Chapter II

Materials and Methods

Experimental animals

The experiments were performed on male Wistar rats. Animals weighing 250-350 g were used to study the effect of test compounds on cortical amino acid neurotransmitters while those aged 14-21 days were used to study the effect of test compounds on the $GABA_A$, glycine and NMDA currents in acutely dissociated rat hippocampal neurons by using the whole-cell patch clamp technique.

Rats were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom and were acclimatized in the laboratory for a week before starting the experiments. All animals were maintained under natural light/dark cycle at control temperature (25°C) and allowed free access to both food (C.P. Food, Thailand) and water. The experiment was carried out between 8 a.m. – 6.00 p.m. Each animal was used only once. Care and handling of the animal was conducted in compliance with the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Chemicals

1. N-Hydroxymethyl-2-propylpentamide (HPP) was kindly supplied by Assist. Prof. Dr. Chamnan Patarapanich and coworker (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand).

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

3. DL-2-Amino-5-phosphonopentanoic acid (AP-5; Sigma, U.S.A.)

4. γ-Amino-n-butylic acid (GABA; Sigma, U.S.A.)

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

6. Calcium chloride-2-hydrate ($CaCl_2.2H_2O$; Riedel de Haën, Germany)

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

8. Chloral hydrate (Witayasom, Thailand)

9. D-Glucose monohydrate (Riedel de Haën, Germany)

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

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

12. Glycine (Sigma, U.S.A.)

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

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

15. L-Homoserine (Sigma, U.S.A.)

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

17. N-methyl-D-aspartate (NMDA; Sigma, U.S.A.)

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

19. Magnesium sulfate-6-hydrate ($MgSO_4.6H_2O$; Riedel de Haën, Germany)

20. 2-Mercaptoethanol (Merck, Germany)

21. Methanol, HPLC grade (Merck, Germany)

22. *O*-Phthaldialdehyde (OPA; Sigma, U.S.A.)

23. Polyethyleneglycol 400 (PEG 400;Witayasom, Thailand)

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

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

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

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

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

29. Sodium dihydrogen phosphate 2 hydrate ($NaH_2PO_4.2H_2O$; Riedel de Haën, Germany)

30. Sodium hydrogen carbonate ($NaHCO_3$; Riedel de Haën, Germany)

31. di-Sodium hydrogen phosphate 2 hydrate ($Na_2HPO_4.2H_2O$; Riedel de Haën, Germany)

32. Sodium hydroxide (NaOH; Riedel de Haën, Germany)

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

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

Drug preparations and administrations

The test substances (VPA and HPP) which are insoluble in water were dissolved in PEG400 and the others which are soluble in water (Chloral hydrate) was dissolved in 0.9% sodium chloride (NSS). Doses of test substances were expressed as milligram of substance/kilogram of body weight (mg/kg B.W.) and they were administered intraperitoneally (i.p.). The volumes of injection was kept at 0.4-0.8 ml.

All drugs in the experiment of whole cell patch-clamp technique were prepared as stock solutions, kept frozen in small microtubes, and diluted to appropriate concentrations in physiological salt solution (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) daily before use. Drugs that cannot be dissolved in PSS was dissolved in dimethyl sulfoxide (DMSO) at a concentration less than 0.1%(v/v) and it was confirmed that this concentration did not alter or evoke any current in separate control experiments.

Rapid drug application was 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 neuron to be recorded. One end (influx arm) of the U-tube was connected to a reservoir of external solution or drug solution hold 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 reversed 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 neuron. Reopening of the valve caused to be resuctioned the

solution surrounding a neuron within 50 ms. GABA, glycine or NMDA was superfused in the absence and presence of the test substances.

Equipments

1. Automatic infusion pump (CMA/100, Carnegie, Sweden)
2. Stereotaxic Instruments (NARISHIGE, Japan)
3. System for freely moving animal (CMA/120, Carnegie, Sweden)
4. HPLC system
   - C_1 Reverse – phase, 250 x 4.6 mm, particle size 5 μm, Spherisorb ODS2. (Attech^{R}, U.S.A.)
   - Guard column with packing material, particle sized 5 μm, Spherisorb ODS2.(Phenominex^{R}, U.S.A.)
   - Fluorescence detector (Water 470, U.S.A.)
   - Pump with gradient system (Water 470, U.S.A.)
   - Analog to Digital Instruments (Maclab$^{TM/4}$, AD Instruments, Australia)
   - Macintosh computer (Model LC 630, Apple computer, Inc., U.S.A.) with software programs (MacLab $^{TM/4}$, AD Instruments; Australia); Chart^{TM} V3.2.8 for data recording system and Peak^{TM} V1.3 for data processing system
   - Laser printer (Laser writer select 360, Apple computer, Inc., U.S.A.)
5. Microdialysis probe; horizontal type, molecular weight cut off 50,000 (Homofilter PNF – 140, Asahi Medical Co., Tokyo Japan)
6. Analog Digital Instruments (MacLab^{TM}/4, ADInstruments, Australia)

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

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

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

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

11. Faraday cage

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

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

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

15. 0.22 μm membrane filter (Millipore, U.K.)

