ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
19 (1999) 205–216

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A method for evaluating chemical selectivity of agonists for glutamate receptor channels incorporated in liposomes based on an agonist-induced ion flux measured by ion-selective electrodes

Hanna Radecka [1], Jun Nakanishi, Ayumi Hirano, Masao Sugawara, Yoshio Umezawa *

Department of Chemistry, School of Science, The University of Tokyo, Hongo, Tokyo 113, Japan

Received 8 January 1998; received in revised form 15 June 1998; accepted 26 June 1998

Abstract

A new method for evaluating chemical selectivity of agonists for the NMDA subtype of glutamate receptor (GluR) channels is described. The method is based on the magnitude of Ca^{2+} release from GluR-incorporated liposomes, which is measured by a Ca^{2+} ion-selective electrode with a thin-layer mode. The partially purified GluRs from rat whole brain were reconstituted into Ca^{2+}-loaded liposomes. Small aliquots (each 50 μl) of the proteoliposomes, in the presence of an antagonist DNQX for blocking non-NMDA subtype, were subjected to potentiometric measurements of Ca^{2+} release under stimulation by three kinds of agonists, i.e. NMDA, L-glutamate and L-CCG-IV. The amount of the Ca^{2+} ion flux through the GluR channel induced by the agonists was found to increase in the order of NMDA < L-glutamate < L-CCG-IV, which was consistent with that of binding affinity of the agonists toward the NMDA subtype. However, the range of selectivity of the relevant agonists was much smaller compared with results based on binding affinities. The present method provides physiologically more relevant values for the agonist selectivity of GluRs as compared to that of the conventional binding assay in the sense that the selectivity is based on the very magnitude of Ca^{2+} flux through the NMDA receptor, i.e. the extent of signal transduction by a given agonist. The evaluation of agonist selectivity based on Na^{+} release was also investigated by using a Na^{+} ion-selective electrode, but agonist-induced Na^{+} release was not detected, because of low permeability of Na^{+} through the NMDA subtype.

Keywords: NMDA receptor; Liposome; Selectivity of agonist; Ca^{2+} ion flux; Ion-selective electrode; Thin-layer potentiometry

1. Introduction

The specific binding of the principal neurotransmitter L-glutamate to glutamate receptor (GluR) ion channels at postsynaptic membranes

* Corresponding author. Fax: +81 3 58022989; e-mail: umezawa@chem.s.u-tokyo.ac.jp

[1] Visiting Scientist from the Chemistry Department, Agriculture Faculty, University of Agriculture and Technology, 10-957 Olsztyn, Poland.

0731-7085/99/$ - see front matter
PII S0731-7085(98)00146-0

in the mammalian central nervous system is known to trigger the opening of its channel through which a large number of ions (Na^+ and Ca^{2+}) are allowed to permeate following their electrochemical potential gradients [1]. The GluRs are categorized into three subtypes based on pharmacological responses, i.e. α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), kainic acid, and *N*-methyl-D-aspartic acid (NMDA) receptors [2]. On the basis of each receptor subtype of GluRs, the evaluation of selectivity of agonists and antagonists is important for elucidating the fundamental process of neuronal signal transduction [3], molecular design of specific agonists [4,5] and structure/function relationships [6,7].

So far, selectivity of agonists for each receptor subtype was described in terms of ligand affinities. The ligand affinities were mostly evaluated with binding assay [4,5,8,9], which is based on competitive binding of an agonist of interest and a radiolabeled reference agonist with their target receptor. In binding assay, 50% inhibitory concentration of agonist (IC_{50}) is commonly used as a measure for the ligand affinity, which is obtained as follows [9]: synaptic membrane fractions were incubated with various concentrations of the sample agonist in the presence of a given concentration of standard radiolabeled agonist. The receptor–agonist complex was then separated from the free agonist and its radioactivity was measured by a scintillation counter. IC_{50} is the concentration of agonist of interest at which the bindings of half of the radiolabeled agonists are prevented. The IC_{50} is related to dissociation constant (K_d) between the receptor and agonist of interest. An alternative method to evaluate ligand binding affinities is functional assay which is based on the potency of agonists to induce the signal-transducing function of receptors, i.e. induction of ion fluxes through whole-cell channels. The ion flux was detected with various tools such as patch-clamp [10], fluorescence indicators (e.g. Fura 2 [8,11,12]) or radiolabeled permeant ions (e.g. $^{45}Ca^{2+}$ [13], $^{14}CH_3NH_3^+$ [14]). Based on thus-detected ion fluxes, the selectivity of agonists has been reported as an agonist concentration required for 50% change in the ion flux, i.e. effective agonist concentrations of half-maximal responses (EC_{50}).

In contrast to the above two approaches, we have proposed, in our previous paper [15], a new approach to evaluate the chemical selectivity of agonists for NMDA subtype based on the very magnitude of the signal transducing response of the receptor rather than ligand binding affinities. The method was based on the integrated channel currents of NMDA subtype embedded in planar bilayer lipid membranes corresponding to the total ion flux through the open channel. The selectivity of agonists thus obtained may be more physiologically relevant in the sense that the selectivity reflected the signal transducing ability of agonist-activated receptor.

In the present paper, a new method for evaluating chemical selectivity of agonists for NMDA subtype of GluRs incorporated in liposomes based on the very magnitude of Ca^{2+} flux was proposed. The principle of the present approach is schematically shown in Fig. 1. By activating the NMDA receptor by three kinds of agonists, NMDA, L-glutamate or (2*S*, 3*R*, 4*S*) isomer of 2-(carboxycyclopropyl)glycine (L-CCG-IV), the Ca^{2+} loaded in the interior of the liposomes is released to the external solution following its concentration gradient through the open channels of NMDA subtype. The amount of Ca^{2+} released is detected by a Ca^{2+} ion-selective electrode (Ca^{2+}-ISE) with a thin-layer mode [16–22]. In contrast to the previous approach based on the integrated channel current, corresponding to the total cation fluxes of Ca^{2+} and Na^+ through NMDA receptor in planar bilayer lipid membranes, the present approach provides Ca^{2+} ion-specific selectivity of GluRs toward agonists, which is achieved by utilizing a Ca^{2+}-ISE. Especially, the high permeability of the Ca^{2+} ion through the NMDA subtype is widely known [23–25], and the selective detection of Ca^{2+} ion flux is significant [26–28], since the Ca^{2+} ion is one of the second messengers inside the cell and regulates various kinds of Ca^{2+}-dependent signal cascades [29]. The selectivity obtained by the present method is physiologically more relevant in the sense that the selectivity is a Ca^{2+} ion-specific one, and that the selectivity is based on the very magnitude of Ca^{2+} ion flux

