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PERMEATION OF CALCIUM THROUGH EXCITATORY AMINO ACID RECEPTOR CHANNELS IN CULTURED RAT HIPPOCAMPAL NEURONES

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SUMMARY

1. *N*-methyl-D-aspartate (NMDA)-, quisqualate- and kainate-induced currents were recorded in cultured rat hippocampal neurones using the whole-cell voltage-clamp technique. To isolate the inward currents carried by Ca^{2+} and other divalent cations (Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+}), both Na^+ and K^+ in the control external solution were replaced with the impermeant cation *N*-methylglucamine (NMG).

2. Replacement of Na^+, K^+ and Ca^{2+} with NMG abolished NMDA-, quisqualate- and kinate-induced inward currents. In Na^+-, K^+-free (abbreviated simply as Na^+-free) solution containing 10 mM-Ca^{2+} NMDA caused prominent inward currents at -60 mV. In this solution with the internal solution containing 165 mM-Cs^+, the reversal potential of the NMDA-induced current was $-5{\cdot}0 \pm 0{\cdot}7$ mV ($n = 36$), indicating a value of $P_{Ca}/P_{Cs} = 6{\cdot}2$ for the ratio of the permeability coefficients of Ca^{2+} and Cs^+ according to the constant-field equation.

3. NMDA elicited inward current responses at -60 mV in Na^+-, Ca^{2+}-free solution containing 10 mM-Sr^{2+}, Ba^{2+}, or Mn^{2+}, but not in Na^+-free, 10 mM-Mg^{2+} solution. On the basis of reversal potential measurements, the permeability sequence of NMDA receptor channels among the divalent cations was determined to be $Ba^{2+}(1{\cdot}2) > Ca^{2+}(1{\cdot}0) > Sr^{2+}(0{\cdot}8) > Mn^{2+}(0{\cdot}3) \gg Mg^{2+}(< 0{\cdot}02)$.

4. The reversal potential of the quisqualate-induced current was more negative than -80 mV in Na^+-free, 10 mM-Ca^{2+} solution, indicating a value of $P_{Ca}/P_{Cs} < 0{\cdot}18$.

5. Kainate-induced current responses were classified into two types. In the type I response the reversal potential of the kainate-induced current was more negative than -80 mV in Na^+-free, 10 mM-Ca^{2+} solution, indicating that the Ca^{2+} permeability of this type of kainate channel is as low as that of the quisqualate channel. In the neurones which showed a type I response, there was a tendency of outward rectification in the current–voltage plots of the kainate response in control solution.

6. In the type II response kainate caused prominent inward currents at -60 mV in Na^+-free, 10 mM-Ca^{2+} solution. The reversal potential was $-23{\cdot}3 \pm 5{\cdot}6$ mV ($n = 17$), indicating a permeability ratio $P_{Ca}/P_{Cs} = 2{\cdot}3$. In the neurones which showed a type II response, a remarkable inward rectification was observed in the current–voltage plots of the kainate response in control solution.

7. Type II kainate channels showed relatively poor selectivity among divalent

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cations. The permeability sequence was $Ba^{2+}(1{\cdot}3) > Ca^{2+}(1{\cdot}0) > Sr^{2+}(0{\cdot}9) > Mg^{2+}(0{\cdot}8) > Mn^{2+}(0{\cdot}7)$.

INTRODUCTION

The ion channels coupled to glutamate and related excitatory amino acid (EAA) receptors are highly permeable to Ca^{2+} in the mammalian central nervous system (CNS) neurones (Dingledine, 1983; Pumain & Heinemann, 1985; MacDermott, Mayer, Westbrook, Smith & Barker, 1986; Mayer & Westbrook, 1987; Ozawa, Nakamura & Yuzaki, 1988). This fact is important for understanding both the physiology and the pathology of the CNS neurones.

Several lines of evidence suggest that the NMDA channel is the major pathway of Ca^{2+} entry among the three types of EAA receptor channels. Whole-cell voltage-clamp experiments by Mayer & Westbrook (1987) on cultured CNS neurones showed that the reversal potential of the current response to NMDA was shifted in the depolarizing direction by raising the extracellular Ca^{2+} concentration. However, the reversal potentials of the quisqualate and kainate responses were much less affected (MacDermott *et al.* 1986; Mayer & Westbrook, 1987). Ascher & Nowak (1988) recorded inward single-channel currents resulting from NMDA receptor activation in 100 mM-Ca^{2+} solution containing no Na^+. These electrophysiological observations have also been substantiated by an optical technique using Arsenazo III (MacDermott *et al.* 1986; Mayer, MacDermott, Westbrook, Smith & Barker, 1987). In cultured spinal neurones voltage clamped near resting potential, the optical response indicated a transient elevation of intracellular Ca^{2+} due to ion flux through NMDA channels. With this method the responses to quisqualate and kainate were accompanied by minimal Ca^{2+} signals. Very recently, however, it has been suggested that both quisqualate and kainate channels permit Ca^{2+} entry on the basis of intracellular Ca^{2+} measurements using the fluorescent indicator Fura-2 (Ogura, Akita & Kudo, 1989).

The purpose of the present study was to measure directly the currents carried by Ca^{2+} and other divalent cations through receptor channels separately activated by NMDA, quisqualate and kainate in cultured rat hippocampal neurones. A preliminary note describing these results has already appeared (Iino, Tsuzuki & Ozawa, 1989).

METHODS

Tissue culture

Hippocampal neurones were cultured by the method of Banker & Cowan (1977). Briefly, embryos on gestation day 17–19 were obtained from maternal rats killed by cervical dislocation under ether anaesthesia. Hippocampi were removed from the embryos and maintained in Ca^{2+}-, Mg^{2+}-free saline containing 0·1% trypsin (DIFCO) for 10 min at 37 °C. The hippocampi were then transferred to Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 15% fetal bovine serum and mechanically dissociated by triturating with a Pasteur pipette. The dissociated cells were plated on collagen-coated cover-slips and grown in DMEM with 15% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultured neurones were used for tight-seal

whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) between 8 and 14 days after plating.

