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ACTIVATION AND DESENSITIZATION OF *N*-METHYL-D-ASPARTATE RECEPTORS IN NUCLEATED OUTSIDE-OUT PATCHES FROM MOUSE NEURONES

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SUMMARY

1. Activation and desensitization of *N*-methyl-D-aspartate (NMDA) receptors were studied in large outside-out patches excised from cultured embryonic neurones dissociated from mouse forebrain. The patches were exposed to rapid changes of NMDA or L-glutamate concentrations in the presence of glycine at concentrations (10–20 μM) saturating the modulatory site of the NMDA receptor.

2. Immediately after formation of the patch the responses to NMDA and L-glutamate showed a slow and small desensitization, even with high concentrations of agonist. During the following hour, the peak response either decreased or remained relatively stable, but in all cases the desensitization increased and accelerated until it stabilized. In this 'stabilized' state, the desensitization produced by high concentrations of NMDA (1 mM) or L-glutamate (300 μM) had an exponential time course, with a time constant of about 30 ms. The ratio of the peak over the steady-state current was in the order of 40 for NMDA and about 30 for L-glutamate.

3. Concentration–response curves were built for the peak and the plateau responses, for NMDA and for L-glutamate. The comparison of these curves indicated that (i) the EC_{50} of the peak (K_{app}) was always higher than the EC_{50} of the plateau (K_{ss}); (ii) the two EC_{50} values for NMDA (K_{app} and K_{ss}) were higher than those for L-glutamate; (iii) the Hill coefficient was close to 1·4 for each of the four curves.

4. The application of NMDA or L-glutamate at a low concentration for 3 s periods reduced the response to a subsequent application of the same agonist at a saturating concentration. The IC_{50} of this 'predesensitization', termed K_{pre}, was lower than the EC_{50} of the steady-state response, K_{ss}.

5. The onset rates of desensitization increased with the concentration of agonist. The EC_{50} of this relation was close to the value of K_{app}.

6. The decay of the currents at the end of a 3 s application of agonist was usually

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well described by the sum of two exponentials both of which were faster for NMDA than for L-glutamate.

7. The recovery from desensitization after a long (3 s) pulse of agonist was approximately exponential, with a time constant of about 0·5 s for NMDA and about 3·5 s for L-glutamate.

8. The results can be interpreted by using a model similar to those used for the nicotinic receptor assuming that (i) the NMDA receptor exists, in the absence of agonist, in both an activatable (R) and two desensitized (D) states; (ii) the R and D states have two equivalent binding sites for NMDA agonists; (iii) the affinity of the R state for the agonist is lower than that of the D states. However, in contrast with the hypotheses made for the nicotinic receptor, it is not possible to assume that the reactions leading to the opening of the channel through activation of the R states are much faster than the reactions connecting the R and D states.

9. The differences between L-glutamate and NMDA responses suggest that L-glutamate differs from NMDA by a higher affinity for the various receptor states as well as by a higher efficacy.

10. The evolution of the NMDA receptor behaviour between the moment at which the patch is formed and the time at which it reaches a stable state suggests that the progressive deepening of desensitization is due to an acceleration of the transition between the doubly liganded R state and the doubly liganded D states. In contrast, both the equilibrium between the non-liganded R and D states and the kinetics of the activation pathway appear relatively stable over time.

INTRODUCTION

In recent years it has become apparent that in the vertebrate CNS most excitatory glutamatergic synapses involve the activation of both NMDA and non-NMDA receptors. The synaptic currents mediated by NMDA receptors are slower than those mediated by non-NMDA receptors and despite recent advances (Lester, Clements, Westbrook & Jahr, 1990; Hestrin, Sah & Nicoll, 1990; Gibb & Colquhoun, 1991) the interpretation of these slow kinetics remains incomplete, in particular because the analysis of single-channel behaviour as a function of agonist concentration has not reached a level of sophistication comparable to that obtained in the case of the nicotinic receptor (see Adams, 1987; Dilger & Brett, 1990). In an effort to contribute to the development of this approach for the NMDA receptor, we have analysed the responses to step concentration changes of NMDA and L-glutamate of non-conventional outside-out patches, that we have called 'nucleated patches' (Sather, MacDonald & Ascher, 1990*b*). These patches provide a larger number of channels than a typical outside-out patch, and thus reduce greatly the amount of averaging of successive responses required to measure the mean amplitude and the time course of the responses. We have attempted to characterize both the activation and the desensitization of the responses elicited by NMDA and by L-glutamate on these patches, and to account for the data by a kinetic model.

This attempt has been only partly successful due to the fact that the responses recorded from nucleated patches, like those recorded in the whole-cell mode and in conventional outside-out patches, change with time. The most marked effect concerns desensitization, which, in nucleated patches, was both accelerated and

enhanced during the hour following the formation of the patch. This progressive evolution complicated both the analysis and the interpretation of the data. Nevertheless we have been able to characterize in some detail the responses obtained after the patch has 'stabilized' (a process which requires a time varying from a few minutes to half an hour) and to propose kinetic models to describe the responses. Furthermore, taking advantage of the regularity of the evolution of the responses after the formation of the patch, we can infer some of the kinetic properties of the NMDA receptor prior to the excision of the membrane, and identify among the kinetic parameters those which appear stable and those which evolve during the transformation of the receptor.

METHODS

All the data were obtained from nucleated patches. Cortical and diencephalic neurones taken from 15- to 16-day-old mouse embryos were cultured as previously described by Ascher, Bregestovski & Nowak (1988) for 2–5 weeks. Embryos were obtained from mice killed by cervical dislocation. The principal steps in making nucleated patches were, as for conventional outside-out patches, to obtain the whole-cell recording configuration and then to excise the patch by carefully withdrawing the patch pipette from the cell (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The major difference in making nucleated patches was the application of suction through the patch pipette during the period of pipette withdrawal. The effect of the suction was to pull the cell nucleus towards the pipette tip so that, as the pipette was slowly withdrawn, the nucleus was gradually extracted from the cell. The complete separation of the nucleated patch from the parent cell took usually 1 or 2 min. Outside-out patches formed in this way (enclosing the cell nucleus) were roughly spherical in shape and 5–8 μm in diameter. Relative to conventional outside-out patches, nucleated patches possessed both larger agonist responses, presumably due to their relatively larger surface, and substantially greater durability, most likely attributable to the structural support provided by the enclosed nucleus. Thus maximum peak responses recorded from nucleated patches were often in the order of 100–300 pA (although the range was very wide) and recordings typically lasted longer than 1 h (up to 4 h in the longest record).