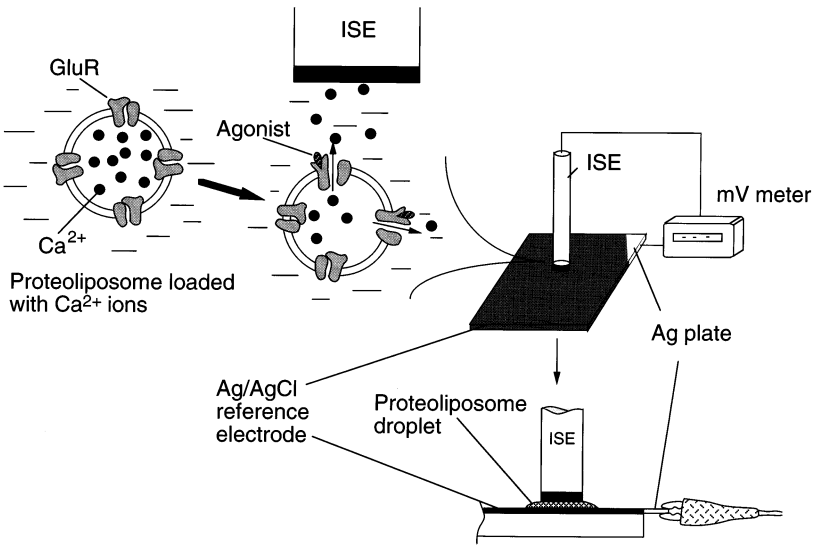

Fig. 1. A schematic diagram of the present method for measuring chemical selectivity of glutamate receptor (GluR) ion channels based on an agonist-induced Ca^{2+} flux from Ca^{2+}-loaded proteoliposomes monitored by a Ca^{2+} ion-selective electrode with a thin-layer mode.

which corresponds to the extent of signal transduction induced by a given agonist. The present approach is, in principle, applicable to distinguish a Na^+ flux from the total ion fluxes (Na^+ and Ca^{2+}) if a Na^+ ion-selective electrode is used. However, the Na^+ flux could not be detected because of the low Na^+ permeability exhibited by the NMDA subtype, as will be described in the present paper.

2. Experimental

2.1. Chemicals

L-α-Phosphatidylcholine (PC, from egg, purity > 99%, in chloroform solution) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (chol) was obtained from Wako Pure Chemicals (Osaka, Japan) and recrystallized three times from methanol. *N*-Methyl-D-aspartic acid (NMDA) and 6,7-dinitroquinoxaline-2,3-(1H,4H)-dione (DNQX) were purchased from Sigma (St. Louis, MO). L-Glutamic acid was from Wako. The (2*S*,3*R*,4*S*) isomer of 2-(carboxycyclopropyl)glycine (L-CCG-IV) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]-cyclohepten-5,10-imine malenate (MK-801) were obtained from Research Biochemicals (Natick, MA). Diethyl-*N*,*N*′-[(4*R*,5*R*)-4,5-dimethyl-1,8-dioxo-3,6-dioxaoctamethylene]bis(12-methylamino-dodecanoate) (ETH1001) was purchased from Fluka (Buchs, Switzerland). Didodecyl bis(12-crown-4),tetrakis[3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate, sodium salt (HFPB) and 2-nitrophenyl octyl ether (*o*-NPOE) were from Dojindo (Kumamoto, Japan). Chloroform of HPLC grade (Wako) was used after passing through an alumina column. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) and bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane (Bistris) were obtained from Dojindo. Other chemicals used were all of analytical grade. Purified water obtained with an ultra-pure water system Milli-RX 12α and Milli-Q Plus (Millipore, Bedford, MA) were used throughout the experiments. Stock solutions of L-glutamate, NMDA and L-CCG-IV were prepared, respectively, by dissolving a weighed amount of each agonist in buffer solution A or buffer solution B (vide infra), stored at 4°C and used within 3 weeks.

2.2. Thin-layer potentiometry

2.2.1. Preparation of Ca^{2+} ISE membrane

The solvent polymeric liquid membranes containing 1 wt.% Ca^{2+} ionophore ETH1001, 66 wt.% plasticizer *o*-NPOE, 33 wt% poly(vinyl chloride) (PVC) and 73 mol% (relative to the ionophore) HFPB as an anionic additive were prepared according to the conventional procedure [30]. A membrane disc of 10 mm diameter was cut out and fixed on one end of a glass tube (o.d. 7 mm and i.d. 6 mm) with Teflon seal tape. As an internal reference solution, 0.01 M $CaCl_2$ solution was used. An Ag/AgCl wire was used as an internal reference electrode.

2.2.2. Preparation of Na^+ ISE membrane

The solvent polymeric liquid membranes were prepared by mixing 3.2 wt% didodecyl-bis(12-crown-4) (Na^+ ionophore), 65 wt% *o*-NPOE, 32 wt% PVC and 1.1 mol% (relative to the ionophore) HFPB. A membrane disc of 6 mm diameter was cut out and fixed on a liquid membrane type ISE body (Denki Kagaku Keiki, Tokyo, Japan). As an internal reference solution, 0.1 M NaCl was used. ISE membrane was conditioned with 0.05 M Na_2SO_4 overnight.