Solutions

Whole-cell recordings were initiated in control external solution which contained (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 2·4; glucose, 10; HEPES, 10 (pH adjusted to 7·4 with NaOH). To release NMDA responses from a Mg^{2+} block (Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984), Mg^{2+} was omitted from the external solution throughout the experiments. Tetrodotoxin (1 μM) and bicuculline (20 μM) were added routinely to the external medium to block Na^+ currents and spontaneous synaptic activities mainly mediated by GABA. After recording a set of current responses induced by excitatory amino acids (EAAs) at various membrane potentials, the control bathing medium was changed to solutions in which Na^+, K^+ and Ca^{2+} were replaced with isotonic *N*-methylglucamine (NMG). The Na^+-, K^+- and Ca^{2+}-free solution will simply be referred to as Na^+-, Ca^{2+}-free solution in this paper. The Na^+-, Ca^{2+}-free solution contained (in mM): NMG, 155; glucose, 10; HEPES, 10 (pH adjusted to 7·4 with HCl). To examine the permeation of Ca^{2+}, and other divalent cations (Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+}), through EAA receptor channels, the divalent cations were included in the Na^+-free solution as chloride salts and the osmolarity of the solutions was maintained by suitably lowering the NMG concentration. The osmolarity of the external solutions ranged between 300 and 312 mosM. Solution exchanges were performed by applying the test solution and simultaneously draining a similar volume with a two-channel peristaltic pump. The bath volume was 0·5 ml and complete replacement of the external solution was performed by the addition of 5 ml of new solutions. All the experiments were performed at room temperature (23–25 °C).

The internal solution used to fill the patch pipettes contained (in mM): CsCl, 150; EGTA, 5; HEPES, 10; and was titrated to pH 7·2 using CsOH (which raised the concentration of Cs^+ by approximately 15 mM). The actual membrane potential was corrected by the liquid-junction potential between the control external solution and the internal solution on the assumption that the junction potential between the internal solution and the interior of the cell was negligible (Hagiwara & Ohmori, 1982). A coarse-tip capillary filled with agar–3 M-KCl was used as the reference electrode to eliminate the liquid-junction potentials between the reference electrode and various external solutions. The reference electrode was always placed down-stream from the preparation.

Drug application

NMDA, quisqualate and kainate were applied to the soma by ionophoresis using high-resistance (100–200 MΩ) electrodes. These electrodes were filled with 100 mM-NMDA, 10 mM-quisqualate and 100 mM-kainate solutions, respectively. To dissolve these EAAs completely, NMG was added to each solution until the pH was raised to 7·4. In most experiments, the EAAs were applied using 100 ms current pulses of 100–500 nA intensity and a retaining current of 1–5 nA was used to prevent leakage. However, the pulse duration was occasionally altered between 20 and 500 ms to obtain current responses of adequate amplitudes for data analysis.

Recordings

The resistance of the patch pipette was between 3 and 5 MΩ in the control external solution. The value of series resistance during the recording of whole-cell currents was estimated as described by Fox, Nowycky & Tsien (1987). The series resistance typically ranged between 6 and 14 MΩ. Because the amplitude of the current response was usually smaller than 550 pA, the series resistance error was expected to be smaller than 8 mV with a value of series resistance 14 MΩ. Membrane potentials were not corrected for these errors. The whole-cell current through the patch electrode was measured by a current–voltage converter with a 10 GΩ feedback resistor. The cut-off frequency of the recording system was 1–3 kHz. The oscilloscope output of the membrane current was fed into a PCM (pulse code modulation) data recorder. The stored data were digitized at 1 kHz with a 12 bit A/D converter in combination with a computer (Micro 11/W73L, ASR, Tokyo, Japan). A set of current responses at various membrane potentials was superimposed using a plotter (YHP 7475A, Yokogawa–Hewlett Packard, San Diego, CA, USA). Data are expressed as mean ± S.D.

RESULTS

NMDA-induced Ca^{2+} currents

Whole-cell recordings from cultured hippocampal neurones were initiated in the control external solution containing no Mg^{2+}. Ionophoretic applications of NMDA caused inward current responses in neurones voltage clamped at −60 mV. The duration of the ionophoretic current was usually 100 ms and the intensity was adjusted so as to make the amplitude of the response 400 ± 150 pA at −60 mV. Once the ionophoretic current intensity was determined, it was fixed during a series of recordings. A series of current responses to NMDA at various membrane potentials in the control solution is shown in Fig. 1*A* and the peak amplitude of the response is plotted against the membrane potential in Fig. 1*E*. The response reversed at about 0 mV. When Na^+, K^+ and Ca^{2+} were removed by exchanging the control solution with Na^+-, Ca^{2+}-free solution, the NMDA-induced inward current was abolished. Even at −90 mV, the direction of the current response was outward (Fig. 1*B* and *E*). This indicates that NMG, which was used as a substitute for Na^+ and K^+, was practically impermeant through NMDA receptor channels. As shown below, NMG was also impermeant through both quisqualate and kainate receptor channels.

On addition of 10 mM-Ca^{2+} to the Na^+-, Ca^{2+}-free solution, NMDA caused a prominent inward current at −60 mV. The first challenge of NMDA produced the largest response. At later applications, the amplitude of the inward current response gradually decreased, reaching a steady level (about 50–80% of the amplitude induced by the first) after five to ten challenges (Fig. 1*C*). After the NMDA-induced inward current had reached the steady level, a series of current responses was recorded at various membrane potentials in Na^+-free, 10 mM-Ca^{2+} solution (Fig. 1*D*). The peak amplitude of the current response is plotted against the membrane potential in Fig. 1*E*. The reversal potential ranged from −3·5 to −7·0 mV in thirty-six cells and the mean value was −5·0 ± 0·7 mV. To estimate the permeability of NMDA receptor channel to Ca^{2+}, the following constant-field equation was used as a first approximation:

$$\frac{P_{Ca}}{P_{Cs}} = \frac{[Cs^+]_i}{[Ca^{2+}]_o} \frac{\exp(EF/RT)[\exp(EF/RT)+1]}{4}, \quad (1)$$

where E is the reversal potential, F, R and T have their usual meanings, and P_{Ca} and P_{Cs} represent the permeability coefficients to Ca^{2+} and Cs^+ (Hodgkin & Katz, 1949; Lewis, 1979; Adams, Dwyer & Hille, 1980). Since the mean value of the reversal potential was −5 mV in the presence of 10 mM-Ca^{2+} externally and 165 mM-Cs^+ internally, P_{Ca}/P_{Cs} was calculated to be 6·2 according to eqn (1). When ionic activities were used instead of ionic concentrations, this value was calculated to be 14·3. In this calculation, activity coefficients for Ca^{2+} and Cs^+ were estimated from the reports by Shatkay (1968) and Bates, Staples & Robinson (1970), respectively.

