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THE ROLE OF DIVALENT CATIONS IN THE *N*-METHYL-D-ASPARTATE RESPONSES OF MOUSE CENTRAL NEURONES IN CULTURE

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SUMMARY

1. Single-channel currents activated by *N*-methyl-D-aspartate (NMDA) agonists were analysed in the presence of various extracellular concentrations of divalent cations in outside-out patches from mouse neurones in primary culture.

2. In nominally Mg^{2+}-free solutions the opening and closing of the channels leads to rectangular current pulses, the mean duration of which varies little with membrane potential. After addition of Mg^{2+}, the single-channel currents recorded at negative potentials appear in bursts of short openings separated by brief closures.

3. The duration of the short openings decreases with increasing Mg^{2+} concentration, while the duration of the short closures is independent of the Mg^{2+} concentration. Depolarization increases the duration of the short openings and decreases the duration of the short closures.

4. The dependence of the burst structure on the Mg^{2+} concentration and on membrane potential is compatible to a first approximation with a model in which Mg^{2+} ions enter the open channel and block it by binding at a deep site. A better approximation requires, however, additional assumptions such as Mg^{2+} permeation and/or interactions between Ca^{2+} and Mg^{2+}.

5. Increasing the extracellular Ca^{2+} concentration from 1 to 100 mM produces three effects on the currents flowing through NMDA channels. It shifts the reversal potential towards a positive value (+30 mV); it reduces the outward current flowing through the NMDA channels at very positive potentials; it reduces the inward current flowing at negative potentials.

6. The interpretation of the effects of Ca^{2+} appears to require three hypotheses: that Ca^{2+} permeates the NMDA channel, that there exists a significant surface potential at the entrance of the NMDA channel in physiological solutions and that both Ca^{2+} and monovalent cations bind to the channel, binding being stronger in the case of Ca^{2+} ions.

7. While Co^{2+} and, to a lesser extent, Mn^{2+} mimic the effects of Mg^{2+} on the NMDA channel, Ca^{2+}, Ba^{2+} and Cd^{2+} do not. The distinction between Mg^{2+}-like and Ca^{2+}-like divalent cations corresponds to a difference in the speed of exchange of the water

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molecules surrounding the cations in solutions. Thus, it is possible that permeation occurs for all the divalent cations, but is slower for those which are slowly dehydrated.

INTRODUCTION

In the vertebrate central nervous system, extracellular Mg^{2+} and Ca^{2+} ions both play a major role in the responses of neurones to amino acids activating the NMDA (*N*-methyl-D-aspartate)-sensitive receptor.

The initial observations on the involvement of Mg^{2+} by Ault, Evans, Francis, Oakes & Watkins (1980) indicated that Mg^{2+} at physiological concentrations decreases the responses to NMDA agonists and that this effect is not related to a reduction of transmitter release, but to a 'non-competitive' postsynaptic blockade. That the blockade is due to entry of Mg^{2+} into the NMDA channel was then suggested by two sets of data. First, recordings of the whole-cell current produced by NMDA agonists showed (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer, Westbrook & Guthrie, 1984) that the Mg^{2+} block increases with hyperpolarization. Secondly, single-channel recordings of the NMDA-induced current (Nowak *et al.* 1984) indicated that Mg^{2+} induces a flickering resembling that produced by local anaesthetics on the ACh-induced currents of cultured striated muscle cells (Neher & Steinbach, 1978).

The Mg^{2+} effects are not mimicked by divalent cations like Ca^{2+}, Ba^{2+} and Cd^{2+} (Ault *et al.* 1980; Mayer *et al.* 1984) and indeed many reports have indicated that both glutamate and NMDA induced Ca^{2+} entry into vertebrate central neurones (Heinemann & Pumain, 1980; Bührle & Sonnhof, 1983; Dingledine, 1983; Zanotto & Heinemann, 1983; Mayer & Westbrook, 1985; Lambert & Heinemann, 1986; Pumain, Louvel & Kurcewicz, 1986; Kudo & Ogura, 1986). While in most cases it was not possible to establish how much of the Ca^{2+} entry occurred directly through the NMDA channels and how much through voltage-dependent Ca^{2+} channels activated by depolarization, direct evidence that the NMDA channel is a major route of Ca^{2+} entry was recently presented by MacDermott, Mayer, Westbrook, Smith & Barker (1986) who used Arsenazo III to monitor Ca^{2+} entry induced by NMDA in neurones voltage clamped near resting potential.

In the analysis presented below, single-channel currents resulting from NMDA receptor activation were recorded in solutions containing various divalent cations and an attempt was made to analyse in more detail both the blocking effects and the permeation of these cations.

Preliminary descriptions of this work have been presented (Nowak *et al.* 1984; Nowak & Ascher, 1985; Ascher & Nowak, 1986).

METHODS

The methods were similar to those described in a preceding paper (Ascher, Bregestovski & Nowak, 1988). The neurones used were taken from 12–15 day mouse embryos and cultured for 1–8 weeks. The agonists were applied in the bath. Most experiments were performed on outside-out patches (Hamill, Marty, Neher, Sakmann & Sigworth, 1981).

Composition of solutions

The single-channel currents were activated either by NMDA (5–10 μM) or L-glutamate (5–20 μM). The internal solution contained (mM): CsCl, 140; K-EGTA, 5; $CaCl_2$, 0·5; HEPES, 10, buffered with KOH at pH 7·2. In the experiments where the extracellular Ca^{2+} concentration was increased, the intracellular Ca^{2+} buffering was increased by using 10 mM-K-EGTA and 1 mM-$CaCl_2$. The external solutions were prepared by mixing in variable proportions a 'Ca^{2+}-free solution' (mM: NaCl, 140; KCl, 2·8; HEPES, 10, buffered to pH 7·2) with a 100 mM-Ca^{2+} solution (mM: $CaCl_2$, 100; KCl, 2·8; HEPES, 10, buffered to pH 7·2). The various solutions will be labelled 0 Ca^{2+}, 1 mM-Ca^{2+} etc. In a few experiments, Ba^{2+} was substituted for Ca^{2+} using a 10 mM-Ba^{2+} solution (mM: NaCl, 126; $BaCl_2$, 10; KCl, 2·8; HEPES, 10; buffered to pH 7·2 with NaOH).

Many acidic amino acids have a high affinity for divalent cations, and therefore are partly complexed in solutions containing high concentrations of divalent cations. Since we did not know the dissociation constants (K) of NMDA, but knew those of L-glutamate (Sillén & Martell, 1964), this last compound was used preferentially to activate NMDA receptors in the experiments in which complexing of the amino acid could be significant. This was the case for Co^{2+} ions which have a very high affinity for glutamic acid ($K = 10^{5·6}$) so that in a solution containing 10 μM-Co^{2+}, addition of 10 μM-glutamate lowers the free Co^{2+} concentration to 6 μM (Fig. 5). For Mn^{2+} the complexing is less important ($K = 10^{3·3}$) but still significant if one uses high concentrations of glutamate (Fig. 6). For the other divalent cations complexation was neglected, except in experiments involving very high concentrations of divalent cations. Thus, in the experiments where we varied the Ca^{2+} concentration from 1 to 100 mM, the free glutamate concentration was adjusted to a constant value of 10 μM. This required a total (free + bound) glutamate concentration of 10·2 μM in 1 mM-Ca^{2+}, 36·9 μM in 100 mM-Ca^{2+}.