2.2.3. Preparation of a plate-shaped reference electrode

A plate-shaped Ag/AgCl reference electrode was prepared by anodic oxidation of silver plate (5×5 cm, 2 mm thick) in 0.1 M KCl solution, according to the procedure described previously [17]. It is important to note that the influence of proteins if any on the potential change of the Ag/AgCl reference electrode is negligible, even though the reference electrode is in direct contact with the proteoliposome suspensions [16,18–21]. It should also be pointed out that the unwanted shifts of the reference potential due to possible variation of chloride ion activity from one sample to another can be eliminated by the use of 10 mM LiCl or 10 mM Bistris–HCl buffers throughout the whole potential measurements.

2.2.4. Calibration of the Ca^{2+} and Na^+ ISEs with a thin-layer mode

The calibration curves for Ca^{2+} and Na^+ were measured in buffer solution A and in buffer solution B, respectively, at room temperature. Buffer solution A was 100 mM Hepes–NaOH (pH 7.4) containing 10 mM LiCl, 20 mM potassium acetate, 13.3 mM K_2SO_4, 0.1 mM EDTA and 20% glycerol, and buffer solution B was 10 mM Bistris–HCl (pH 7.4) containing 230 mM sucrose, 0.10 mM NH_4Cl, 5.0 μM glycine, 10 μM DNQX and 10% glycerol. Before calibrating Ca^{2+} and Na^+ ISEs in Ca^{2+}- and Na^+-containing solutions, both the membranes were washed with buffer solution A and B, respectively, until steady potentials were obtained. The potential was measured with a Denki Kagaku Keiki mV meter model IOL-50.

For preparing its calibration curve for Ca^{2+}, a 50-μl portion of buffer solution A containing 1.0×10^{-6} M $CaSO_4$ was dropped onto the plate reference electrode placed in a horizontal position. The Ca^{2+} ISE was lowered in an upright position on the droplet. The potential was measured after waiting for 3 min for the steady potential (within ± 1 mV min^{-1}) to be attained. The Ca^{2+} ISE was pulled up and both the Ca^{2+} ISE and the plate reference electrode were rinsed with Milli-Q water. Next, 50 μl of buffer solution A containing 1.0×10^{-5} M $CaSO_4$ was dropped onto the plate reference electrode and the potential was measured in the same way. The whole procedure was repeated for each 50-μl droplet of buffer solution A containing increased concentrations of $CaSO_4$. The calibration curve was thus prepared in the Ca^{2+} concentration range from 1.0×10^{-6} to 1.0×10^{-2} M. The potential versus log *C* plot with the Ca^{2+} ISE showed a Nernstian response in the concentration range between 1.0×10^{-5} and 1.0×10^{-2} M (Fig. 2a).

The calibration curve for Na^+ was prepared basically in the same manner as for Ca^{2+} except that 40 μl of buffer solution B containing a given concentration of Na_2SO_4 (from 5.0×10^{-6} to 5.0×10^{-4} M) was used. The potential versus log *C* plot with the Na^+ ISE showed a Nernstian response in the concentration range of Na^+ from 3.2×10^{-5} to 1.0×10^{-3} M (Fig. 2b).

2.3. Isolation of glutamate receptors (GluRs)

The synaptic plasma membranes containing glutamate receptor ion channel proteins were obtained from whole brains of six anesthetized adult male SD rats as described in our previous paper [15]. The synaptic membranes were finally suspended in 3 ml of Ca^{2+} solution (10 mM $CaSO_4$, 10 mM LiCl, 20 mM potassium acetate, 0.1 mM EDTA, 20% glycerol, 0.1 mM dithiothreitol, 100 mM Hepes–NaOH, pH 7.4) or Na^+ solution (10 mM NaCl, 210 mM sucrose, 0.1 mM EDTA, 5 μM glycine, 10% glycerol, 0.1 mM dithiothreitol, 10 mM Bistris–HCl, pH 7.4).

The total amount of extracted proteins was determined by Bradford's method [31] using Coomassie Brilliant Blue G-250 dye (Bio-Rad protein assay kit, Bio-Rad Laboratories, Hercules, CA) in the same manner as described in our previous paper [15]. The protein suspension was diluted with Ca^{2+} solution or Na^+ solution, respectively, to give a concentration of ca 2 mg protein ml^{-1}.

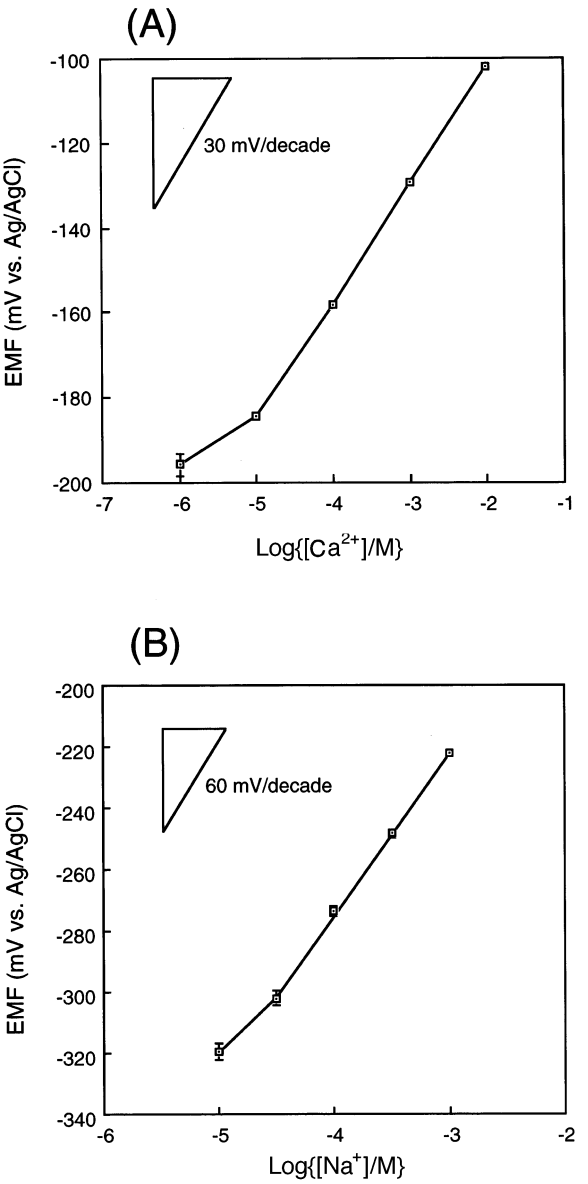

Fig. 2. Calibration curves for Ca^{2+} (A) in buffer solution A (see Section 2) and Na^+ (B) in buffer solution B with Ca^{2+} and Na^+ ion-selective electrodes, respectively, with a thin-layer mode ($n = 3$). Potentials were measured with respect to a Ag/AgCl plate reference electrode.

