ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 453–460

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

A single-channel method for evaluation of very magnitudes of Ca^{2+} ion fluxes through ε4/ζ1 *N*-methyl-D-aspartate receptor channels in bilayer lipid membranes

Masaki Wakabayashi [a], Ayumi Hirano [a], Masao Sugawara [a,*], Shigeo Uchino [b,1], Sadayo Nakajima-Iijima [b]

[a] *Department of Chemistry, College of Humanities and Sciences, Nihon University, Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan*

[b] *Pharmaceuticals Discovery Laboratory, Yokohama Research Center, Mitsubishi Chemical Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227-8502, Japan*

Received 7 April 2000; received in revised form 1 August 2000; accepted 2 August 2000

Abstract

A single-channel method for evaluating agonist selectivity in terms of the very number of Ca^{2+} ions passed through the ε4/ζ1 *N*-methyl-D-aspartate (NMDA) receptor ion channel in bilayer lipid membranes (BLMs) is described. The number of Ca^{2+} passed through the single-channel was obtained from single-channel recordings in a medium where the primary permeant ion is Ca^{2+}. The recombinant ε4/ζ1 NMDA channel was partially purified from Chinese hamster ovary cells expressing the channel and incorporated in BLMs formed by the tip-dip method. It was found that the ε4/ζ1 channel in BLMs is permeable to Ca^{2+} and Na^{+}, but the number of Ca^{2+} passed through the channel is much fewer than that of Na^{+}. The integrated Ca^{2+} currents induced by three typical agonists NMDA, L-glutamate and L-CCG-IV were obtained at concentration of 50 μM, where the integrated currents for all the agonists reached their saturated values. The integrated Ca^{2+} currents obtained are $(3.1 \pm 0.21) \times 10^{-13}$ C/s for NMDA, $(4.6 \pm 0.31) \times 10^{-13}$ C/s for L-glutamate and $(5.7 \pm 0.25) \times 10^{-13}$ C/s for L-CCG-IV, respectively, suggesting that the three kinds of agonists have different efficacies to induce permeation of Ca^{2+}. The range of the agonist selectivity thus obtained is much narrower than that of binding affinities for the NMDA receptors from rat brain. The present method is able to detect Ca^{2+} permeation with a detection limit of $\approx 10^5$ Ca^{2+} ions/s. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: NMDA receptor; Bilayer lipid membrane; Agonist selectivity; Ca^{2+} permeation; Single-channel recording

* Corresponding author. Tel.: +81-3-33291151; fax: +81-3-53179433.

E-mail address: sugawara@chs.nihon-u.ac.jp (M. Sugawara).

[1] Present address: Department of Neurochemistry, National Institute of Neuroscience, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.

0731-7085/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.

PII: S0731-7085(00)00467-2

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is a cation channel that plays a key role in brain development, synaptic plasticity, memory formation and neurotoxicity [1,2]. Among the cations, which are permeable through the open pore of the NMDA receptors, Ca^{2+} works as a second messenger that regulates various kinds of intracellular signal cascades [3,4]. The NMDA receptor is formed by assembly of the ζ1 subunit with any one of four ε1–4 subunits (named for the mouse form) [5–8] and the assemblies have their own regional distributions in the brain [5,6,9]. Expression of cDNAs encoding the NMDA receptors has shown that the ε1–3/ζ1 channels in *Xenopus* oocytes are permeable to Ca^{2+}. However, no studies have explicitly described about whether the mouse ε4/ζ1 channel is permeable to Ca^{2+}, although the Ca^{2+} permeability of the NR1/NR2D channels (rat equivalents of the ε4/ζ1 channels) are known to be comparable to those of the NR1/NR2A-C (ε1–3/ζ1) channels [10].