When the Ca^{2+} concentration in the external solution was changed from 5 to 50 mM in Na^+-free solution, the reversal potential of the NMDA-induced response shifted in the positive direction (Fig. 1*F* and *G*). Although the relation between the reversal potential and the Ca^{2+} concentration could be roughly described by the constant-

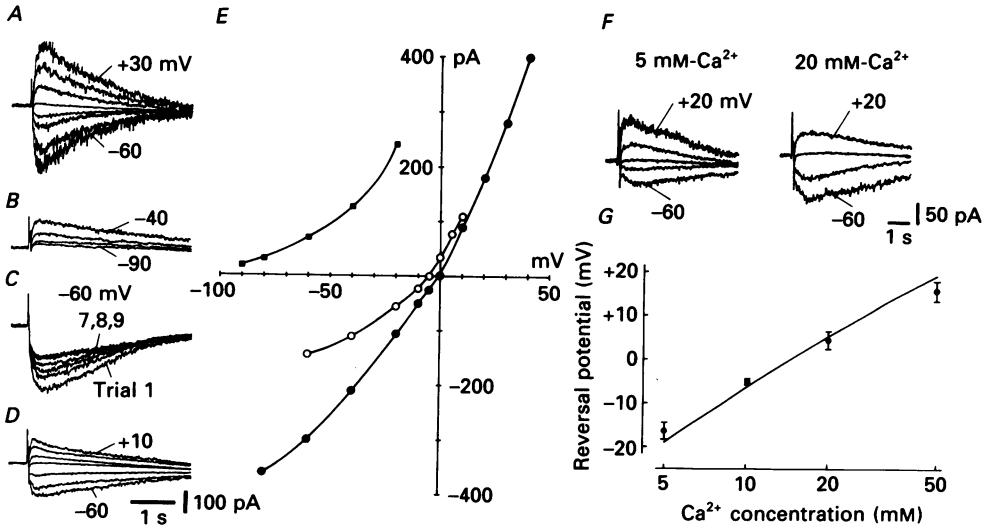

Fig. 1. Membrane current responses to NMDA and their dependence on the membrane potential. *A*, *B* and *D*, NMDA-induced currents at various membrane potentials in the control (*A*), Na^+-, Ca^{2+}-free, (*B*) and Na^+-free, 10 mM-Ca^{2+} (*D*) solutions. The membrane potentials (in mV) are +30, +20, +10, 0, −10, −20, −40, −60 in *A*, −40, −60, −80, −90 in *B* and +10, +5, 0, −5, −20, −40, −60 in *D*. *C*, superimposed current traces at −60 mV evoked by the 1st to the 9th challenge of NMDA immediately after Na^+-free, 10 mM-Ca^{2+} solution had replaced the Na^+-free, Ca^{2+}-free solution. *E*, relation between the peak amplitude of the NMDA-induced current and the membrane potential in the control (●), Na^+, Ca^{2+}-free (■) and Na^+-free, 10 mM-Ca^{2+} (○) solutions. All the traces in *A–D* were taken from the same neurone. *F*, NMDA-induced currents at various membrane potentials in Na^+-free solutions containing 5 and 20 mM-Ca^{2+}. The membrane potentials (in mV) are +20, 0, −15, −30, −60 in 5 mM-Ca^{2+} solution, and +20, +5, −40, −60 in 20 mM-Ca^{2+} solution. In this neurone, the reversal potential shifted from −15 to +5 mV when the Ca^{2+} concentration was raised from 5 to 20 mM. *G*, reversal potentials as a function of the external Ca^{2+} concentration. Each point and error bar represent the mean ± S.D. of the reversal potential obtained from ten, thirty-six, eighteen and ten cells for 5, 10, 20 and 50 mM-Ca^{2+} solutions, respectively. The continuous line through the data points was drawn according to the constant-field voltage equation:

$$E_{rev} = \frac{RT}{F} \ln\left\{\frac{1}{2}\left[-1 + \sqrt{\left(1 + \frac{16 P_{Ca}[Ca^{2+}]_o}{P_{Cs}[Cs^+]_i}\right)}\right]\right\}. \quad (2)$$

A constant value of $P_{Ca}/P_{Cs} = 5{\cdot}7$ was calculated by regression analysis, giving the best fit of eqn (2) to the plots of the reversal potentials, and was used to draw the continuous line.

field equation, the shift of the reversal potential was slightly smaller as compared with the predicted value at higher Ca^{2+} concentrations (see Discussion).

Permeation of divalent cations through NMDA receptor channels

It has been suggested that Sr^{2+}, Ba^{2+} and Mn^{2+} are permeant through NMDA receptor channels, since a depolarizing shift of the NMDA reversal potential occurred during applications of these divalent cations at 20 mM to a solution containing 105 mM-Na^+ and 0·3 mM-Ca^{2+} (Mayer & Westbrook, 1987). We re-examined this

point by recording directly the NMDA-induced currents carried by these divalent cations in Na^+-free solutions containing one of them at 10 mM. In Fig. 2*A–D*, a set of NMDA-induced currents at various membrane potentials was first recorded in Na^+-free, 10 mM-Ca^{2+} solution (dashed lines). Then, the other set of responses was recorded after Ca^{2+} had been replaced with either isomolar Sr^{2+} (*A*), Ba^{2+} (*B*), Mn^{2+} (*C*) or Mg^{2+} (*D*). When Sr^{2+} replaced Ca^{2+} in Na^+-free solution, the reversal potential always shifted in the negative direction. The mean value of the reversal potential in Na^+-free, 10 mM-Sr^{2+} solution was $-9{\cdot}9 \pm 1{\cdot}0$ mV ($n = 7$), whereas the corresponding value in 10 mM-Ca^{2+} solution was $-4{\cdot}9 \pm 0{\cdot}2$ mV. On replacement of Ca^{2+} with isomolar Ba^{2+}, the reversal potential shifted in the positive direction to $-1{\cdot}8 \pm 2{\cdot}4$ mV ($n = 7$) from $-4{\cdot}9 \pm 0{\cdot}3$ mV.