2.4. Preparation of Ca^{2+}- and Na^+-loaded proteoliposomes

The diluted synaptic membranes (1.5 ml) was transferred into a pear-shaped flask coated with a PC-chol (weight ratio, 4:1) lipid film and sealed with nitrogen. The lipid film was prepared as follows: 2.5 mg of PC and 0.6 mg of cholesterol in a pear-shaped flask were dissolved in 1.5 ml of chloroform. Chloroform was roughly evaporated with a rotary evaporator under reduced pressure, and completely evaporated with vacuum pump for 1 h. In order to load the ion of interest inside the liposomes, the protein suspension and the lipid film were vigorously vortexed and then sonicated in an ice bath for 5 min. The proteoliposome suspension thus obtained was divided into small portions in separate microcentrifuge tubes and stored at 4°C under nitrogen until use.

The trapped volumes of the Ca^{2+}- and Na^+-loaded proteoliposomes, determined according to the procedure described by Oku et al. [32], were 2.6 and 3.1% of the total volume, respectively.

2.5. Potentiometric measurement

2.5.1. For Ca^{2+} release

In order to establish a sufficient outward Ca^{2+}

concentration gradient between exterior and interior solutions of liposomes, the Ca^{2+} concentration outside liposomes was expected to be minimized. For this purpose, a Ca^{2+}-loaded proteoliposome suspension was centrifuged ($7000 \times g$) and the resulting supernatant was replaced with the same volume of buffer solution A free of Ca^{2+}. This procedure was repeated again. Prior to the potentiometric measurement, one-fifth volume of buffer solution A containing 2.5×10^{-5} M glycine and 6.0×10^{-5} M DNQX was mixed with proteoliposome suspension in order to enhance the agonist-induced ion flux and antagonize the response of non-NMDA subtype of GluRs.

Following the above pretreatments, a 50-μl aliquot of the proteoliposome suspension was dropped on the Ag/AgCl plate reference electrode, then the Ca^{2+} ISE was brought into contact with this droplet. After 3 min, a 5.0-μl portion of buffer solution A containing 1.1×10^{-7} M of each agonist (NMDA, L-glutamate or L-CCG-IV) was injected to the droplet to bring its concentration to 1.0×10^{-8} M. The droplet was mixed well by repeatedly lowering and lifting the Ca^{2+}-ISE, and allowed to stand for 3 min for potential recording. Then successive addition of each 5.0-μl buffer solution A containing each agonist (1.1×10^{-6}, 1.2×10^{-5} and 1.3×10^{-4} M) to the same proteoliposome droplet was carried out to reach agonist concentrations of 1.0×10^{-7}, 1.0×10^{-6} and 1.0×10^{-5} M, respectively; the potentials at 3 min after each addition of the agonist solutions were recorded, respectively. A similar procedure was performed by adding buffer solution A free of agonist for the background Ca^{2+} leakage measurement, under otherwise identical conditions.

During these potentiometric measurements, each injection of the agonist solutions increased the volume of proteoliposome sample droplet causing diluted Ca^{2+} concentrations in the exterior of the liposomes. The increased amount (moles) rather than concentration of Ca^{2+} in the exterior of liposomes was therefore used as a measure of Ca^{2+} release.

On the basis of the amount of initially loaded Ca^{2+} and the amount of Ca^{2+} released, the decreased amount of Ca^{2+} inside the liposomes under stimulation by 1.0×10^{-5} M L-glutamate was estimated to be 30% of the initial value.

2.5.2. For Na^+ release

The method for measuring the Na^+ release induced by L-glutamate and L-CCG-IV using a Na^+ ISE was basically the same as that for the Ca^{2+} release, except that the incubation with DNQX and glycine was omitted since buffer solution B already contained 10 μM DNQX and 5 μM glycine.

2.6. Procedure for evaluating agonist selectivity based on Ca^{2+} release

The net Ca^{2+} release (ΔCa) induced by each agonist is defined as follows, taking into account the background Ca^{2+} leakage (Δb):

$$\Delta Ca = \Delta a - \Delta b \quad (1)$$

where Δa is the increased amount of Ca^{2+} by increasing the concentration of each agonist from 1.0×10^{-8} to 1.0×10^{-5} M and Δb is the background Ca^{2+} leakage by injecting the same volumes of buffer solution A containing no agonists. The agonist selectivity was evaluated with the ratio of ΔCa between two agonists of interest which was obtained by using each 50-μl proteoliposome sample pretreated at the same time. This is because, within the same proteoliposome preparation, the number of GluRs was constant (see Section 3.4).

3. Results and discussion

3.1. Characteristics of Ca^{2+}-loaded proteoliposomes

Prior to the addition of the agonist solutions, the residual Ca^{2+} concentration in the exterior aqueous phase of the proteoliposome suspension was found to be ca 10^{-4} M ($n = 24$). This relatively high concentration of Ca^{2+} is ascribed to incomplete removal of Ca^{2+} and agonist irrele-

