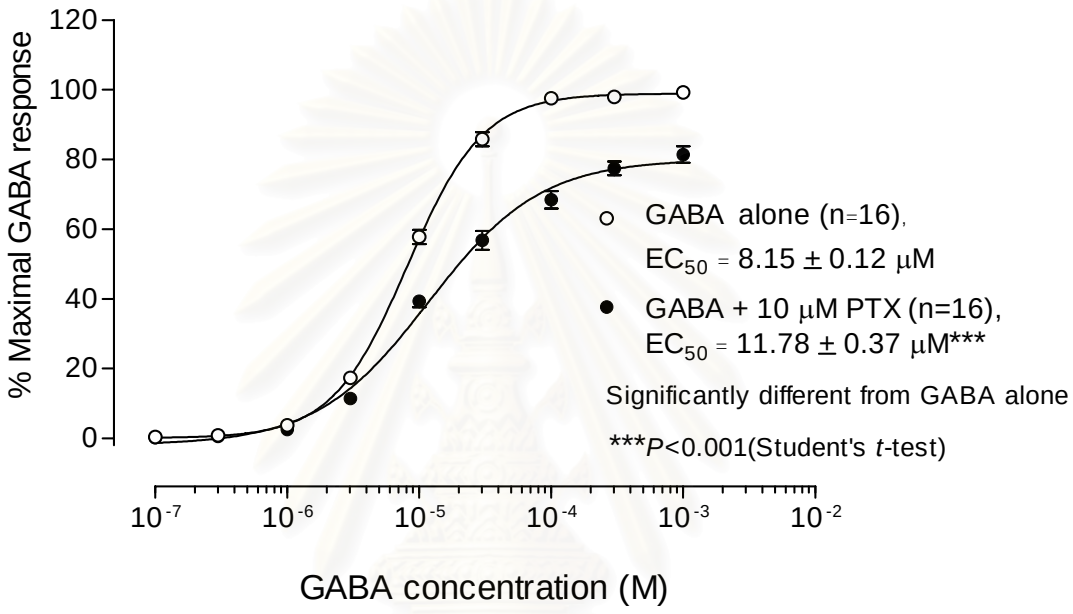

Figure 3.7 The GABA concentration-response curves demonstrating the inhibition of the $GABA_A$ currents by 10 μM picrotoxinin (PTX). PTX shifted the GABA concentration-response curve to the right with reduction in maximal GABA response.

2.2 Effect of pentobarbital sodium (PB) on the GABA-induced inward currents.

Pentobarbital sodium, in the absence of GABA, directly activated inward currents (n=14) that could be inhibited by the $GABA_A$ receptor antagonist bicuculline methochloride (Figure 3.8).

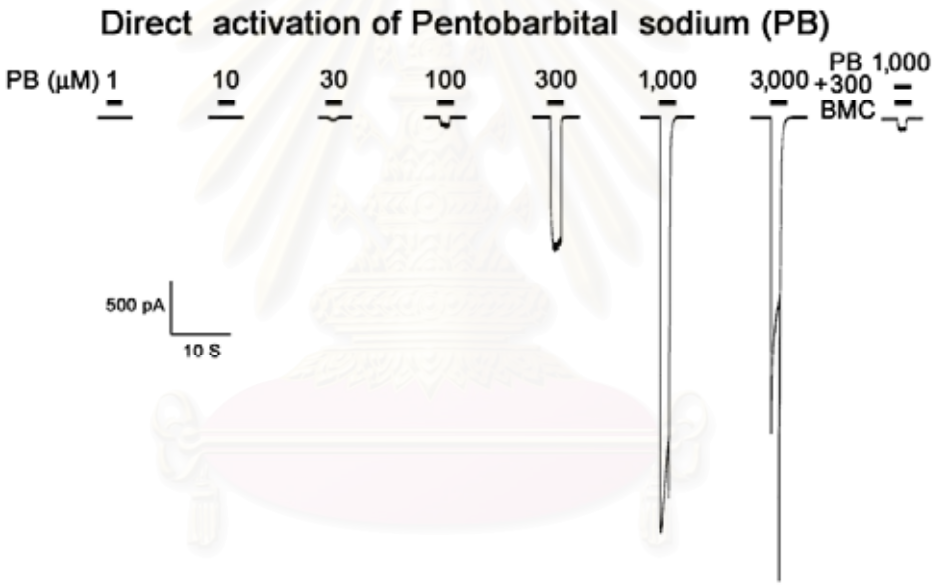

Figure 3.8 Representative current traces demonstrating the directly activated-inward currents of 1-3,000 μM pentobarbital sodium that could be inhibited by bicuculline methochloride (BMC).

Coapplication of 3 μM GABA with 1-1,000 μM PB produced the potentiation of the $GABA_A$ currents in concentration-dependent manner (n=13) (Figure 3.9). The inward currents induced by increasing concentrations of GABA on control and increasing concentrations of GABA in the presence of 10 and 30 μM PB are shown in figure 3.10 and 3.11, respectively.

The potentiation of the $GABA_A$ currents by PB resulted in a leftward shift of the GABA concentration-response curves. GABA in the presence of 10 and 30 μM PB decreased the GABA EC_{50} values to 7.41 $\pm$ 0.14 μM (Dunnett's test, $P<0.001$, n=12) and 4.78 $\pm$ 0.26 μM (Dunnett's test, $P<0.001$, n=11) respectively, compared with 10.39 $\pm$ 0.19 μM (n=23) of control (Figure 3.12).

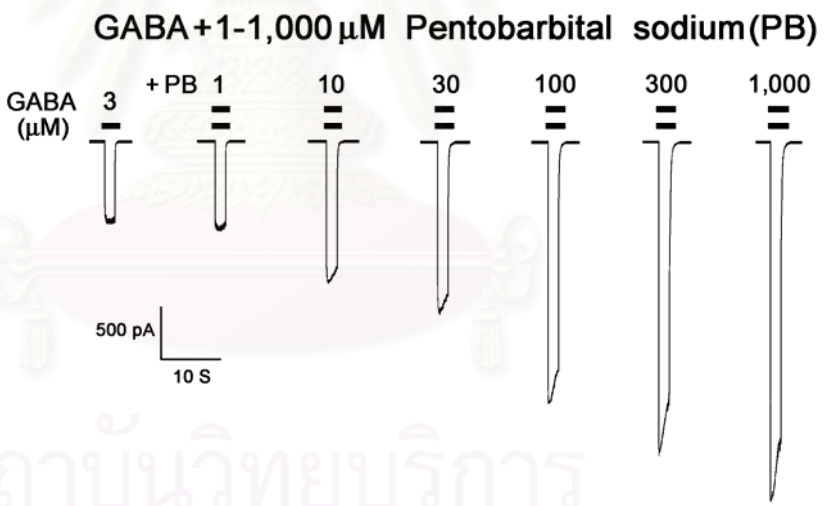

Figure 3.9 Representative current traces demonstrating the potentiation of 1-1000 μM pentobarbital sodium induced by 3 μM GABA.

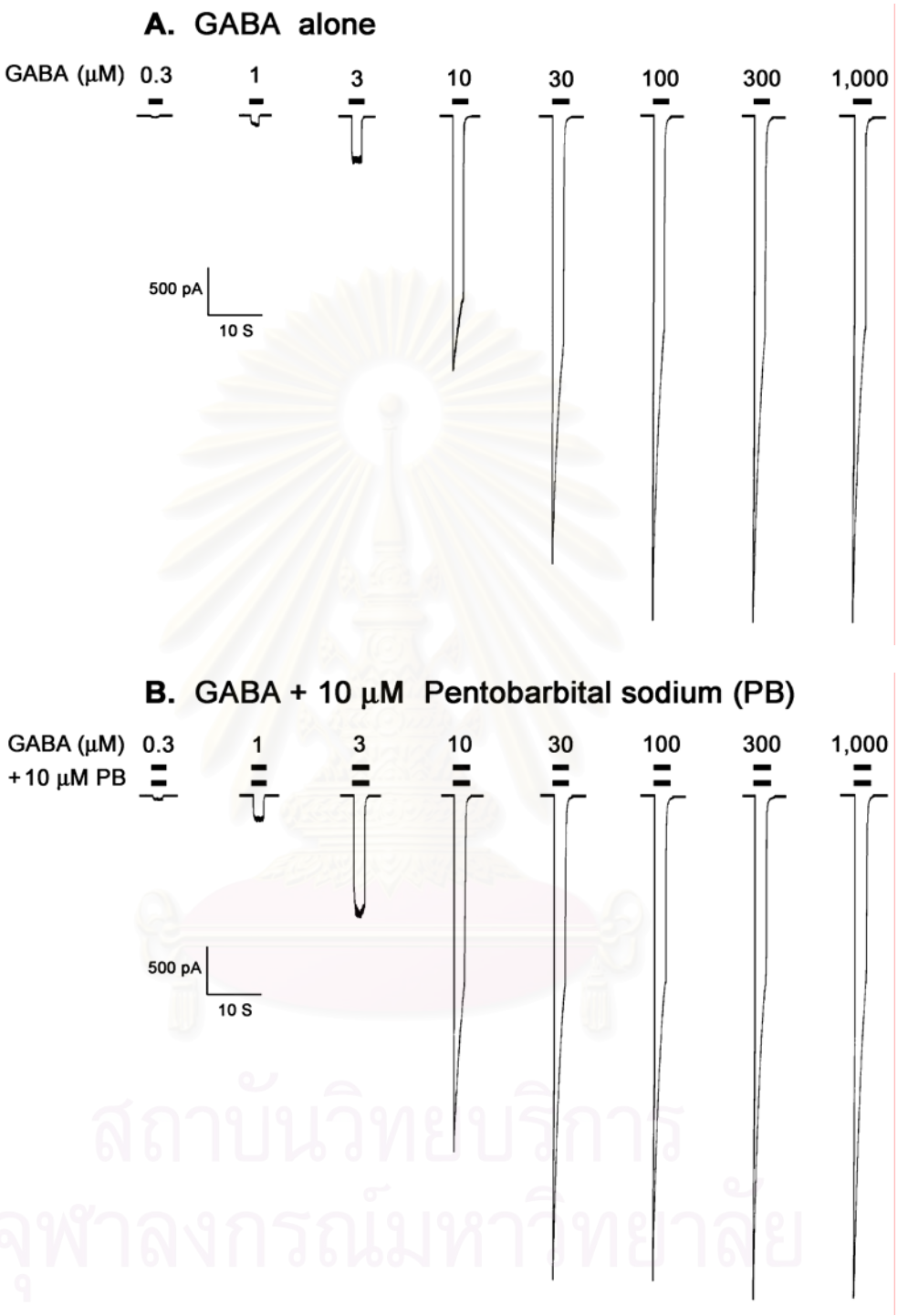

Figure 3.10 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 10 μM pentobarbital sodium (PB).

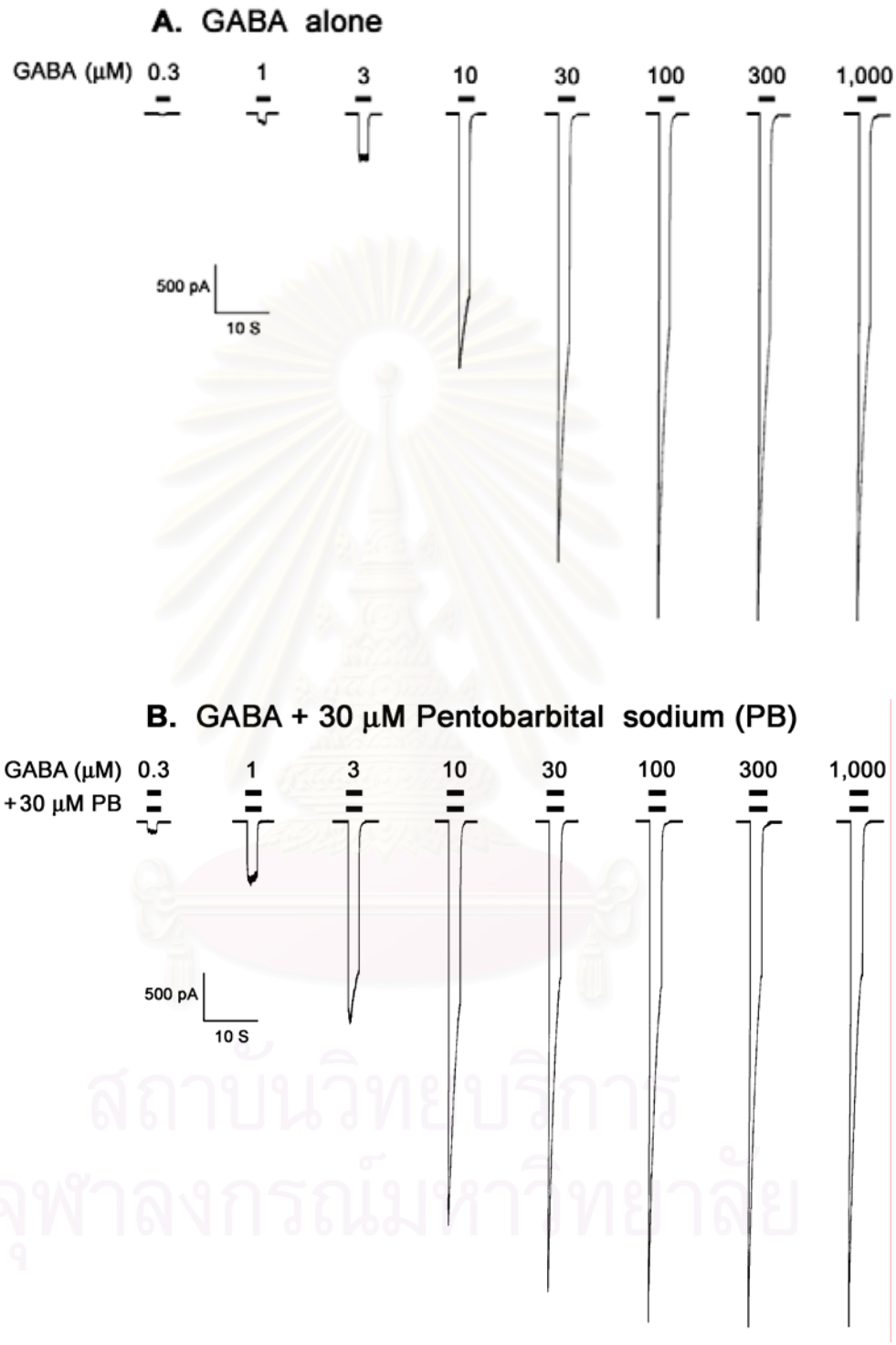

Figure 3.11 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 30 μM pentobarbital sodium (PB).

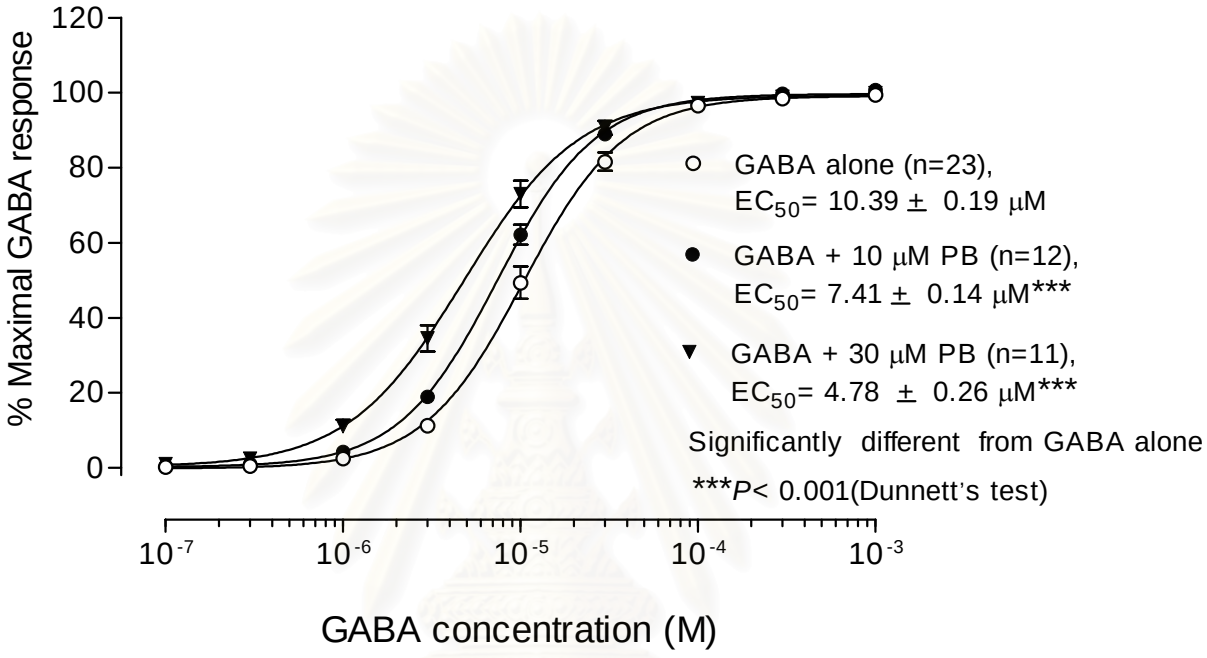

Figure 3.12 The GABA concentration-response curves demonstrating the potentiation of the $GABA_A$ currents by 10 and 30 μM pentobarbital sodium (PB).