Ascher & Nowak (1988) have shown by recording single-channel currents that Mn^{2+} mimics the blocking effects of Mg^{2+}, but that Mn^{2+} itself can go through NMDA receptor channels. In accordance with their observation, we could detect small but significant inward currents at potentials more negative than -30 mV in Na^+-free, 10 mM-Mn^{2+} solution (Fig. 2*C*). The reversal potential was $-28{\cdot}6 \pm 2{\cdot}0$ mV ($n = 6$). In contrast, no NMDA-induced inward current was detected at potentials more positive than -90 mV in Na^+-free, 10 mM-Mg^{2+} solution (Fig. 2*D*). On the basis of reversal potential measurements, the permeability ratios of Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+} relative to Ca^{2+} were determined using eqn (1). The permeability sequence was Ba^{2+} ($1{\cdot}22 \pm 0{\cdot}16$, $n = 7$) > Ca^{2+} ($1{\cdot}00$) > Sr^{2+} ($0{\cdot}75 \pm 0{\cdot}04$, $n = 7$) > Mn^{2+} ($0{\cdot}29 \pm 0{\cdot}03$, $n = 6$) $\gg$ Mg^{2+} ($< 0{\cdot}02$, $n = 6$).

As described above, the permeability sequence among alkali-earth cations derived from the constant-field theory was $Ba^{2+} > Ca^{2+} > Sr^{2+}$. However, the slope conductance of the NMDA-induced response decreased when Ba^{2+} substituted for Ca^{2+} and increased following the replacement of Ca^{2+} with Sr^{2+} (Fig. 2*A* and *B*). Since this occurred in the whole potential range tested, it is possible that either the binding of NMDA to the receptor or the open probability of the NMDA channel is affected by the presence of different species of the divalent cations. Furthermore, it cannot be excluded that the amount of NMDA reaching the receptor site may be affected by the presence of these divalent cations.

Quisqualate-induced currents

Figure 3 shows quisqualate-induced currents at various membrane potentials in control (*A*), Na^+-, Ca^{2+}-free (*B*) and Na^+-free 10 mM-Ca^{2+} (*C*) solutions. The peak amplitude of the current response is plotted against the membrane potential in Fig. 3*D*. The current responses in the control solution reversed at potentials slightly more negative than 0 mV. Inward current could not be detected at potentials more positive than -80 mV after the control solution had been replaced with Na^+-, Ca^{2+}-free solution. In contrast to the NMDA response, the inward current was also absent after addition of 10 mM-Ca^{2+} to the Na^+-free solution. Since quisqualate caused no inward current even at -80 mV in Na^+-free, 10 mM-Ca^{2+} solution, P_{Ca}/P_{Cs} was estimated to be less than 0·18 (0·41 when ionic activities were used instead of ionic concentrations). This is less than 3% of the corresponding value for the NMDA receptor.

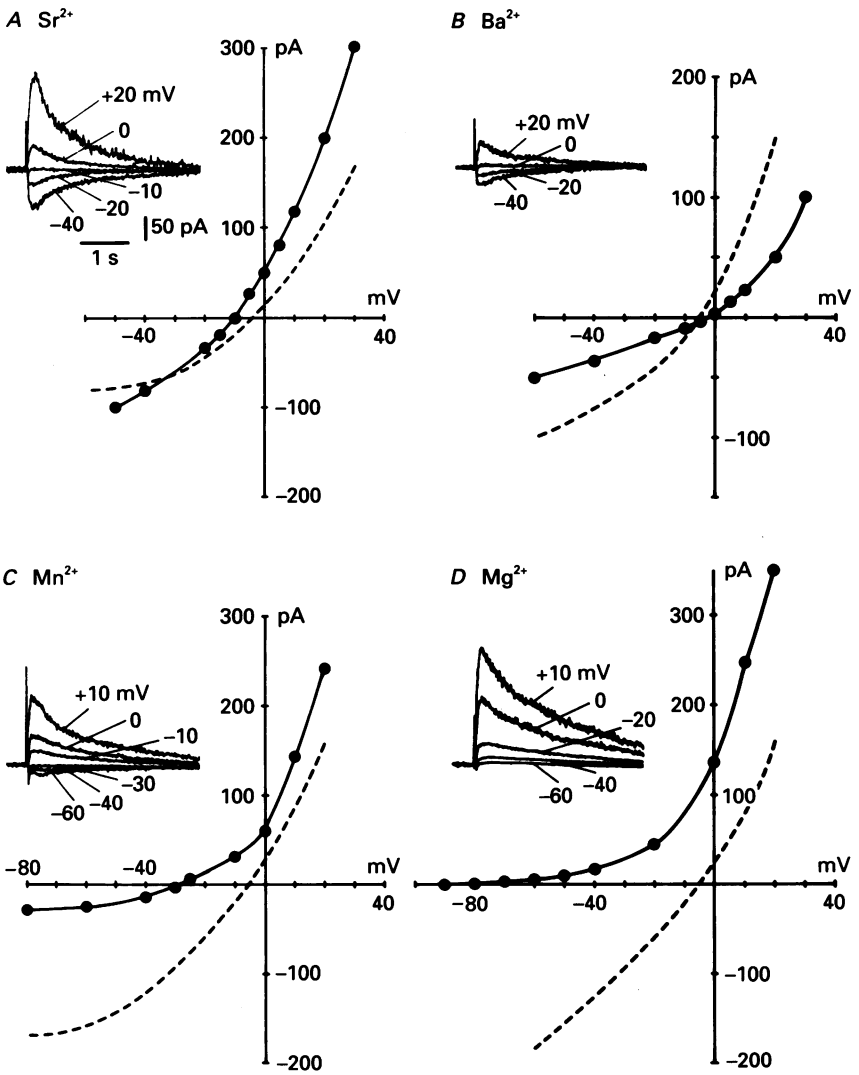

Fig. 2. NMDA-induced membrane currents in Na^+-free solutions containing 10 mM-Sr^{2+} (*A*), Ba^{2+} (*B*), Mn^{2+} (*C*) and Mg^{2+} (*D*). In each graph the peak amplitude of the NMDA-induced current is plotted against the membrane potential. Similar current–voltage plots of responses to NMDA in Na^+-free, 10 mM-Ca^{2+} solution in the corresponding neurones are shown by dashed lines. Insets represent superimposed current traces evoked by NMDA at various membrane potentials.