Owing to the key role of Ca^{2+} in regulating intracellular signaling cascades, it is important to quantify Ca^{2+} permeation through the NMDA receptor. The common way for quantifying the Ca^{2+} permeability is based on reversal potential shifts, which leads to the estimation of relative permeabilities of Ca^{2+} over monovalent ions [11–14]. Recently, a more direct approach for evaluating the Ca^{2+} permeability has been reported based on simultaneous measurements of whole-cell current and Ca^{2+} influx [15–17]. On the other hand, only limited studies have been reported on evaluation of agonist selectivity for the NMDA receptor on the basis of Ca^{2+} fluxes. Varney et al. compared the L-glutamate- and NMDA-induced Ca^{2+} influxes through recombinant human NR1/NR2A-B (corresponding to ε1–2/ζ1 in mouse) NMDA receptor channels expressed in mammalian cell lines by using fura-2 fluorometry [18]. They reported that L-glutamate is more efficacious than NMDA for inducing the Ca^{2+} influx. In our previous paper, we evaluated the agonist selectivity for the native NMDA receptors partially purified from rat whole brain by quantifying the magnitudes of Ca^{2+} effluxes from the receptor-incorporated liposomes with a Ca^{2+} ion-selective electrode in a thin-layer mode [19]. However, in each of these studies, only the relative magnitudes of Ca^{2+} fluxes were accessible, and no absolute number of Ca^{2+} ions passed through the channel was given because the cell or liposome membranes contained multiple channels, whose number scattered from one membrane to another.

In the present study, we describe an electrochemical method for evaluating the very magnitudes of Ca^{2+} ion fluxes through a single NMDA receptor channel in bilayer lipid membranes (BLMs). The method is based on single-channel recordings in media where only Ca^{2+} is a permeable cation. Three typical agonists, i.e. NMDA, L-glutamate and (2S, 3R, 4S) isomer of 2-(carboxycyclopropyl)glycine (L-CCG-IV) are used as stimulants for the ε4/ζ1 channel. The recombinant ε4/ζ1 channel is partially purified from Chinese hamster ovary (CHO) cells expressing the ε4/ζ1 channel and incorporated in planar BLMs formed by the tip-dip method. Among the four kinds of ε1–4/ζ1 channels, the ε4/ζ1 channel is of particular interest, as the predominant expression of the ε4/ζ1 subunit mRNA in the embryonic and early postnatal brain suggests an important role of ε4-containing receptors in brain development [9] and the ε4/ζ1 channel has the highest affinity to L-glutamate [8]. We demonstrate that the ε4/ζ1 channel is permeable to Ca^{2+} and Na^{+} and the very number of Ca^{2+} ions passed through the channel is a new and useful measure of the agonist selectivity.

2. Experimental

2.1. Materials

L-α-Phosphatidylcholine (PC, purity > 99%, 25 or 50 mg/ml chloroform solution) and L-α-phosphatidylethanolamine (PE, 10 mg/ml chloroform solution) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, L-glutamate and tetrodotoxin (TTX) were obtained from Wako Pure Chemicals Co. Cholesterol was recrystallized three times from methanol. NMDA and

concanavalin A (type IV) were purchased from Sigma Chemical Co. (St. Louis, MO). L-CCG-IV and (5R,10S)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,b]-cyclohepten-5,10-imine hydrogen maleate (MK-801) were from Research Biochemicals Inc. (Natick, MA). Other chemicals used were all of analytical reagent grade. Milli-Q water (Millipore reagent water system, Bedford, MA) was used throughout the experiments. Lipid solutions of 1 mg/ml PC:PE:cholesterol (3:1:1, w/w) in chloroform/*n*-hexane (1:1, v/v) and 2 mg/ml PC:PE:cholesterol (3:1:1, w/w) in chloroform were prepared and stored under nitrogen at −20°C until use.

2.2. Purification of the ε4/ζ1 NMDA receptor

The ε4/ζ1 NMDA receptor expressed in the CHO cells by heat induction was purified in the same manner as described in our previous paper [20], except that the CHO cells were washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $NaHPO_4$ and 1.5 mM KH_2PO_4). The total protein concentration ranged from 0.12 and 0.27 mg/ml protein among three experiments. The protein suspension was stored at 4°C under nitrogen until use.