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

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

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

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

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

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

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

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

Experimental methods for microdialysis study

Experimental animals

Male Wistar rats weighing 250-350 g. were divided into 6 groups of 5 animals each for determination of the effect of test substances on the levels of aspartate, glutamate, glycine and GABA in rat cerebral cortex. Two groups were used as control (NSS and PEG400, 0.4 ml i.p.). The other 4 groups were used for testing the effect of test substances (VPA 220, 440 mg/kg B.W. i.p. and HPP 80, 160 mg/kg B.W. i.p.).

Microdialysis technique

This technique was modified from Benveniste and Huttermeier (1990).

Microdialysis probe implantation

Rats were anesthetized with chloral hydrate (350 mg/kg B.W. i.p.) with supplementary doses as required to maintain surgical anesthesia. The anesthetized animals were then placed in a stereotaxic apparatus (Narishige, Japan). The surface of microdialysis probe (0.2 mm outer diameter, acrylic polymer with 50,000 molecular weight cut off) was totally covered with epoxy resin except the area of 5 mm in length that contacted the cerebral cortex of the rat. After the appropriate area of the skull was exposed, the probe was implanted transversely into the cerebral cortex at coordination of 2 mm rostral to the bregma and 1-1.5 mm inferior to the cerebral surface according to a

stereotaxic atlas of rat brain (Pellegrino, Pelligrino and Cushman, 1979) and was fixed by polycarboxylate cement. After microdialysis probe implantation, the rats were allowed at least 24 hours for recovery before the experiment was started.

Collection of cerebrospinal fluid (CSF) samples

The rat was placed in the collecting sample instrument (CMA/120, Carnegie, Sweden) which allowed freely moving (Figure 4). One side of the probe was connected to a constant flow infusion pump (CMA/100, Carnegie, Sweden) by polyethylene tube and the other side was placed into a collecting tube. The perfusion fluid for this microdialysis experiment was artificial cerebrospinal fluid (aCSF) with composition of 120 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 15 mM $CaCl_2$, 1 mM $MgSO_4$ and 6 mM glucose, pH 7.4 (Benveniste and Huttemeier, 1990). The aCSF was continuously perfused at the rate of 2 μl/min. Dialysate collected during the equilibration period of 60 min was discarded. After that the first three sucessive dialysate samples were collected at 20 min interval for basal amino acid level determination. The test substance was then injected intraperitoneally and the dialysate was collected at 20, 40, 60, 80, 100, 120, 140, 160 and 180 min. The dialysate samples were determined for amino acid levels by high performance liquid chromatography (HPLC) technique.

At the end of each experiment, the brain was exposed and removed to confirm the appropriate position of microdialysis probe by sectioning the specimen with a sharp blade and inspected visually. The data was valid only when the right position of microdialysis probe was confirmed.

Analysis of rat cortical amino acid levels

The experimental method used to determine the levels of rat cortical amino acid by pre-column fluorescence derivatization with *O*-Phthaldialdehyde (OPA) was first published by Lindorth and Mopper (1979). The mobile phase used was

gradient run between 0.05 M phosphate buffer, pH 7.3 in triple distilled water and methanol (HPLC grade). Both of the mobile phases were degassed with continuous helium gas. For gradient run, the mobile phase gradient was increased from 20% to 60% methanol in one linear step at the increment rate of 2%/min for 20 minutes. The rate of mobile phase was 1 ml/min. At the end of the run, initial condition was restored by the reversed methanol gradient run from 60% to 20% at the rate of 10%/min. A delay period of about 10 minutes was required for column equilibration.

The solution of OPA was maintained by an addition of 4 μl 2-mercaptoethanol every 4 days. The derivatization procedure was performed by mixing 10 μl of homoserine solution (internal standard) and adding 50 μl of OPA solution at room temperature. Then 50 μl injection to HPLC (Figure 5) was made after a precise 2 min incubation period.