When both the duration and intensity of the ionophoretic current for applying quisqualate were increased (> 400 ms, > 400 nA), inward current responses occasionally occurred in Na^+-free, 10 mM-Ca^{2+} solution. However, these responses were completely abolished by the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (DL-APV) (150 μM). They were also depressed by 1 mM-Mg^{2+} in a voltage-dependent manner at membrane potentials more negative than −40 mV.

It has been shown that quisqualate produces predominantly single-channel openings with small conductances (< 20 pS) in outside-out patches from large cerebellar neurones, and granule cells and hippocampal neurones, but that it can also activate, though at low frequencies, 30–50 pS channels (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Cull-Candy, Howe & Ogden, 1988). It is therefore possible that high concentrations of quisqualate activate a significant population of 40–50 pS NMDA channels, thereby inducing inward Ca^{2+} currents detectable in whole-cell recordings.

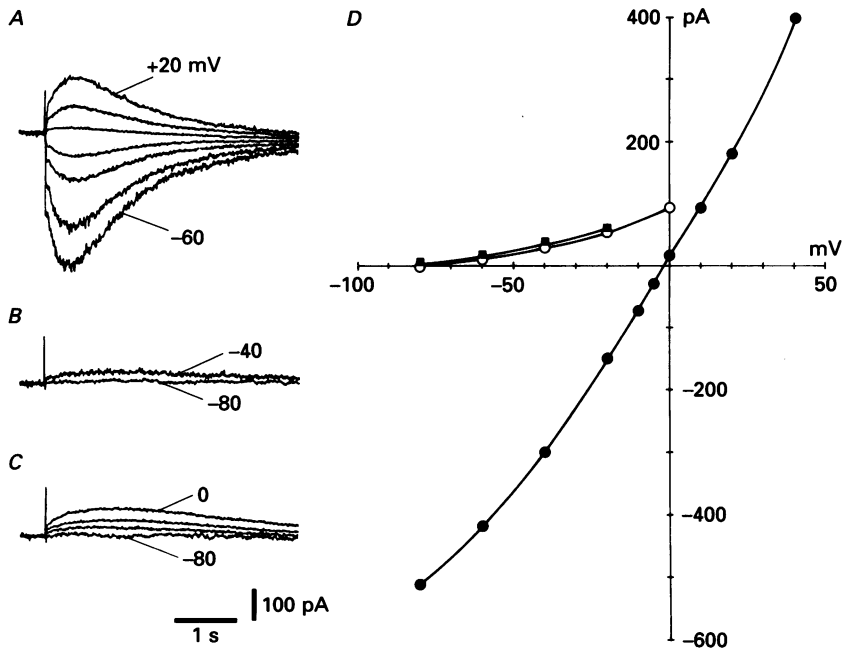

Fig. 3. Membrane current responses to quisqualate and their dependence upon the membrane potential. *A–C*, quisqualate-induced currents at various membrane potentials in the control (*A*), Na^+-, Ca^{2+}-free (*B*) and Na^+-free, 10 mM-Ca^{2+} (*C*) solutions. The membrane potentials (in mV) are +20, +10, 0, −10, −20, −40, −60 in *A* and 0, −20, −40, −80 in *C*. Quisqualate was applied ionophoretically using a current pulse of 20 ms duration and 100 nA intensity. *D*, relation between the peak amplitude of the quisqualate-induced current and the membrane potential in the control (●), Na^+-, Ca^{2+}-free (■) and Na^+-free, 10 mM-Ca^{2+} (○) solutions. All the traces were taken from the same neurone.

Two types of kainate responses

Two different types of kainate responses were found in cultured hippocampal neurones. Figure 4*A–D* shows the type of kainate receptor response (referred to as 'type I' in this paper) observed in the majority of cells tested. In the control solution, the kainate response reversed at potentials slightly more negative than 0 mV and the current–voltage plot, obtained by plotting the peak amplitude of the current response against the membrane potential, showed outward rectification. In Na^+-, Ca^{2+}-free solution, kainate caused no inward current at −80 mV. No inward current response could be induced by adding 10 mM-Ca^{2+} and the current–voltage plot of the response in Na^+-free, 10 mM-Ca^{2+} solution was almost similar to that in Na^+-, Ca^{2+}-

free solution. This indicates that the permeability to Ca^{2+} of the type I kainate receptor is as low as that of the quisqualate receptor.

These properties of the kainate receptor are similar to those reported previously in the mammalian central neurones (Mayer & Westbrook, 1984, 1987; MacDermott