2.3 Effect of diazepam (DZP) on the GABA-induced inward currents.

Diazepam, concentration up to 1,000 μM, did not induce inward current in the absence of GABA (n=14) (Figure 3.13 A). Diazepam, concentrations from 0.001-10 μM, produced the potentiation of the $GABA_A$ currents in the concentration-dependent manner with maximal potentiation at 1 μM (n=16) (Figure 3.13 B). The inward currents induced by increasing concentrations of GABA on control and increasing concentrations of GABA in the presence of 0.3 and 1 μM diazepam are shown in figure 3.14 and 3.15, respectively.

The potentiation of the $GABA_A$ currents by diazepam resulted in a leftward shift of the GABA concentration-response curves. DZP at a concentration 0.3 and 1 μM decreased the GABA EC_{50} values to 3.50 $\pm$ 0.29 μM (Dunnett's test, $P<0.001$, n=13), and 2.95 $\pm$ 0.33 μM (Dunnett's test, $P<0.001$, n=9), respectively, compared with 6.04 $\pm$ 0.16 μM (n=22) of control (Figure 3.16).

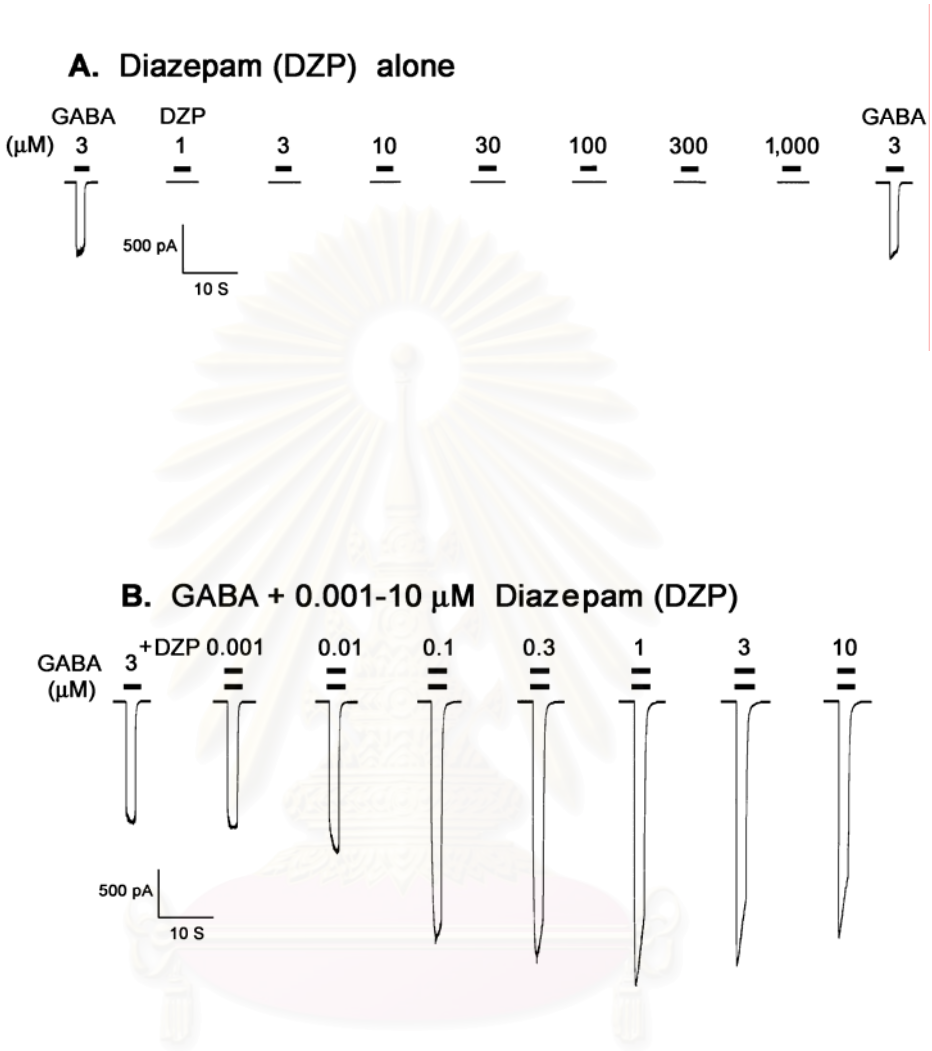

Figure 3.13 Representative current traces demonstrating the direct effect and potentiation of diazepam (DZP) on the $GABA_A$ currents. (A): DZP, up to 1,000 μM, did not induce inward current in the absence of GABA. (B): Coapplication of 0.001-10 μM diazepam in the presence of 3 μM GABA produced the concentration-dependent manner. Note that the maximal potentiating effect of the $GABA_A$ currents by DZP exhibited at 1 μM DZP.

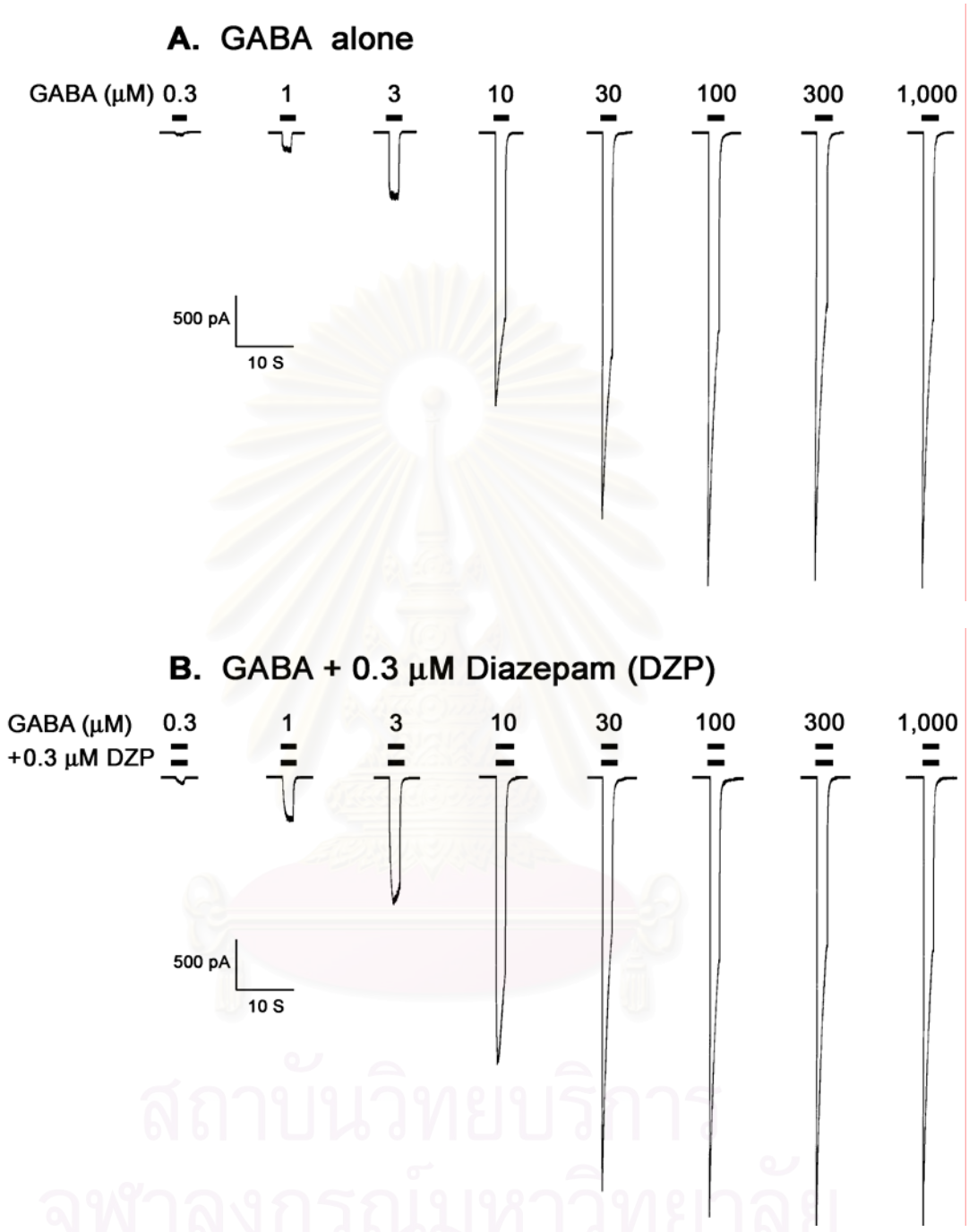

Figure 3.14 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 0.3 μM diazepam (DZP).

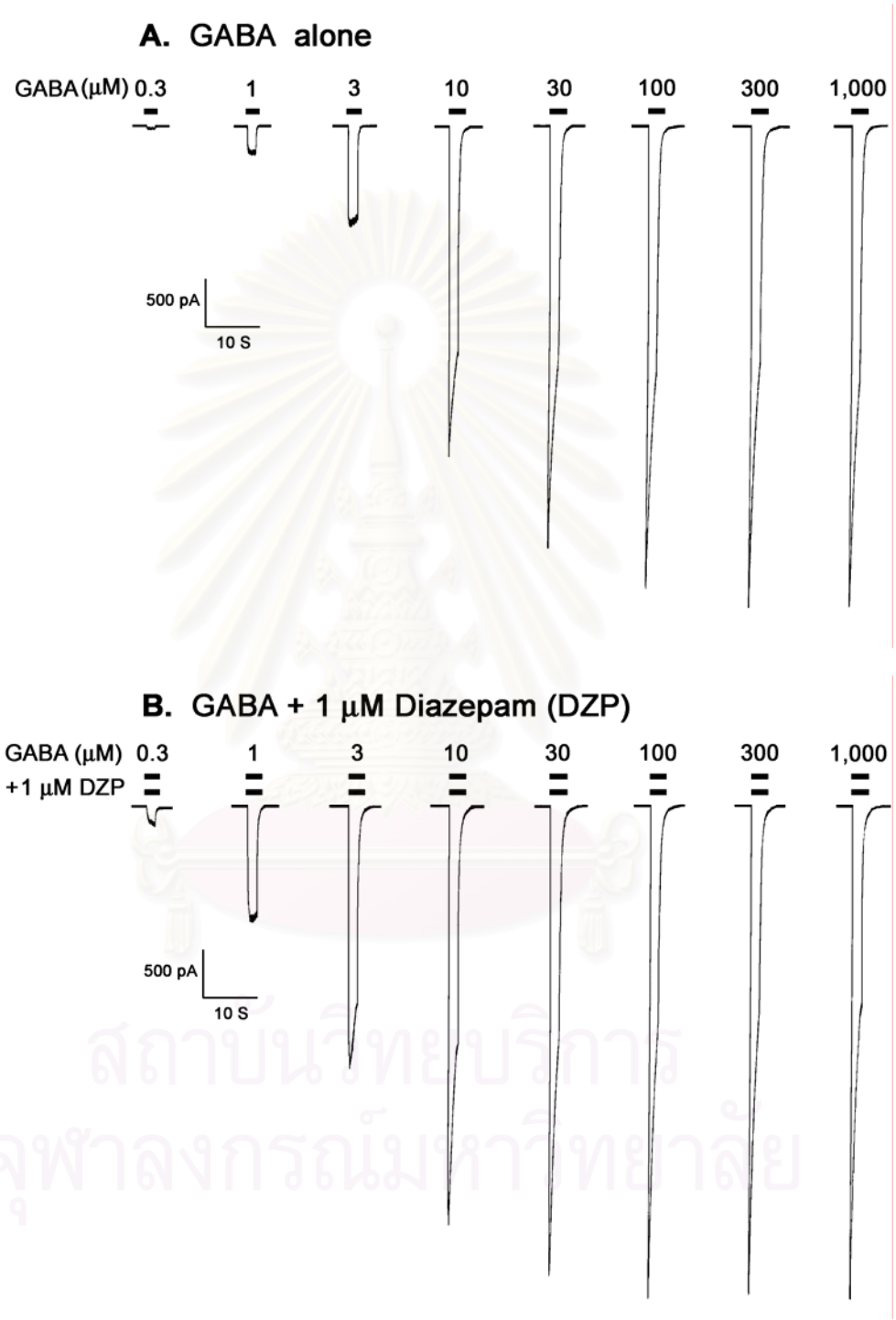

Figure 3.15 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 1 μM diazepam (DZP).

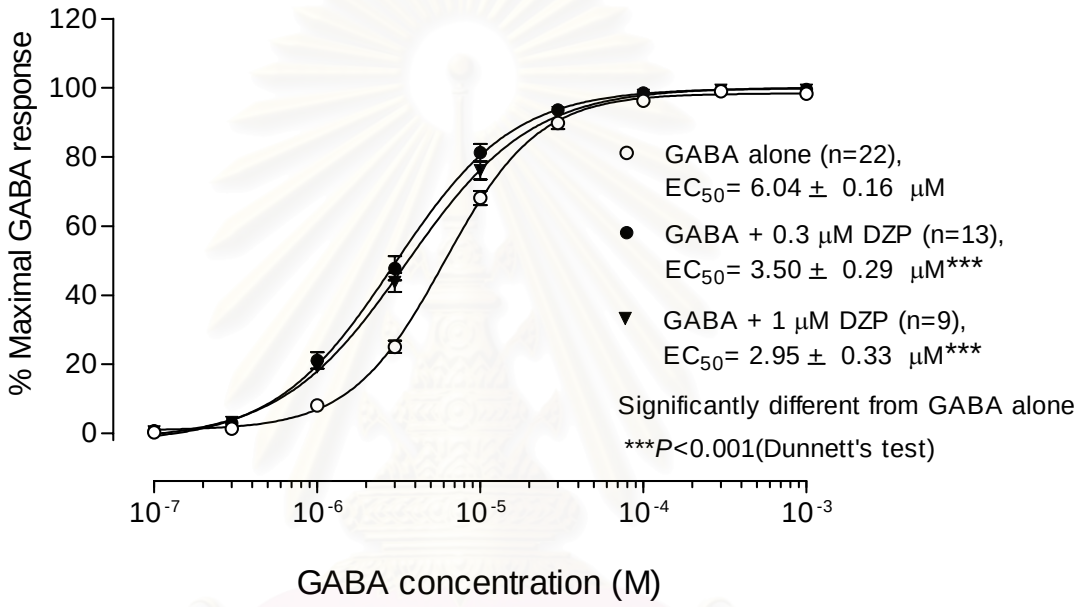

Figure 3.16 The concentration-response relationship demonstrating the potentiation of the $GABA_A$ currents by 0.3 and 1 μM diazepam (DZP).

3. Neuronal response to glycine.

The inward currents evoked by a rapid application of increasing concentrations of 1-3,000 μM glycine demonstrated dose-dependent manner. The glycine concentration producing a maximal current amplitude was about 1,000 μM (Figure 3.17).