Residual Mg^{2+} concentration. Mayer & Westbrook (1984) have indicated that 'nominally Mg^{2+}-free solutions' may in fact contain up to 5 μM-Mg^{2+}. We have not checked this point in detail, but do not believe that the free Mg^{2+} concentration was high enough to affect the NMDA channels. Our major argument in favour of this claim is the observation (described in the results section) that the bursts observed in 'Mg^{2+}-free' solutions did not resemble those observed in the presence of Mg^{2+}. When Mg^{2+} blocks the NMDA channel, the mean duration of the short gaps within bursts has a characteristic value which does not depend on the Mg^{2+} concentration but varies with membrane potential. Although some short gaps were observed in Mg^{2+}-free solutions (Nowak *et al.* 1984; Ascher *et al.* 1988) they were shorter than the gaps observed in the presence of Mg^{2+} and did not change with potential.

Single-channel analysis

Single-channel current amplitudes were evaluated as described by Takeda & Trautmann (1984). For the analysis of the bursts produced by the divalent cations which block the NMDA channel (Mg^{2+}, Co^{2+}, Mn^{2+}) the records were digitized at a sampling rate of 10 kHz while filtering at half the sampling frequency. Openings and closings of the channels were detected as the crossing of a threshold chosen as the mid-point between the closed and the open state. From the digitized records three types of histograms were formed: open-time (t_o), closed-time (t_c) and burst-duration (t_b) histograms. The problem of overlap of several channels was avoided by selecting data with a low density of events, using low agonist concentrations. In such conditions the closed-time histograms recorded at low agonist concentrations contained long 'interburst intervals' the duration of which (measured in seconds) varied with the agonist concentration but also from patch to patch. These intervals were neglected in the present study, and the histograms which were fitted were those corresponding to the short closed times (in the millisecond range), which will be abbreviated as 'closed times'.

For the construction of the burst-durations histograms, closures shorter than five times the mean closed time, $\bar{t}_c$, were neglected. We verified that the value obtained in this way was not altered if the value of the 'neglected' closed times was increased, as expected from the clear separation between short and long closed times in the closed-times histograms. Fitting an exponential to the burst-durations histograms allowed the calculation of the mean burst duration, $\bar{t}_b$, and of the total number of bursts, N_b.

The evaluation of $\bar{t}_o$ from the open-times histogram can become erroneous if $\bar{t}_c$ is very short because missed closures ('missed gaps' of Colquhoun & Sakmann, 1981) lead to an overevaluation of the open time. Similarly, $\bar{t}_c$ will be overstimated if $\bar{t}_o$ becomes very short (as is the case in high Mg^{2+} concentrations, as will be seen). In an attempt to estimate the importance of this deviation.

we corrected the value of $\bar{t}_o$ in some cases where it was sufficiently high to consider that most openings were detected. In such a case Colquhoun & Sigworth (1983) have shown that the 'true' open time, μ_o, is given by

$$\mu_o = (t_b - t_c N)/(N+1),$$

where N is the number of closures per burst obtained by dividing the total number of closures, N_c, by the total number of bursts, N_b. The value of N_c was obtained from the closed-time histograms. For the construction of these histograms, we neglected the first bins of the histograms up to a threshold of 0·3 ms. Assuming that the distributions do not deviate from a single exponential for short intervals, one can calculate the number of missing events (Takeda & Trautmann, 1984) and from there the total number of closures, N_c, which would have been observed if the time resolution of the analysis was zero. A further complication came from the fact that in many cases the histograms of the burst durations were not exponential, introducing an uncertainty in the correction. In those cases we used for the value of $\bar{t}_b$ the arithmetic mean of the burst duration. Given all the approximations involved, it was satisfactory that the value of μ_o differed by less than 20% – in many cases by less than 10% – from the value of $\bar{t}_o$ estimated by a direct fit of the open-times histogram.

RESULTS

Mg^{2+}-induced flickering of the NMDA current

Figure 1 illustrates some NMDA single-channel records obtained in various extracellular Mg^{2+} concentrations, $[Mg^{2+}]_o$, at −60 mV. The currents recorded in control (Mg^{2+}-free) solutions, in 10 μM-Mg^{2+} and in 50 μM-Mg^{2+} have the same amplitude (2·7 pA) but differ by the fact that in the presence of Mg^{2+} the current is characterized by bursts of short openings. In 100 μM-Mg^{2+}, the single-channel current appears smaller, but since this is associated with an increased frequency of closures during the burst, it is likely that the reduction of the single-channel current is only the result of the limited frequency response of the recording. Figure 1 also illustrates NMDA single-channel currents recorded in 100 μM-Mg^{2+} at +40 mV, where little flickering was observed.

Figures 2, 3 and 4 present the results of the quantitative analysis of single-channel currents such as illustrated in Fig. 1. As indicated in the Methods section, we measured three parameters: the open time (t_o), the (short) closed time (t_c) and the burst duration (t_b) which is only unambiguously defined when the channel opens infrequently. The histograms of the first two values were usually well fitted with a single exponential, which allowed accurate measurement of the mean open time ($\bar{t}_o$) and the mean closed time ($\bar{t}_c$).

Figure 2 illustrates the contrast between the effects of increasing $[Mg^{2+}]_o$ on $\bar{t}_o$ and $\bar{t}_c$. The increased flickering illustrated in Fig. 1 appears to result entirely from a shortening of the mean open time $\bar{t}_o$ which, at −60 mV, decreases from 5·8 ms in Mg^{2+}-free solution to 3·2 ms for $[Mg^{2+}]_o$ = 10 μM, 1·9 ms for $[Mg^{2+}]_o$ = 20 μM, 1 ms for $[Mg^{2+}]_o$ = 50 μM and 0·55 ms for $[Mg^{2+}]_o$ = 100 μM. The relation between $1/\bar{t}_o$ and $[Mg^{2+}]_o$ appears linear between 0 and 100 μM-Mg^{2+}, with a slope of $1{\cdot}6 \times 10^7$ s^{-1} M^{-1}. In contrast $\bar{t}_c$ appears independent of the Mg^{2+} concentration and close to 0·7 ms.

These data are compatible with a scheme of open-channel block (Adams, 1976) which can be written (Neher & Steinbach, 1978)

$$R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} R^* \quad \text{and} \quad R^* + Mg^{2+} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} RMg,$$

where R and R* are the closed and open state of the channel in the absence of Mg^{2+}, RMg the channel blocked by Mg^{2+} and β, α, k_+ and k_- the reaction rate constants. In such a scheme, $\bar{t}_o$ and $\bar{t}_c$ are given by (Neher & Steinbach, 1978)

$$\bar{t}_o = (\alpha + k_+[Mg^{2+}]_o)^{-1},$$

$$\bar{t}_c = (k_-)^{-1}.$$

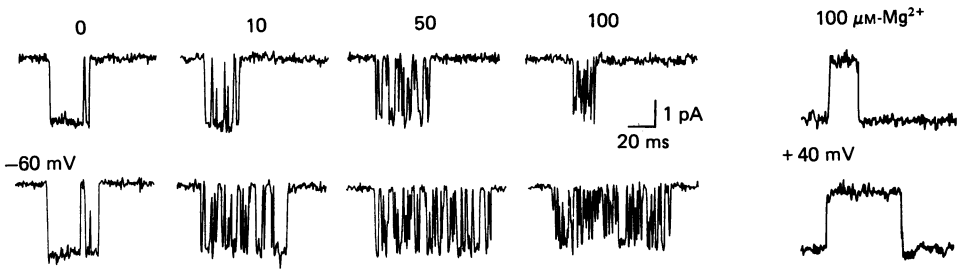

Fig. 1. Single-channel currents induced by NMDA (10 μM) in the absence of extracellular Mg^{2+} and in the presence of increasing concentrations of Mg^{2+}. Two examples of long openings are illustrated in each case. The eight records on the left were obtained at −60 mV; the two records on the right were obtained at +40 mV. In the first column, no Mg^{2+} was present. The concentration of Mg^{2+} was then increased to 10 μM (second column), 50 μM (third column) and 100 μM (fourth and fifth columns). Notice that at −60 mV, increasing the Mg^{2+} concentration increases the flickering. No flickering is detected at +40 mV even in 100 μM-Mg^{2+}.