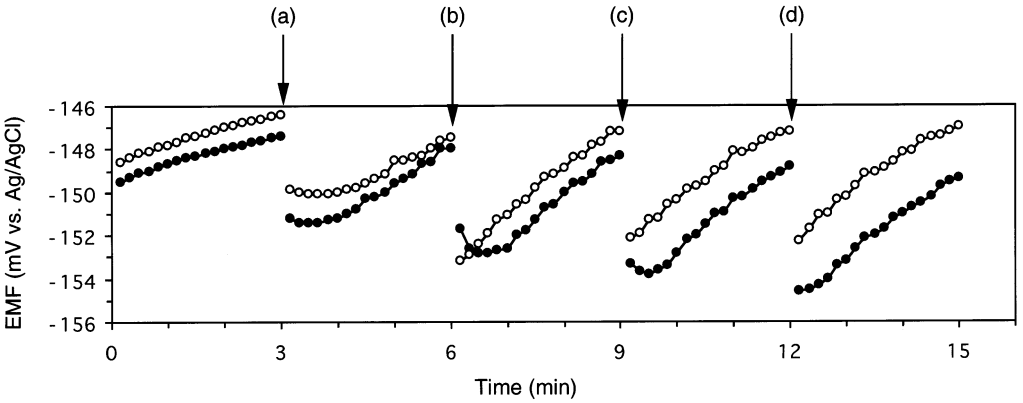

Fig. 3. Potential–time profiles of the L-glutamate-triggered Ca^{2+} release and the background Ca^{2+} leakage from Ca^{2+}-loaded liposomes embedded with GluRs. (○) Five μl of buffer solution A containing L-glutamate was injected at each arrow in order that the final concentrations of L-glutamate in the sample droplet become: (a) 1.0×10^{-8} M; (b) 1.0×10^{-7} M; (c) 1.0×10^{-6} M; and (d) 1.0×10^{-5} M, respectively. (●) Five μl of buffer solution A containing no agonist was injected at each arrow. The initial volume of each proteoliposome droplet was 50 μl.

vant leakage of Ca^{2+} from the liposomes. The concentration of Ca^{2+} inside the liposomes was determined to be ca 10^{-2} M by a Ca^{2+} ISE after osmotic rupture with water of the proteoliposomes, together with taking into account the measured value of the trapped volume (2.6%) of proteoliposomes. A concentration gradient of Ca^{2+} as great as 10^2 was thereby established, directed from the interior to the exterior of liposomes. Such a high concentration (ca 10^{-2} M) of internal Ca^{2+} has been demonstrated to cause inactivation of the NMDA receptor using an inside-out patches technique [33]. However, in the present study, a sufficient concentration gradient of Ca^{2+} across liposome membranes was necessary as a driving force for agonist-induced Ca^{2+} efflux. Thus we used ca 10^{-2} M of Ca^{2+} as an internal solution of the liposomes, although such a high concentration is a compromise for attaining efficient activity of GluRs.

Under the present experimental conditions, where agonists are injected to the exterior aqueous phase of the proteoliposomes, only GluRs whose agonist binding site face the outside of the liposomes are subjected to stimulation by each agonist; the resulting Ca^{2+} flux therefore is directed outwards following its own concentration gradient. The direction of this Ca^{2+} flux is in a way reversed from the actual post-synaptic membrane system where the Ca^{2+} flux is directed from the extracellular to the cytoplasmic side. It is, however, noted that the channel current observed in the planar lipid bilayer incorporated with GluRs is basically linear and symmetrical with the change in magnitude and polarity of the applied membrane potentials [15], indicating that the difference in the direction of the ion flux in this case is irrelevant to the extent of ion permeation through the NMDA receptor.

3.2. Potential–time profiles of a Ca^{2+} ISE for Ca^{2+} release

A typical potential–time profile, monitored by a Ca^{2+} ISE with a thin-layer mode, of the L-glutamate-triggered Ca^{2+} release and the background Ca^{2+} leakage is shown in Fig. 3. The arrows indicate the time at which each 5-μl aliquot of buffer solution A with (○) or without (●) L-glutamate was injected into a proteoliposome droplet, placed beforehand on the plate reference electrode. Because of agonist irrelevant leakage of Ca^{2+} from liposomes, the potential when the Ca^{2+} ISE was brought into contact with sample droplet (time = 0) varied from one sample to another. This variation was minimized by comparing each proteoliposome sample pretreated at the same time. Within the same prote-

oliposome preparation, the potential of adding L-glutamate (○) was larger than that of simply injecting buffer solution A (●). The difference in the potentials demonstrates that the agonist-induced Ca^{2+} release in fact occurred. Also, the time course in the observed potentials simply reflects the effect of dilution of the proteoliposome sample droplet as a lowering of starting potentials upon each consecutive injection of the L-glutamate solution. For accurately evaluating the amount of Ca^{2+} release from proteoliposomes, the observed potentials 3 min after the injection of each agonist were used, when complete mixing of the sample droplet was expected to be attained. Using thus-measured potentials together with corrected volumes of the sample droplets, the amount of Ca^{2+} release was obtained. The GluRs in their open state may pass not only Ca^{2+} but also Na^{+}; however, the Ca^{2+} ISE employed here essentially detects a Ca^{2+} ion flux: the potentiometric selectivity coefficient $K^{pot}_{Ca,Na}$ is known to be $10^{-3.7}$ [30].

3.3. Effect of antagonists and agonist concentration dependence

The effect of antagonists, DNQX for non-NMDA subtype and MK-801 for NMDA subtype, on L-glutamate-induced Ca^{2+} release was examined in the concentration range from 1.0×10^{-8} to 1.0×10^{-5} M L-glutamate. For comparison, the background Ca^{2+} leakage was also examined in the same proteoliposomes preparation. The amount of Ca^{2+} existing outside the liposomes was plotted against the concentration of L-glutamate or injected volume of buffer solution A, as shown in Fig. 4.

As described in the previous section, the amount of Ca^{2+} release (curve B) was greater than that of the background Ca^{2+} leakage (curve C). On the other hand, in the presence of antagonists DNQX and MK-801, L-glutamate-induced Ca^{2+} release (curve A) was suppressed to a level identical to the background Ca^{2+} leakage (curve C). These results demonstrate that the difference between the amount of L-glutamate-induced Ca^{2+} release and the background Ca^{2+} leakage is due to embedded GluRs which, in a channel-open state, allowed permeation of Ca^{2+}.

Fig. 5 shows the concentration-dependent Ca^{2+} release for three kinds of agonists and the background Ca^{2+} leakage in the presence of 10 μM DNQX (non-NMDA subtype antagonist [34]).