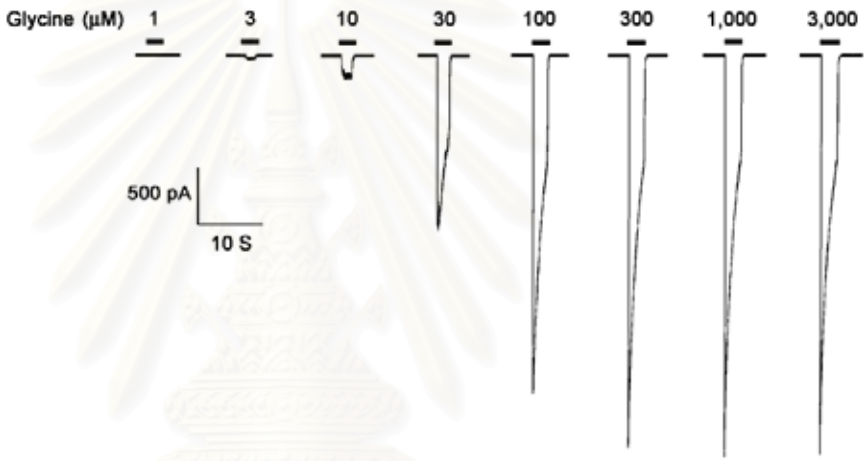

Figure 3.17 Representative current traces demonstrating whole-cell glycine currents induced by increasing concentrations of 1-3,000 μM glycine to an acutely dissociated hippocampal pyramidal neurone from male Wistar rat aged 21-27 days. Drug applications were separated by at least 1-2 min interval and the duration was indicated by the solid line above the current traces. All record current traces are from the same neurone. Holding potential (V_H) was –20 mV.

The EC_{50} values and Hill coefficient of the log.concentration-response relationship obtained from the control neurones were 31.66 $\pm$ 0.18 μM and 1.65 $\pm$ 0.11 respectively (n=16) (Figure 3.18).

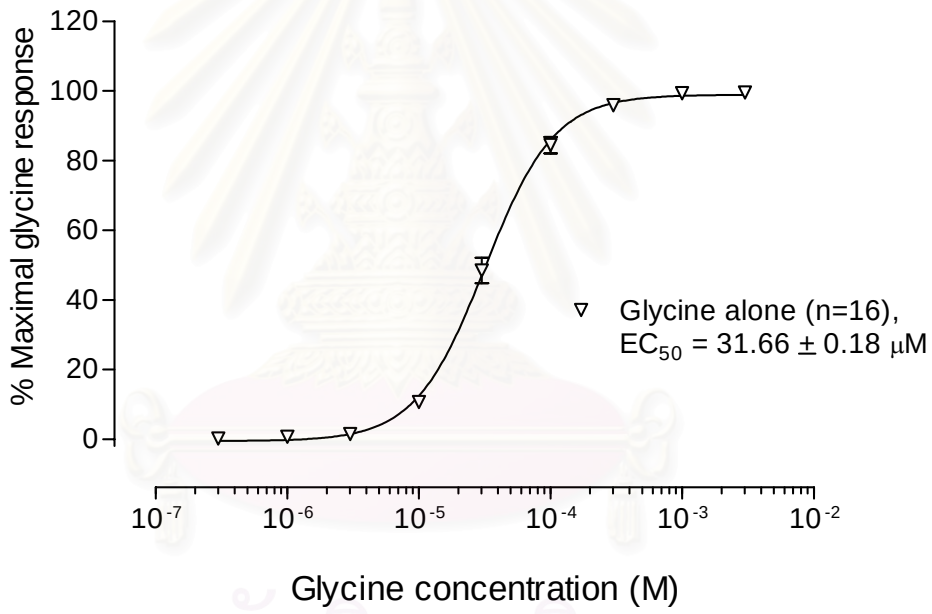

Figure 3.18 Glycine log.concentration-response relationship in acutely dissociated rat hippocampal pyramidal neurones. Each point is the mean $\pm$ S.E.M. of the current response, expressed as percentage of the maximal response.

The inward currents induced by glycine could be antagonized by 5 μM strychnine sulfate, a competitive antagonist of glycine at the glycine receptor (Figure 3.19).

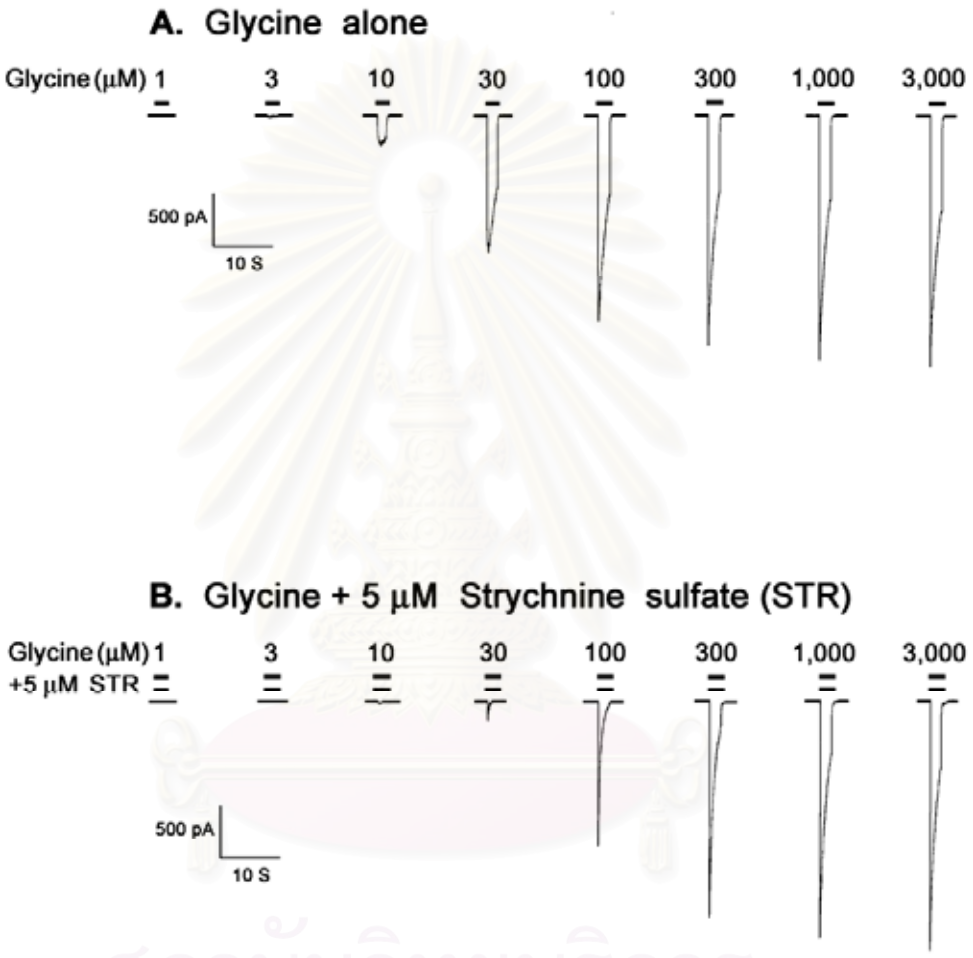

Figure 3.19 Representative current traces demonstrating the inhibition of the glycine currents by 5 μM strychnine sulfate (STR).

Coapplication of 30 μM glycine with 1-20 μM strychnine sulfate produced the inhibition of the glycine currents in the concentration-dependent manner (n=9) (Figure 3.20).

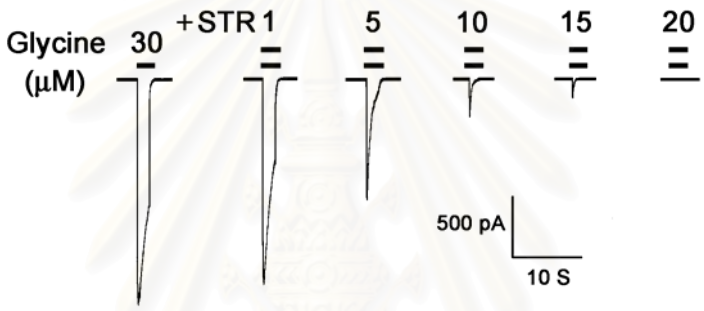

Figure 3.20 Representative current traces demonstrating inhibition of the glycine currents by high concentrations of strychnine sulfate (STR).

Strychnine shifted the glycine concentration-response curve to the right without change in maximal glycine response. Strychnine at a concentration 5 μM increased the glycine EC_{50} values to 113.50 ± 0.15 μM (Student's *t*-test, *P*<0.001, n=9), compared with 31.05 ± 0.26 μM (n=9) of control (Figure 3.21).

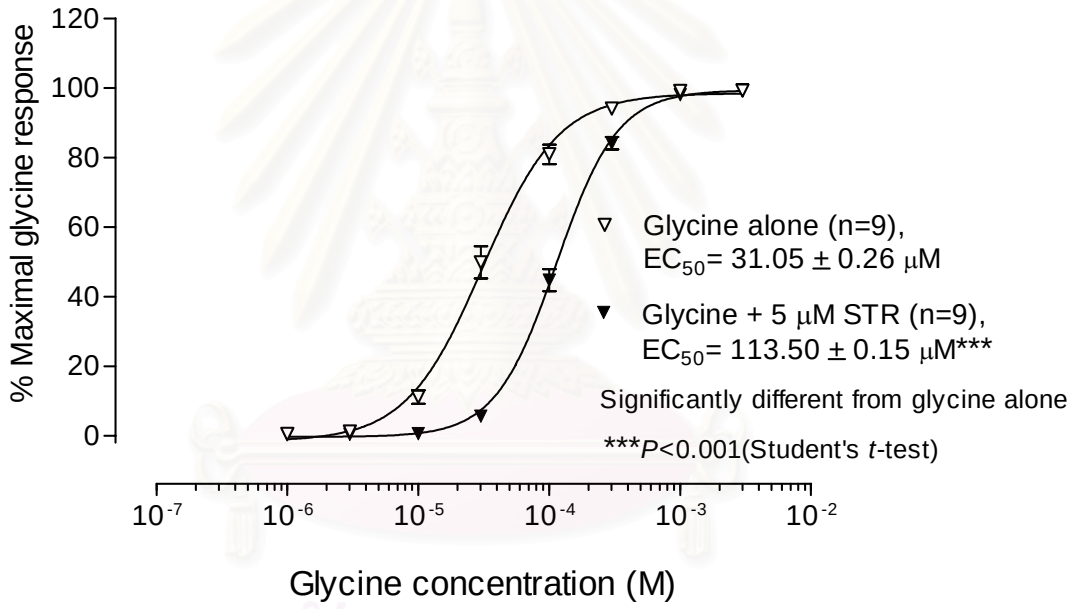

Figure 3.21 The glycine concentration-response relationship demonstrating the inhibition of the glycine currents by 5 μM strychnine sulfate (STR).

4. Effects of valproic acid (VPA) on the inward current-induced by GABA and glycine.

4.1 Effect of valproic acid (VPA) on the GABA-induced inward currents.

Valproic acid (VPA), concentration up to 5,000 μM, did not induce inward current in the absence of GABA (n=10) (Figure 3.22 A). Coapplication of 3 μM GABA with 30-5,000 μM VPA produced the concentration-dependent potentiation of the $GABA_A$ currents (n=16) (Figure 3.22 B). The inward currents induced by increasing concentrations of GABA on control and increasing concentrations of GABA in the presence of 1,000 and 3,000 μM VPA are shown in figure 3.23-3.24, respectively.

The potentiation of the $GABA_A$ currents by VPA resulted in a leftward shift of the GABA concentration-response relationship. GABA in the presence of 1,000 and 3,000 μM VPA decreased the GABA EC_{50} values to 10.27 $\pm$ 0.25 μM (Dunnett's test, $P<0.05$, n=8) and 6.41 $\pm$ 0.23 μM (Dunnett's test, $P<0.001$, n=8), respectively, compared with 11.14 $\pm$ 0.18 μM of control (n=16) (Figure 3.25).

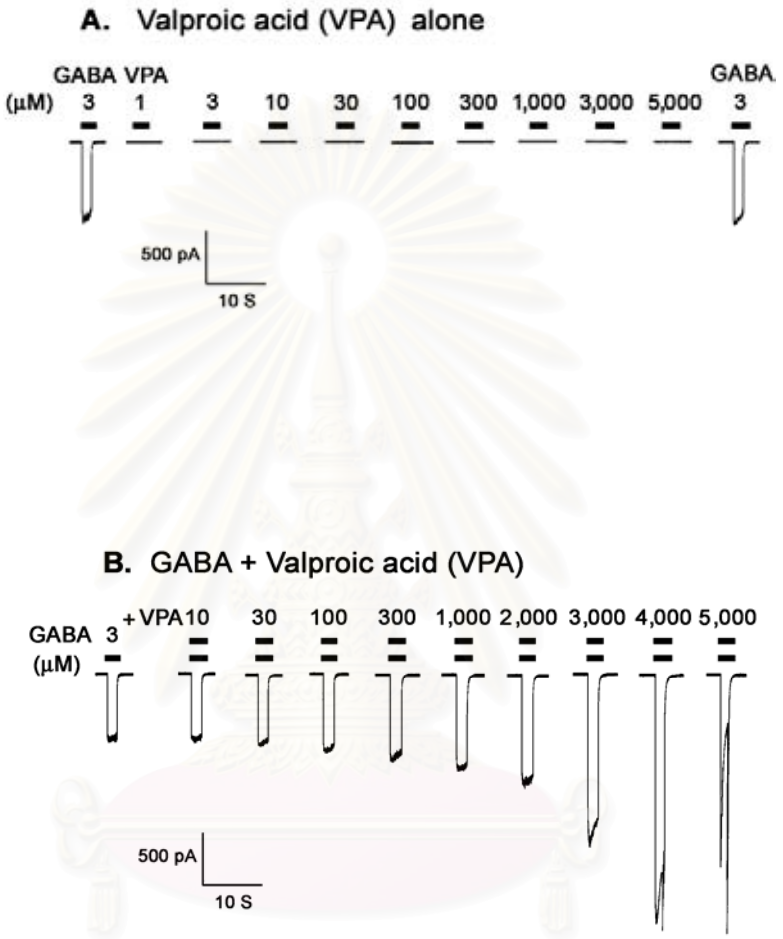

Figure 3.22 Representative current traces demonstrating the effect of valproic acid (VPA) on the $GABA_A$ currents. (A): VPA, concentration up to 5,000 μM, did not induce inward current in the absence of GABA. (B): Coapplication of 1-5,000 μM VPA in the presence of 3 μM GABA potentiated the GABA-induced inward currents in the concentration-dependent manner.