From the mean values of four experiments similar to that of Fig. 2 we calculated, at −60 mV, $k_+ = 1{\cdot}8 \pm 0{\cdot}9$ (S.D.) $\times 10^7$ M^{-1} s^{-1} and $k_- = 1400 \pm 155$ (S.D.) s^{-1}.

Figure 3 illustrates the voltage dependence of k_- and k_+. For k_- the relation can be described by an exponential relation

$$1/\bar{t}_c = k_- = 5{\cdot}4 \times 10^3 \,(\exp(V/47)),$$

where k_- is expressed in s^{-1} and V in mV.

The voltage dependence of k_+ can be calculated from the variation with membrane potential of the slope of the relation between $1/\bar{t}_o$ and the Mg^{2+} concentration (Fig. 2). Figure 3 indicates that the dependence is approximately described by a relation:

$$k_+ = 6{\cdot}1 \times 10^5 \exp(-V/17),$$

where k_+ is in M^{-1} s^{-1} and V is in mV.

The equilibrium dissociation constant for the binding reaction of Mg^{2+} to the channel can then be calculated (see Fig. 4) as

$$K_{Mg} = k_-/k_+ = 8{\cdot}8 \times 10^{-3} \exp(V/12{\cdot}5),$$

where K_{Mg} is given in M. As can be seen in Fig. 4, at −60 mV a mean value is 72 μM.

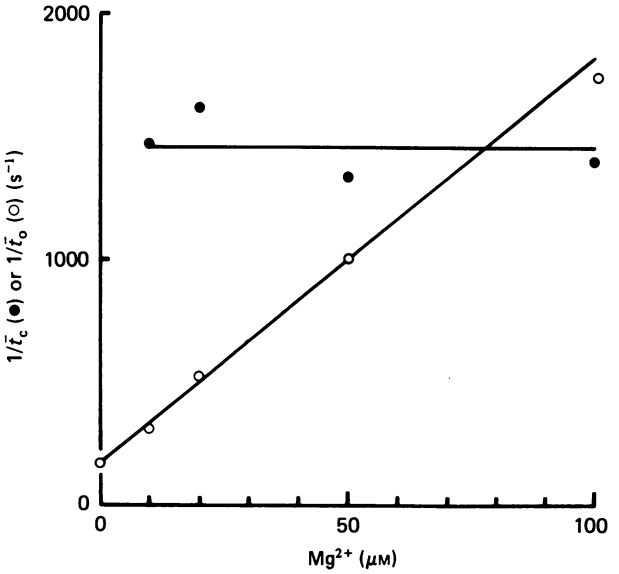

Fig. 2. Dependence of the mean open time and of the mean closed time on the Mg^{2+} concentration. NMDA was applied at 10 μM. The membrane potential was −60 mV. The values plotted on the ordinate correspond either to the inverse of the mean closed time ($1/\bar{t}_c$) (●) or to the inverse of the mean open time ($1/\bar{t}_o$) (○). The value of $1/\bar{t}_c$ in the absence of Mg^{2+} (not illustrated) was 3400 s^{-1}. The significance of this value (corresponding to very brief interruptions) has been discussed by Ascher *et al.* (1988). The slope of the relation between $1/\bar{t}_o$ and the Mg^{2+} concentration is $k_+ = 1{\cdot}6 \times 10^7$ M^{-1} s^{-1}. The value of $1/\bar{t}_o$ for $[Mg^{2+}]_o = 0$ is 170 s^{-1} (Ascher *et al.* 1988).

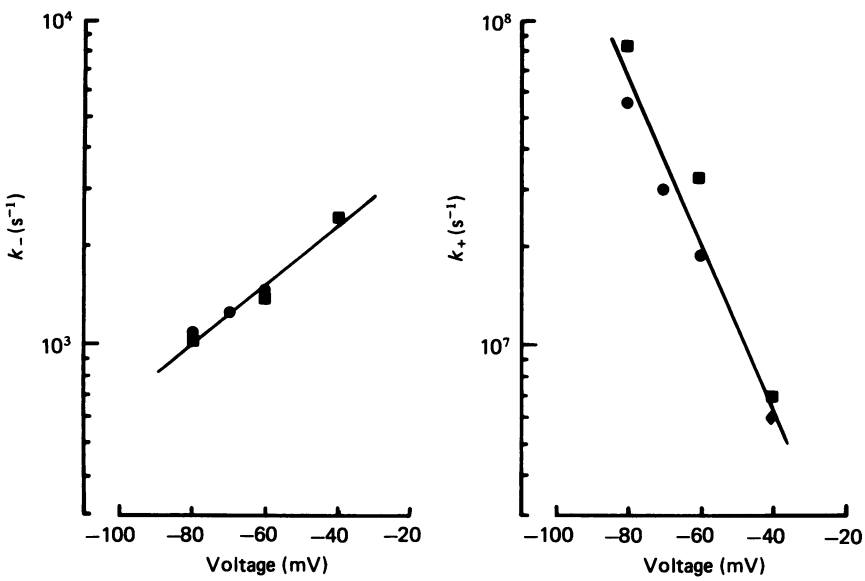

Fig. 3. Dependence on membrane potential of the forward and backward Mg^{2+} binding rates. Left, variations of k_- (obtained as the inverse of the mean closed time). Right, variation of k_+ (obtained from the slope of the relation between the inverse of the mean open time and the Mg^{2+} concentration). Each symbol corresponds to a different experiment. Note the difference in the sign of the two slopes, as well as in their absolute values.

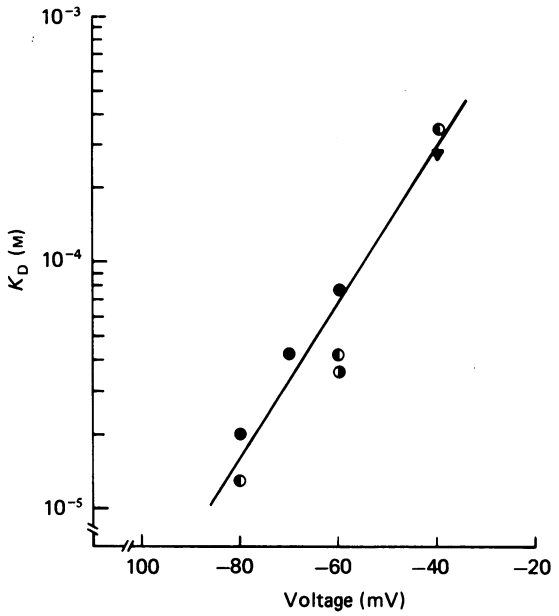

Fig. 4. Voltage dependence of the dissociation constant of the blocking reaction of Mg^{2+}. The dissociation constant K_D was calculated as the ratio of the two rate constants k_-/k_+, the variation of which is illustrated in Fig. 3. Different symbols refer to different experiments.