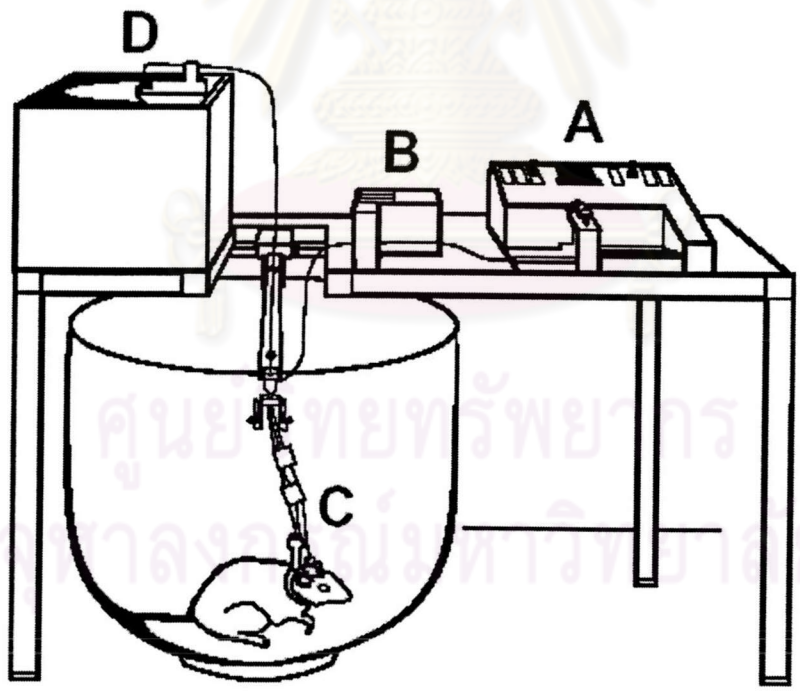

Figure 4 Diagram for freely moving animal system with microdialysis infusion pump (A), syringe selector (B), microdialysis probe (C) and a collecting sample instrument (D)

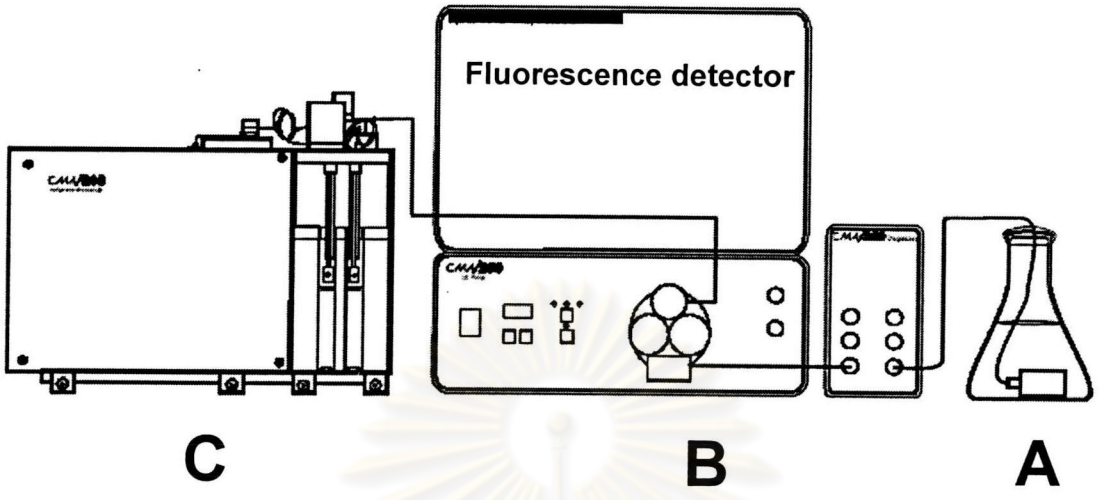

Figure 5 Diagram for HPLC system with mobile phase (A), HPLC Pump (B), Sample injector (C) and Fluorescence detector.

Experimental methods for whole-cell patch clamp technique

Dissociation of hippocampal neurons

Hippocampal neurons were acutely dissociated from male Wistar rats aged 14-21 days by the method of Sooksawate and Simmonds (1998). Rats were killed by cervical dislocation and decapitated. The skull bones were removed with a rongeur and used fine scissors for dissected dura. Brains were rapidly removed with a stainless steel spatula, placed iced cold (~4^{0}C) physiological salt solution (PSS) for 3 min, then 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 25^{0}C.

