CHAPTER II

MATERIALS AND METHODS

1. Materials

1.1 Experimental Animals

20 *Xenopus laevis* (*Xenopus Express*™, USA) were obtained commercially. The frogs were maintained below 20 °C and exposed to a continuos light-dark cycle of 12 hrs each.

1.2 Instruments

1) Analog to Digital Converter (MacLab™, ADInstruments, Australia)
2) Automatic Injector (Nanoject, Drummond Scientific Company, USA)
3) Borosilicate Glass Capillary tubes (GD-1.5, Narishige, Japan)
4) Faraday Cage
5) Fiber Illuminator (C-FI115, Nikon, Japan)
6) Incubator (SuperCool, Memmert, Sweden)
7) MAXIscript™ Kit (Ambion, Inc., USA)
8) Qaigen Plasmid Kit (Qaigen, Inc., USA)
9) Macintoch Computer (LC630, ADInstruments, Australia)
   Software: Chart™ V3.5.6
10) Micromanipulator (M330IL, World Precision Instruments, USA)
11) pH Meter (210, Orion, USA)
12) Steroscopic Zoom Microspope (SZ-ST, Olympus, Japan)
13) Vertical ground Bath Clamp (VG-2A-X100, Axon Instruments, USA)
14) Vertical Micropiptte Puller (PP-83, Narishige, Japan)

1.3 Compounds

2-amino-5-phosphonopentanoate (AP5)	(Sigma, USA)
Calcium chloride dihydrate	(Merck, Germany)
Calcium dinitrate tetrahydrate	(Merck, Germany)
Collagenase from *Clostridium histolyticum*	(Wako, Japan)
Dimethyl dichlorosilane	(Sigma, USA)
Dimethyl sulfoxide (DMSO)	(Merck, Germany)
Glycine(Gly)	(Sigma, USA)
Glutamate(Glu)	(Sigma, USA)
Magnesium sulfate heptahydrate	(Merck, Germany)
N-(2-propylpentanoyl)urea(VPU)	
Penicillin G sodium	(General Drug House, Thailand)
Sodium chloride	(Merck, Germany)
Sodium hydrogencarbonate	(Merck, Germany)
Sodium hydroxide pellets	(Merck, Germany)
Spermine	(Sigma, USA)
Streptomycin	(General Drug House, Thailand)
Tricaine	(Sigma, USA)
Tris-(hydroxymethyl)aminomethane hydrochloride	(USB, USA)
Valproic acid(VPA)	(Sigma, USA)

(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.)

1.4 cDNAs

pN60 containing the 4.2 kb NR1 subunit cDNA (Moriyoshi et al., 1991) inserted into pBluescript (Stratagene, CA, USA) was generously provided by Dr. Shigetada Nakanishi, Kyoto University, Japan. This cDNA

corresponds to the predominant splice variant found in the CNS, NR1A. (Durand et al., 1992). cDNA encoding the NR2B subunit which lacks the 21-amino acid insert encoded by exon-5 was the generous gift of Dr. Peter Seeburg, University of Heidelburg, Germany.

2. Methods

2.1 Isolation of single oocyte

Oocytes were surgically removed from the frog without the animal needing to be killed. A healthy-looking frog was selected that had not been operated on for at least 3 months. The frog was then placed in a plastic box that contains an ice/water slurry.

Once anesthetized, the frog was placed on its back in a tray containing ice. A small abdominal incisor was made with shape blade, and the underlying fascia was also cut. Bundles of oocytes were gently teased out with forceps. After enough cells had been removed, the remaining ovary was pushed back into the abdomen, and the excision was closed. First the fascia was stitched together, and then the skin is closed. The frog was then transferred to a container containing only water.

Once the frog had recovered, it should be transferred to a colony tank. The oocytes are placed in modified Barth's solution (MBS) culture media (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)4H_2O$, 0.14 mM $CaCl_2 2H_2O$, 0.82 mM $Mg(SO_4)7H_2O$, 7.5 mM Tris-(Hydroxymethyl) aminomethane, pH7.4).

Follicular cell layers were removed manually after incubation for 2 hours at 23 °C in Ca^{2+}-free MBS containing 2.0 mg/ml collagenase. Finally,

oocytes were kept in MBS added 0.01 mg/ml Penicillin and Streptomycin at 18 °C.