Patch pipettes, which had resistances of 5–8 MΩ, were coated along their shanks with beeswax to reduce pipette capacitance, and filled, in most cases, with a solution containing (mM): 84 CsF, 7 CsCl, 7 EGTA and 7 HEPES; CsOH was used to adjust the pH to 7·2 (CsF solution). In some experiments patch pipettes were filled with a solution containing (mM): 98 KCl, 7 EGTA, and 7 HEPES; KOH was used to adjust the pH to 7·2 (KCl solution). No differences in NMDA receptor properties were observed between recordings obtained with one or the other of these two pipette solutions; however, the longevity of recordings was increased with the CsF solution. These pipette solutions, 30% hypo-osmotic relative to solutions bathing the external surface, were preferred to equiosmotic pipette solutions which caused nucleated patches to swell and develop leak currents.

During recordings, patches were bathed in a Mg^{2+}-free solution containing (mM): 140 NaCl, 2·8 KCl, 1 $CaCl_2$, and 10 HEPES; NaOH was used to adjust the pH to 7·2. Drugs were applied to the patch by means of a 3- or 4-barrel fast-perfusion system (Johnson & Ascher, 1987; Sather, Johnson, Henderson & Ascher, 1990*a*; J. W. Johnson & P. Ascher, in preparation). A saturating concentration of glycine (10 μM-glycine in experiments with NMDA; usually 20 μM-glycine and 2 μM-CNQX in experiments with L-glutamate) was added to all fast-perfusion solutions which, with the exception of other added drugs, were identical to the bathing solution. Nucleated patches were placed directly in front of the glass perfusion barrels, which were square in cross-section and 500 μm wide. Solutions continuously flowed by gravity from all barrels in the assembly and solution changes were accomplished by lateral, stepwise displacements of the perfusion barrels so as to exchange the barrel facing the patch for another one. With this system, the composition of the solution flowing past the patch could be controlled before, during and after application of a drug. The speed with which solution changes could be accomplished was estimated by measuring the speed of block by Mg^{2+} of NMDA-activated current. In Fig. 1, a single exponential function with a time constant of 2 ms described well the block produced by a sub-saturating concentration of Mg^{2+} (160 μM). Thus, the time constant obtained for the onset of the response to a sub-saturating concentration of NMDA (50 μM, plus 10 μM-glycine) (10 ms) was not affected by external solution

exchange times. These exchange times, however, probably interfered with the onset of the responses produced by high concentrations of NMDA (1 mM) or L-glutamate (300 μM). For this reason, the peak responses were calculated by extrapolating to $t = 0$ the decay of the currents as described below.

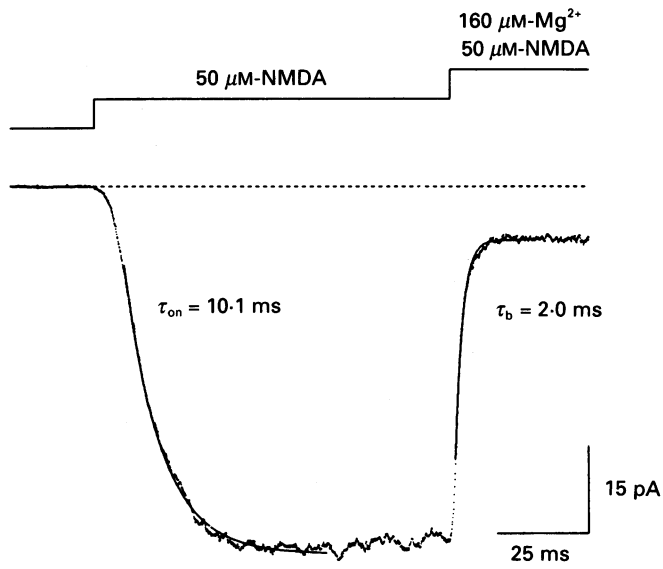


Fig. 1. Adequacy of the speed of exchange of solutions bathing a nucleated patch determined by comparison of the rate of onset of NMDA-activated current with its rate of block by Mg^{2+}. The data trace (dotted) represents the ensemble average of 120 individual responses, which were filtered at 3 kHz and sampled at 10 kHz. Sub-saturating concentrations of NMDA (50 μM) and Mg^{2+} (160 μM) were used so that the kinetics of changes in patch current resulting from application of these agents reflected a combination of only the speed of solution exchange and activation or block kinetics. Smooth curves drawn through the data represent single exponential functions fitted to the onset of the response to NMDA (τ_{on} = 10·1 ms) and to the block by Mg^{2+} (τ_b = 2·0 ms). The data were obtained shortly after break-in. Between the applications of NMDA, the patch was continuously bathed in 0 Mg^{2+} and 10 μM-glycine, flowing from the 'control' barrel. The holding potential was −50 mV and the pipette was filled with the CsF solution.

Nucleated patches were voltage clamped at −50 mV using a List EPC-7 patch-clamp amplifier. The voltage-clamp current was usually filtered at one-half the sampling rate, digitized and later analysed using the Strathclyde Electrophysiology Software. Ten to fifty records were usually used to generate average responses. Figure 2 shows an example of this procedure. On the left is shown an individual response to 1 mM-NMDA; during the steady, desensitized period of the response, single-channel events can be distinguished. On the right of the figure is shown the average response to 1 mM-NMDA. Superimposed on the average response is a fitted single exponential function. The 'observed peak current' was measured directly as the average peak current while the 'extrapolated peak current' was measured by extrapolating the fitted exponential function to time zero, i.e. the time of response onset. The 'steady-state current' was calculated by extrapolating the function to $t = \infty$. Fits of curves to the data were generated using an error-minimization routine (Levenberg–Marquardt algorithm). Error bars shown in the figures represent standard error of the mean.

The data used to construct concentration–response curves for NMDA or L-glutamate were recorded when the responses had reached an approximately stable shape. Nevertheless, each new agonist concentration was tested alternately with a reference concentration, allowing for correction, by linear extrapolation, when some rundown of the response occurred. The term rundown will be

used below to characterize a condition in which the response peak decreased, but in which there was no change in the characteristics of desensitization (ratio of the peak current over the steady-state current; time constant of the decay). The concentration–response curves were fitted with the Hill equation in the form

$$\frac{I}{I_{max}} = \frac{[A]^{n_H}}{[A]^{n_H}+[K]^{n_H}},$$

where I is the measured current, I_{max} the maximal current, [A] the agonist concentration, K the apparent dissociation constant and n_H the Hill coefficient.