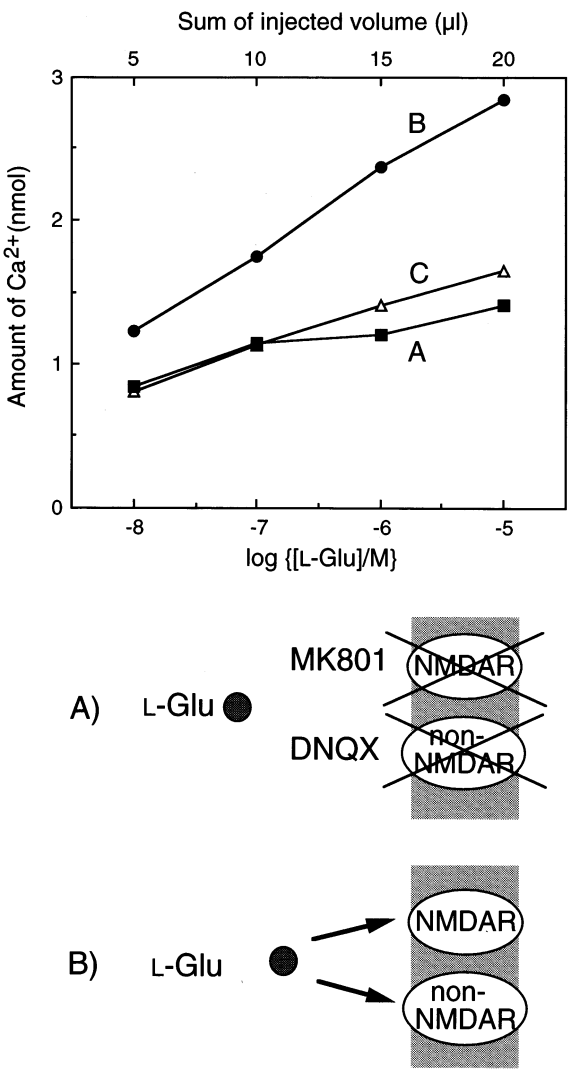

Fig. 4. Effect of GluR antagonists on L-glutamate-induced Ca^{2+} release from Ca^{2+}-loaded liposomes embedded with GluRs. The amount of Ca^{2+} released was obtained 3 min after L-glutamate injection and plotted against the concentration of L-glutamate with (A) or without (B) 5 μM DNQX and 5 μM MK-801. (C) Background Ca^{2+} leakage was obtained by injecting buffer solution A without L-glutamate. The bottom figures represent NMDA and non-NMDA subtypes being blocked by MK-801 and DNQX (A), and activated by L-glutamate (B).

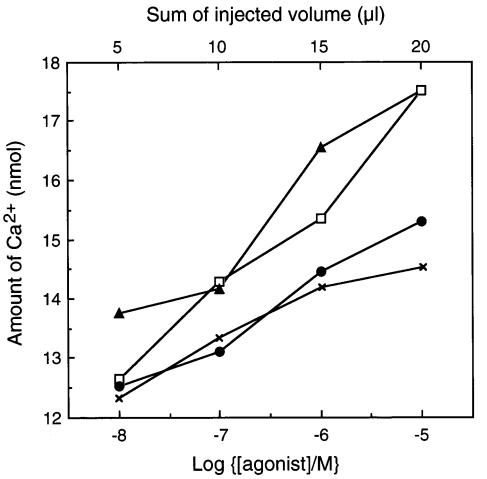

Fig. 5. Dependence of the Ca^{2+} release from Ca^{2+}-loaded liposomes embedded with GluRs on the concentrations of agonists: (▲) L-glutamate, (□) L-CCG-IV, (●) NMDA or (×) no agonist. Potentials 3 min after injection of buffer solution A containing each agonist were measured. All measurements were performed using each 50-μl of proteoliposomes from the same preparation.

NMDA and L-CCG-IV also induced Ca^{2+} release like L-glutamate. On the basis of the concentration-dependent Ca^{2+} release, we used the increased amount of Ca^{2+} (Δa) from 1.0×10^{-8} to 1.0×10^{-5} M of agonist concentration for evaluating the selectivity of each agonist. Fig. 5 also indicates that the background Ca^{2+} leakage was not negligible as compared to the agonist-induced Ca^{2+} release. Therefore, the net Ca^{2+} release (ΔCa) was obtained by subtracting the background Ca^{2+} (Δb) leakage as described in Section 2.

3.4. Evaluation of agonist selectivity for NMDA subtype of glutamate receptors

Table 1 shows the ΔCa values of three kinds of agonists and their ratio within the same proteoliposomes preparation. Although the absolute value of ΔCa induced by an agonist of interest varied from one proteoliposome preparation to another, the ratio of ΔCa between each agonist, NMDA/L-glutamate and L-CCG-IV/L-glutamate, was found to be almost constant among different proteoliposome preparations. This is simply because, within the same proteoliposome preparation, the number of GluRs was constant. It should be noted that, since the potential often happened to jump suddenly by several mV during measurements, seemingly due to the rupture of proteoliposomes, all the data obtained were checked by Q-test [35] before calculating the mean values and standard deviations. The probability of successful measurements after the Q-test was 50% out of 24 measurements.

The selectivity of agonists evaluated with the present approach was found to increase in the order of NMDA < L-glutamate < L-CCG-IV. This selectivity order is consistent with that of binding affinity of the agonists toward the NMDA subtype. However, the range of selectivity of the relevant agonists was much smaller (NMDA:L - glutamate:L - CCG - IV = 0.58 ± 0.31:-1.0:1.6 ± 0.4) compared with the result based on binding affinities (NMDA:L-glutamate:L-CCG-IV = 0.022:1.0:17 [8]). This suggests that the present method based on the Ca^{2+} flux through the NMDA receptor subtype gives a physiologically more relevant agonist selectivity toward NMDA receptor subtype compared to the assay based on binding affinity: the present approach is based on the very magnitude of Ca^{2+} ion passed through the NMDA receptors, which reflects not only the binding affinity for the agonists but also the ability of signal transduction and amplification. Interestingly, the selectivity ratio of the relevant agonists based on the Ca^{2+} release was practically in agreement with that based on the total ion flux (Ca^{2+} and Na^{+}), i.e. the selectivity ratio NMDA:L-glutamate:L-CCG-IV = 0.47 ± 0.57:1.0:2.9 ± 1.5 [15].