2.3. Formation of BLMs by the tip-dip method and incorporation of a single ε4/ζ1 NMDA receptor

The BLMs were formed by the tip-dip method [21] with glass pipets having an outer tip diameter of ≈2 μm. The pipets were made from borosilicate glass (GC150F-7.5, Clark Electromedical Instruments, Pangbourne, UK) using a four-pull technique with a Sutter micropipet puller model P-97 (Sutter Instrument Co., Novato, CA). Pipets having resistance of 7–11 megaohm in pipet solution (vide infra) were used. A Teflon chamber used for measurements had a compartment of 1 cm^2 surface area and 500 μl volume. The solution used for chamber was 56 mM $CaCl_2$, 5.2 mM Hepes/$Ca(OH)_2$, 10 μM glycine, 31 μg/ml concanavalin A, 10 μM TTX, 0.14 mM sucrose and 50 μM agonist, pH 7.2 (abbreviated as Ca^{2+} solution) or 0.17 M NaCl, 5.2 mM Hepes/NaOH, 10 μM glycine, 31 μg/ml concanavalin A, 10 μM TTX, 0.14 mM sucrose and 50 μM agonist, pH 7.2 (abbreviated as Na^+ solution). The solution used for filling pipets (abbreviated as pipet solution) was 56 mM $CaCl_2$, 5.2 mM Hepes/$Ca(OH)_2$, 10 μM glycine, 31 μg/ml concanavalin A, 10 μM TTX, 0.14 mM sucrose and ca. 12–50 mg/ml protein, pH 7.2. When the Na^+ solution was used as chamber solution, the pipet solution was the same as the chamber solution except that 50-μM agonist was replaced by ca. 12–50 mg/ml protein. The chamber and pipet solutions were incubated for 40–60 min on an ice bath. After a BLM was formed, the single ε4/ζ1 NMDA receptor was incorporated into the BLM at an applied potential of +80 mV (pipette side). The identification of the ε4/ζ1 channel incorporated in BLMs was performed in terms of blocking effect of an antagonist MK-801 on agonist-induced channel currents with BLMs formed by the folding method [20].

2.4. Current recordings and data analysis

Channel currents were recorded with an EPC-7 amplifier (List Electronic, Darmstadt, Germany) and filtered at 1.0 kHz by an eight-pole low-pass filter of Bessel type. Data were degitized at 10 kHz and stored online using an IMC-P5K86 computer in which pCLAMP version 6.0.3 (Axon Instruments Inc., Burlingame, CA) was installed. Data acquisition was continued for 20 s with a sampling interval of 100 μs. All the recordings were done at room temperature (20–25°C). The criterion used for ascertaining single-channel currents was the same as described previously [20], i.e. no double openings are seen in entire time duration at high agonist concentration (50 μM). The magnitude of integrated current was also used for distinguishing single-channel recordings from multi-channel ones [20]. The integrated currents, corresponding to the number of Ca^{2+} ions passed through the ε4/ζ1 channel, were obtained by integrating the single-channel currents with respect to time. The single-channel conductance was obtained as a chord conductance. The fraction of time [AR_c] for which the channel is closed and that [AR_{ot}] for which the receptor is open to