Dependence of burst duration on $[Mg^{2+}]_o$ and membrane potential

The value of the mean burst duration is expected to vary with the Mg^{2+} concentration in a way which gives useful information about the type of 'channel-block' model which best describes the data. As discussed in the Methods section, many of the histograms of the burst duration were not well fitted with a single exponential. This could be due either to the fact that we did not collect long enough samples or it could reflect complexity in the relation between $\bar{t}_b$ and $[Mg^{2+}]_o$. In selecting the arithmetic mean from the best experiment, we found $\bar{t}_b$ values of 17 ms in Mg^{2+}-free solution, 13 ms in 10 μM-Mg^{2+}, 13 ms in 20 μM-Mg^{2+}, 17 ms in 50 μM-Mg^{2+}, and 7 ms in 100 μM-Mg^{2+}. A similar trend was observed in three other experiments: $\bar{t}_b$ showed no clear change up to 50 μM-Mg^{2+}, and decreased at higher Mg^{2+} concentrations.

In spite of the low accuracy due to the use of the arithmetic mean, these values allow some conclusions concerning the kinetics of NMDA response, which are considered in the Discussion.

Effects of Co^{2+}, Mn^{2+} and Cd^{2+}

Ault *et al.* (1980) have indicated that Co^{2+} and Mn^{2+} mimic the effects of Mg^{2+} on NMDA responses, and Mayer *et al.* (1984) have shown that Cd^{2+}, which mimics Mg^{2+}, Co^{2+} and Mn^{2+} in blocking many Ca^{2+} conductances, does not block the NMDA responses. We have extended these observations at the single-channel level. Figure 5 illustrates the 'Mg^{2+}-like' effects of 6 μM-free Co^{2+}, while Fig. 6 illustrates the

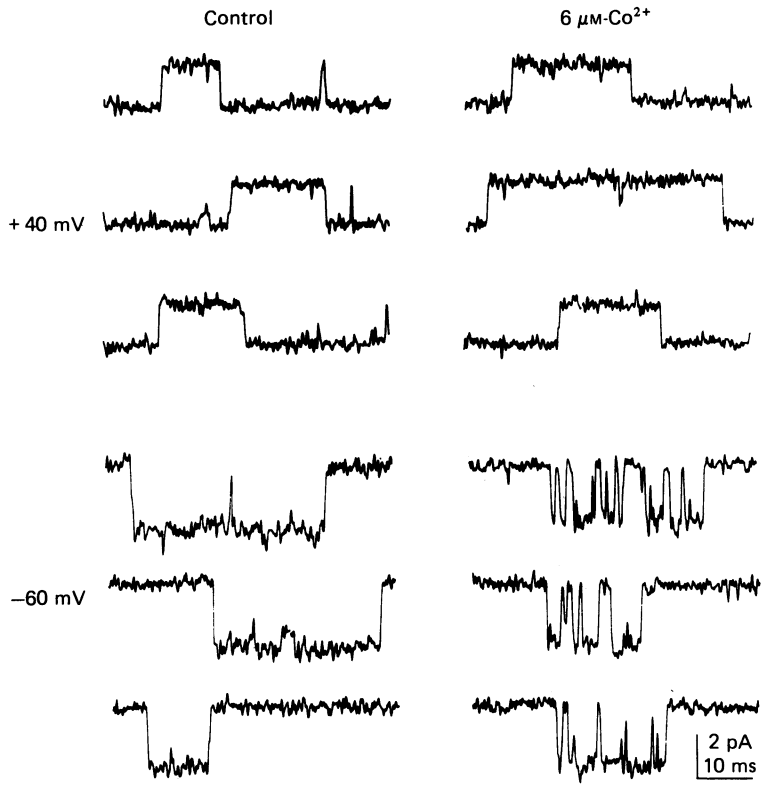

Fig. 5. Co^{2+} mimics the effects of Mg^{2+}. Outside-out patch. Single-channel currents were recorded at +40 mV (upper traces) and −60 mV (lower traces) in the presence of 10 μM-glutamate (left) and after addition to 10 μM-glutamate of 10 μM-$CoCl_2$ (right). The calculated value of the free-Co^{2+} and free-glutamate concentrations (using the complexing constant of $10^{5.6}$ M^{-1} l given by Sillén & Martell (1964) was 6 μM.

flickering induced by 8·2 and 85·2 μM-Mg^{2+}. In contrast Cd^{2+} did not induce any significant flickering at 100 μM (not shown).

The flickering induced by Co^{2+} resembles that produced by Mg^{2+}. The analysis of two experiments where Co^{2+} was applied at 6 and 14 μM led to values of k_- very similar to those obtained with Mg^{2+} (about 1300 s^{-1} at −60 mV), to slightly higher values for k_+ (mean value of $5{\cdot}3 \times 10^7$ M^{-1} s^{-1} at −60 mV) and consequently to a slightly higher affinity (24 μM at −60 mV).

The flickering observed with 8·2 μM-Mn^{2+} is quite different from that observed with a comparable concentration of Mg^{2+}, and appears mostly as an increase of the noise recorded during the opening of the channel. This suggests that the mean closed time, $\bar{t}_c$, is shorter than in the case of Mg^{2+} or Co^{2+} (too short to be measured directly). The strong reduction of the single-channel current at 85·2 μM-Mn^{2+} can be explained by the fact that both $\bar{t}_c$ and $\bar{t}_o$ are now small.

Effects of $[Ca^{2+}]_o$ *on the I–V relation of NMDA currents*

It was shown previously (Nowak *et al.* 1984; Cull-Candy & Ogden, 1985), that in the usual Mg^{2+}-free solution ($[Ca^{2+}]_o$ = 1 mM) the single-channel currents associated

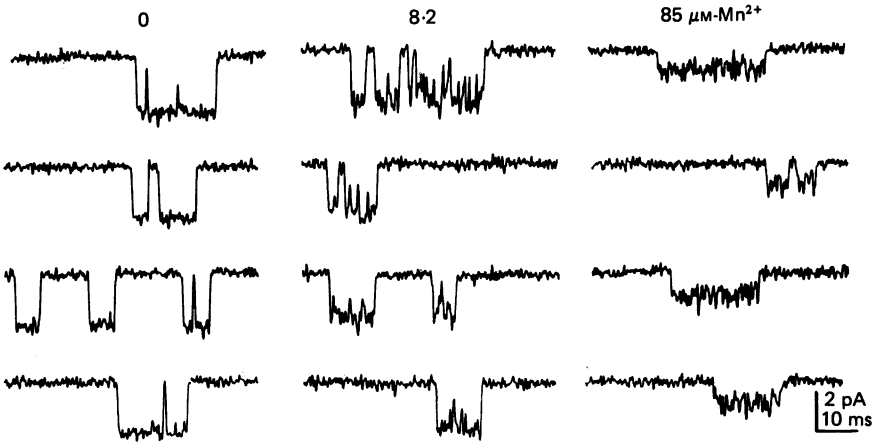

Fig. 6. Effects of Mn^{2+} on the NMDA channels. Outside-out patch. Holding potential: −60 mV. Glutamate was applied at a concentration of 100 μM in the absence of Mn^{2+} (left) then in the presence of 10 μM-Mn^{2+} (8·2 μM-free Mn^{2+}) (centre) and 100 μM-Mn^{2+} (85 μM-free Mn^{2+}) (right).