The experiments were performed at room temperature. *N*-Methyl-D-aspartic acid (NMDA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonovaleric acid (APV) were obtained from Tocris or Cambridge Research Biochemicals; L-glutamate and glycine were obtained from Prolabo.

RESULTS

Characterization of the responses to NMDA and to L-glutamate

For nucleated patches pre-equilibrated in a saturating concentration of glycine, application of NMDA or L-glutamate elicited responses which desensitized (Fig. 2). As discussed below, this desensitization increased with time until it reached an approximately stable amplitude. To characterize the responses during their evolution as well as after stabilization, we took advantage of the fact that the decay of the current after its peak could be reasonably well described by a simple exponential function of the form

$$I(t) = (I_p - I_{ss}) \exp(-t/\tau_D) + I_{ss},$$

where $I(t)$ is the current, I_p the extrapolated peak current, I_{ss} the steady-state current and τ_D the time constant of desensitization.

In many cases a better fit of the decay of the response required two exponentials, and furthermore the rising phase of the responses (in particular for L-glutamate) was often not negligible relative to the duration of the decay. A more accurate fit of the responses would require fitting both the onset and the decay of the response (Clements & Westbrook, 1991). However, because of the marked variability of the responses and because our solution exchange system did not allow us to characterize accurately the exact moment of the concentration change, we did not attempt such an elaborate fit. Nearly all the results discussed below will be presented assuming that, to a first approximation, desensitization is well described by a single exponential process, and that, at saturating concentrations of agonist, the extrapolated peak, I_p, gives a reliable indication of the number of activatable receptors.

When L-glutamate was used as an agonist, it was necessary to identify the NMDA receptor-mediated component in the total response to L-glutamate. Although the dissociation constant of L-glutamate is about two orders of magnitude lower for NMDA receptors (in the micromolar range) than for non-NMDA receptors (100 μM range) (Verdoorn & Dingledine, 1988; Trussell & Fischbach, 1989; Patneau & Mayer, 1990), the presence of the latter receptors in our patches could not be neglected when examining the responses to high concentrations of L-glutamate. Figure 3*A* illustrates this problem. The records presented were recorded before the time-dependent changes in NMDA receptor kinetics had progressed very far, allowing a clear distinction between the rapidly desensitizing non-NMDA component and the slowly desensitizing NMDA component. The slow component of the response to 100 μM-L-

glutamate was similar to that of the 50 μM response, but the peak response was much larger with 100 μM- than with 50 μM-L-glutamate. The trace on the right, produced by subtracting the response to 50 μM-L-glutamate from the response to 100 μM-L-glutamate, primarily represents the non-NMDA receptor-activated current.

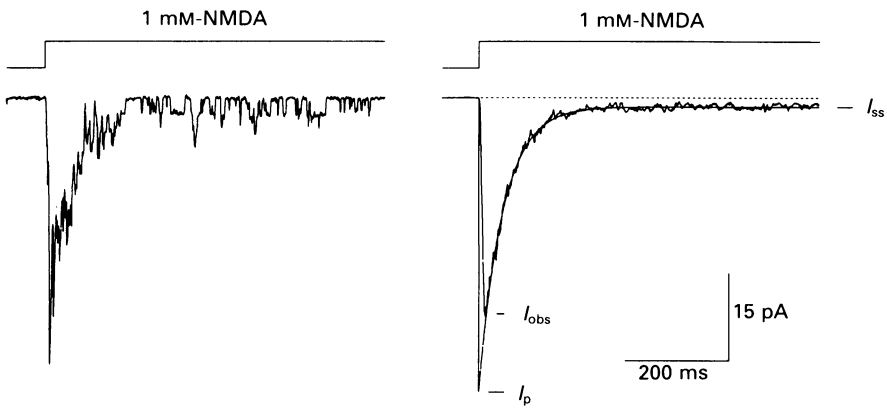


Fig. 2. Method of analysis of agonist-activated currents recorded from nucleated patches. An example of the patch current activated by a single application of 1 mM-NMDA is shown on the left, and the ensemble average patch current, composed of responses to twenty-two similar applications of NMDA, is shown on the right. The straight lines at the top of the figure indicate the timing of NMDA applications. The small size of the NMDA-activated current recorded from this patch allowed single-channel current to be resolved in the individual record once the steady, desensitized response level was reached. Superimposed on the ensemble average current is a single exponential function (smooth curve) fitted to the decay phase of the response. The peak current (I_p) obtained by extrapolating the fitted exponential to the time of response onset was -50 pA, and the observed peak current (I_{obs}) was -37 pA. The time constant of desensitization was 45 ms and the steady, desensitized current level (I_{ss}) was $-1{\cdot}6$ pA. The control and the NMDA solution both contained 10 μM-glycine. The holding potential was -50 mV and the patch pipette contained the CsF solution. The data on the left and right were obtained from the same patch, were filtered at 1 kHz and were sampled at 2 kHz.

To eliminate the non-NMDA components of the L-glutamate responses, we used in most cases CNQX (2 μM) and raised the glycine concentration from the usual 10 μM to 20 μM in order to overcome the blocking effect of CNQX at the glycine binding site on the NMDA receptor (Birch, Grossman & Hayes, 1988; see Thomson, 1990). Addition of 1 mM-$MgCl_2$ and 50 μM-APV to this solution (to block NMDA components) showed that no significant non-NMDA response remained. It could be argued, however, that these pharmacological treatments could alter activation and desensitization. Therefore in some experiments we evaluated the contribution of the non-NMDA component to the total response by eliminating the NMDA component. For this purpose (Fig. 3*B*) we used a solution containing 1 mM-$MgCl_2$ and 50 μM-APV and lacking added glycine. The non-NMDA L-glutamate responses (records on the right) could be subtracted from the total responses (records on the left) to yield the NMDA component (not shown). Unlike the case on the left, on the right the peak of the responses increased sharply when the dose of L-glutamate was increased from 200 to 500 μM, consistent with its identification as non-NMDA receptor-activated current. In part *C* of Fig. 3, the bottom two records of part *B* (responses to 500 μM-