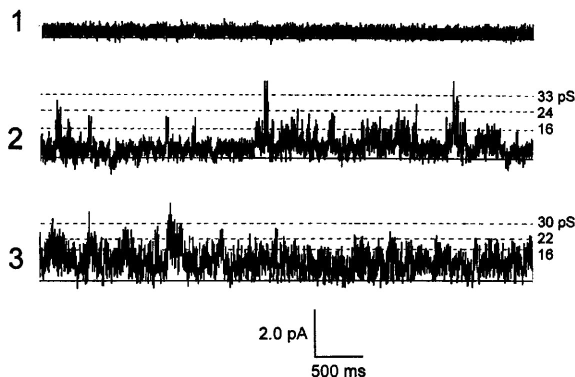

Fig. 1. Examples of single-channel currents from the BLMs containing the ε4/ζ1 NMDA receptor channel, activated by 50 μM L-glutamate in Ca^{2+} (record 2) and Na^{+} (record 3) solutions. Record 1 shows a current trace obtained before the ε4/ζ1 channel was incorporated in the BLM. Applied potential was + 80 mV. Solution condition: (1 and 2) 56 mM $CaCl_2$, 5.2 mM Hepes/$Ca(OH)_2$, 10 μM glycine, 31 μg/ml concanavalin A, 10 μM TTX, 0.14 mM sucrose, pH 7.2 and (3) 0.17 M NaCl, 5.2 mM Hepes/NaOH, 10 μM glycine, 31 μg/ml concanavalin A, 10 μM TTX, 0.14 mM sucrose, pH 7.2.

any conductance level, i.e. the fractions of total open time, in the recording time were calculated according to the following equations [22].

$$[AR_c] = \frac{T_c}{T_r} \quad (1)$$

$$[AR_{ot}] = \frac{T_o}{T_r} \quad (2)$$

Where T_r is the total recording time, T_o the total open time and T_c the total closed time.

3. Results and discussion

3.1. Single-channel currents in Ca^{2+} and Na^{+} media

The ε4/ζ1 channel is expected to permeable to Na^{+} and Ca^{2+} ions, since the rat equivalent NR1/NR2D channels having more than 99% amino acid homology with the ε4/ζ1 channel have been shown to be permeable to both ions. To test this, the single-channel recordings were performed in Na^{+} and Ca^{2+} solutions (vide supra). Examples of recordings of the ε4/ζ1 single-channel activity in the BLMs bathed in Ca^{2+} and Na^{+} solutions containing 50 μM L-glutamate are shown in Fig. 1. As can be seen on the record 2, where the ε4/ζ1 channel in the Ca^{2+} solution was activated by 50 μM L-glutamate, the channel openings with the maximum conductance level of ≈ 30 pS, accompanied by a few lower levels of ≈ 16 and ≈ 24 pS, are seen. Occasionally, a conductance level of ≈ 12 pS was also observed. The channel opening was also seen in the Na^{+} solution (record 3). The single-channel conductance levels did not vary between the Ca^{2+} and Na^{+} media. However, the integrated current in the Ca^{2+} solution was significantly smaller by 54% (27% in the number of ions) than that in the Na^{+} solution under the identical ionic strength. The smaller integrated current in the Ca^{2+} solution was due to the shorter total open time of the channel (Table 1). The Ca^{2+}-dependent decrease in the integrated current is consistent with the finding by Mishina et al. [8] that an increase in

Table 1
Magnitudes of L-glutamate-induced integrated single-channel currents for the ε4/ζ1 channel and fractions of the total open time obtained in Ca^{2+} and Na^{+} media

Medium[a]		Ion flux[b] ($\times 10^{-13}$ C/s)	Fraction of time (%)[c]		Mean open time[c] (ms)
Ca^{2+}(mM)	Na^{+}(M)		$[AR_c]$	$[AR_{ot}]$	
56[d]	0	4.6 ± 0.31 (3)	64	36	2.2
0[d]	0.17	8.5 ± 0.88 (4)	28	72	3.8

[a] Ion strength was adjusted to be 0.17.
[b] Values are expressed as mean ± S.D. Values in brackets refer to the number of BLMs studied.
[c] Mean value from three and four measurements for the Ca^{2+} and Na^{+} solutions, respectively.
[d] See Section 2 for solution composition.