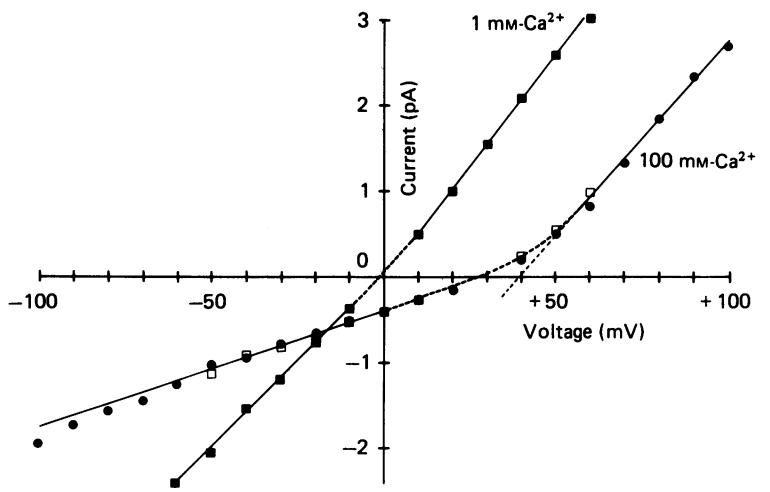

Fig. 7. Current–voltage relations of single NMDA channels in low and high Ca^{2+}. Outside-out patches. Single-channel currents were obtained in solution containing 1 mM-Ca^{2+} and in a Na^+-free solution containing 100 mM-Ca^{2+}. Results are from two different outside-out patches. One of them was analysed in both 1 mM-Ca^{2+} (■) and 100 mM-Ca^{2+} (□) during application of NMDA. The other (●) was only studied in the 100 mM-Ca^{2+} solution, and channels were opened by glutamate. In this case, the total concentration of glutamate was adjusted (to 10·2 μM in 1 mM-Ca^{2+}, to 36·9 μM in 100 mM-Ca^{2+}) in order to obtain in all cases a concentration in free glutamate of 10 μM. In the case of NMDA no correction was attempted and the total concentration of NMDA was 10 μM. The *I–V* curve recorded in 1 mM-Ca^{2+} was slightly atypical inasmuch as its slope at positive potentials (52 pS) was different from its slope at negative potentials (44 pS). This difference was encountered in only a few cases. The thin dashed line extrapolates to the abscissa the linear part of the outward current–voltage relation.

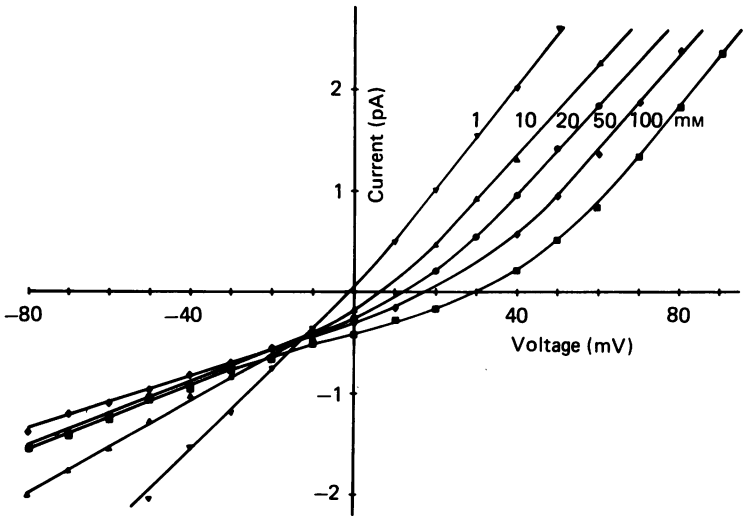

Fig. 8. Current–voltage relations of single NMDA channels in solutions containing different amounts of Na^+ and Ca^{2+}. Outside-out patches. Single-channel currents were produced either by NMDA (10 μM) or glutamate (10 μM). Results from different experiments have been pooled for 100 mM-Ca^{2+} ($n = 2$, ■), 20 mM-Ca^{2+} ($n = 4$, ●) and 10 mM-Ca^{2+} ($n = 9$, ▲). The data for 1 mM-Ca^{2+} (▼) and 50 mM-Ca^{2+}(◆) are from a single experiment.

with the activation of the NMDA receptor invert close to 0 mV and vary linearly as a function of membrane potential. For the main conductance state, the slope of the *I–V* relation is about 50 pS (see Ascher *et al.* 1988).

To study the role of extracellular Ca^{2+} in the NMDA response we analysed the NMDA single-channel currents both in low-Ca^{2+} and in high-Ca^{2+} solutions. Two experiments were performed in nominally Ca^{2+}-free solutions (no EGTA added). In both cases the *I–V* relations were linear in the range where they were analysed (−60 to +60 mV), and the inversion potential could not be distinguished from that measured in 1 mM-Ca^{2+} solution. The slope of the *I–V* relation was 56 pS in one case and 55 pS in the other. Although these values are slightly higher than the mean values found in the 1 mM-Ca^{2+} solution, more experiments would be required to conclude that decreasing $[Ca^{2+}]_o$ from 1 mM to 0 actually increases the single-channel conductance.

For experiments using a high external Ca^{2+} concentration the 1 mM-Ca^{2+} solution was replaced with one of four solutions: 10, 20, 50 or 100 mM-Ca^{2+} (see Methods). In none of these solutions did we observe bursts comparable to those observed with Mg^{2+} or Co^{2+}.

Figure 7 illustrates the *I–V* curve obtained in the 100 mM-Ca^{2+} solutions. The *I–V* relation crosses the abscissa around +30 mV instead of 0 mV. Away from the reversal potential the *I–V* relation tends towards linearity, but the slopes of the two asymptotes are quite different: for the inward current, the asymptote has a slope of 14 pS, while for the outward current the slope is 46 pS.

Figure 8 illustrates the family of curves which was obtained when $[Ca^{2+}]_o$ was

increased from 1 to 100 mM. Three major effects can be observed: (i) a monotonic shift of the reversal potential from 0 to 30 mV; (ii) a progressive decrease of the inward current slope which reached an apparent limit of about 15 pS; (iii) a parallel shift of the outward I–V relation.

The fact that a substantial inward current is recorded at negative potentials in isotonic Ca^{2+} (100 mM-Ca^{2+}, Na^+-free) solution indicates that the NMDA channel is permeable to Ca^{2+}, at least when Na^+ is absent. The shift of the reversal potential in high-Ca^{2+} solution suggests that the permeability to Ca^{2+} is higher than to Na^+. However, as will be discussed later, this conclusion cannot be quantitatively validated until one is able to evaluate the surface potential existing at the mouth of the NMDA channel. An indication that this potential is substantial is given by the 'third' effect, the shift of the outward I–V relation.

The effect of Ca^{2+} on the outward current (which must be carried by Cs^{2+}) can be explained by assuming that, in low-Ca^{2+} solution, there is a negative surface potential on the outer side of the NMDA channel. When $[Ca^{2+}]_o$ increases, this surface potential becomes less negative and may even become positive. This increases the potential barrier against which Cs^{2+} ions have to move, and therefore causes a shift to the right of the I–V relation, as seen in Fig. 8. The change of surface potential can be calculated from this shift. The elementary current at large positive potentials may be expressed as

$$I_{Cs} = \gamma (V - \Psi),$$

where γ is the NMDA channel conductance in Ca^{2+}-free solution, V the membrane potential and Ψ a 'correction' potential which increases when $[Ca^{2+}]_o$ increases. The value of Ψ for each value of $[Ca^{2+}]_o$ can be obtained by extrapolating to the abscissa the outward current asymptote (Fig. 9). The values of Ψ have been plotted in Fig. 9 in inverse co-ordinates. The approximately linear relation obtained in these co-ordinates suggests that Ψ is related to $[Ca^{2+}]_o$ by a hyperbolic relation

$$\Psi = \Psi_{max} \frac{[Ca^{2+}]_o}{K_{Ca} + [Ca^{2+}]_o},$$

with $\Psi_{max} = 68$ mV and $K_{Ca} = 57$ mM (K_{Ca}, equilibrium dissociation constant of Ca^{2+}).