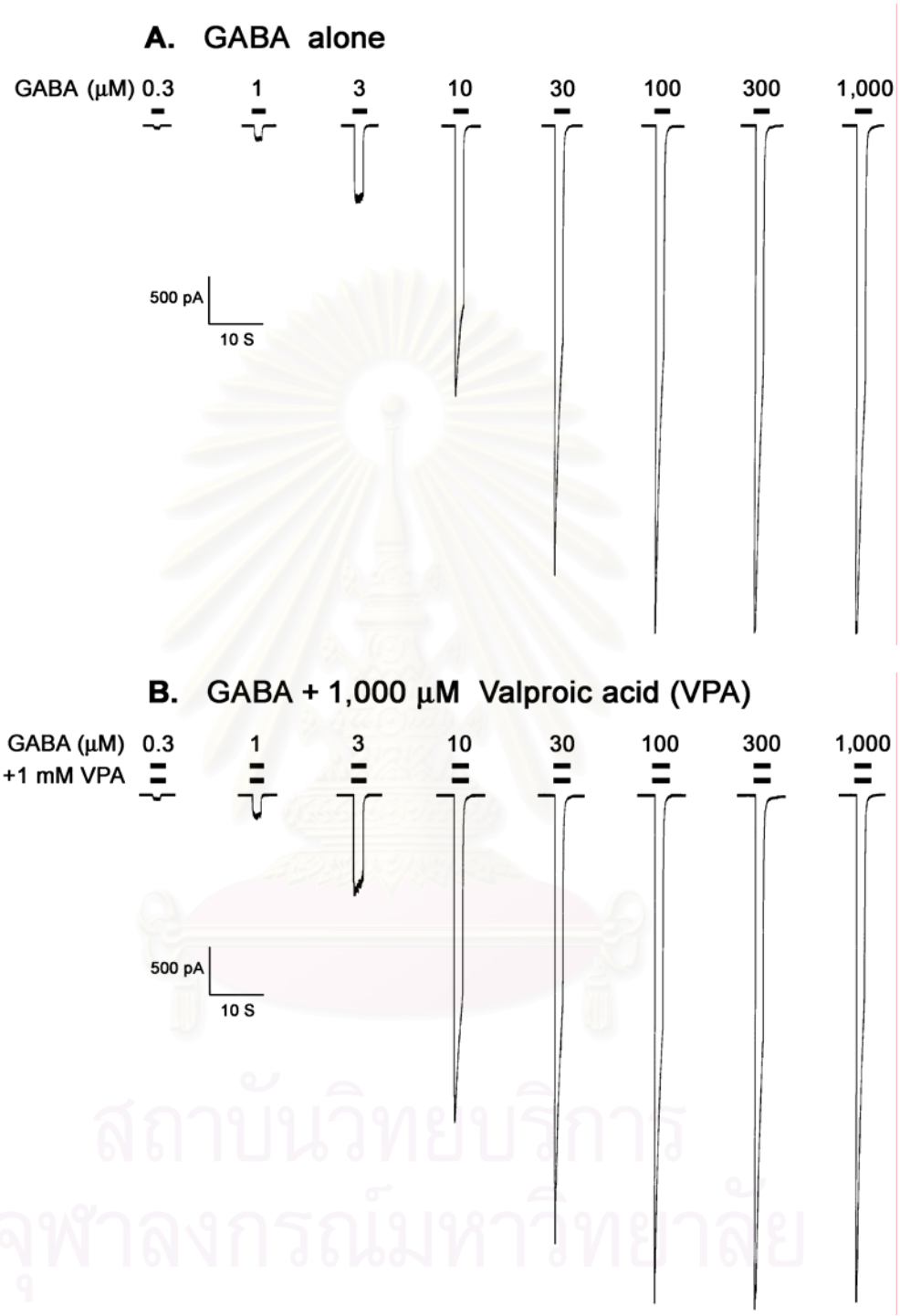

Figure 3.23 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 1,000 μM valproic acid (VPA).

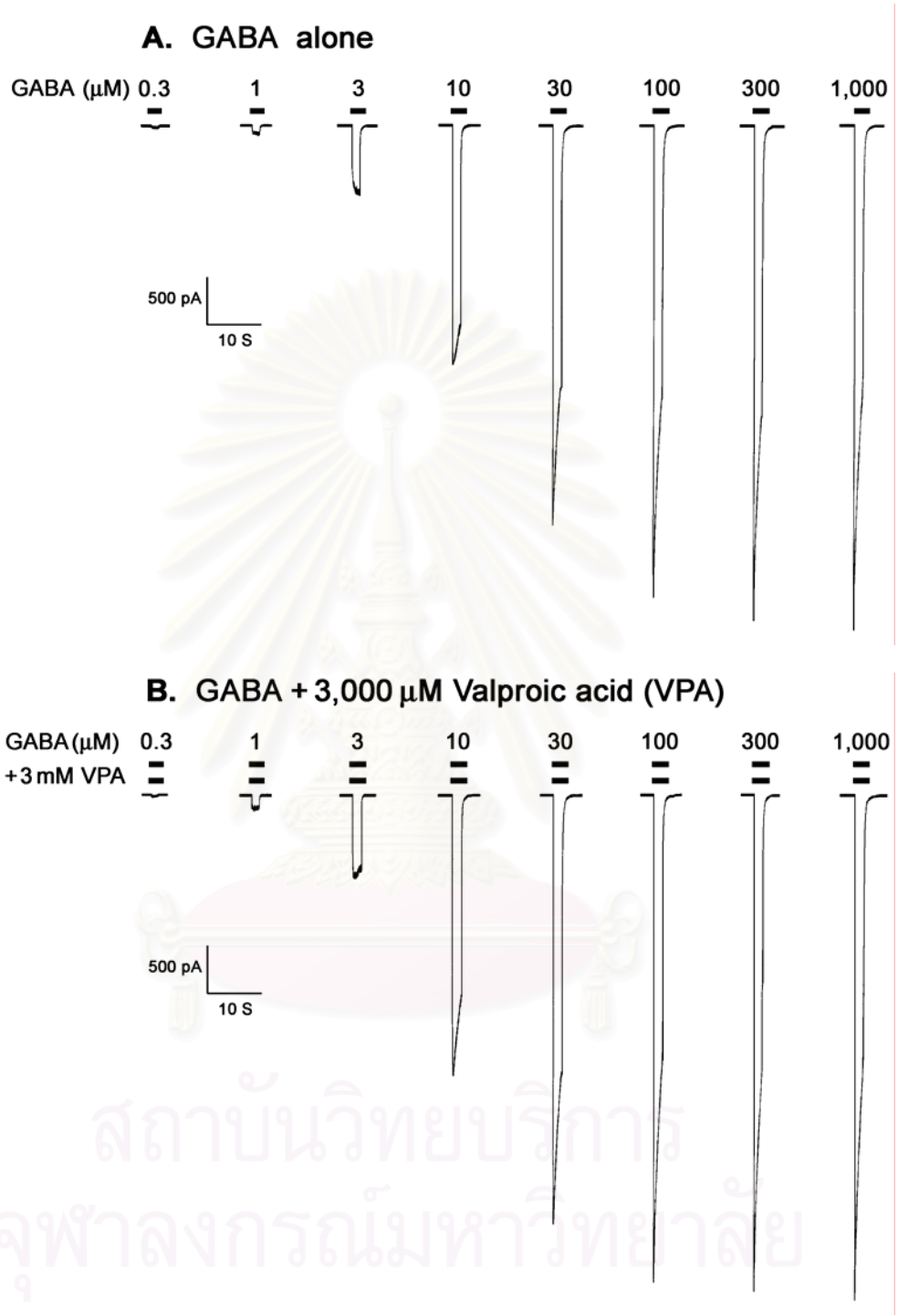

Figure 3.24 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 3,000 μM valproic acid (VPA).

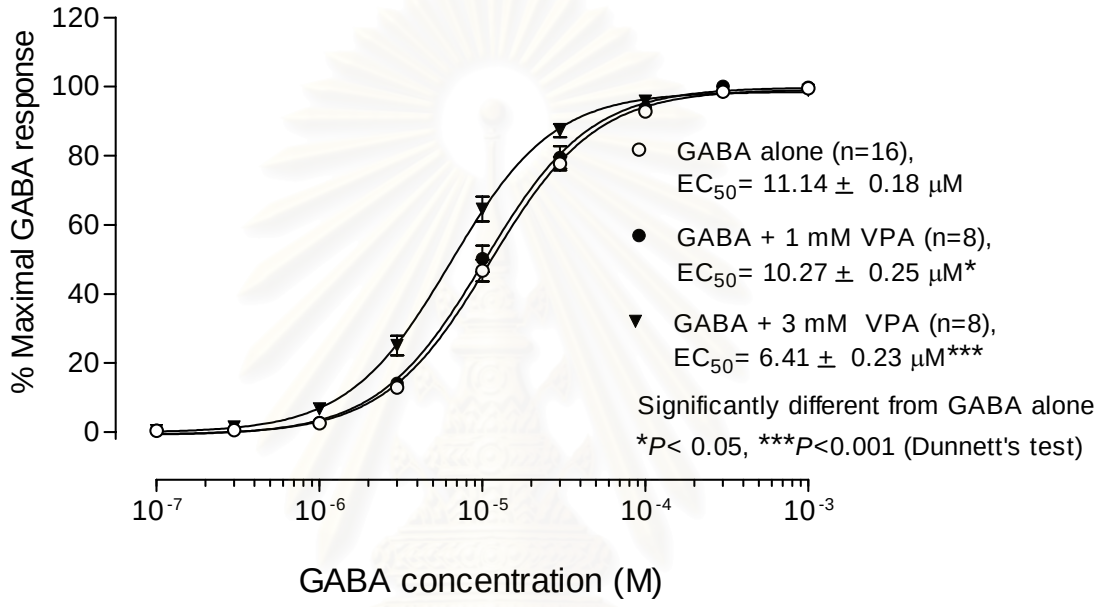

Figure 3.25 The GABA concentration-response relationship demonstrating the potentiation of the $GABA_A$ currents by 1,000 and 3,000 μM valproic acid (VPA).

4.2 Effect of valproic acid (VPA) on the glycine-induced inward currents.

A concentration range of 1-5,000 μM VPA with 30 μM glycine, applied to a recorded neurones, did not affect the glycine-induced inward currents (n=7) (Figure 3.26). The inward currents induced by increasing concentrations of 1-3,000 μM glycine on control and increasing concentrations of 1-3,000 μM glycine in the presence of 3,000 μM VPA are shown in figure 3.27.

The EC_{50} values for glycine log.concentration response relationship was not significant difference (Student's *t*-test, *P*=0.816) from 32.57 $\pm$ 0.20 μM (n=9) in the absence of VPA compared with 32.64 $\pm$ 0.22 μM (n=9) in the presence of 3,000 μM VPA (Figure 3.28).

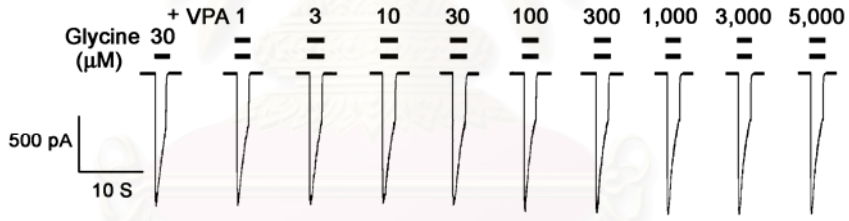

Figure 3.26 Representative current traces demonstrating that 1-5,000 μM VPA did not affect the glycine currents induced by 30 μM glycine.

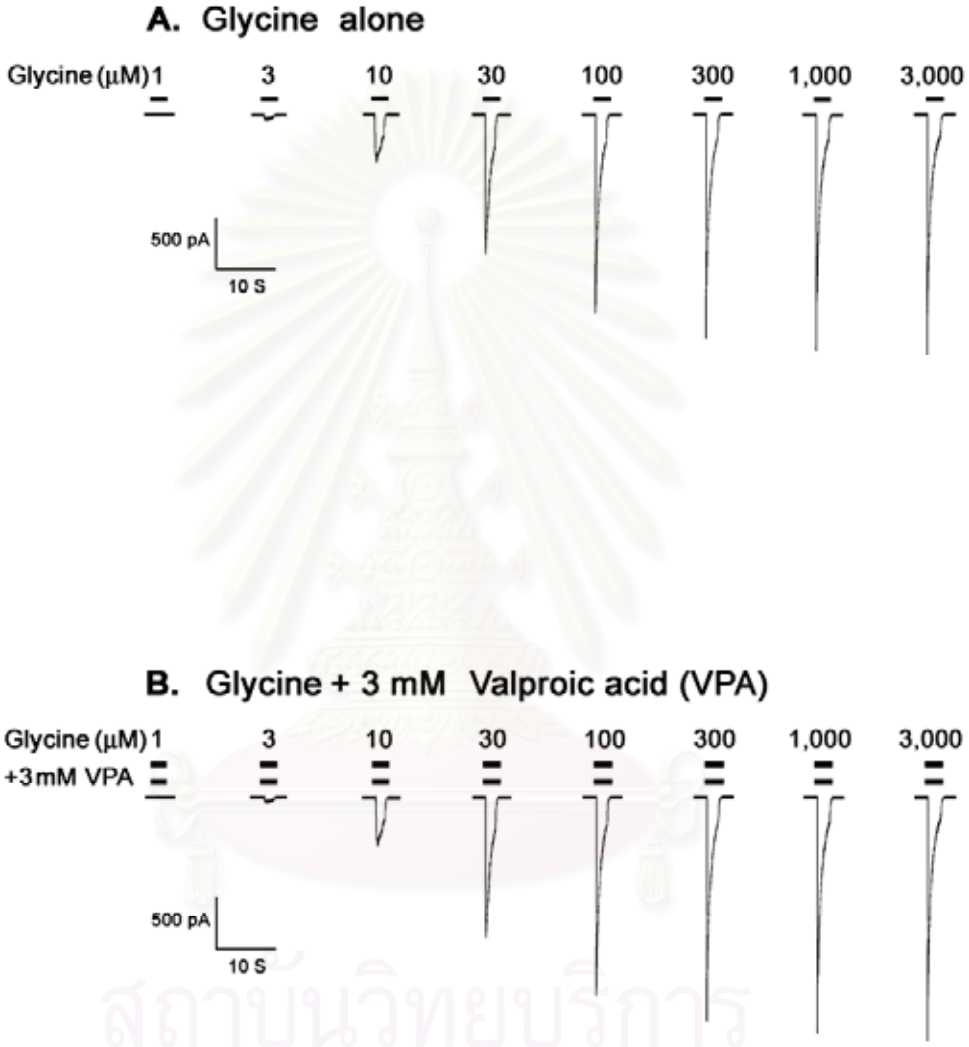

Figure 3.27 Representative current traces demonstrating that 3,000 μM VPA did not affect the inward current induced by increasing concentrations of glycine.

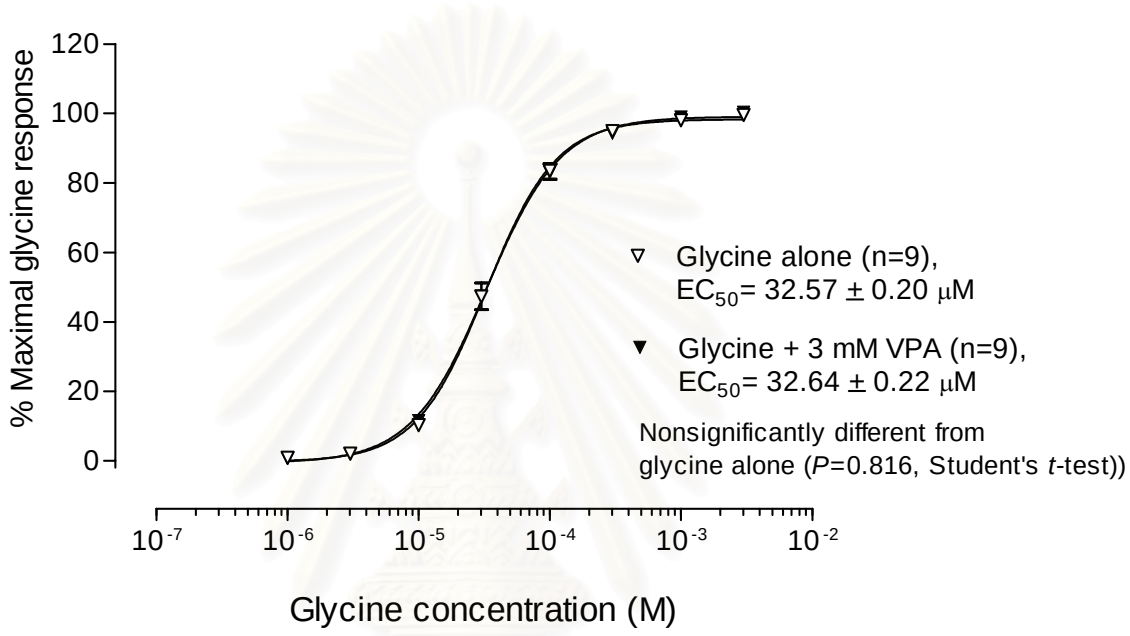

Figure 3.28 The effect of 3,000 μM VPA on the glycine concentration-response relationship. Coapplication of 3,000 μM VPA with increasing concentrations of 1-3,000 μM glycine did not affect the glycine currents.

5. Effects of n-(2-propylpentanoyl) urea (VPU) on the inward current-induced by GABA and glycine.

5.1 Effect of VPU on the GABA-induced inward currents.

In the absence of GABA, n-(2-propylpentanoyl) urea (VPU), up to 300 μM, did not induce inward current (n=14) (Figure 3.29 A). However, coapplication of 3 μM GABA with 1-300 μM VPU produced the concentration-dependent potentiation of the $GABA_A$ currents (n=14) (Figure 3.29 B). The inward currents induced by increasing concentrations of GABA on control and increasing concentrations of GABA in the presence of 100, 200 and 300 μM VPU are shown in figure 3.30-3.32, respectively.