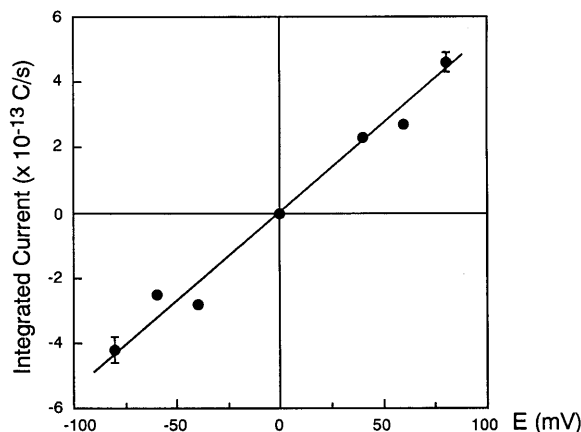

Fig. 2. Dependence of the integrated single-channel current for the ε4/ζ1 channel activated by 50 μM L-glutamate on the applied potential. Error bars mean ± S.D. Solution condition was the same as for record 2 in Fig. 1.

extracellular Ca^{2+} concentration decreases the whole-cell current evoked by application of L-glutamate to *Xenopus* oocytes expressing the ε4/ζ1 channel. The Ca^{2+}-dependent channel current demonstrates that the ε4/ζ1 channel is permeable to Ca^{2+}, although the permeation of Ca^{2+} is much lower than that of Na^{+}. In the Ca^{2+} solution, the currents are practically carried by Ca^{2+}, because concentration of protons is about 10^{-6} order lower than that (56 mM) of Ca^{2+} and the Hepes zwitterions at pH 7.2, which are much larger than the narrow portion of channel pore (about 5.5 Å in diameter for NMDA receptor) [23,24], are impermeable. The time integration of the current, therefore, gives the number of Ca^{2+} ions passed through the ε4/ζ1 channel at its open state.

Averaging the integrated currents at 50 μM L-glutamate from three preparations of BLMs yielded $(4.6 \pm 0.31) \times 10^{-13}$ C/s, corresponding to 1.4×10^{6} Ca^{2+} ions/s, with the R.S.D. of 6.7% ($n = 3$). On the basis of the precision of the present approach, one can detect a difference of 10^{5} Ca^{2+} ions/s, if agonists evoke different extent of Ca^{2+} permeation. The detection limit for the integrated Ca^{2+} currents, defined as three times the background integrated current obtained from BLMs containing no channels, was 3.3×10^{5} Ca^{2+} ions/s. This detection limit is comparable to detection limits of the methods utilizing Ca^{2+} indicators, Ca^{2+}-ion selective electrodes and fiber optic Ca^{2+} sensors. The detection limits of these methods range from 10^{-8} to 10^{-7} M Ca^{2+} ions (from 10^{5} to 10^{8} in the number of Ca^{2+} ions) for cells having diameter of several 10–100 μm [25–28].

3.2. Voltage dependence

The dependence of the L-glutamate-induced integrated Ca^{2+} current on the magnitude of applied potential was investigated in the potential range from −80 to +80 mV. As shown in Fig. 2, the integrated current–voltage profile was linear over the whole potential range examined. This shows that the permeation of Ca^{2+} through the open pore of the channel occurred symmetrically. In the present study, an applied potential of +80 mV was used, because the success probability of single-channel recording was higher at the positive applied potential. But the reason for the higher success probability at the positive potential is not known yet.

3.3. Agonist concentration dependence

The single-channel activity of the NMDA receptor is known to consist of the two processes, i.e. binding of an agonist to the channel and transition from the bound state to the open state of the channel [29]. The entry of Ca^{2+} ions is allowed only when the channel is in its open state. The agonist-concentration dependence of the integrated single-channel currents from the BLMs containing the single ε4/ζ1 channel is shown in Fig. 3. The channel activity of the ε4/ζ1 channel was observed only in the presence of L-glutamate. The integrated Ca^{2+} current increased with the increase in L-glutamate concentration and reached its plateau above 30 μM, reflecting saturation of the fraction of total open time $[AR_{ot}]$ at L-glutamate concentration above 30 μM (data not shown). The EC_{50} value for L-glutamate was 0.7 μM, which is close to the reported one (0.4 μM) for the same channel expressed in *Xenopus* oocytes [7]. Based on the EC_{50} values and assuming that the stoichiometry of the binding between