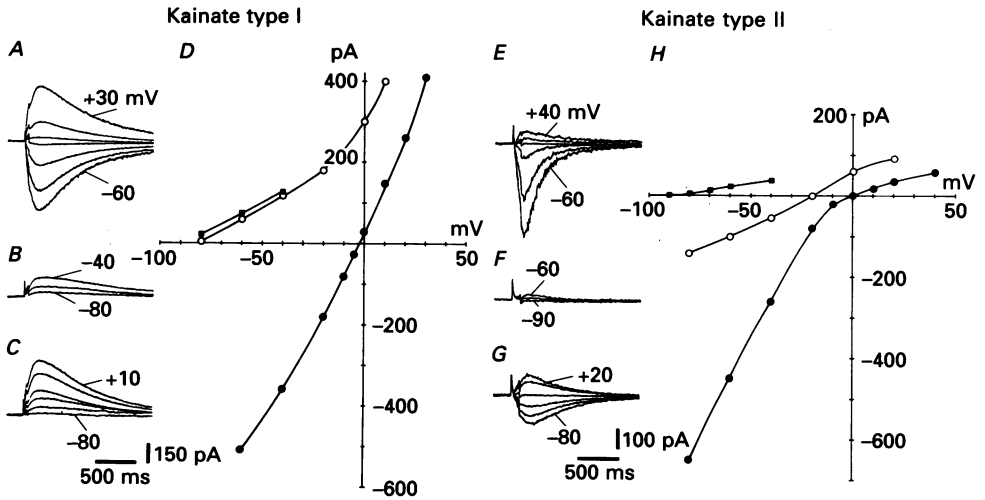

Fig. 4. Current responses to kainate. *A–C*, type I current responses to kainate at various membrane potentials in the control (*A*), Na^+-, Ca^{2+}-free (*B*) and Na^+-free, 10 mM-Ca^{2+} (*C*) solutions. The membrane potentials (in mV) are +30, +10, 0, −5, −20, −40, −60 in *A*, −40, −60, −80 in *B* and +10, 0, −20, −40, −60, −80 in *C*. *D*, relation between the peak amplitude of the kainate-induced current and the membrane potential in the control (●), Na^+-, Ca^{2+}-free (■) and Na^+-free, 10 mM-Ca^{2+} (○) solutions. All the traces in *A–C* were taken from the same neurone. *E–G*, type II current responses to kainate at various membrane potentials in the control (*E*), Na^+-, Ca^{2+}-free (*F*) and Na^+-free, 10 mM-Ca^{2+} (*G*) solutions. The membrane potentials (in mV) are +40, +20, 0, −20, −40, −60 in *E*, −60, −80, −90 in *F* and +20, 0, −20, −40, −60, −80 in *G*, *H*, relation between the peak amplitude of the kainate-induced current and the membrane potential in the control (●), Na^+-, Ca^{2+}-free (■) and Na^+-free, 10 mM-Ca^{2+} (○) solutions. Note a remarkable inward rectification in the current response in the control solution. All the traces in *E–G* were taken from the same neurone.

et al. 1986; Mayer *et al.* 1987). However, we found a second type of kainate receptor response in seventeen out of the sixty-one cells tested. The current response of this type of kainate receptor is shown in Fig. 4*E–H*. In the control solution, kainate caused inward currents at negative membrane potentials. Although the kainate response reversed at about 0 mV, the amplitude of the outward current response recorded at positive potentials was much smaller than expected (Fig. 4*E*). The peak amplitude of the kainate-induced current is plotted against the membrane potential in the control solution in Fig. 4*H* (●). There is a remarkable inward rectification in the current–voltage plot. This type of kainate response will hereafter be referred to as the type II kainate response. In the cells showing a type II response, kainate caused no inward current at potentials more positive than −90 mV in Na^+-, Ca^{2+}-free solution (Fig. 4*F* and *H*). In Na^+-free, 10 mM-Ca^{2+} solution, however, kainate induced prominent inward currents at −40 to −80 mV (Fig. 4*G* and *H*). In this solution the reversal potential of kainate response ranged between −14 and −33 mV

($-23{\cdot}3 \pm 5{\cdot}6$, $n = 17$). From the mean value of the reversal potential, P_{Ca}/P_{Cs} was calculated to be 2·3 according to eqn (1). This value was 5·3 when ionic activities were used instead of ionic concentrations.

Figure 5 shows the effect of raising the Ca^{2+} concentration (in the Na^+-free

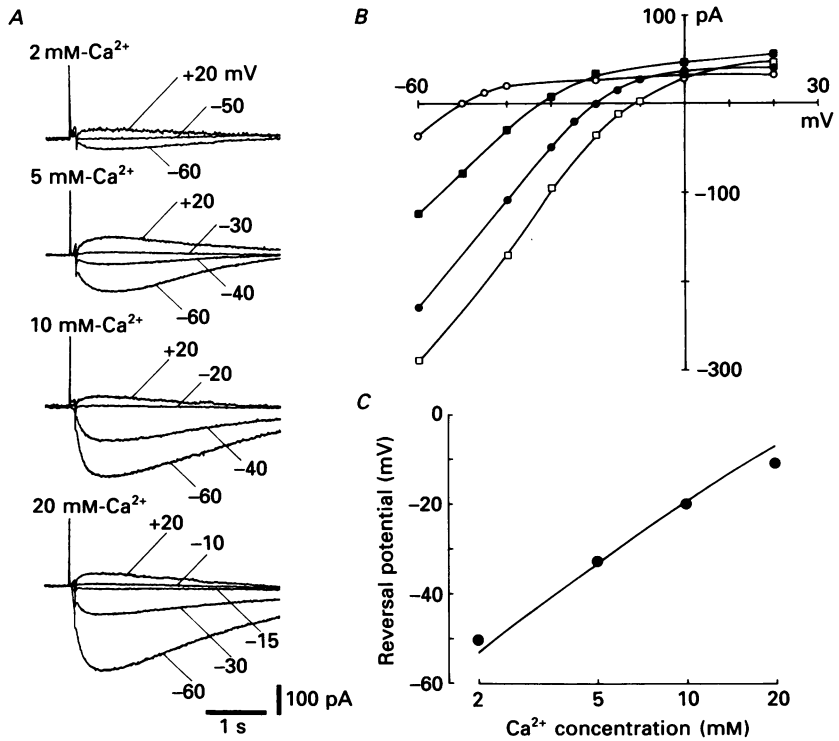

Fig. 5. Effects of changing the external Ca^{2+} concentration on type II current responses to kainate. *A*, current responses to kainate at various membrane potentials in Na^+-free solutions containing 2, 5, 10 and 20 mM-Ca^{2+}. The current traces were obtained from the same neurone. *B*, relation between the peak amplitude of the kainate-induced current and the membrane potential in the Na^+-free solutions containing 2 mM- (○), 5 mM- (■), 10 mM- (●) and 20 mM- (□) Ca^{2+}. *C*, reversal potentials estimated in *B* were plotted as a function of the external Ca^{2+} concentration. The continuous line through the data points was based on eqn (2). A constant value of $P_{Ca}/P_{Cs} = 2{\cdot}8$, calculated by the same method as described in the legend of Fig. 1, was used to draw the line.

solution) on the current responses caused by activation of type II kainate receptors. Kainate-induced currents at various membrane potentials were recorded in Na^+-free solutions containing 2, 5, 10 and 20 mM-Ca^{2+} in a neurone showing a type II response (Fig. 5*A*). The peak amplitude of the current responses in each solution is plotted against the membrane potential (Fig. 5*B*). As the concentration of external Ca^{2+} was increased, the amplitude of the inward current at −60 mV increased, and the reversal potential shifted in the positive direction (Fig. 5*C*). As also seen in the NMDA response, the shift of the reversal potential was slightly smaller than predicted at higher Ca^{2+} concentrations (see Discussion).