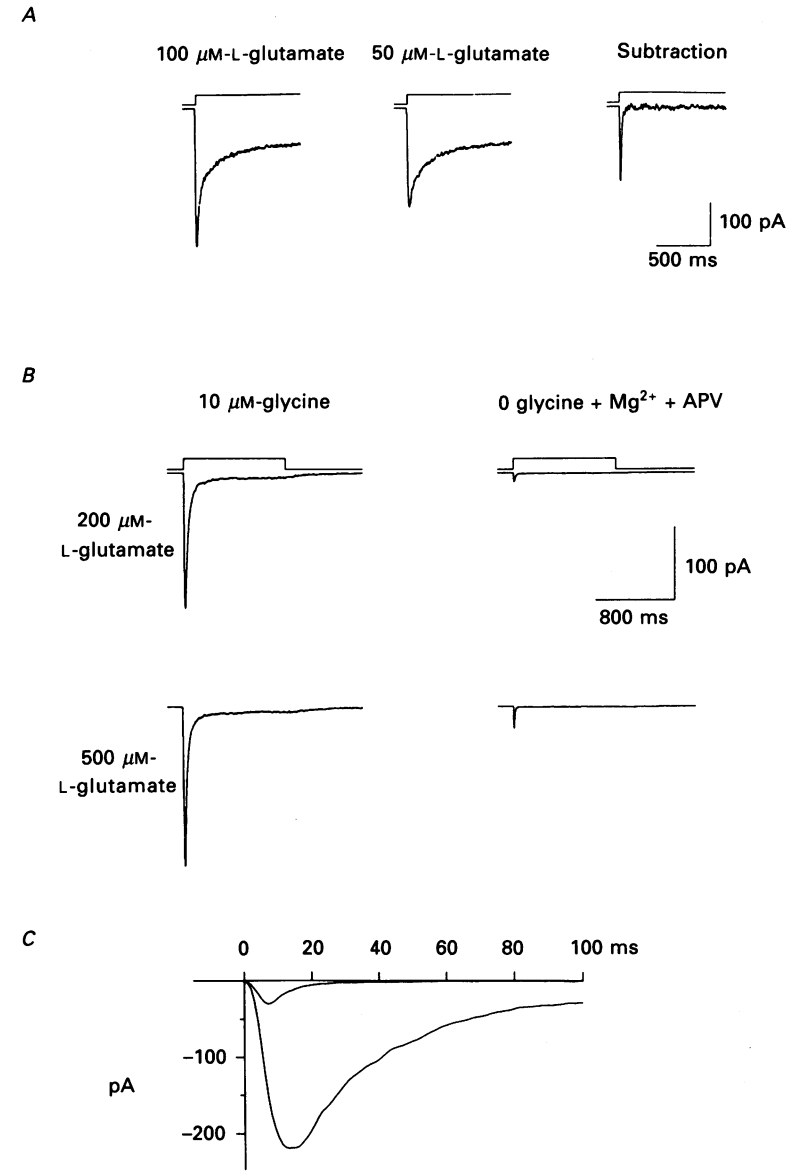


Fig. 3. Measurement of NMDA receptor activation by L-glutamate. *A*, high concentrations of L-glutamate produced responses consisting of two components. The traces illustrated were obtained before the NMDA desensitization had reached its stable state, allowing clear visual identification of the slow NMDA desensitization and the fast non-NMDA one. Subtraction of the 50 μM response from the 100 μM response yielded the rapidly desensitizing component (τ_D = 13 ms) shown on the right. The glycine concentration was 10 μM. *B*, responses to the indicated concentrations of L-glutamate (left) and to the application of the same concentrations of L-glutamate in solutions containing 1 mM-$MgCl_2$, 50 μM-APV and no added glycine (right). *C*, superimposition of the total response to 500 μM-L-glutamate and of the non-NMDA receptor-mediated response to 500 μM-L-glutamate recorded from the same patch (same data as in the lower pair of traces in *B*). *A–C*, patch pipette contained the CsF solution. *B* and *C* were recorded from a different patch than *A*.

L-glutamate) have been superimposed to compare their amplitudes as a function of time. The non-NMDA current desensitized so rapidly and so completely that its presence had little effect on the parameters of the NMDA response (desensitization time constant, extrapolated peak current, steady current).

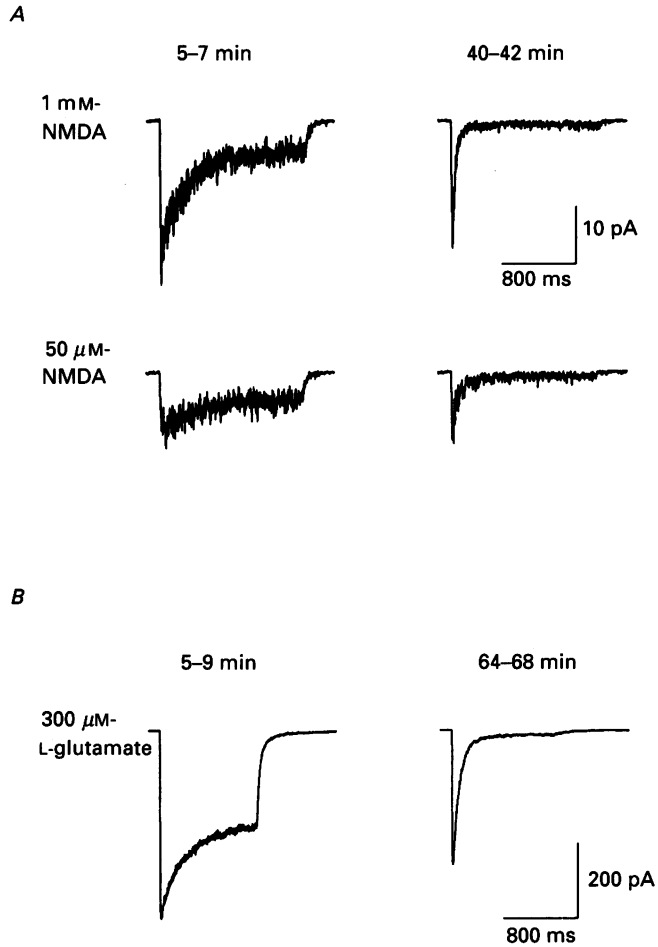


Fig. 4. Comparison of NMDA receptor-mediated currents soon after the break-in and later in the experiment. The records shown in both *A* and *B* represent the average of eight to ten individual responses obtained during the indicated time periods. Time zero was taken as the start of the whole-cell recording, i.e. the time when the cytoplasmic membrane surface was first exposed to the pipette solution. No data were obtained during the first 5 min of these recordings because the nucleated patches were being formed during this time. *A*, responses elicited with either 1 mM- (top) or 50 μM- (bottom) NMDA. Both the control and the NMDA solutions contained 10 μM-glycine. Data of all four traces were recorded from the same patch, with each application alternating between 1 mM- and 50 μM-NMDA. The pipette contained the KCl solution. *B*, responses elicited with 300 μM-L-glutamate. The control and the test solutions contained 20 μM-glycine and 2 μM-CNQX, and the pipette contained the CsF solution. The holding potential was −50 mV in both *A* and *B*. *A* and *B* were from different patches.