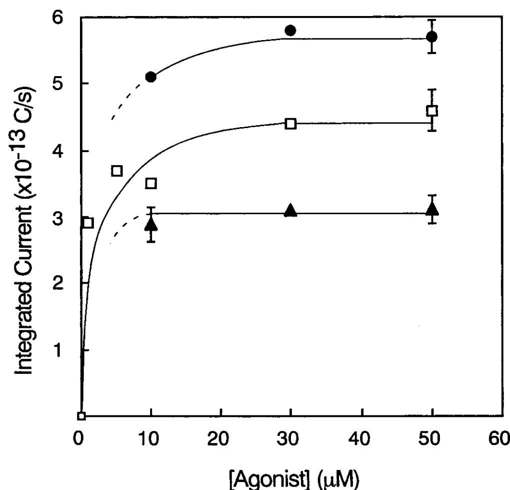

Fig. 3. Dependence of the integrated single-channel currents from the BLMs containing ε4/ζ1 NMDA receptor on the concentration of agonists: (●) L-CCG-IV, (□) L-glutamate and (▲) NMDA. Error bars mean ± S.D. Applied potential was +80 mV. Solution condition was the same as for record 2 in Fig. 1.

the ε4/ζ1 channel and L-glutamate is 1:1, it is calculated that the ε4/ζ1 channel is occupied by L-glutamate at 50 μM for 99% of the time. Similarly, the integrated single-channel currents of the ε4/ζ1 channel activated by NMDA and L-CCG-IV exhibited their maximum values above 30 μM, showing saturation of the agonist binding to the ε4/ζ1 channel. The comparison of the integrated currents among the agonists were performed at concentration of 50 μM, where the integrated Ca^{2+} currents reflect the number of Ca^{2+} ions passed through the channel that is oscillating between the bound, but closed state and its open state.

3.4. Agonist selectivity

Based on the above results, the very magnitudes of the agonist-induced integrated Ca^{2+} currents were compared among three kinds of agonists, i.e. NMDA, L-glutamate and L-CCG-IV. As given in Table 2, L-glutamate was more efficacious than NMDA, with NMDA eliciting 67% of the integrated Ca^{2+} current by L-glutamate. L-CCG-IV was more efficacious by 20% than L-glutamate for inducing Ca^{2+} current. The order of Ca^{2+} permeation was L-CCG-IV > L-glutamate > NMDA. This order was the same as that of the fraction of total open time, i.e. L-CCG-IV (55%) > L-glutamate (36%) > NMDA (26%). These results show that the three agonists have their own efficacy to induce Ca^{2+} permeation through the ε4/ζ1 channel. The agonist selectivity in terms of Ca^{2+} permeation was parallel to that in 0.16 M Na^{+} solution containing 1.9 mM Ca^{2+} [22], except that the magnitudes of the agonist-induced integrated Ca^{2+} currents were 77–85% (38–43% in the number of Ca^{2+}) of those in 0.16 M Na^{+} solution containing 1.9 mM Ca^{2+}. The attenuated Ca^{2+} permeation is explained by a decrease (ca. 10%) in the total open time [AR_{ot}] in the Ca^{2+} solution. These results show that if one considers the relative amount of permeated ions as a measure of agonist selectivity, as described in

Table 2
Comparison of the integrated single-channel currents through the single ε4/ζ1 channel in BLMs

	Integrated single-channel current (×10^{-13} C/s)[a]		
	NMDA	L-Glu	L-CCG-IV
56 mM Ca^{2+}[b]	3.1 ± 0.21 (3)	4.6 ± 0.31 (3)	5.7 ± 0.25 (3)
0.16 M Na^{+} + 1.9 mM Ca^{2+}[c]	3.8 (2)[d]	6.0 ± 0.36 (4)[d]	6.7 ± 0.92 (3)[d]

[a] Applied potential: +80 mV. Agonist concentration: 50 μM. Values are expressed as mean ± S.D. Values in brackets refer to the number of BLMs studied.