2.2 Injection of oocyte

2.2.1 Preparation of mRNA

Plasmids were linearized with *Not*I (NR1A) or *Sal*I (NR2B) and transcribed in vitro with T7 (NR1A) or SP6 (NR2B) RNA Polymerase using the mMessage mMachine Transcription Kit (Ambion, TX, USA). (Buller and Monaghan, 1997) RNA stocks were diluted in sterile distilled water to final concentration of 0.4-0.5 μg/μl.

2.2.2 Injection of mRNA

On the day of surgery, selected oocytes were injected with recombinant receptors. Grass capillary tubes were pulled to a fine tip on a vertical micropipette puller and broken back. Oocytes were injected in the vegatal pole with mRNAs (4 ng of NR1A plus 20 ng of NR2B; 25-nl total volume) with a microinjector. NR1A and NR2B RNAs were injected in a ratio of 1:5 to minimize the formation of homomeric NR1 receptors.

2.2.3 Incubation

After injection, the oocytes were incubated in MBS prior to physiological assay (3-8 days). The oocytes were stored at 18°C in small petri dishes, and the solution is changed daily. During this latter process, the cells should be inspected daily under a microscope, and unhealthy ones discarded.

2.3 Application of test substance

VPU was dissolved in 100% Dimethylsufoxide (DMSO, Merke, Germany). The final DMSO concentration (≤ 0.1%) had no pharmacological effect when applied alone (Leewanich, 1998). Other chemicals were dissolved in the buffer solution. All drugs were applied via the perfusion system.

2.4 Electrophysiological Recording

Electrophysiological recording were performed 3 to 8 days postinjection and were carried out at room temperature (22-25°C). Two electrode voltage-clamp recording were obtained with a Geneclamp (Axon Instrument) amplified with 3 M KCl-filled microelectrodes (0.5-5 mΩ). Only oocytes with resting membrane potential more negative than -30 mV were used for the experiments.

The recording chamber was continuously perfused by gravity driven with Mg^{2+}-free MBS at 1 ml/min and all drugs were dissolved in the same solution, unless otherwise indicated, at a holding potential of –70 mV. Drugs were applied until the peak of the response was observed. A wash out period for recovery was 5-10 min, depending on the concentration of drugs applied (Leewanich, 1998). Currents from TEVC amplifier were changed from analog to digital by MacLab™ (ADInstruments, Australia), and recorded by Macintosh computer using Chart™ V 3.5.6 (ADInstruments, Australia).

The control responses were measured before and after each drug application to take into account possible shifts in the control currents as the recording proceeded. The results are presented as percentages of control responses in order to compensate for variability of the level of receptor in different oocytes. Before recording of response currents of each oocyte, The

control currents of 300 μM glutamate and 10 μM glycine were examined three times and calculated the average value as 100% response.

The effects of test substances on the concentration response curve of glutamate were investigated. The same group of oocytes (4-6 oocytes) was tested in the absence and presence of the test substances at the same concentration of glutamate and glycine. Then, the results of several glutamate concentrations were summarized and presented as concentration response curve of glutamate

2.5 Data analysis

Experimental values are presented as mean±standard error of the mean (S.E.M.). To combine data from different oocytes, the glutamate and glycine responses on each oocyte were expressed as percentages of the maximal response, or responses to 300 μM glutamate and 10 μM glycine of the same oocyte.

Dose-response curves for agonist were fit using GraphPad PRISM™ Version 3.0 (GraphPad Software, U.S.A.) according to the equation

$$I = I_{max}/[1+(EC_{50}/A)^n]$$

Where I is the current response, I_{max} is the maximal current response, n is the Hill coefficient, A is the agonist concentration and EC_{50} is the agonist concentration producing a half-maximal response.

Antagonist inhibition curves were determined in the presence of 300 μM glutamate and 10 μM glycine and were fit according to the equation:

$$I = I_{max} - I_{max}/[1+ (IC_{50}/A)^n]$$

Where I_{max} is the current response in the absence of antagonist, A is the antagonist concentration and IC_{50} is the antagonist concentration producing a half-maximal inhibition.

Significance was tested by *Pair* Student's *t*-test and 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.

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