As will be considered in the discussion, the presence of a large surface potential on the outer surface complicates the interpretation of the shift of the reversal potential produced by variations of $[Ca^{2+}]_o$. In low $[Ca^{2+}]_o$ the surface concentrations of the divalent cations can be expected to be much higher than the bath concentrations, while in high $[Ca^{2+}]_o$ the difference will be reduced. The relative fluxes of Na^+ and Ca^{2+} will therefore depend not only on the relative permeabilities of the two ions, but also on their concentrations at the entrance of the channel.

High-Ba^{2+} solutions

Given the evidence for the presence of a strong surface potential in low-Ca^{2+} solutions, it could be expected that other divalent cations (provided that they did not show the Mg^{2+} effects) would mimic some of the effects of Ca^{2+} ions. That this is the case is illustrated by the results of an experiment where 10 mM-Ba^{2+} was added to the '0 Ca^{2+}' solution (Fig. 10). The shift of the reversal potential to positive values

and the parallel shift of the outward I–V relation resemble the effects observed with the 10 mM-Ca^{2+} solution. However, it is clear that the effects observed at negative potentials cannot be deduced from those observed at positive potentials: while the effects of 10 mM-Ba^{2+} on the outward current are approximately equivalent to those of 15 mM-Ca^{2+}, in the inward current region the depression of the inward current corresponds to that produced by nearly 50 mM-Ca^{2+}. These results indicate that the

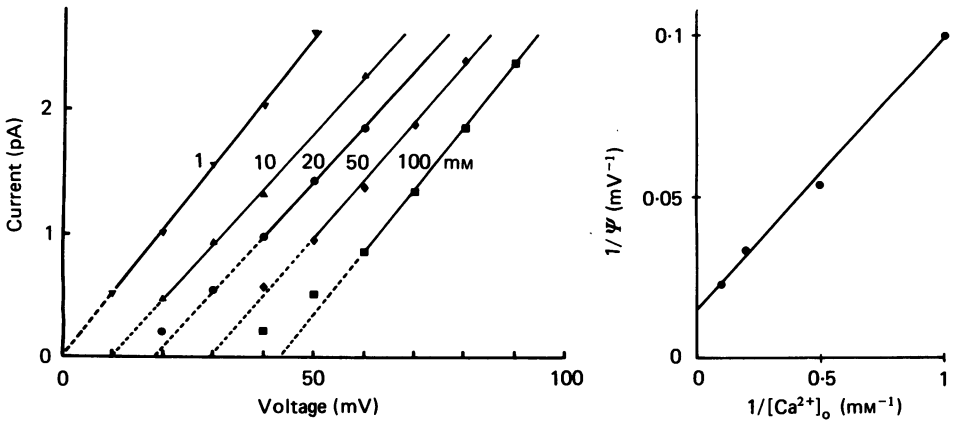

Fig. 9. Calculation of the changes of surface potential produced by altering the extracellular calcium concentration, $[Ca^{2+}]_o$. Left, data from Fig. 8 have been replotted to indicate how the upper limb of the current–voltage relation was extrapolated to the voltage axis to calculate the value of Ψ, the difference between the inner and outer surface potentials (see text). Right, $1/\Psi$ and $1/[Ca^{2+}]_o$ are linearly related. The maximum change of Ψ, deduced from the value of $1/\Psi$ extrapolated for $1/[Ca^{2+}]_o = 0$, is 68 mV. The intersection with the abscissa (not shown) gives $K_{Ca} = 57$ mM.

explanation of the effects from the Ca^{2+}–Ba^{2+} substitution must include not only the consequences of the surface potential change, but also changes in the relative permeability of the channel to Na^+, K^+, Ca^{2+} and Ba^{2+}, and probably interactions between these ions.

Reversal potential of quisqualate and kainate responses

The reversal potential of these responses in high-Ca^{2+} solutions have not been studied in as much detail as the NMDA responses, and only in the whole-cell recording mode. We think that it is worth mentioning, however, that when the external Ca^{2+} was increased from 1 to 100 mM, the reversal potential shifted from 0 mV (Nowak *et al.* 1984; Mayer & Westbrook, 1984; Ascher & Nowak, 1988) towards negative potentials. This shift is opposite to that observed for the NMDA response, but identical to that observed for the ACh response at the vertebrate neuromuscular junction (Katz & Miledi, 1969; Lewis, 1979; Adams, Dwyer & Hille, 1980) and for glutamate at arthropod neuromuscular junction (e.g. Jan & Jan, 1976). This suggests that the Ca^{2+} permeability of the quisqualate- and kainate-activated channels is smaller than that of the NMDA channel. It does not exclude the possibility that Ca^{2+} ions cross the quisqualate and/or kainate channels, as they cross

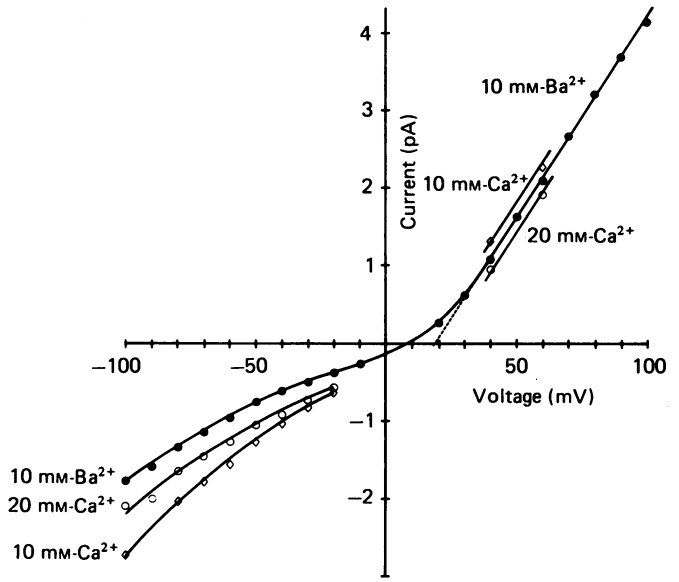

Fig. 10 Current–voltage relation of NMDA channels in Ba^{2+}-containing solutions. Outside-out patch. In the 10 mM-Ba^{2+} solution, containing 10 μM-Ba^{2+} and nominally 0 Ca^{2+}, the I–V relation of the NMDA channel was obtained with 10 μM-NMDA. The values of the currents in 10 mM-Ca^{2+} and 20 mM-Ca^{2+} are taken from Fig. 8, and correspond to mean values from nine experiments (10 mM-Ca^{2+}) and four experiments (20 mM-Ca^{2+}). In the experiment illustrated here for 10 mM-Ba^{2+}, the current at +40 mV was 1·08 pA, and −0·97 pA at −60 mV. In three other experiments the values obtained at +40 mV were 1·16, 1·0 and 0·98 pA; the values obtained at −60 mV were −1·12, −1·10 and −0·96 pA.

ACh channels or glutamate channels at neuromuscular junctions, but it predicts a smaller Ca^{2+} entry than during a NMDA response of comparable size, as indeed observed recently by MacDermott *et al.* (1986).