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^{0}C and continuously oxygenated throughout the procedure. After the enzymatic treatment, the slices 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 neurons from surrounding tissue. The neurons suspension was allowed to stand for 10 min before the supernatant containing dissociated neurons was taken off, leaving only 0.3-0.5 ml containing big lumps. The neurons were suitable for electrophysiological recording after being allowed to adhere to the base of recording chamber for 30-40 min.

Electrophysiological measurements

The effects of HPP on $GABA_A$ and glycine receptors were investigated in acutely dissociated rat hippocampal neurons using the whole-cell application of the patch clamp technique (Hamill et al., 1981; Figure 6). Whole-cell membrane currents will be recorded from the somata of the neurons 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 will be 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) was ranged from 3-5 MΩ.

Membrane current was measured with a patch clamp amplifier (Axopatch 200B; Axon Instruments, U.S.A.) and 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, neuron 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).

The effects of HPP on NMDA receptors were investigated in acutely dissociated rat hippocampal neurons using the whole-cell application of the patch clamp technique using the same procedure as the effects of HPP on $GABA_A$ and glycine receptors (Hamill et al., 1981). During whole-cell membrane currents recording, neuron was superfused with Mg^{2+}-free and 2 μM glycine oxygen-saturated PSS and the patch-pipette was filled with the Mg^{2+}-free intrapipette solution.

To confirm the response of GABA-induced inward current, bicuculline methochloride and picrotoxinin was co-applied with 3 μM GABA. Diazepam was used to confirm the potentiation response of GABA-induced inward current. Neuronal response of glycine-induced inward current was confirmed by using strychnine sulfate co-application with 30 μM glycine. Finally, DL-2-amino-5-phosphonopentanoic acid was used to confirm NMDA-induced inward current by co-applied with 100 μM NMDA

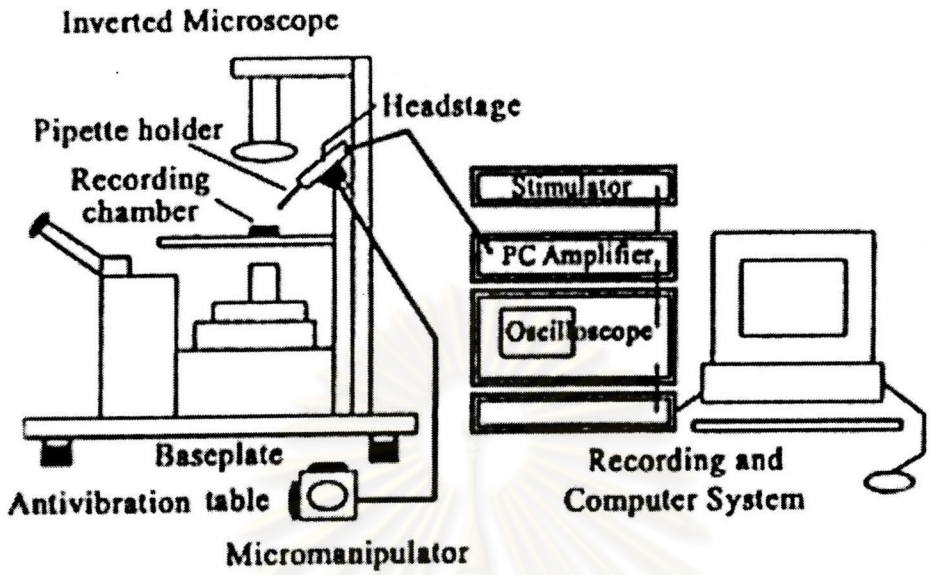

Figure 6 Diagram for the whole-cell application of the patch-clamp technique.

Calculation and statistical analysis

Statistical analysis in microdialysis experiment was carried out using SPSS/PC+(1991) software. All numerical data are expressed as mean ± standard error of the mean (S.E.M.). Analysis of variance (oneway ANOVA followed by Duncan's Multiple range Test) was used to compare the data between various groups ($p<0.05$).

Experimental values from whole-cell patch clamp experiment were presented as mean±standard error of the mean (S.E.M.). To combine data from different neurons, the GABA, glycine and NMDA responses on each neuron were expressed as percentages of the maximal response of the same neuron.

For concentration-response analysis, data was 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 $GABA_A$, glycine or NMDA currents.
I_{min} and I_{max}	are the minimal and the maximal currents recorded in a given neuron, respectively.
EC_{50}	is the concentration of GABA, glycine or NMDA eliciting 50% of the maximal currents.
$[X]$	is the GABA, glycine or NMDA concentration.
H	is the Hill coefficient.

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