[b] See Section 2 for solution composition.

[c] Measured in 0.16 M NaCl, 1.9 mM $CaCl_2$, 2.6 mM KCl, 5.2 mM Hepes/NaOH (pH 7.2), 10 μM glycine, 31 μg/ml concanavalin A, 0.14 mM sucrose, 10 μM TTX.

[d] Data taken from [22].

our previous paper [19], the selectivity ratio gives an identical value for both the Ca^{2+} and Na^{+} media, although the number of ions passed through the channel is different between the two media. Thus, the evaluation of the very magnitude of the permeated ions is important for obtaining the agonist selectivity.

It will be interesting to compare the agonist selectivity obtained above with that based on the Ca^{2+} effluxes from liposomes containing NMDA receptor channels purified from rat whole brain [19]. The selectivity by the latter approach is obtained only as a ratio of the Ca^{2+} effluxes because of the presence of multi-channels. It is well recognized that the rat NMDA receptor subunits (NR1 an NR2) are equivalent to the mouse ζ1 and ε subunits, respectively [1]. The ratio of the mean values of the integrated Ca^{2+} currents ($n = 3$) listed in Table 2 among the three agonists was NMDA: L-glutamate: L-CCG-IV = 0.67: 1.0: 1.2, which is close to that (0.58: 1.0: 1.6) obtained for rat NMDA receptor by the Ca^{2+} efflux method. The important feature of the results obtained by the two approaches is that the range of the selectivity ratio is much narrowed as compared with that of the binding affinity toward the rat NMDA receptor subtype [30].

The permeation of Ca^{2+} through the native NMDA receptor from rat whole brain is known to be higher than that of Na^{+} [19]. In contrast, the above results indicate that the permeation of Ca^{2+} through the mouse ε4/ζ1 channel is much lower than that of Na^{+}. The present results suggest that the reported high permeation of Ca^{2+} through the NMDA receptors in neurons is elicited by NMDA receptor types other than the ε4/ζ1 (NR1/NR2D) channel.

4. Conclusion

The results obtained above demonstrates that the single-channel approach is useful for the evaluation of very number of Ca^{2+} ions passed through the ε4/ζ1 channel in BLMs activated by different kinds of agonists. The method allows for the evaluation of the efficacy of agonists to induce permeation of Ca^{2+} ions in the order of 10^5–10^6 ions/s at a single-channel level. As far as we know, this is the first example of demonstrating the permeation of Ca^{2+} through the ε4/ζ1 channel on the single-channel level. The very number of Ca^{2+} evaluated is a more physiologically relevant measure of the agonist selectivity in the sense that the signal transduction ability of the channel is directly obtained and can be compared among different receptor types. The approach described here is generally applicable to other receptor channels, including the ε1–3/ζ1 channels, if the targeted channels are Ca^{2+}-conductive ones.

Acknowledgements

The authors thank H. Kanda, M. Sutani, K. Sakai and M. Seino for their experimental helps in protein isolation. This work was supported from Grants for Scientific Research by the Ministry of Education, Science and Culture, Japan. Supports from the New Energy and Industrial Technology Development Organization (NEDO) was also acknowledged. Finally, one (A.H.) of the authors thanks the Japan Society for the Promotion of Science for research fellowships.