DISCUSSION

Our main experimental results can be summarized by three points: Mg^{2+} ions block the NMDA channel, Ca^{2+} ions permeate it, and to a first approximation divalent cations can be categorized as Mg^{2+} like (Co^{2+}, Mn^{2+}) or Ca^{2+} like (Ba^{2+}, Cd^{2+}). We shall discuss successively these three sets of results.

Block by Mg^{2+}

The simplest way to account for the Mg^{2+} block is to assume (Woodhull, 1973; Neher & Steinbach, 1978) that Mg^{2+} ions block the open channel at a site situated in the membrane electrical field. If one knows the voltage dependence of the equilibrium dissociation constant of Mg^{2+} block (K_{Mg}), it is possible to calculate the fraction (δ) of the membrane voltage at the blocking site and the value of K_o, the dissociation constant in the absence of a transmembrane voltage, by the relation

$$K_{Mg} = K_o \exp(\delta z F V / RT),$$

where z, F, R and T have their usual meaning.

From the data presented in the Results section (Fig. 4) one calculates $K_0 = 8{\cdot}8 \times 10^{-3}$ M and $\delta = 1$. This places the Mg^{2+} binding site on the cytoplasmic side of the channel.

Two pieces of evidence, however, indicate that the Mg^{2+} block cannot be described by such a simple picture. The first concerns the voltage dependence of the unidirectional rate constants of Mg^{2+} block, the second the dependence of the burst duration on the Mg^{2+} concentration.

Voltage dependence of the Mg^{2+} block. The blocking and unblocking rates of Mg^{2+} block have opposite voltage dependencies (as predicted by the simple theory of channel block), but the blocking rate is nearly three times more sensitive to membrane potential (e-fold increase for 17 mV hyperpolarization) than the unblocking rate (e-fold decrease for 47 mV hyperpolarization). This difference was not observed in the classical cases studied by Neher & Steinbach (1978), Sine & Steinbach (1984) and French, Worley & Krueger (1984), but there have been other reports of very asymmetrical voltage dependencies of the blocking and unblocking rates, e.g. by Yamamoto & Yeh (1984) in the study of blockage of the Na^+ channels by aminoacridine, and by Lansman, Hess & Tsien (1986) in the study of the blockage of Ca^{2+} channels by various divalent cations. This last study is particularly interesting because it includes an analysis of a blocking effect of Mg^{2+} ions which resembles the effect of Mg^{2+} on NMDA channels, even though it occurs at much higher concentrations (appreciable block is detected only at $[Mg^{2+}]_o$ higher than 1 mM).

As indicated by Lansman *et al.* (1986), the asymmetry can be explained by assuming that Mg^{2+} ions are both 'blockers' and 'permeators'. In such a case, hyperpolarization will have two opposing effects on the unblocking rate. The effect expected if Mg^{2+} ions are blockers is a reduction of the probability that Mg^{2+} ions leave their binding site. The effect expected if Mg^{2+} ions are permeant is an increase of the probability that they leave their binding site to be swept through the open channel. The combination of the two effects can reduce (NMDA channels) or abolish (Ca^{2+} channels) the voltage dependence of the Mg^{2+} unblocking.

This hypothesis, however, does not explain the very high voltage dependence of the blocking rate (e-fold for 17 mV). If one assumed for the access of Mg^{2+} to its binding site an outer barrier with a peak situated at half-distance between the outer surface and the binding site, the voltage dependence of the forward rate could not be larger than e-fold for 25 mV. A possible explanation of the high voltage dependence is that other ions, for example Ca^{2+}, interact with Mg^{2+} by repulsion effects. This could increase the voltage sensitivity of the rate of binding of Mg^{2+} as well as reduce the voltage sensitivity of the unbinding of Mg^{2+}.

Mayer & Westbrook (1987*a*, *b*) have presented data suggesting that when the extracellular Ca^{2+} concentration is increased, one can observe a relief of Mg^{2+} block at very negative potentials. We have not analysed single-channel currents in the conditions where such an effect occurs, but the observation agrees with the hypothesis of a Ca^{2+}–Mg^{2+} interaction.

Mg^{2+} concentration and burst duration. The simple open-channel block scheme (Neher & Steinbach, 1978) predicts that increasing $[Mg^{2+}]_o$ should increase the burst duration, whereas the experimental results indicate no increase and even a decrease

at high Mg^{2+} concentrations. To account for this possibility we have suggested (Nowak *et al.* 1984) that Mg^{2+} produces, in addition to the 'fast channel block', a slower, long-lasting block (see, e.g. Ascher, Marty & Neild, 1978). Such a block could produce an apparent shortening of $\bar{t}_b$, inasmuch as the apparent end of the burst would in fact correspond to the entry of the channel into a long-lived blocked state. This scheme could explain the observed decrease in opening frequency, since the channels in the long-lived blocked state would not be available for opening. However, the study of Neher (1983) of the effects of high concentrations of QX 222 on ACh-activated channels suggests another possibility, in which the reduction of $\bar{t}_b$ would not be an apparent reduction but a true one, and could be explained by assuming that blocked channels can close through pathways which short cut the open state.

The evaluation of these schemes has not been pursued, mostly because accurate evaluation of bursts durations required long and stable recordings which we were not able to obtain. A possible reason of the variability observed was recently uncovered when it was found (Johnson & Ascher, 1987) that variations of the speed of perfusion of the bath could produce significant variations in the responses to NMDA through variations of the concentration of glycine released by the cultured cells. Experiments with controlled glycine concentrations should allow a reinvestigation of this problem.

Ca^{2+} permeability and Ca^{2+} conductance

The presence of an inward current at negative potentials in 100 mM-Ca^{2+} clearly indicates that Ca^{2+} can permeate the NMDA channel. The quantitative evaluation of the Ca^{2+} permeability or of the Ca^{2+} conductance is, however, complicated. We have attempted to describe the data by using the Goldman–Hodgkin–Katz (GHK) equations, but, as described in the Appendix, have found that they are inadequate. We have not tried any other model, but can state that a satisfactory solution will probably have to incorporate two features: a large surface potential and binding of permeant ions inside the channel.

The presence of a surface potential seems required to explain that the outward current–voltage relation shifts to the right when $[Ca^{2+}]_o$ is increased (Figs 7–9; Jahr & Stevens, 1987). As a consequence, it is likely that in physiological conditions Ca^{2+} ions are more concentrated at the entrance of the channel than in the bulk solution.

Binding of Ca^{2+} ions inside the channel could explain the apparent contradiction between the shift of the reversal potential in high Ca^{2+} (which suggests that the Ca^{2+} permeability is higher than the Na^+ permeability) and the fact that the NMDA-induced current at negative potentials is smaller in isotonic Ca^{2+} than in isotonic Na^+ (which suggests that the Ca^{2+} conductance is smaller than the Na^+ conductance). A similar situation was encountered by Hess, Lansman & Tsien (1986) who compared the Ca^{2+} currents of heart cells in control solutions and in isotonic Ca^{2+} solutions. The reversal potential measurements suggested that Ca^{2+} is more permeable than Cs^+, but the single-channel conductance was larger in Na^+ than in Ca^{2+}. It is tempting to interpret these observations, as well as those that we have made on NMDA currents, by assuming that permeation involves binding of the permeant cations, and that this

binding is stronger for the divalent than for the monovalent cations. When reversal potentials are measured in biionic conditions, the ions which bind more tightly will appear to have a higher permeability coefficient. In single-channel current measurements, on the other hand, the ions which bind more tightly will give rise to a smaller single-channel conductance (see also Hagiwara, Toyama & Hayashi, 1971; Hamill, Bormann & Sakmann, 1983).