Time-dependent change of the responses

Desensitization was apparent in the first responses recorded after obtaining a nucleated patch. However the recording was usually started 1–5 min after the breakthrough into the whole-cell recording configuration, so that, given the

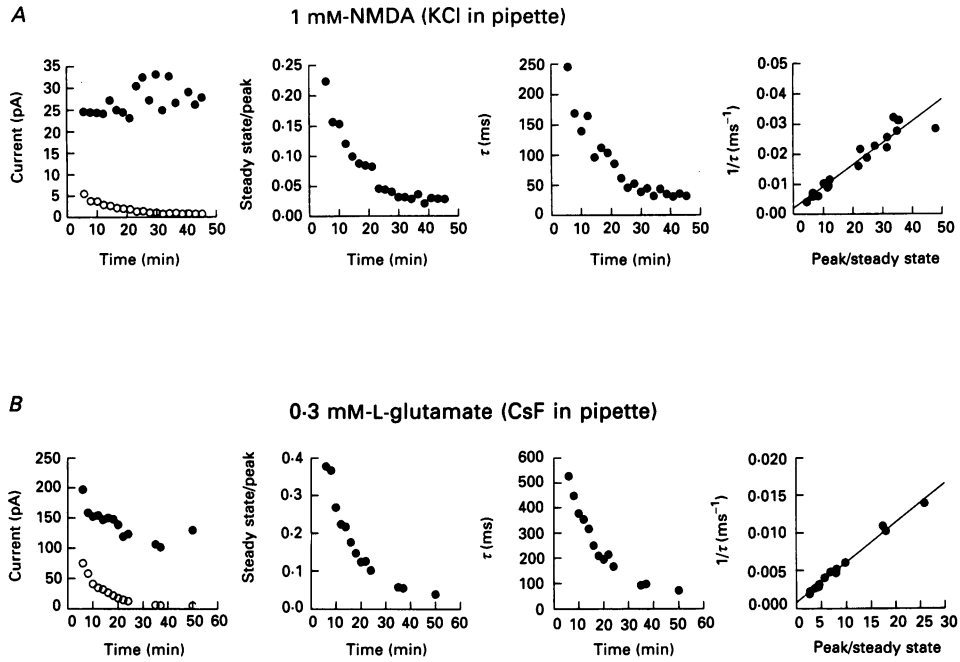


Fig. 5. Time course of changes in kinetic behaviour of NMDA receptor-mediated currents. *A*, 1 mM-NMDA. Control and test solutions contained 10 μM-glycine, and the pipette contained the KCl solution. *B*, 300 μM-L-glutamate. Control and test solutions contained 20 μM-glycine and 2 μM-CNQX, and the pipette contained the CsF solution. For both *A* and *B*, peak (●) and steady-state currents (○), steady-state/peak current ratios, and the time constants of desensitization were plotted against time in the first three columns. Time zero indicates the point at which the cell-attached patch was ruptured and whole-cell recording was initiated. During the first minutes following breakthrough into the whole-cell configuration, the process of making the nucleated patches interfered with data recording. The last column illustrates the linear correlation between the peak/steady-state current ratio and the inverse of the time constant of desensitization. For NMDA (upper graph) the slope of the line was 0·7 s^{-1}; for L-glutamate (lower graph) it was 0·5 s^{-1}.

progressive increase of desensitization described below, the presence of desensitization in the patch does not imply its presence in the intact cell (see Discussion). The initial degree of desensitization was highly variable but in all cases, with time (over a period lasting from a few minutes to half an hour), the responses evolved until they reached an approximately stable and highly consistent shape.

To characterize the evolution of desensitization, the values of I_p, I_{ss} and τ_D were plotted as a function of time after formation of the patch. Figures 4 and 5 illustrate examples of such an evolution, both for responses to NMDA recorded from patches dialysed with the KCl solution and for responses to L-glutamate recorded with the

CsF solution. In the case of Fig. 4*A* two concentrations of NMDA were alternately applied, and in both cases the speed and the extent of desensitization increased with time. In the case of Fig. 4*B* the desensitization underwent a similar but more pronounced change, because the initial response showed less desensitization. This difference was not linked with the use of CsF instead of KCl, but to the fact that the

TABLE 1. The peak responses to NMDA (1 mM) (I_p, NMDA) and to 300 μM-L-glutamate (I_p, L-glutamate) were measured in the first minutes after formation of the patch (A) and 30–40 min later, after stabilization of the shape of the responses (B) in six different patches

	I_p, NMDA		I_p, L-glutamate		I_p, L-glutamate/I_p, NMDA	
Expt	A	B	A	B	A	B
1	323	368	451	523	1·4	1·4
2	58	82	244	210	4·2	2·6
3	26	21	37	32	1·4	1·5
4	265	316	473	513	1·8	1·6
5	378	339	520	544	1·4	1·6
6	120	177	173	120	1·4	1·6
Mean	195	217	316	323	1·9	1·7

The results (expressed in pA) are presented in the first four columns. The mean values, tabulated in the last line, indicate that the peak response changed little with time. The last two columns indicate the ratio of the response to L-glutamate over the response to NMDA, both at the beginning (A) and at the end (B) of the analysis. The ratio was always larger than 1.

cell used, and its nucleus, were much larger, leading to larger currents but probably also to a slower exchange of the patch internal solution with the pipette solution.

Figure 5 illustrates graphically the evolution of the responses in two experiments similar to those from which the records of Fig. 4 were selected. In both patches the extrapolated peak current (I_p) fluctuated with time with a tendency to decrease in the second patch. In both patches, the steady-state (plateau) current (I_{ss}) decreased markedly and regularly over a period of about 30 min, until it reached an approximately steady value. As a result, the ratio of the steady-state over the peak current, I_s/I_p, decreased. In parallel, the time constant of the desensitization, τ_D, decreased, at approximately the same rate.

The behaviour illustrated in the two examples of Fig. 5 was typical inasmuch as the ratio I_{ss}/I_p and the time constant τ_D decreased progressively with time and they did so with a similar rate. However, this rate was variable: the change often occurred much more rapidly than in the examples illustrated, and the stabilized condition was sometimes obtained within a few minutes as is the case in classical outside-out patches. The behaviour of the peak current was also variable. In the first example of Fig. 5, I_p changed relatively little with time (see also Fig. 4*A*). This was often the case, but in many other substances, as in the second example of Fig. 5, I_p tended to decrease progressively, and in still others, it showed an initial increase during the first minutes after the formation of the patch before starting to decrease.