The potentiation of the $GABA_A$ currents by VPU resulted in a leftward shift of the GABA concentration-response relationship. VPU at a concentration 100, 200 and 300 μM decreased the GABA EC_{50} values to 6.43 $\pm$ 0.20 μM (Dunnett's test, $P<0.001$, n=15), 5.63 $\pm$ 0.20 μM (Dunnett's test, $P<0.001$ n=15), 5.25 $\pm$ 0.19 μM (Dunnett's test, $P<0.001$, n=14), respectively, compared with 8.52 $\pm$ 0.11 μM of control (n=44) (Figure 3.33).

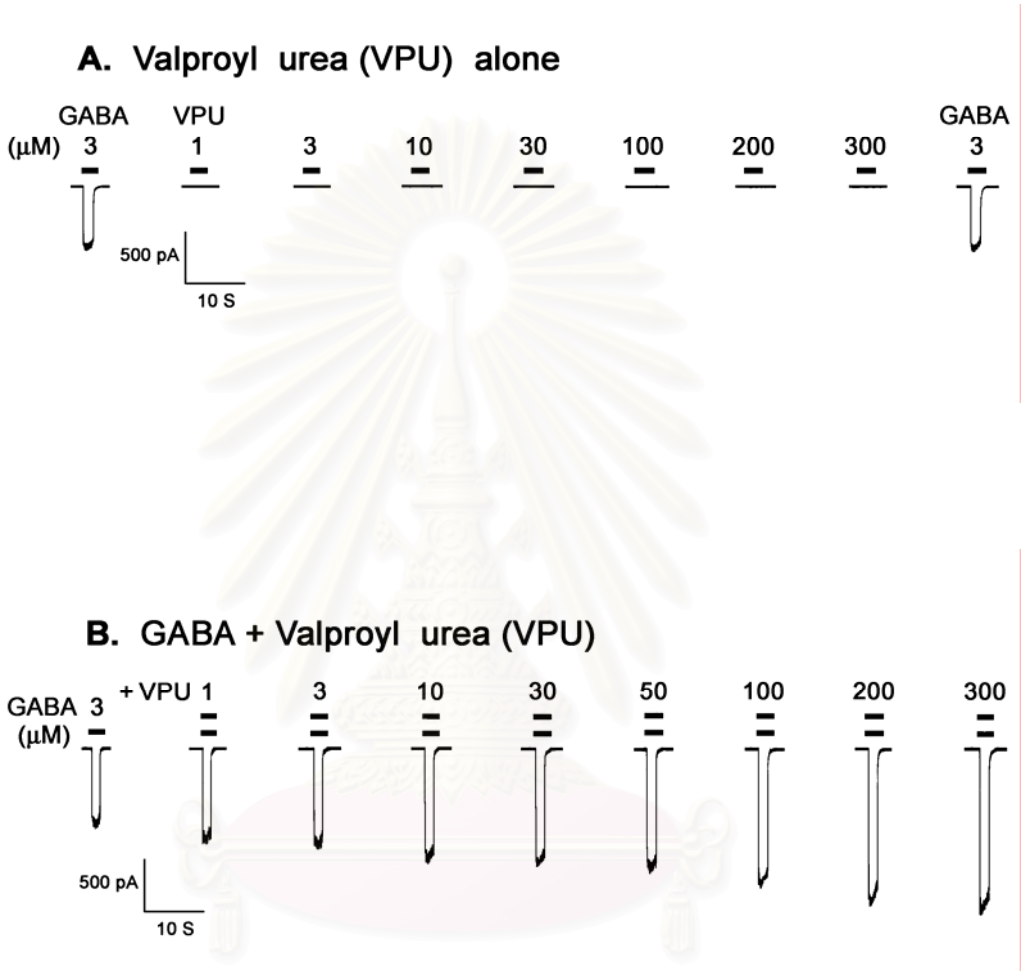

Figure 3.29 Representative current traces demonstrating the direct effect and potentiation of VPU on the $GABA_A$ currents. (A): VPU, concentration up to 300 μM, did not induce inward current in the absence of GABA. (B): Coapplication of 1-300 μM VPU in the presence of 3 μM GABA potentiated the GABA-induced inward currents in the concentration-dependent manner.

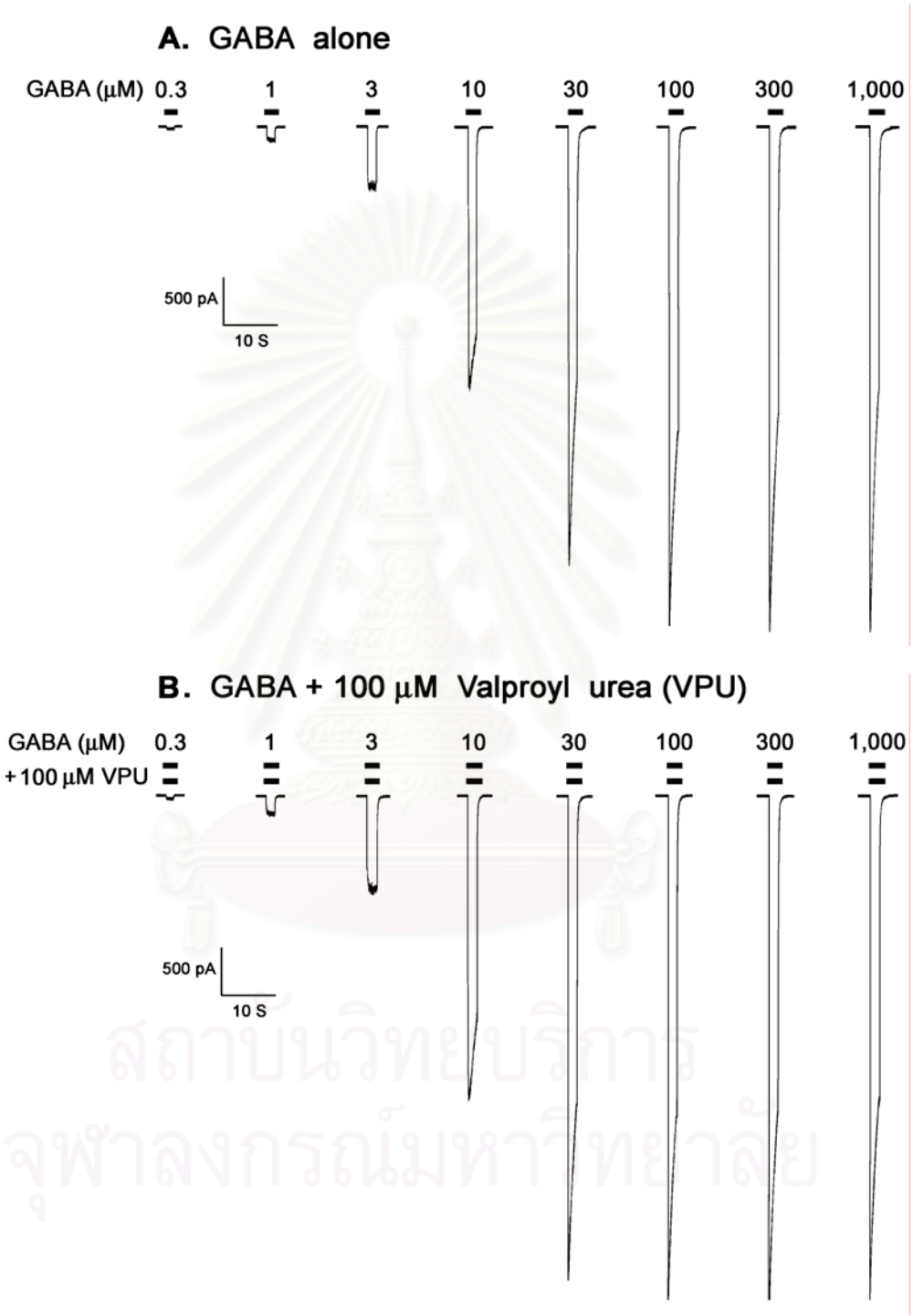

Figure 3.30 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 100 μM valproyl urea (VPU).

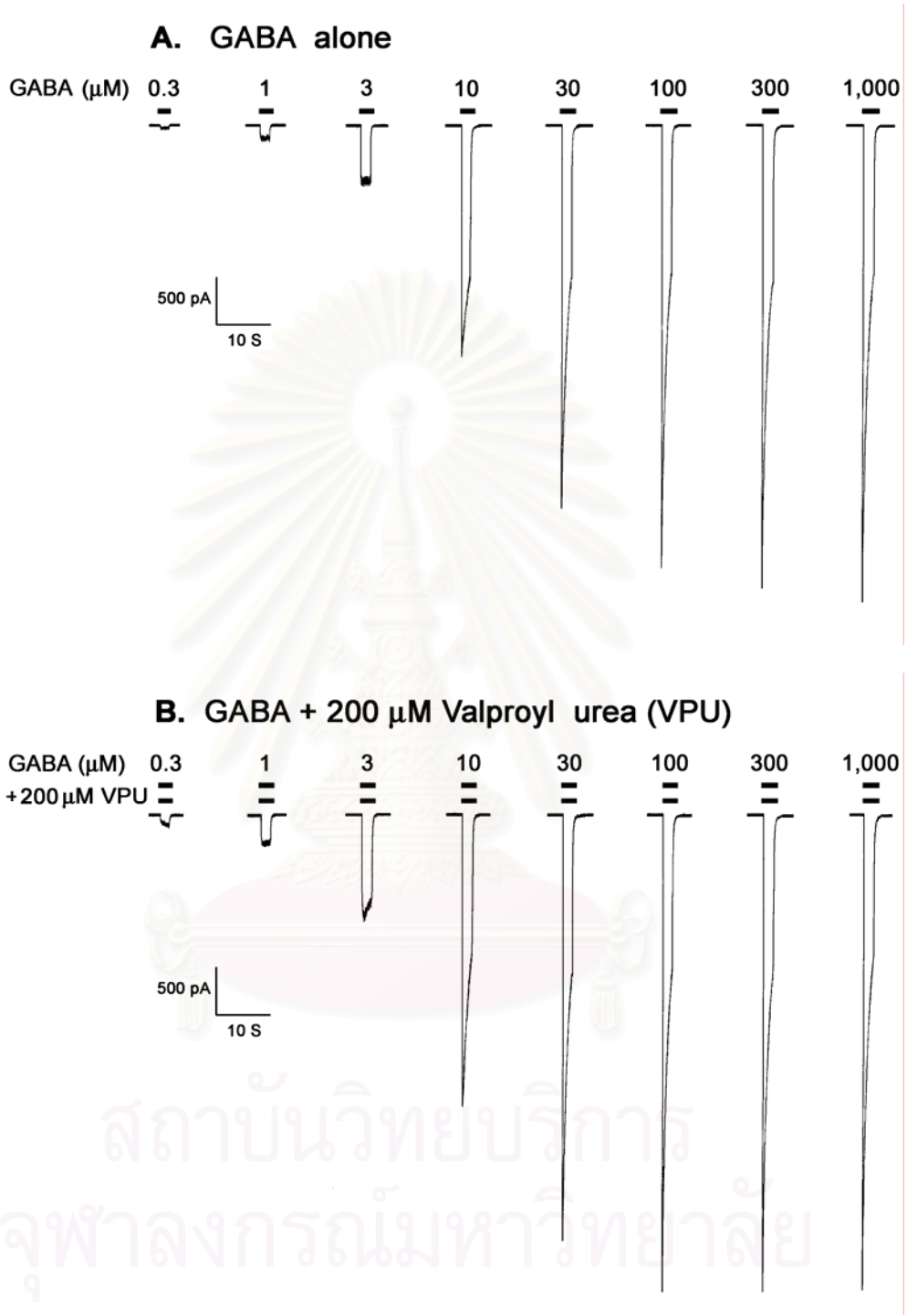

Figure 3.31 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 200 μM valproyl urea (VPU).

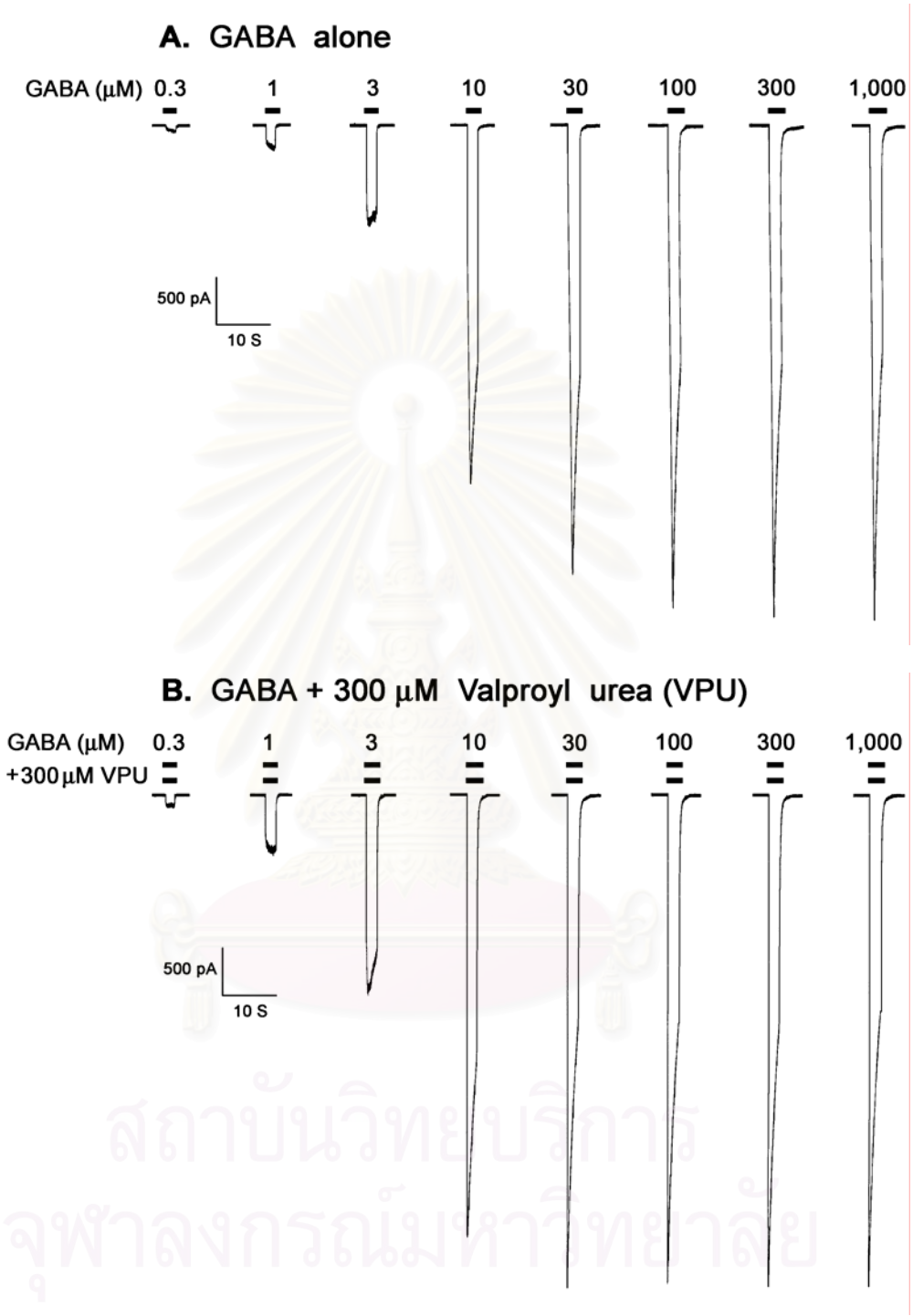

Figure 3.32 Representative current traces demonstrating the potentiation of the $GABA_A$ currents by 300 μM valproyl urea (VPU).