Permeant and impermeant cations

Ault *et al.* (1980) noted that the depressant actions of Mg^{2+} on the NMDA depolarizations were mimicked by Mn^{2+}, Co^{2+} and Ni^{2+}. Mn^{2+} was somewhat less potent than Mg^{2+}; Co^{2+} and Ni^{2+} were more potent than Mg^{2+}. On the other hand, Ca^{2+}, Sr^{2+} and Ba^{2+} had only very weak Mg^{2+}-like actions.

The results of the present paper, as well as those of Mayer *et al.* (1984), confirm the distinction between two families of divalent cations (those which appear to block the NMDA response, those which do not) and add Cd^{2+} to the family of 'non-blocking' divalent cations. This is an important addition since the two categories presented by Ault *et al.* (1980) could also fit the distinction between the divalent cations which block the Ca^{2+} channel and those which do not. The fact that Cd^{2+} does not block the NMDA channel indicates that the NMDA channel is fundamentally different from the Ca^{2+} channel.

The NMDA channel is also completely different from most of the cationic channels having a reversal potential close to 0 mV. Most of these channels are permeable to Na^+, K^+ and Ca^{2+} but also to Mg^{2+} (see e.g. Katz & Miledi, 1969; Jan & Jan, 1976; Adams *et al.* 1980; Miledi, Parker & Schalow, 1980; Eusebi, Miledi, Parker & Stinnakre, 1985). An exception to this 'non-specific cationic selectivity' is the channel closed by light to photoreceptors which seems impermeant to both Ca^{2+} and Mg^{2+} (Haynes, Kay & Wau, 1986).

The NMDA channel thus appears to occupy a unique position among cationic channels, distinct from those selective for monovalent cations as well as from those which accept both monovalent and divalent cations. For the NMDA channel, the selectivity appears to cut between Mg^{2+}-like cations and Ca^{2+}-like cations. Recently, Nelson, Worley, Rogers & Lederer (1985) have described a cationic channel from heart muscle which appears to be blocked by Mg^{2+} in a voltage-dependent way, and seems insensitive to Ca^{2+} and Ba^{2+}. This channel is at this time the only example of a channel resembling the NMDA channel.

A clue to the difference between permeant and blocking ions may be found in the measurements (Diebler, Eigen, Ilgenfritz, Maas & Winkler, 1969) of the speed of exchange of the water molecules surrounding cations in solutions. These studies show a difference of three orders of magnitude between two families, one comprising Ba^{2+}, Ca^{2+}, Sr^{2+} and Cd^{2+}, the other Co^{2+}, Mg^{2+} and Ni^{2+}. As noted by Hille (1984): 'the slow replacement of waters around ions such as Ni^{2+}, Co^{2+}, Mg^{2+} could be a major factor reducing the permeability of such ions in the smallest ionic channels'. Thus, the NMDA 'channel blockers' may be able to cross the NMDA channel, but only after dehydration. Their blocking effect would be the consequence of the fact that they take more time to dehydrate than the 'permeant' divalent cations.

Functionally, it is unlikely that the passage of Mg^{2+} ions through the NMDA channel has a great physiological importance. The key action of Mg^{2+} is likely to be

its blocking role, which confers to the NMDA system its voltage sensitivity. In contrast, the direct evaluation of Ca^{2+} entry through the NMDA channel appears essential both for the understanding of permeation through the channel and for the evaluation of the function of the NMDA receptor.

APPENDIX

At first glance it is tempting to use the constant field hypothesis to describe the I–V relation of NMDA currents because it is one of the few simple theories which predicts that the I–V relation in high Ca^{2+} should have two linear asymptotes, as indicated by our experimental data. The GHK equations also allow one to deduce from reversal potential measurements the ratio of two ionic permeabilities in the conditions of a biionic potential. The situation in the 100 mM-Ca^{2+} solution corresponds approximately to such conditions, where it can be considered that the internal solution contains only one permeant cation, Cs^+ ($[Cs^+]_i = 140$ mM), and the external solution only one permeant cation, Ca^{2+} ($[Ca^{2+}]_o = 100$ mM).

If one could use the GHK equation directly the ratio of the relative permeabilities to Ca^{2+} and Cs^+, P_{Ca} and P_{Cs} would be given by (Jan & Jan, 1976; Lewis, 1979)

$$\frac{P_{Ca}}{P_{Cs}} = \frac{[Cs^+]_i}{4[Ca^{2+}]_o}\left(\exp\frac{V_r F}{RT}\right)\left(1+\exp\frac{V_r F}{RT}\right),$$

where V_r is the reversal potential of the NMDA current. $V_r = 30$ mV leads to $P_{Ca}/P_{Cs} = 5$. From the fact that in 1 mM-Ca^{2+} the reversal potential is 0 one can deduce that $P_{Na}/P_{Cs} = 1$ and therefore that $P_{Ca}/P_{Na} = 5$. This value should then be corrected (to 7·5) to account for the fact that the activity coefficient in the 100 mM-Ca^{2+} solution is only two-thirds of that in the 1 mM-Ca^{2+} (140 mM-Na^+) solution.

However, we cannot use the GHK equations in this simple form, since we know that there exists a significant surface potential in low-Ca^{2+} solutions. GHK equations can be modified to account for surface potentials (see e.g. Frankenhaeuser, 1960; Lewis, 1979). In order to do this, one has to assume both an internal (Ψ_i) and an external (Ψ_o) surface potential, and to replace in the 'current' equations the terms $[Cs^+]_i$ and $[Ca^{2+}]_o$ by terms representing the surface concentrations of Cs^+ and Ca^{2+}, $[Cs^+]_{is}$ and $[Ca^{2+}]_{os}$, with

$$[Cs^+]_{is} = [Cs^+]_i \exp(-\Psi_i F/RT),$$

$$[Ca^{2+}]_{os} = [Ca^{2+}]_o \exp(-\Psi_o F/RT,$$

Replacing $[Cs^+]_i$ and $[Ca^{2+}]_o$ by $[Cs^+]_{is}$ and $[Ca^{2+}]_{os}$ leads to expressions which define both the reversal potential and the limiting slopes of the I–V relation as a function of Ψ_o, Ψ_i, $[Ca^{2+}]_{os}$ and $[Cs^+]_{is}$. None of these relations can be solved without a separate knowledge of Ψ_o and Ψ_i which cannot be obtained from our data. However the relations can be combined to define a term Λ such that

$$\Lambda = \frac{4P_{Ca}[Ca^{2+}]_{os}}{P_{Cs}[Cs^+]_{is}},$$

and Λ can be expressed as a function of U, defined by

$$U = (F/RT)(V - \Psi_o + \Psi_i).$$

The term Λ can be calculated in two ways from the experimental data. It is the ratio of the limiting slopes of the I–V relation since, when V becomes large, the slope of the I–V relation tends towards $F P_{Cs} [Cs^+]_{is} U$ for the outward current, and towards $4 F P_{Ca} [Ca^{2+}]_{os} U$ for the Ca^{2+} inward current. But Λ can also be calculated from the reversal potential V_r by the relation

$$\Lambda = \exp(U_r)(1 + \exp(U_r)),$$

where $U_r = F(V_r - \Psi_o + \Psi_i)/RT$.

From the data of Fig. 8 one can calculate the two values of Λ. The value obtained from the ratio of the limiting slopes is 0·3, while that obtained from the reversal potential is 1·1. The difference between these two Λ values appears quite large and indicates that the insertion of surface potential corrections in the GHK equations is not sufficient to fit the experimental data in a coherent way.

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