In a series of experiments we compared the response produced in the same patch by saturating concentrations of NMDA (1 mM) and L-glutamate (300 μM). We found a systematic difference between the two responses: the peak response to L-glutamate

was always larger than the peak response to NMDA. The results of these experiments are summarized in Table 1 which also illustrated that, on average, the peak current remained constant through the wash-out.

. *Analysis of peak and steady current concentration–response relations*

During the initial phase of recording, it was not possible to build concentration–response curves for the steady state because the rate of evolution in

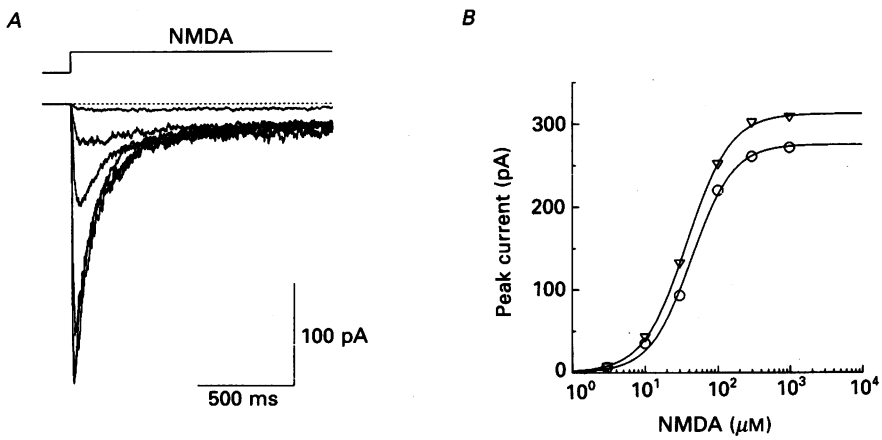


Fig. 6. Currents activated by application of various concentrations of NMDA. *A*, superimposed averaged responses. NMDA concentrations ranged from 3 to 1000 μM. All records were recorded from one patch after 30 min of dialysis. The glycine concentration was 10 μM. *B*, concentration–response plots constructed from the data in *A*. Both the observed (○) and extrapolated (▽) peak currents are shown. For the observed peak currents, the Hill coefficient of the fitted curve was 1·5 and the EC_{50} was 43 μM; the corresponding values for the fit to the extrapolated peak currents were 1·4 and 37 μM. CsF pipette solution.

response behaviour was too great relative to the amount of time needed to collect the ten to fifty individual responses used in generating mean responses. Once a quasi-stable condition of response behaviour was reached, however, reliable concentration–response relations could be constructed for individual patches. An example from such a patch of a family of superimposed mean responses elicited with a range of NMDA concentrations is shown in Fig. 6*A*. In Fig. 6*B*, both the observed (○) and extrapolated (▽) peak currents from part *A* have been plotted *versus* agonist concentration. There were only small differences between the Hill coefficients (n = 1·5 and 1·4, observed and extrapolated) and the EC_{50} values (43 and 37 μM, observed and extrapolated) obtained by fitting the Hill equation to the two curves. In the analyses described below, the concentration–response relations for peak currents activated by NMDA or L-glutamate were constructed using the extrapolated values. This had the advantage, in the case of L-glutamate, of eliminating the non-NMDA components even in the absence of CNQX (Fig. 3*C*).

The mean NMDA concentration–response relations for peak currents and steady

currents are shown in Fig. 7*A*. The Hill coefficients obtained for the steady and peak currents were very similar to one another, with values of 1·3 and 1·4, respectively. The steady currents can be seen to approach saturation at lower concentrations of NMDA than the peak currents. The EC_{50} values derived from the fitted curves in Fig. 7*A* were 5·7 and 57 μM for steady and peak currents, respectively.

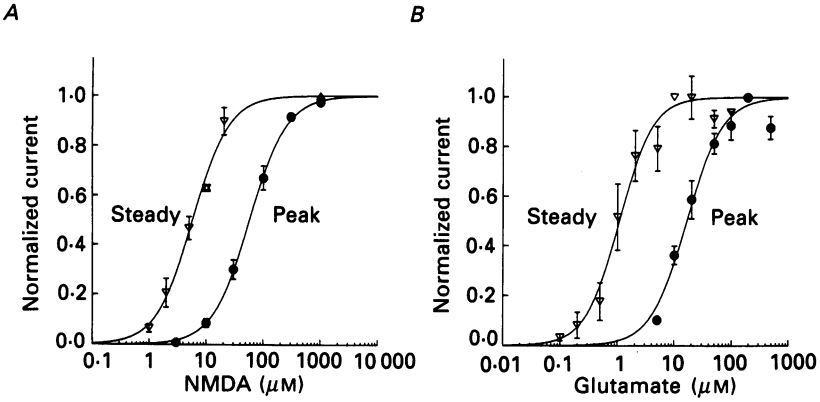


Fig. 7. Mean concentration–response relations describing peak and steady-state currents activated by application of NMDA or L-glutamate. *A*, peak (●) and steady-state (△ and ▽) relations for NMDA. The peak current data were obtained from a total of four patches. Separate curves were initially obtained for each patch, the responses of each patch were then normalized relative to the maximum value of the curve fit to these responses, the normalized values from the different patches were averaged together, and finally a Hill curve was fitted to these normalized, averaged data. The values of the Hill coefficient and EC_{50} describing the fit to the peak currents were 1·3 and 57 μM, respectively. The steady-state data were obtained from a total of three patches in which the data were normalized to the values obtained for 10 μM-NMDA, plus a separate series of experiments, in which the mean ratio between steady-state responses to 10 μM- and 1 mM-NMDA was determined (indicated by △). Since 1 mM-NMDA was supramaximal for the steady-state response, and the data set from each patch included a 10 μM response, this ratio was used to normalize the data sets. The values of the Hill coefficient and EC_{50}, derived from the curve fit to the normalized mean steady-state data, were 1·4 and 5·7 μM, respectively. Patches were continuously bathed in 10 μM-glycine. *B*, peak (●) and steady-state (▽) relations for L-glutamate. The peak current data were derived from a total of three patches. Data from each of these three patches were used in calculating the average shown in the plot, except for the mean response to 5 μM-L-glutamate, for which data were available from only two of the patches. For each patch, the peak current data were normalized relative to the response to 200 μM-L-glutamate since this was in all three cases the largest response. The Hill curve fitted to the peak currents yielded values of the Hill coefficient and EC_{50} of 1·4 and 16·5 μM, respectively. The steady-state data for L-glutamate were obtained from eight patches. For each test concentration, normalized mean currents shown were calculated using data from two to eight patches, and in most cases four patches. Since steady-state responses to 10 μM-L-glutamate were available for each of the eight patches, the data from the different patches were initially scaled to their 10 μM response amplitudes before being averaged together. The Hill coefficient and EC_{50} values describing the fitted curve were 1·5 and 1·1 μM, respectively. Seven of the eight patches were studied in 10 μM-glycine, and the other one in 20 μM-glycine and 10 μM-CNQX.