These results indicate that type II kainate receptor channels are permeable to Ca^{2+}. However, it might be possible that kainate induces inward Ca^{2+} currents by

activating NMDA receptors. To test this possibility, kainate and NMDA were applied alternately to a neurone showing a type II kainate response and the effects of NMDA receptor antagonists on both kainate- and NMDA-induced inward currents in Na^+-free, 10 mM-Ca^{2+} solution were studied (Fig. 6). Addition of 1 mM-

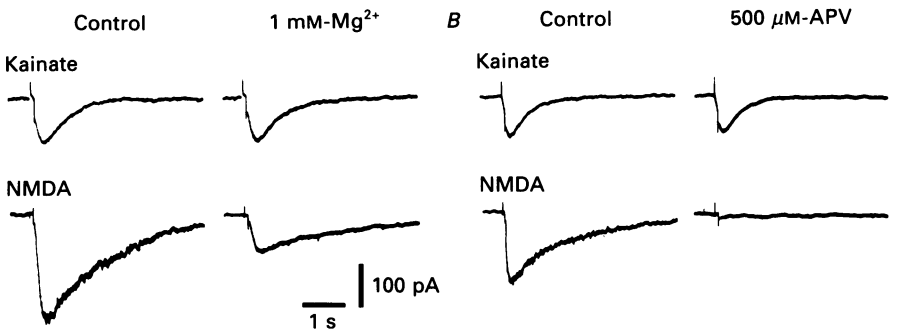

Fig. 6. Insusceptibility of kainate-induced Ca^{2+} currents to NMDA antagonists. Both kainate- and NMDA-induced current responses were induced at −60 mV in Na^+-free, 10 mM-Ca^{2+} solutions in the same neurone. *A*, effects of Mg^{2+} (1 mM). *B*, effects of DL-APV (500 μM). Note that both Mg^{2+} and APV affected only NMDA responses, but not kainate responses.

Mg^{2+} to the external solution never affected kainate-induced currents, whereas it depressed NMDA-induced currents at −60 mV (Fig. 6*A*). High concentrations (500 μM) of DL-APV, which completely suppressed NMDA responses, had no effect on kainate-induced currents. We therefore conclude that Ca^{2+} is capable of passing through the type II kainate receptor channel, which is independent of the NMDA receptor.

Permeation of divalent cations through kainate receptor channels

The relative permeability of the type II kainate receptor to divalent cations (Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+}) was determined by measuring reversal potentials in Na^+-free solutions containing one of these divalent cations at 10 mM (Fig. 7). In these experiments, the reversal potential was first determined in Na^+-free, 10 mM-Ca^{2+} solution by plotting the peak amplitude of the kainate response against the membrane potential (dashed lines). After the solution had been replaced with Na^+-free solution containing either 10 mM-Sr^{2+} (*A*), Ba^{2+} (*B*), Mn^{2+} (*C*) or Mg^{2+} (*D*), the reversal potential was measured in the same neurone (continuous lines). From the shift of the reversal potential the permeability ratios of Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+} relative to Ca^{2+} were calculated using eqn (1) on the assumption that P_{Cs} was constant. The relative permeabilities were $0{\cdot}90 \pm 0{\cdot}07$ ($n = 6$) for Sr^{2+}, $1{\cdot}27 \pm 0{\cdot}13$ ($n = 5$) for Ba^{2+}, $0{\cdot}71 \pm 0{\cdot}07$ ($n = 7$) for Mn^{2+} and $0{\cdot}79 \pm 0{\cdot}06$ ($n = 6$) for Mg^{2+}. Thus, the type II kainate receptor shows a much weaker selectivity among the divalent cations than the NMDA receptor. The permeability sequence for the type II kainate receptor, $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$, also differs from that of the NMDA receptor ($Ba^{2+} > Ca^{2+} > Sr^{2+} > Mn^{2+} \gg Mg^{2+}$).

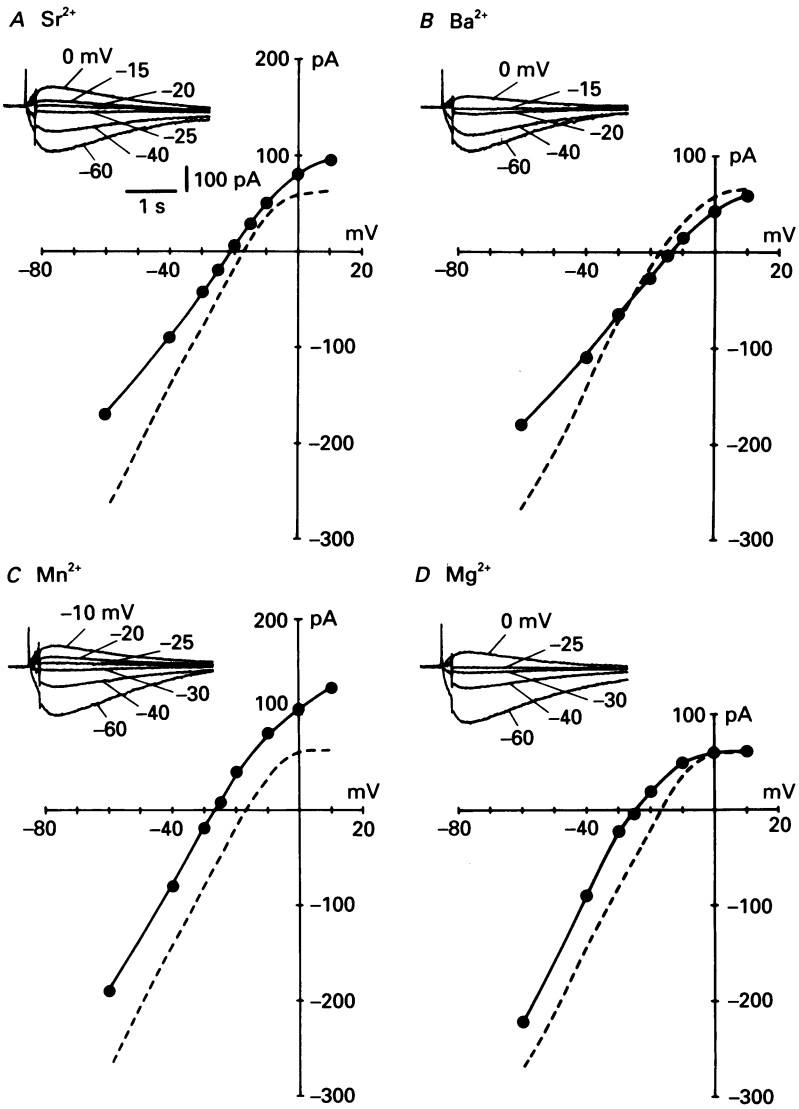

Fig. 7. Kainate-induced type II current responses in Na^+-free solutions containing 10 mM-Sr^{2+} (*A*), Ba^{2+} (*B*), Mn^{2+} (*C*) and Mg^{2+} (*D*). In each graph the peak amplitude of the kainate-induced current is plotted against the membrane potential. Similar current–voltage plots in Na^+-free, 10 mM-Ca^{2+} solution are shown by dashed lines for each neurone. Insets represent superimposed current traces evoked by NMDA at various membrane potentials.