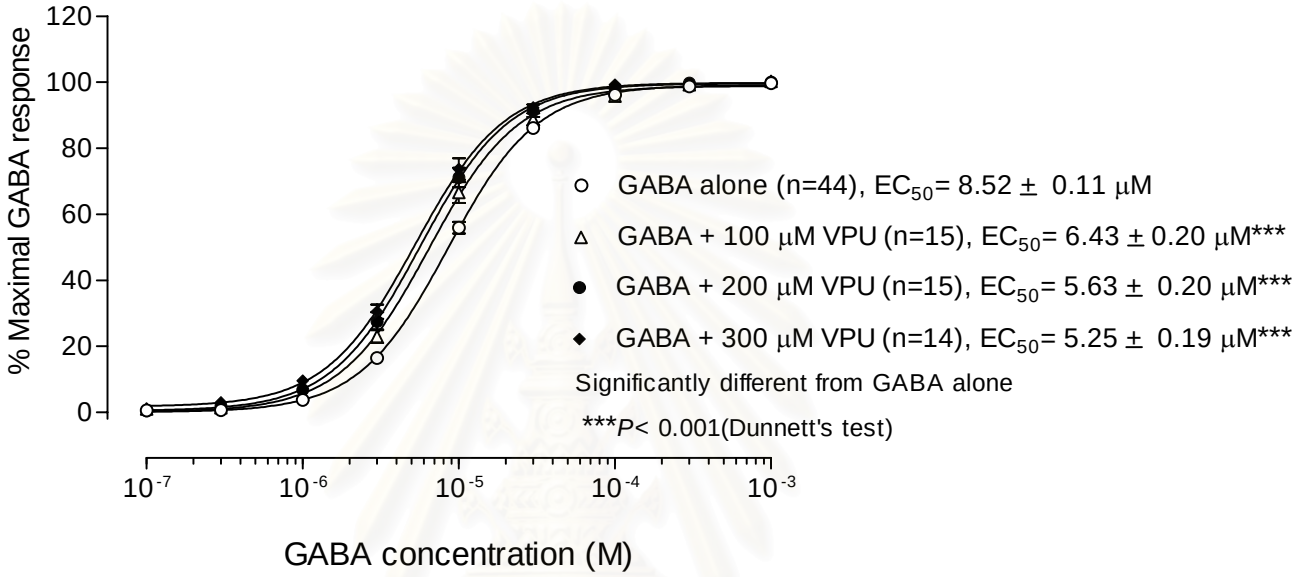

Figure 3.33 The GABA concentration-response relationship demonstrating the potentiation of the $GABA_A$ currents by 100, 200 and 300 μM valproyl urea (VPU).

The potentiations of the $GABA_A$ currents by VPU, VPA, DZP and PB are shown in figure 3.34. The maximal potentiations of the $GABA_A$ currents by VPU (at 300 μM), VPA (at 4,000 μM), DZP (at 1μM) and PB (at 300 μM) were 196.92 ± 9.01%(n=14), 328.33 ± 14.80% (n=16), 196.10 ± 9.2% (n=16) and 537.40 ± 26.26% (n=13), respectively.

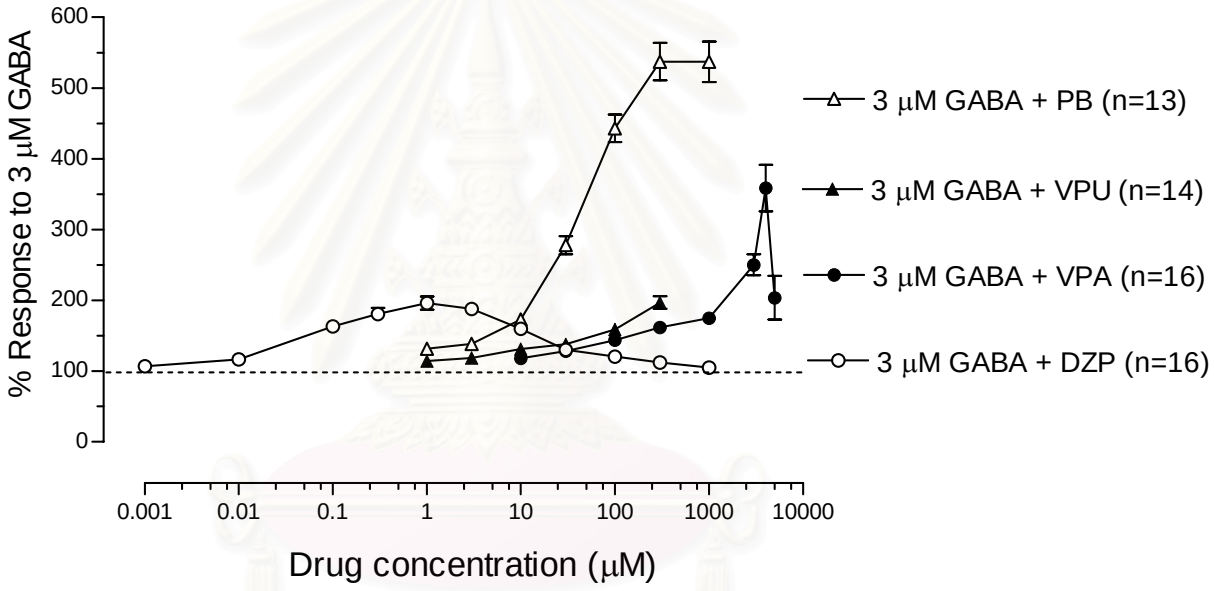

Figure 3.34 The concentration-dependent potentiations of the $GABA_A$ currents by pentobarbital sodium (PB), valproyl urea (VPU), valproic acid (VPA) and diazepam (DZP).

5.2. Effect of VPU on the glycine-induced inward currents.

A concentration range of 1-300 μM VPU with 30 μM glycine, applied to a recorded neurones, did not affect the glycine-induced inward currents (n=9) (Figure 3.35). The inward currents induced by increasing concentrations of 1-3,000 μM glycine on control and 1-3,000 μM glycine in the presence of 300 μM VPU are shown in figure 3.36.

The EC_{50} values for glycine log.concentration-response relationship was not significant difference (Student's *t*-test, *P*=0.275) from 32.99 $\pm$ 0.19 μM (n=9) in the absence of VPU compared with 32.67 $\pm$ 0.21 μM (n=9) in the presence of 300 μM VPU (Figure 3.37).

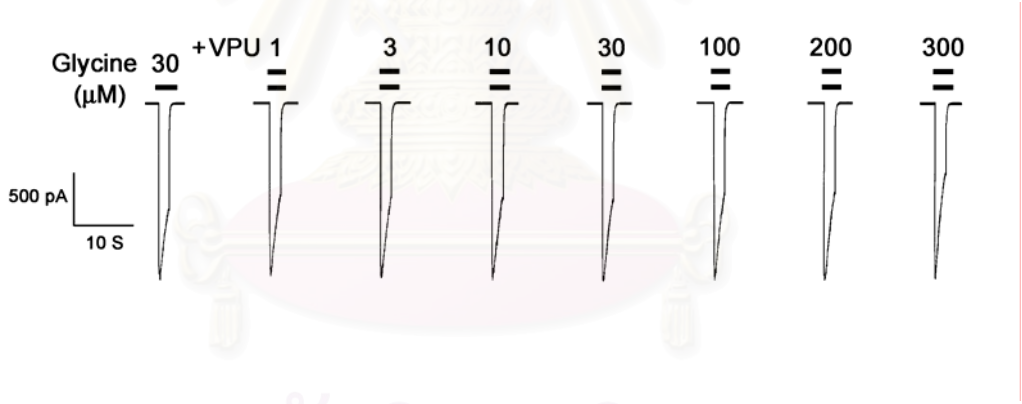

Figure 3.35 Representative current traces demonstrating that 1-300 μM VPU did not affect the glycine currents induced by 30 μM glycine.

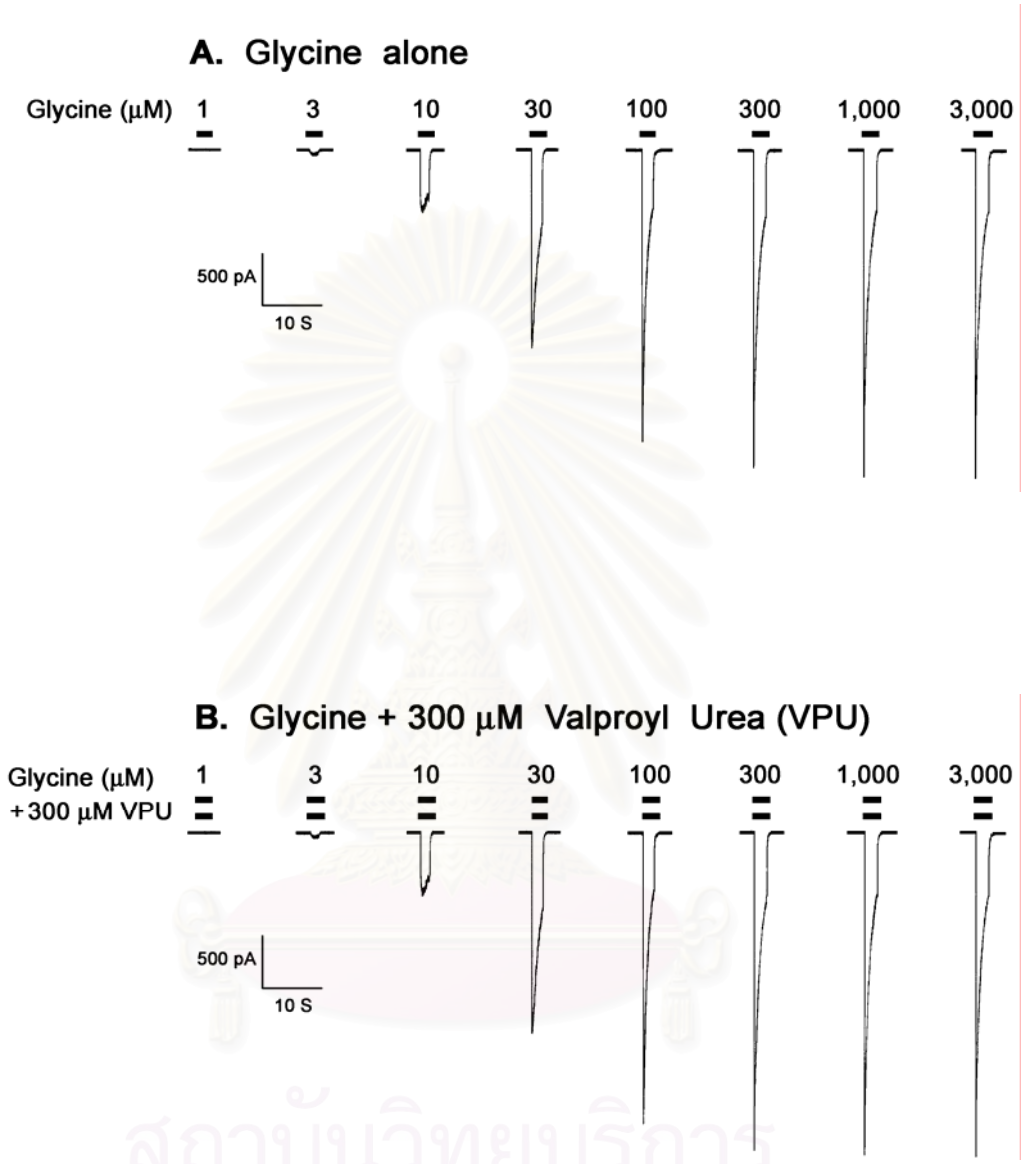

Figure 3.36 Representative current traces demonstrating that 300 μM VPU did not affect the inward current induced by increasing concentrations of glycine.

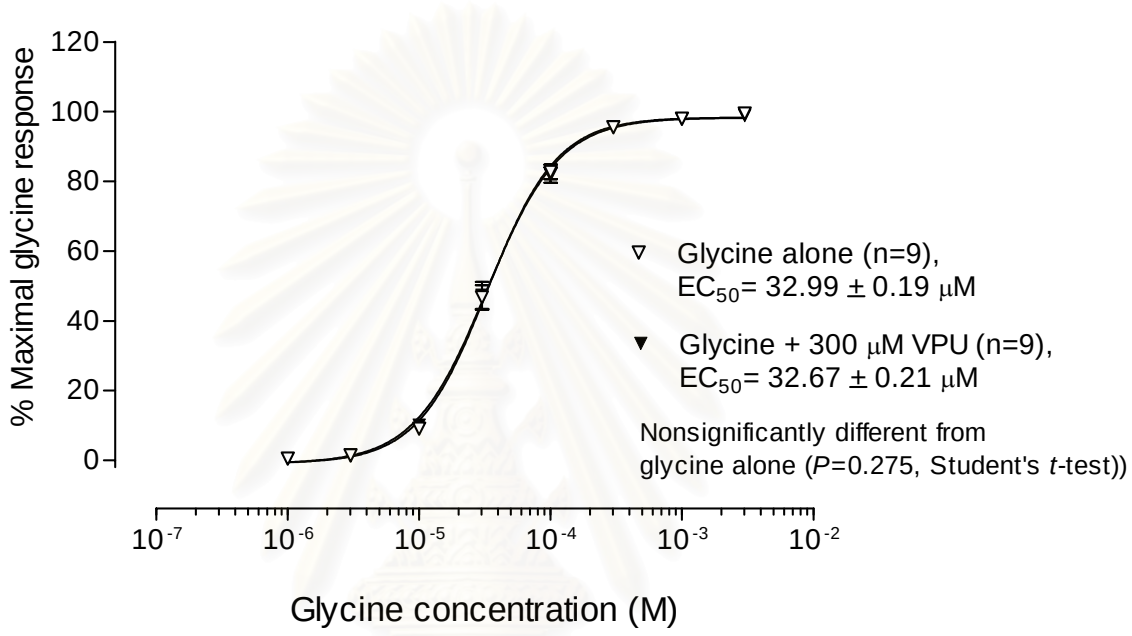

Figure 3.37 The effect of 300 μM VPU on the glycine concentration-response relationship. Coapplication of 300 μM VPU with increasing concentrations of 1-3,000 μM glycine did not affect the glycine currents.

6. Effect of Flumazenil on the potentiation of the $GABA_A$ currents by VPU.

Flumazenil (FMZ), an antagonist of benzodiazepine sites on the $GABA_A$ receptor, could not directly activate inward current and did not affect the inward current induced by 3 μM GABA (Figure 3.38).

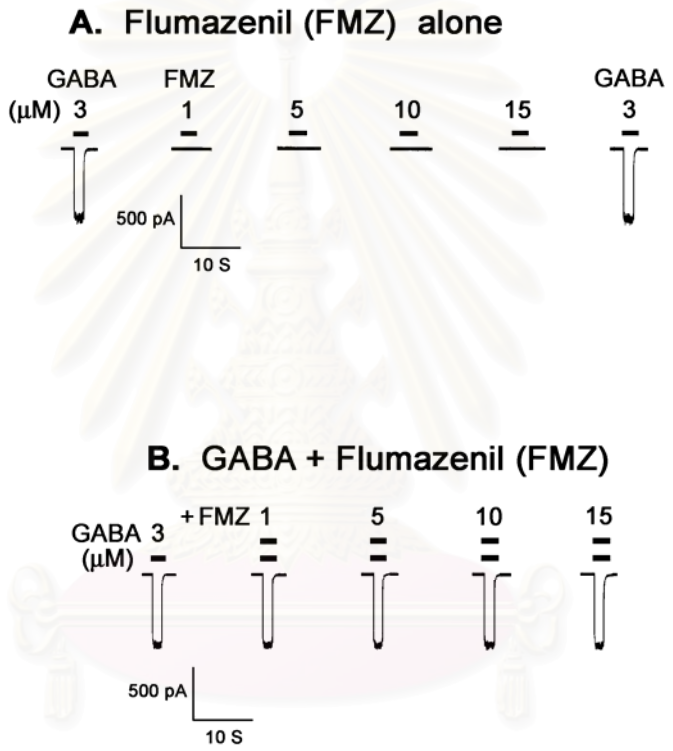

Figure 3.38 Representative current traces demonstrating the effect of flumazenil on the $GABA_A$ currents. (A): Flumazenil, concentration up to 15 μM, did not induce inward current in the absence of GABA (n=3). (B): In addition, FMZ in the presence of 3 μM GABA did not affect the inward current induced by 3 μM GABA (n=6).