The mean L-glutamate concentration–response relations for peak currents (●) and steady currents (▽) are shown in Fig. 7*B*. These concentration–response relations were constructed in essentially the same manner as the corresponding relations for NMDA. However, for both peak and steady currents, a sag in the curve was evident

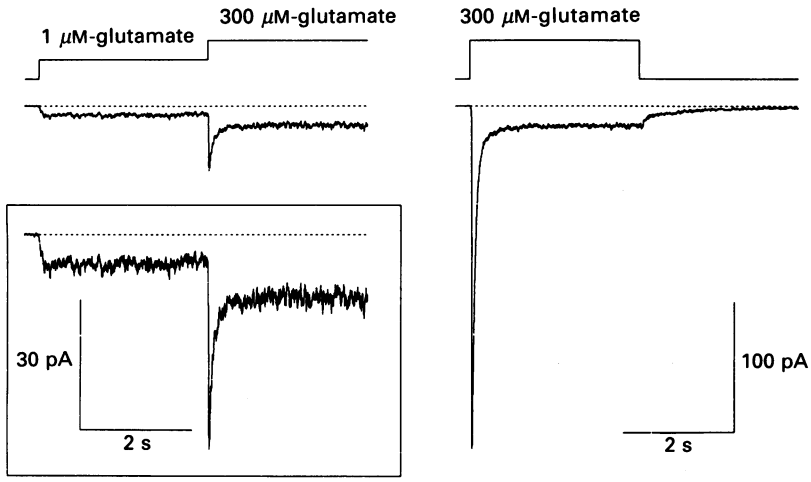


Fig. 8. A predesensitization experiment. On the left are shown averaged responses to predesensitizing (1 μM) and test (300 μM) concentrations of L-glutamate. The averaged control response (300 μM-L-glutamate) is shown on the right. Predesensitized test responses alternated with control responses, so that one of every two records was a control. Due to a recording artifact, the number of individual responses included in the ensemble average test response ($n = 16$) was less than that of the ensemble average control response ($n = 22$). In the example illustrated, the application of 1 μM-L-glutamate for 3 s reduced the test response amplitude to 15% of control. The patch was not exposed to L-glutamate during the 12 s preceding each application of either the predesensitizing or control doses. This interval appears sufficient for a nearly complete recovery of desensitization (see Fig. 11). The solutions contained 20 μM-glycine and 2 μM-CNQX.

at high concentrations of L-glutamate. The data were consequently normalized relative to the largest responses measured, which occurred at 200 μM-L-glutamate for the peak current and at 10 μM-L-glutamate for the steady current. The concentration–response relation for the steady current represents a composite of responses obtained in two overlapping concentration ranges: 0·1–10 and 10–100 μM.

The Hill coefficients were 1·4 for the peak currents and 1·5 for the steady currents, values similar to those found in the case of NMDA. The EC_{50} values for steady and peak currents were 1·1 and 16 μM.

Predesensitization

A commonly used alternative to comparison of peak and steady current as a means to study desensitization is the analysis of 'predesensitization' experiments. In this method, the desensitization produced by the application of a 'predesensitizing' concentration of agonist prior to the addition of a high test concentration is

measured as the fractional reduction of the test response amplitude relative to the control response recorded in the absence of the predesensitizing concentration. The relation between the information derived from a predesensitization experiment and that obtained from steady current measurements depends upon the nature of the

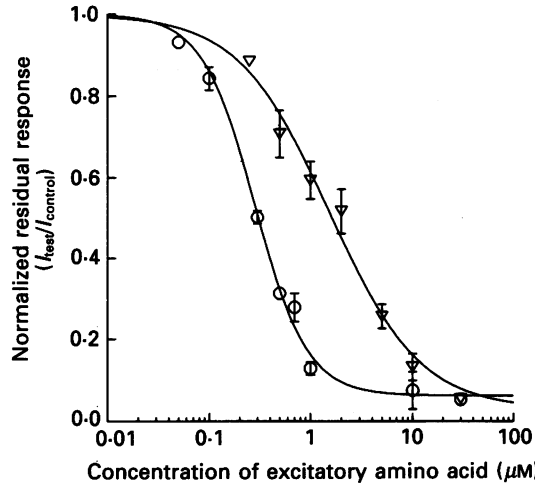


Fig. 9. Concentration–inhibition relationships describing predesensitization of NMDA receptors by NMDA or L-glutamate. The ratio between test and control response amplitudes is plotted as a function of the predesensitizing concentration (applied for 3–5 s). The relationship for NMDA (▽) was based on data obtained from four patches. Test and control responses were elicited with 1 mM-NMDA. All solutions contained 10 μM-glycine. The Hill coefficient of the curve fitted to the NMDA data was 1·0, the IC_{50} was 1·5 μM and the normalized steady-state current (the current remaining after exposure to a maximally desensitizing concentration of NMDA) was 0·03. The relationship for L-glutamate (○) was constructed using data obtained from three patches. Each of the values shown in the plot is based upon measurements from one to three patches, with most of the values representing averages obtained from all three patches. Test and control responses were obtained with 300 μM-L-glutamate. All solutions contained 20 μM-glycine and 2 μM-CNQX. The Hill coefficient of the curve fitted to the L-glutamate data was 1·7, the IC_{50} was 0·3 μM and the normalized steady current was 0·06. Receptors were allowed to recover from desensitization for 3 or 3–12 s in the cases of NMDA and L-glutamate, respectively, before each application of either predesensitizing or control doses of agonist. These intervals were selected after analysis of the recovery of desensitization (Fig. 11).

kinetic scheme which describes the behaviour of the receptor under study. Hence predesensitization measurements can be used to help distinguish between potential kinetic models.