DISCUSSION

In the present study, we have recorded Ca^{2+} currents through NMDA receptor channels in cultured rat hippocampal neurones bathed in solutions in which the alkali metal cations were replaced with the impermeant cations NMG. The results indicate that the NMDA receptor channel is highly permeable to Ca^{2+}, showing a P_{Ca}/P_{Cs} value of 6·2 in Na^+-free, 10 mM-Ca^{2+} solution. Mayer & Westbrook (1987)

have shown that raising the external Ca^{2+} concentration, with Na^+ held constant at 105 mM, shifts the reversal potential of the NMDA response in the positive direction in cultured mouse central neurones. They have calculated a value of $P_{Ca}/P_{Na} = 4{\cdot}03$ using the constant-field equation on the assumption that Na^+, K^+ and Cs^+ are equally permeant. Thus, the present result agrees roughly with the previous data with respect to Ca^{2+} permeation. However, the permeability sequence among divalent cations through the NMDA channel was $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mn^{2+} \gg Mg^{2+}$ in the present experiment. This differs from that reported by Mayer & Westbrook (1987), $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mn^{2+} \gg Mg^{2+}$. Since replacement of Ca^{2+} with Ba^{2+} shifted the reversal potential of the NMDA response in the positive direction with no exception in all the seven neurones tested, we conclude that the permeability of the NMDA channel to Ba^{2+} is higher than that to Ca^{2+} according to the reversal potential criterion.

The values of P_{Ca}/P_{Cs} for both quisqualate and the type I kainate receptor channels were less than 3% of the ratio for the NMDA channel. This result is in good agreement with the previous reports (MacDermott *et al.* 1986; Mayer & Westbrook, 1987; Ascher & Nowak, 1988). In the present study, however, we found an additional type of kainate receptor channel. This type II kainate channel was characterized by a significant permeability to Ca^{2+} and the remarkable inward rectification in the current–voltage plot of the kainate response. Analysis of noise and single-channel currents recorded in outside-out membrane patches in rat cerebellar neurones has indicated that kainate activates multiple types of channels (Cull-Candy *et al.* 1988; Cull-Candy & Usowicz, 1989). Kainate produces predominantly small-amplitude openings of 8 and 15–18 pS and large-amplitude openings of 30–50 ps at lower frequencies. Kainate also produces channel openings that are too small (< 1 pS) to be detected as discrete channel events in cerebellar neurones. Obviously study is needed to characterize single-channel properties of both type I and type II kainate responses in hippocampal neurones and to clarify the relevance of these two types of kainate responses to the different types of kainate channels revealed in cerebellar neurones.

At present, we have not conducted systematic studies on morphological and functional identification of the neurone which exhibits the type II kainate response. So far we have not encountered the type II kainate response in relatively mature neurones resembling young hippocampal pyramidal cells with thick dendrite-like processes. The type II response occurred rather frequently in smaller cells with thin and short outgrowing processes. It remains to be elucidated whether the type II kainate receptor appears at a certain stage of the cell development irrespective of neurone species or in a specific group of neurones irrespective of age.

The ionic selectivity of channels has been most commonly estimated using the constant-field equation. To facilitate the comparison with previous works on the permeation of divalent cations in a variety of channels (Adams *et al.* 1980; Denkin, 1983; Mayer & Westbrook, 1987; Ozawa *et al.* 1988), we have also utilized this equation to describe the selectivity of EAA receptor channels to divalent cations as a first approximation. The analysis of NMDA single-channel currents by Ascher & Nowak (1988) has demonstrated that in isotonic Ca^{2+} solution Ca^{2+} carries much smaller currents than expected from the high P_{Ca} value calculated by the use of the constant-field equation. Probably each Ca^{2+} ion that binds tightly inside the NMDA

channel retards the permeation of Ca^{2+} itself and other permeant cations. In other words, the saturation effect of the divalent cation capable of binding tightly to the channel violates the independence law, thus causing the deviation of the current–voltage plot from the prediction by the constant-field current equation. The deviation is even more obvious in the current–voltage plot of the type II kainate response. The reversal potential is near 0 mV in the control solution, indicating that P_{Cs} is almost equal to P_{Na} according to the constant-field voltage equation. Yet, the outward current carried by Cs^+ through this channel is much smaller than the inward current predominantly carried by Na^+ when the absolute value of the voltage driving force is equal. Probably, Cs^+ driven into the channel from the cell interior binds inside the channel, thereby blocking the outward movement of Cs^+ itself. However, it is expected that reversal potential measurements are not so sensitive to the blocking effects of these permeant cations, since these effects reduce the effective number of active channels, but do not affect reversal potential of active channels (see Hille, 1984). Thus, the permeability ratio obtained from the present reversal potential measurements gives useful information about the selectivity of EAA receptor channels to divalent cations.

The slope of the reversal potential changes against the external Ca^{2+} concentration predicted by the constant-field equation was too steep to fit the data points both in NMDA and type II kainate responses (Figs 1*G* and 5*C*). This could be due to the fact that the ionic concentration was used instead of ionic activity. We therefore calculated the activity of Ca^{2+} in each solution (Shatkay, 1968) and replotted the reversal potential against the activity of Ca^{2+}. However, it turned out that this could only account for a small part of the deviation. Similar deviation was found in reversal potentials of acetylcholine-induced currents against the external Ca^{2+} concentration in frog endplates (Adams *et al.* 1980). The deviation was explained as follows: if the channel has a negative surface potential (ψ), divalent cations will be concentrated at the membrane surface by a factor of $\exp(-2\psi F/RT)$. At low ionic strength, ψ is negative and the advantage, $\exp(-2\psi F/RT)$, is large, whereas at higher ionic strength, ψ is less negative and the advantage is smaller. Therefore, reversal potentials in higher Ca^{2+} solution deviate to more negative values than expected simply by the constant-field equation. This explanation could be at least partly applicable to the present result, since the presence of a negative surface potential has been demonstrated in the NMDA channel (Ascher & Nowak, 1988).

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