3.5. Characteristics of Na^{+}-loaded proteoliposomes and Na^{+} release

The NMDA receptor is known to allow permeation of Na^{+} as well as Ca^{2+}. We also tried to use a Na^{+} release as in the Ca^{2+} case as a measure of the selectivity of agonists to activate the NMDA receptor. The Na^{+}-loaded liposomes were prepared as described in Section 2. The initial Na^{+} concentration outside the liposomes was ca 10^{-4} M, as measured by a Na^{+} ISE. The

Table 1
Chemical selectivity of NMDA, L-glutamate (L-Glu), and L-CCG-IV toward NMDA receptor subtype based on the amount of agonist-induced Ca^{2+} release from Ca^{2+}-loaded liposomes embedded with GluRs

Proteoliposome preparation no.	$\Delta Ca^{a,b}$ (10^{-9} mol Ca^{2+})			Selectivity ratio[c]	
	L-Glu	NMDA	L-CCG-IV	NMDA/L-Glu	L-CCG-IV/L-Glu
1	1.5	0.55	2.7	0.37	1.8
2	1.8	1.8	3.7	1.0	2.1
3	1.6	0.27	2.2	0.17	1.4
4	1.5	1.1	—[d]	0.73	—[d]
5	1.7	0.79	—[d]	0.46	—[d]
6	1.8	1.3	—[d]	0.72	—[d]
7	0.75	—[e]	1.8	—[e]	2.4
8	0.93	—[e]	1.4	—[e]	1.5
9	1.1	—[d]	1.3	—[d]	1.2
10	0.72	—[d]	0.73	—[d]	1.0
				Mean 0.58 ± 0.31 ($n = 6$)[f]	Mean 1.6 ± 0.4 ($n = 7$)[f]

[a]ΔCa (the net Ca^{2+} release) is defined in Section 2. Potential 3 min after injection of each agonist solution was used for obtaining ΔCa.
[b]Measured in 100 mM Hepes/NaOH (pH 7.4), 10 mM LiCl, 20 mM CH_3COOK, 13.3 mM K_2SO_4, 0.1 mM EDTA and 20% glycerol (buffer solution A) containing 5 μM glycine and 10 μM DNQX.
[c]The net Ca^{2+} release induced by NMDA or L-CCG-IV relative to that of L-Glu within the same proteoliposome preparation.
[d]Data were rejected by Q-test with confidence limit of 95%.
[e]Not measured.
[f]Confidence limit of 95%.

concentration of Na^+ inside the liposomes was determined to be ca 10^{-2} M by a Na^+ ISE after osmotic rupture with water of the proteoliposomes. This concentration was comparable to that of the entrapped Ca^{2+} in the above study.

The Na^+ release under stimulation by L-glutamate and L-CCG-IV in the concentration range from 0 M (buffer solution B) to 9.3×10^{-6} M was examined, together with the background Na^+ leakage by adding buffer solution B free of agonists. The results obtained are shown in Fig. 6. Unlike the case of Ca^{2+} release, there was no significant difference between the Na^+ release induced by agonists and the background Na^+ leakage, i.e. the agonist-induced Na^+ release through the open channels of the NMDA subtype was not observed, although the background Na^+ leakage (2.01 ± 0.74 nmol; normalized as 50 μl proteoliposome suspension, $n = 4$) was comparable to that of Ca^{2+} (2.17 ± 0.78 nmol, $n = 3$), and the lower detection limits of Ca^{2+} and Na^+ ISEs were almost identical. The most probable reason for the above result is that the permeability of Na^+ through the NMDA receptor is lower than that of Ca^{2+}. The permeability ratio (P_{Ca}/P_{Na}) of Ca^{2+} to Na^+ through NMDA receptors in biological membranes has been reported to be ~ 6 [23–25]. Our result, together with this fact, indicates that the Na^+ flux through the NMDA subtype is much smaller than the Ca^{2+} flux, if compared under identical conditions, as used in the present study.

4. Conclusion

The present method utilized a Ca^{2+} flux through NMDA receptor subtype as a new measure of the efficacy of the agonist to activate the NMDA receptor subtype. The method relied on thin-layer potentiometry with a Ca^{2+} ion-selective electrode. The present approach provides a Ca^{2+} ion-specific selectivity toward GluRs, in contrast to the method based on total integrated currents used in our previous paper. The observed selectivity order for three typical agonists was

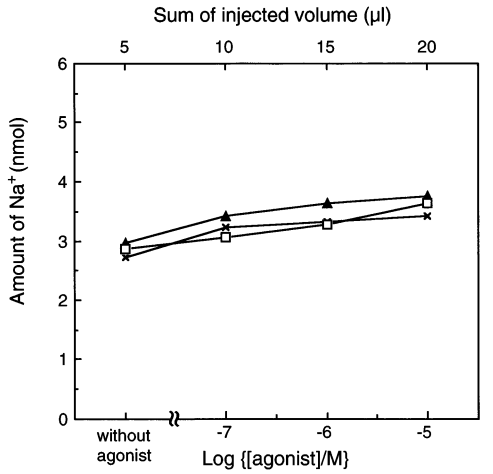

Fig. 6. Dependence of the Na^+ release from Na^+-loaded liposomes embedded with GluRs on the concentrations of agonists: (▲) L-glutamate, (□) L-CCG-IV or (×) no agonist. Potentials 3 min after injection of buffer solution B containing each agonist were emeasured. All measurements were performed using each 40-μl of proteoliposomes from the same preparation.

consistent with that of the binding affinity of the agonists towards the NMDA receptor subtype. However, the range of selectivity of the agonists was much smaller compared with results based on binding assay. This suggests that the present approach provides a method for evaluating a physiologically more relevant Ca^{2+} ion-specific selectivity of GluR ion channel proteins toward agonists as compared to the conventional binding assay, since the selectivity is based on the signal-transducing ability of the GluRs. The present approach can, in principle, be applied to any other ion channels, if an appropriate ionophore for its ISE membrane is chosen in order to distinguish an ion of interest from other permeant ions.