The potentiation of the $GABA_A$ currents induced by 3 μM GABA could be inhibited in the concentration-dependent manner by 5-15 μM flumazenil (n=5) (Figure 3.39 A). However, flumazenil concentration up to 15 μM, did not affect the potentiation of the $GABA_A$ currents by 300 μM VPU (n=7) (Figure 3.39 B).

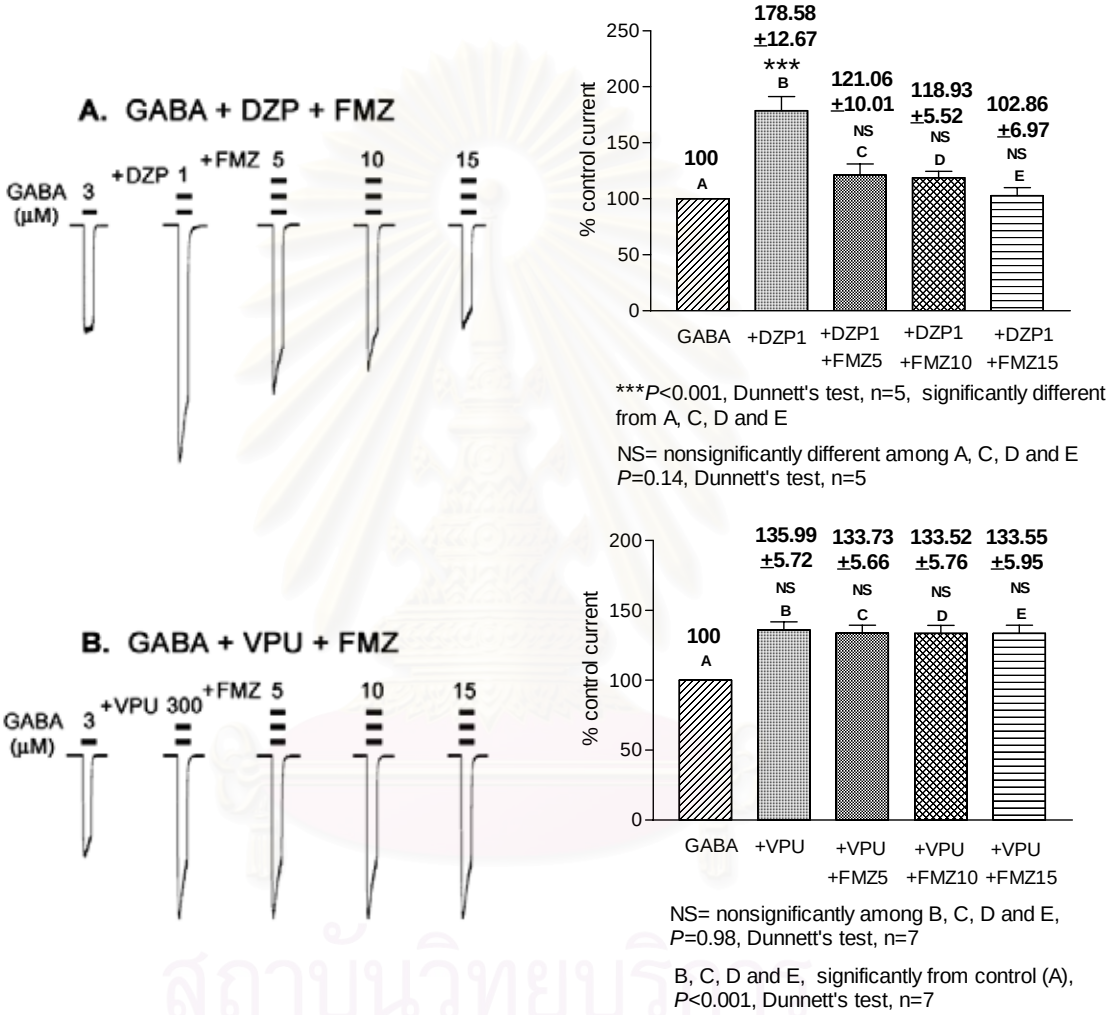

Figure 3.39 Representative current traces demonstrating the effect of flumazenil (FMZ) on the potentiation of the $GABA_A$ currents by DZP and VPU. (A): FMZ, concentration 15 μM completely inhibited the potentiation of the $GABA_A$ currents by 1 μM DZP (n=5). (B): FMZ, concentration up to 15 μM, did not affect the potentiation of the $GABA_A$ currents by 300 μM VPU (n=7).

7. Effects of VPU on the direct activation and potentiation of the $GABA_A$ currents by PB.

7.1 Effect of VPU on the direct activation of the membrane currents by PB.

Pretreatment of 300 μM VPU and coapplication of 300 μM VPU with increasing concentrations of 10-1,000 μM PB, resulted in a decreased current response compared with the PB elicited response alone (n=14) (Figure 3.40).

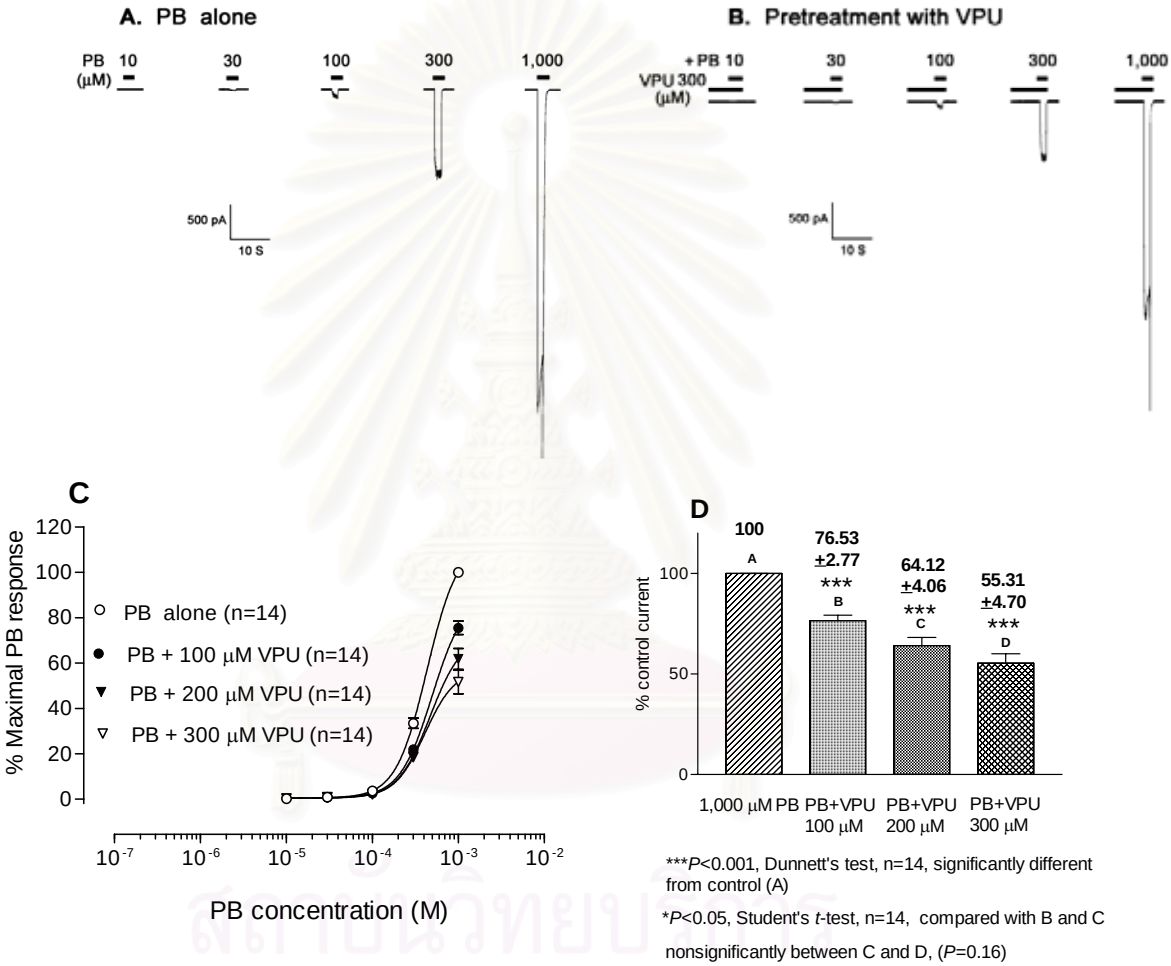

Figure 3.40 Representative current traces demonstrating the effect of 300 μM VPU on the direct activated inward currents by PB. (A): Increasing concentrations of 10-1,000 μM PB elicited the membrane currents. (B): Pretreatment and coapplication with 300 μM VPU in the presence of 10-1,000 μM PB. (C): Effect of VPU on PB concentration-response curve. (D): Inhibition of 1,000 μM PB induced currents by VPU.

7.2 Effect of VPU on the potentiation of the $GABA_A$ currents by PB.

Coapplication of 300 μM VPU and 10 μM PB in the presence of 3 μM GABA produced the currents greater than the potentiation of the $GABA_A$ currents by 300 μM VPU or 10 μM PB (n=15) (Figure 3.41).

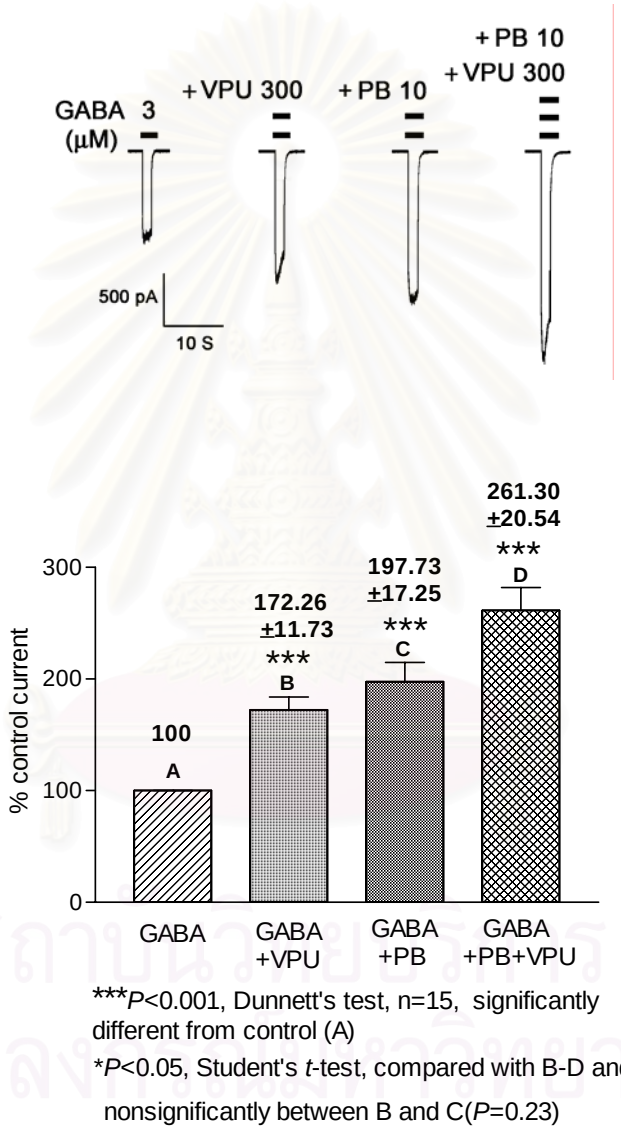

Figure 3.41 Representative current traces and histograms demonstrating the effect of 300 μM VPU on the potentiation of the $GABA_A$ currents by 10 μM PB.

CHAPTER IV

DISCUSSION

1. Antiepileptic drug development.

Despite the availability of several antiepileptic drug (AEDs), only 70% of the patients benefit from current AEDs and many of these patients experience adverse effects. There is a clear need for develop AEDs with higher anticonvulsant efficacy and less toxicity than existing drug (Löscher, 2002; Porter and Rogawski, 1992).

There are at least three preclinical strategies which are used for development of new anticonvulsant drugs: (1) random screening of newly synthesized chemical compounds of diverse structural categories for anticonvulsant activity in animal models, (2) structural variation of known anticonvulsant drugs, and (3) mechanism-based rational drug development, base on knowledge of the basic pathophysiological events involved in seizures or epilepsy (Löscher, 1999).

The most important strategies of rational design of anticonvulsant drugs have been (1) enhancement of GABA-mediated neuronal inhibition, (2) diminution of glutamate-mediated neuronal excitation and (3) modulation of Na^{+}, K^{+} and particularly Ca^{++} ion channels (Dichter, 1994; Löscher, 1999). However, the most successful of these rational strategies has been pharmacological enhancement of GABAergic neurotrans mission (Löscher, 1999).

Valproic acid is one of the major AEDs, however, its anticonvulsant potency is less than the other three major AEDs: phenobarbital, phenytoin and carbamazepine. In addition, valproic acid caused two rare but severe side effects: teratogenicity and hepatotoxicity. Therefore, several laboratories have attempted to develop improved

derivatives of valproic acid with higher potency but lower toxicity (Bialer, 1999; Bialer et al.,1994).

N-(2-propylpentanoyl)urea or valproyl urea is a monoureide analogue of valproic acid which was synthesized in the laboratory of the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. According to the previous study in animal models, VPU has demonstrated a good prospect of being a potent broad spectrum AEDs with higher margin of safety and lower side effects than VPA (Tantisira et al., 1997). Extensive studies on its precise mechanism(s) of actions have to be accomplished before a definite conclusion.

2. Acutely dissociation of hippocampal neurones.

The method employed to dissociate hippocampal neurones in the present study was prepared as described previously by Sooksawate and Simmonds (1998). To avoid possible interference by a general anesthetic, no anesthetic was given prior to decapitation. Pronase and thermolysin, a proteolytic enzyme, were used for enzymatic neuronal dissociation. The mechanical isolation with very gentle pipetting after the enzymatic treatment loosened the intercellular connections and single intact neurones can be routinely dissociated. Most dissociated neurones preserve their morphological structure, the electrical and the chemical properties of the dissociated neurones are well maintained (Kaneda et al., 1988).

The neurones for whole-cell patch clamp in the present study were chosen on the pyramidal-shaped cells, termed pyramidal neurones, and deemed healthy. The dissociated neurone, has a phase-bright smooth surface and internal structure was transparent (Kaneda et al., 1988; Stansfeld and Mathie, 1993).

3. Buffering system, solution and drug application.

HEPES-Tris buffering system was employed to stabilize pH at 7.4 throughout the course of experiment. The bicarbonate buffer continuous bubbling with a 100% O_2 slightly shifts of pH from 7.4 to 7.5 (Kaneda et al.,1988) and it is well recognized that the function of the $GABA_A$ receptors can be modulated by extracellular pH (Chesler and Kaila, 1992; Huang and Dillon, 1999; Pasternack et al., 1996). Cesium chloride was employed as the predominant internal cation to suppress the various K^+ conductance of the cell membrane (Marty and Neher, 1995; Peters et al., 1988).

To minimized current rundown, high ATP (4 mM) was include in the intra pipette solution, in order to have ATPase activity (Marty and Neher, 1995). Stable recording could be maintained about 20 min, although a rundown was occasional detected. To monitoring for rundown of the whole-cell $GABA_A$ or glycine currents over successive drug applications, a control concentration of 3 μM GABA or 30 μM glycine was applied before and after test concentrations. Neurones with current rundown over the lifetime of the recording were not included in this present analysis.

Drugs were applied rapidly to the recording neurones using the U-tube method (Fenwick et al., 1982; Sooksawate and Simmonds, 1998). The tip of the U-tube was usually 300 μm away from the neurone, a position which allowed rapid as well as uniform drug application and preserved the mechanical stability of the neurone. The external solution surrounding a recorded neurone could be exchanged within 50 ms, so that an accurate peak response could be obtained before desensitization develops (Hara et al., 1993; Sooksawate and Simmonds, 1998). Drug application were separated by at least 1-2 min intervals to ensure both edequate wash out of drug from the bath and recovery of the receptors from desensitization.