References

[1] C.J. McBain, M.L. Mayer, Physiol. Rev. 74 (1994) 723–760.
[2] T.V.P. Bliss, G.L. Collingridge, Nature 361 (1993) 31–39.
[3] A. Ghosh, M.E. Greenberg, Science 268 (1995) 239–247.
[4] R.C. Malenka, R.A. Nicoll, Science 285 (1999) 1870–1874.
[5] H. Meguro, H. Mori, K. Araki, E. Kushiya, T. Kutsuwada, M. Yamazaki, T. Kumanishi, M. Arakawa, K. Sakimura, M. Mishina, Nature 357 (1992) 70–74.
[6] T. Kutsuwada, N. Kashiwabuchi, H. Mori, K. Sakimura, E. Kushiya, K. Araki, H. Meguro, H. Masaki, T. Kumanishi, M. Arakawa, M. Mishina, Nature 358 (1992) 36–41.
[7] K. Ikeda, M. Nagasawa, H. Mori, K. Araki, K. Sakimura, M. Watanabe, Y. Inoue, M. Mishina, FEBS Lett. 313 (1992) 34–38.
[8] H. Mori, M. Mishina, Neuropharmacology 34 (1995) 1219–1237.
[9] M. Watanabe, Y. Inoue, K. Sakimura, M. Mishina, NeuroReport 3 (1992) 1138–1140.

[10] H. Monyer, N. Burnashev, D.J. Laurie, B. Sakmann, P.H. Seeburg, Neuron 12 (1994) 529–540.
[11] P. Ascher, L. Nowak, J. Physiol. 399 (1988) 247–266.
[12] M.L. Mayer, G.L. Westbrook, J. Physiol. 394 (1987) 501–527.
[13] M. Iino, S. Ozawa, K. Tsuzuki, J. Physiol. 424 (1990) 151–165.
[14] N. Spruston, P. Jonas, B. Sakmann, J. Physiol. 482 (1995) 325–352.
[15] R. Schneggenburger, Z. Zhou, A. Konnerth, E. Neher, Neruon 11 (1993) 133–143.
[16] N. Burnashev, Z. Zhou, E. Neher, B. Sakmann, J. Physiol. 485 (1995) 403–418.
[17] R. Schneggenburger, Biophys. J. 70 (1996) 2165–2174.
[18] M.A. Varney, C. Jachec, C. Deal, S.D. Hess, L.P. Daggett, R. Skvoretz, M. Urcan, J.H. Morrison, T. Moran, E.C. Johnson, G. Veliçelebi, J. Pharmacol. Exp. Ther. 279 (1996) 367–378.
[19] H. Radecka, J. Nakanishi, A. Hirano, M. Sugawara, Y. Umezawa, J. Pharm. Biomed. Anal. 19 (1999) 205–216.
[20] A. Hirano, M. Sugawara, Y. Umezawa, S. Uchino, S. Nakajima-Iijima, Biosens. Bioelectron. 15 (2000) 173–181.
[21] R. Coronado, R. Latorre, Biophys. J. 43 (1983) 231–236.
[22] A. Hirano, M. Wakabayashi, M. Sugawara, S. Uchino, S. Nakajima-Iijima, Anal. Biochem. 283 (2000) 258–265.
[23] A. Villarroel, N. Burnashev, B. Sakmann, Biophys. J. 68 (1995) 866–875.
[24] M.M. Zarei, J.A. Dani, J. Neurosci. 15 (1995) 1446–1454.
[25] R.Y. Tsien, Ann. Rev. Neurosci. 12 (1989) 227–253.
[26] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Nature 388 (1997) 882–887.
[27] S. Shalom, A. Strinkovski, G. Peleg, S. Druckmann, A. Krauss, A. Lewis, M. Linial, M. Ottolenghi, Anal. Biochem. 244 (1997) 256–259.
[28] W. Tan, R. Kopelman, S.L.R. Barker, M.T. Miller, Anal. Chem. 71 (1999) 606A–612A.
[29] R.A.J. Lester, C.E. Jahr, J. Neurosci. 12 (1992) 635–643.
[30] M. Kawai, Y. Horikawa, T. Ishihara, K. Shimamoto, Y. Ofune, Eur. J. Pharmacol. 211 (1992) 195–202.