Acknowledgements

The authors thank J. Yamamichi for his experimental help in protein isolation and Dr K. Tohda for preparing ion-selective electrodes. One of the authors (H.R.) acknowledges Japan Society for the Promotion of Science for the Invitation Fellowship Programs for Research in Japan (long term) for foreign researchers. This work was supported from Grants for Scientific Research by the Ministry of Education, Science and Culture, Japan. The supports from Suntory Institute for Bioorganic Research are also acknowledged.

References

[1] C.J. MacBain, M.L. Mayer, Physiol. Rev. 74 (1994) 723–760.
[2] J.C. Watkins, R.H. Evans, Annu. Rev. Pharmacol. Toxicol. 21 (1981) 165–204.
[3] R.A.J. Lester, J.D. Clements, G. Tong, G.L. Westbrook, C.E. Jahr, in: G.L. Collingridge, J.C. Watkins (Eds.), The NMDA Receptor, Oxford University Press, Oxford, 1994, pp. 206–218.
[4] D.J. Kyle, R.J. Patch, E.W. Karbon, J.W. Ferkany, in: P. Krogsgaard-Larsen, J.J. Hansen (Eds.), Excitatory Amino Acid Receptors, Ellis Horwood, Chichester, 1992, pp. 121–161.
[5] D.E. Jane, H.J. Olverman, J.C. Watkins, in: G.L. Collingridge, J.C. Watkins (Eds.), The NMDA Receptor, Oxford University Press, Oxford, 1994, pp. 31–104.
[6] K.A.F. Gration, R.L. Ramsey, P.N.R. Usherwood, in: B. Sakmann, E. Neher (Eds.), Single-Channel Recording, Plenum Press, New York, 1983, pp. 377–388.
[7] S.G. Cull-Candy, I. Parker, in: B. Sakmann, E. Neher (Eds.), Single-Channel Recording, Plenum Press, New York, 1983, pp. 389–400.
[8] M. Kawai, Y. Horikawa, T. Ishihara, K. Shimamoto, Y. Ohfune, Eur. J. Pharmacol. 211 (1992) 195–202.
[9] D.E. Murphy, J. Schnider, C. Boehm, J. Lehmann, M. Williams, J. Pharmacol. Exp. Ther. 240 (1987) 778–784.
[10] D.K. Patneau, M.L. Mayer, J. Neurosci. 10 (7) (1990) 2385–2399.
[11] J. Benavides, Y. Claustre, B. Scotton, J. Neurosci. 8 (10) (1988) 3607–3615.
[12] Y. Kudo, K. Akita, M. Ishida, H. Shinozaki, Brain Res. 567 (1991) 342–345.
[13] M.J. Brammer, S. Richmond, J.Z. Xiang, P. Adamson, I. Hajimohammadreza, M.A. Silva, I.C. Campbell, Neurosci. Lett. 128 (1991) 231–234.
[14] A.M. Ly, E.K. Michaelis, Biochemistry 30 (1991) 4307–4316.
[15] M. Sugawara, A. Hirano, M. Rehák, J. Nakanishi, K. Kawai, H. Sato, Y. Umezawa, Biosensors Bioelectron. 12 (1997) 425–439.
[16] K. Shiba, Y. Umezawa, T. Watanabe, S. Ogawa, S. Fujiwara, Anal. Chem. 52 (1980) 1610–1613.
[17] K. Chiba, K. Tsunoda, Y. Umezawa, H. Haraguchi, S. Fujiwara, K. Fuwa, Anal. Chem. 52 (1980) 596–598.
[18] Y. Umezawa, in: Proceedings of the International Meeting on Chemical Sensors, Kodansha & Elsevier, Tokyo and Amsterdam, 1983, pp. 705–710.

[19] Y. Umezawa, S. Sofue, Y. Takamoto, Talanta 31 (1984) 375–378.
[20] H. Abe, M. Kataoka, T. Yasuda, Y. Umezawa, Anal. Sci. 2 (1986) 523–527.
[21] Y. Umezawa, M. Sugawara, in: T. Seiyama (Eds.), Chemical Sensor Technology, vol. 1, Kodansha, Tokyo, 1988, pp. 141–152.
[22] H. Radecka, Y. Umezawa, in: A. Mulchandoni, K.R. Rogers (Eds.), Methods in Molecular Biology, ch. 32, Humana Press, Totowa, 1997.
[23] M.L. Mayer, G.L. Westbrook, J. Physiol. 394 (1987) 501–527.
[24] P. Ascher, L. Nowak, J. Physiol. 399 (1988) 247–266.
[25] M. Iino, S. Ozawa, K. Tsuzuki, J. Physiol. 424 (1990) 151–165.
[26] R. Schneggenburger, Z. Zhou, A. Konnerth, E. Neher, Neuron 11 (1993) 133–143.
[27] M. Rogers, J.A. Dani, Biophys. J. 68 (1995) 501–506.
[28] N. Burnashev, Z. Zhou, E. Neher, B. Sackmann, J. Physiol. 485 (1995) 403–418.
[29] A. Ghosh, M.E. Greenberg, Science 268 (1995) 239–247.
[30] P. Anker, E. Wieland, D. Ammann, R.E. Dohner, R. Asper, W. Simon, Anal. Chem. 53 (1981) 1970–1974.
[31] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[32] N. Oku, D.A. Kendall, R.C. Macdonald, Biochim. Biophys. Acta 691 (1982) 332–340.
[33] L. Vyklicky Jr., J. Physiol. 470 (1993) 575–600.
[34] T. Honoré, S.N. Davies, J. Drejer, E.J. Fletcher, P. Jacobsen, D. Lodge, F.E. Nielsen, Science 241 (1988) 701–703.
[35] D.B. Rorabacher, Anal. Chem. 63 (1991) 139–146.