4. GABA-activated currents.

In the present study, whole cell $GABA_A$ currents were made in nearly symmetrical chloride ion concentrations ($[Cl^-]_0$ =150 mM, $[Cl^-]_i$ = 156.8 mM), therefore, GABA evoked inward currents at negative membrane potential. In figure 3.2 shows a typical recording of the whole-cell currents in response to GABA. At a concentration of 0.3 μM GABA and above, currents were observed. Peak current amplitude increased with increasing GABA concentration and a maximal current amplitude saturated at 300-1,000 μM as previous study (Hevers and Lüddens, 2002).

The desensitization characteristic of the current observed at high concentration were similar to those observed previously in acutely dissociated hippocampal neurone (Itier et al., 1996) and other neurone types (Nakagawa et al., 1991; Shirasaki et al., 1992). The GABA EC_{50} and Hill coefficient obtained in the present study were similar to previous studies with 6.3 μM and 1.25 (Itier et al., 1996) or 11.0 μM and 1.7 (Tietz et al., 1999) which obtained from acutely dissociated hippocampal neurones.

GABA-elicited currents in the present study could be antagonized by $GABA_A$ receptor antagonists, bicuculline and picrotoxinin. Bicuculline, a competitive antagonist, at a concentration of 5 μM shifted the GABA concentration-response curve to the right without change in maximal response (Figure 3.5). Picrotoxinin, a noncompetitive antagonist, at a concentration of 10 μM shifted the GABA concentration-response curve to the right with reduction in its maximum (Figure 3.7) as reported previously (Akaike et al., 1985).

5. Neuronal responses to pentobarbital sodium and diazepam.

5.1 Pentobarbital sodium (PB)

Pentobarbital sodium is generally accepted that it has three different mechanisms of action on the $GABA_A$ receptor channels: (1) potentiation of the current response elicited by GABA (2) agonist effect at the $GABA_A$ receptor channels and (3) blockage of the $GABA_A$ receptor channel currents ((Franks and Lieb, 1994 ; Peters et al., 1988).

In whole-cell patch clamp study, most studies reported some degrees of direct activation of neurones with concentrations of pentobarbital over 30 μM (Peters et al., 1988; Schultz and Macdonald, 1981). At high concentration exceeding millimolar values PB blocked the $GABA_A$ currents, leading to a reduced peak response, follow by a transient rebound in the current upon the termination of the drug application and amplitude greater than the initial response (off-effect) (Akaike et al., 1987; Rho et al., 1996). The current directly activated by pentobarbital sodium was similar to the current activated by GABA except that the pentobarbital response had a somewhat slower onset (Rho et al., 1996). Antagonism of receptor activation by pentobarbital sodium on the $GABA_A$ receptor by bicuculline confirmed that this action was via direct opening of the $GABA_A$ receptor chloride channel (Rho et al., 1996). Also, at high concentration (>1,000 μM) of pentobarbital, the current was partially blocked during coapplication in the presence of GABA (Figure 3.8).

5.2 Diazepam (DZP)

Diazepam modulated GABA-evoked current by producing: (1) an increase in peak current; (2) accelerated rate of current onset at non-saturating GABA concentration; (3) accelerated rate of current decay or desensitization during GABA application; (4) no change in the decay or deactivation of currents after brief pulse

applications of GABA; (5) prolongation of deactivation currents after step application of GABA without changing the exponential time constants for deactivation (Lavoie and Twyman, 1996).

Diazepam itself did not induce any current at the wide concentration ranges (Study and Barker, 1981) as well as this present study (Figure 3.13 A). The potentiation of the GABA-induced current was observed after application of diazepam at a concentration as low as 0.01 μM DZP. This potentiation was maximal at a concentration 1 μM DZP (Figure 3.13 B). At higher concentration, the potentiation was less than that obtained with 1 μM of diazepam, as observed in previous studies in acutely dissociated hippocampal neurones (Eghbali et al., 1997; Itier et al., 1996). With increasing concentrations, diazepam reduced peak, leading to bell-shaped concentration response curve (Yakushiji et al., 1989).

6. Potentiation of the $GABA_A$ currents by valproyl urea (VPU).

In vivo study, intraperitoneally administered, valproyl urea demonstrated anticonvulsant activity in bicuculline test in mice but the median effective dose (ED_{50}) of valproyl urea in protection was relatively high (>300 milligram/kilogram body weight) (Sooksawate, 1995). In microiontophoretic study in rats Khongsombat (1997) reported that valproyl urea depressed spontaneous firing of both cortical and cerebellar Purkinje neurones. This depressant effect of valproyl urea could be antagonized by bicuculline.

The results obtained in the present study demonstrated that valproyl urea alone at a concentration up to 300 μM, a limit of its aqueous solubility, did not elicit any measurable currents in acutely dissociated hippocampal neurones (Figure 3.29 A). Coapplication range of 1-300 μM valproyl urea potentiated the $GABA_A$ currents induced by 3 μM GABA but a maximum was not saturated due to its solubility (Figure 3.29 B). Results from the analysis of GABA log.concentration-response curves show that 100-300 μM valproyl urea significantly decreased the GABA EC_{50} values (Figure 3.33). These results

show that valproyl urea could potentiate the $GABA_A$ currents without the direct activation effect on this receptor.

7. Potentiation of the $GABA_A$ currents by valproic acid (VPA).

Valproic acid (VPA) demonstrates to have broad spectrum of anticonvulsant activities in a wide variety of animal models. They have three different mechanisms of action on GABAergic neurotransmission: (1) an inhibitory effect of valproate on GABA degradation (2) an enhancement of GABA synthesis or (3) direct potentiation of postsynaptic GABAergic function (Löscher, 1999).

The iontophoretic application of sodium valproate (0.5 M) potentiated the inhibitory response to GABA on cortical cells *in vivo* (Schmutz et al., 1979) and in spinal cord neurones in cell culture (Macdonald and Bergy, 1979). However, the concentrations required to potentiate GABA response could obtained only with very high valproate concentrations, compared to a concentrations equivalent the clinically useful therapeutic range in cerebrospinal fluid.

In the present study, valproic acid up to 5,000 μM did not induce any direct effect on the $GABA_A$ currents (Figure 3.22 A). However, at high concentration, valproic acid potentiated the inward current induced by 3 μM GABA with maximal potentiation effect at 4,000 μM (Figure 3.22 B and 3.34).

Result from the analysis of GABA log.concentration-response curve show that 1 mM and 3 mM valproic acid significantly decreased the GABA EC_{50} without change in maximal response. However, the $GABA_A$ potentiating effect of valproic acid required higher concentration in comparison to valproyl urea or other potentiators tested (Figure 3.34).

8. Glycine-activated current.

Applications of increasing concentrations of 1-3,000 μM glycine activated the inward currents. Its amplitude appeared to saturate at 3,000 μM. The glycine EC_{50} value and the Hill coefficient obtained from the log.concentration response curve were 31.66 ± 0.18 μM and 1.65 ± 0.11, respectively (Figure 3.18), similar to the previous studies obtained from acutely dissociated hippocampal neurones (Ye et al., 1999), and ventral tegmental neurones (Ye, 2000).

In this study to ascertain that glycine activated the strychnine-sensitive glycine receptors, the currents recorded were characterized by strychnine inhibition. Strychnine sulfate at a concentration 20 μM could completely inhibit the glycine currents induced by 30 μM glycine (Figure 3.20). Moreover, 5 μM strychnine significantly decreased the glycine EC_{50} values without reduction in its maximal response (Figure 3.21). VPU at a concentration 300 μM and 3,000 μM VPA did not affect the glycine log.concentration-response curves (Figure 3.36 and 3.28).

Valproyl urea and valproic acid did not affect the inward currents induced by 30 μM glycine. Tantisira et al.(1997) reported that valproyl urea and valproic acid were ineffective in strychnine induced convulsion in mice. Thus, it is clearly that intervention at the strychnine-sensitive glycine receptor site does not account for the anticonvulsant activity of valproyl urea and valproic acid.

9. Dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) was used to dissolve drugs and chemicals in the present experiment. Application of DMSO at a final concentration 0.1% had no effect on the $GABA_A$ and the glycine currents. The lack of effect of 0.1% DMSO on the GABA and the glycine response is consistant with previous observations made in a variety of neuronal preparations (Itier et al., 1996; Tietz et al., 1999; Ye, 2000). At concentration ≥ 0.5% DMSO could inhibit the currents induced by 3 μM GABA (Figure appendix 1). It has also reported

that at high concentration DMSO inhibited the $GABA_A$ currents in recombinant $GABA_A$ receptors in *Xenopus* oocyte (Sigel et al., 1998).

10. Possible site(s) of action of valproyl urea on the $GABA_A$ receptor

The $GABA_A$ receptor complex containes numerous binding sites for agonists and allosteric modulators of the receptor. Two experiments were performed to determine whether valproyl urea modulated the $GABA_A$ receptor activity by binding to the two well-characterized allosteric sites, benzodiazepine and barbiturate, on the $GABA_A$ receptor complex.

10.1 benzodiazepine site

The first experiment examined the potential interaction of valproyl urea with the benzodiazepine site using flumazenil, a benzodiazepine antagonist. Flumazenil demonstrated high affinity for the benzodiazepine site on the $GABA_A$ receptor. It lacked of intrinsic activity and was considered as a pure antagonist which used as a control in experimentals characterizing benzodiazepine (Herling and Shannon, 1982; Hunkeler et al., 1981). In this study, flumazenil could not directly activate membrane currents in the absence or potentiated the inward currents in the presence of GABA. However, 15 μM flumazenil could completely inhibit the potentiation of the $GABA_A$ currents by 1 μM diazepam. As shown in figure 3.39 B, 15 μM flumazenil did not, however, influence the valproyl urea induced potentiation of the whole-cell $GABA_A$ currents. It is, therefore, unlikely that valproyl urea induced the GABA potentiation via an interaction with the benzodiazepine recognition site on the $GABA_A$ receptor because flumazenil did not block the effect of valproyl urea.

10.2 barbiturates site

The second experiment examined the potential interaction of VPU with the barbiturate binding site of the $GABA_A$ receptor. Unfortunately, antagonist for the barbiturate site is not available (Garrett and Gan, 1998; Rho et al., 1996). So, an indirect approach was used to examine the potential interaction of VPU to this site.

The effect of VPU on the direct activation of the membrane currents by PB was investigated. Pretreatment with 300 μM VPU and coapplication of 300 μM VPU in the presence of 10-1000 μM PB resulted in a response less than the currents induced by PB alone (Figure 3.40). This experiment suggests that VPU may exert its action on the direct activation of PB at the $GABA_A$ receptor by two possible mechanisms, (1): VPU may compete with PB at direct activation barbiturate site of the $GABA_A$ receptor and decrease the effect of PB, (2): VPU may bind to a specific site on the $GABA_A$ receptor and acts as a negative modulator of PB at barbiturate site.

The present study examined also whether the modulation of GABA induced currents by VPU and PB were related. In this experiment, a concentration of 10 μM PB was chosen because at this concentration PB only potentiated the $GABA_A$ currents without any direct activation. The effect of VPU on the potentiation of the $GABA_A$ currents by PB was investigated by measuring GABA-induced currents in the presence of 300 μM VPU or 10 μM PB and 300 μM VPU plus 10 μM PB. Enhancement of 300 μM VPU plus 10 μM PB on GABA-induced currents was greater than the potentiation of the $GABA_A$ currents by VPU or PB applied with GABA. Since the concentration of PB used in this experiment was low (10 μM), one possible mechanism is that VPU might act at the same site as PB via binding to the non-occupied site of PB on the $GABA_A$ receptor. The effects of both drugs might accumulate to enhance the $GABA_A$ currents greater than the effect of each drug applied alone. The other possible mechanism is that VPU might act at other site on the $GABA_A$ receptor and enhances the potentiation of the $GABA_A$ currents by PB.

The potentiation of the $GABA_A$ receptor by VPU may, at least in part, contributes to its mechanism of anticonvulsant action. The effects on other receptor involved in convulsion would also be interesting to investigate.

CHAPTER V

CONCLUSION

In the present study, a concentration range of 1-300 μM, valproyl urea (VPU) did not directly induce inward currents in acutely dissociated rat hippocampal pyramidal neurones. The $GABA_A$ currents could be enhanced by VPU, in a concentration dependent manner, as well as pentobarbital sodium (PB) and diazepam (DZP). The GABA potentiation effect of VPU required higher concentration in comparison to PB and DZP which had maximal potentiation effects at 300 μM and 1 μM, respectively. Valproic acid (VPA), a reference drug, did not directly elicit inward currents. However, at high concentrations (30-5,000 μM), VPA could potentiate the $GABA_A$ currents with maximal potentiation effect at 4,000 μM. VPU and VPA did not affect the glycine currents.

Flumazenil, a benzodiazepine antagonist, could not inhibit the potentiation of the $GABA_A$ currents by VPU. However, VPU could inhibit the inward currents induced by PB. Moreover, coapplication of VPU with PB increased the potentiation of the $GABA_A$ currents by each of these drugs applied with GABA. These results show that the effect of VPU on the $GABA_A$ receptor may have some interaction directly or indirectly with the barbiturate site(s) on the $GABA_A$ receptor channel. Therefore, the clear mechanism(s) of the interaction should be interesting to investigate.

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Appendix

Effect of Dimethyl sulfoxide (DMSO) on the $GABA_A$ currents.

Dimethyl sulfoxide (DMSO), concentration up to 2% (v/v), did not induce membrane current and 0.1% (v/v) in the presence of 3 μM GABA did not affect the $GABA_A$ currents. However, at high concentration (>0.5%) DMSO decreased the $GABA_A$ currents (Figure appendix 1).

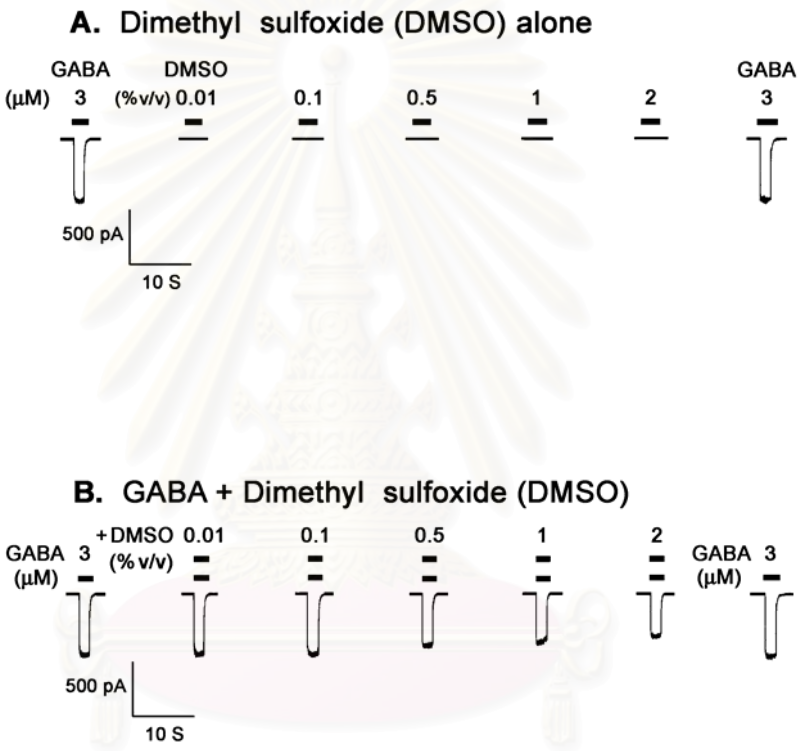

Figure appendix 1. Representative current traces demonstrating the effect of DMSO on the $GABA_A$ currents. (A): When applied alone, DMSO did not induced membrane current (n=8). (B): High concentration (>0.5%) DMSO in the presence of 3 μM GABA decreased the $GABA_A$ currents (n=11).

Biography

Lieutenant Suthep Jenthet was born on May 13rd, 1962 in Samutprakarn, Thailand. He was graduated in Bachelor of Public Health (Health Management) in 1988 from Sukhothai Thammathirat University, and Bachelor of Education (Nursing Education) in 1994 from Srinakharinwirot University, Thailand. Since graduation, he has been appointed as naval nurse in Surgical Unit, Somdejprapinklao Hospital, Naval Medical Department, Royal Thai Navy.