An example of a recording acquired in a predesensitization experiment is shown in Fig. 8, where application of 1 μM-L-glutamate for 3 s reduced the test response peak to about 15% of the control response amplitude (test and control, 300 μM-L-glutamate). By varying the concentration of the predesensitizing pulse, the relations illustrated in Fig. 9 were generated. The Hill coefficient estimated for NMDA was 1·0, and that estimated for L-glutamate, 1·7.

The IC_{50}s were 1·6 μM for NMDA and 0·3 μM for L-glutamate. These IC_{50} values, which will be termed below K_{pre}, were clearly smaller than the EC_{50} values, termed

K_{ss}, derived from steady current measurements (5·7 μM for NMDA and 1·1 μM for L-glutamate). This difference between K_{pre} and K_{ss} is well illustrated in Fig. 8. One micromolar L-glutamate predesensitized 80% of the NMDA receptors that could be activated at the peak of the control response, whereas the steady current elicited by 1 μM-L-glutamate was 50% of the maximal steady current.

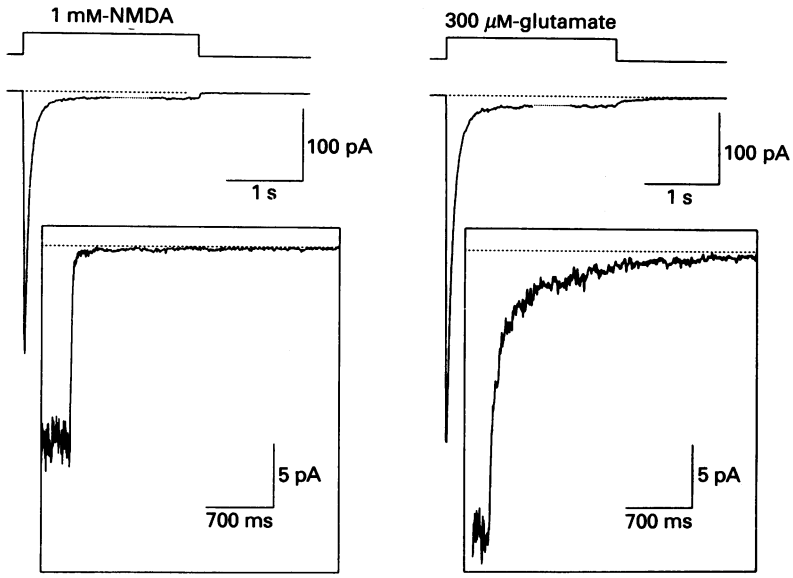


Fig. 10. Off-relaxations. Upper traces (low gain): currents recorded before, during and after 3 s applications of NMDA (1 mM) (left) and L-glutamate (300 μM) (right). The enlarged records (insets) illustrate at higher gain and higher sampling rate the decay of the response after the end of the application of NMDA or L-glutamate (the traces start 200 ms before the end of these applications). The upper traces correspond to the average of about eighteen records, while the lower traces correspond to the average of about thirty records. All records were taken during a 5 min period starting 35 min after the formation of the patch. At this time the patch had not completely reached its 'stable' level of desensitization. This took another 15 min, and at that time the steady-state current was too small to allow an accurate measurement of the off-rate constants. The decay of the currents was in both cases well fitted by a sum of two exponentials with time constants of 11 and 200 ms for NMDA, and 54 and 620 ms for L-glutamate. The slow component represented 5% of the total current in the case of NMDA, and 40% for L-glutamate.

As mentioned before, the IC_{50} of predesensitization was measured on patches that had reached an approximately stable state. Immediately after formation of the patch, the IC_{50} could not be accurately measured but was clearly much higher and in particular concentrations of 1·6 μM-NMDA or of 0·3 μM-L-glutamate produced no detectable predesensitization.

Agonist dependence of the desensitization rate

Data collected to build concentration–response relations for peak currents also contained information about the time constants of desensitization, which decreased when the agonist concentration was increased. The minimal values, obtained for

saturating concentrations of agonists (1 mM-NMDA, 300 μM-L-glutamate) were very similar and close to 30 ms (Table 2). At lower concentrations of agonists the desensitization was slower: time constants in the range of 500 ms were measured with 3 μM-NMDA or 5 μM-L-glutamate. The concentration at which the half-maximal rate was obtained was in the same range as the K_{app} measured for the peak responses, i.e. about 30 μM for NMDA and about 10 μM for L-glutamate.

Off-relaxations

When after a very brief application of L-glutamate or NMDA, one returns to an agonist-free solution, the decay of current is expected to reflect primarily the kinetics of dissociation of the agonist from the activated state. Lester *et al.* (1990) were the first to analyse this decay in conditions where a very brief (5 ms) pulse of L-glutamate (100 μM) was applied on an outside-out patch. They reported that the decay of the current as biexponential. The major component (the fastest one) had a mean time constant of 87 ms and a second component had a mean time constant of 260–600 ms. In similar experiments we analysed the decay of the currents at the end of long pulses (3 s) of either NMDA (1 mM) or L-glutamate (300 μM). In all cases, the NMDA-induced current decayed much faster than the L-glutamate-induced current. This is illustrated in Fig. 10 which shows the off-relaxations for NMDA (1 mM) and L-glutamate (300 μM). The two time constants describing the decay of the current were 11 and 200 ms for NMDA, and 56 and 620 ms for L-glutamate. Similar values were obtained in six other experiments. In those experiments the main component for NMDA had a time constant of 10–30 ms, and the second one was barely measurable. For L-glutamate the time constant of the first component ranged from 20 to 90 ms, that of the second one from 300 to 800 ms, and the two components had similar amplitudes. The values of the time constants are similar to those obtained after short pulses by Lester *et al.* (1990).

Recovery from desensitization

The above analysis of the decay of the current at the end of a long desensitizing agonist application was completed by an analysis of the speed at which the system returns to the 'activatable' state. This was tested in double-pulse experiments like the one illustrated in Fig. 11. Paired pulses of NMDA at saturating (1 mM) concentration were applied, the first pulse (control) producing a steady level of receptor desensitization and the second pulse (test) measuring the amount of recovery from desensitization that occurred during the interval between the two pulses in the pair. Varying the duration of the interval between the end of the first pulse and the beginning of the second one provided the data needed to estimate the time course of recovery.

Superimposed in Fig. 11 are eight pairs of pulses with interpulse intervals ranging from (nominally) 50 to 2000 ms. Due to a slight decline in response amplitude over the course of the experiment, the data are displayed in normalized form, i.e. each pair of responses has been scaled so that the control responses were of a constant size. On this normalized scale, the difference in peak amplitude between the control and test responses was equal to the fraction of receptors remaining desensitized at the time

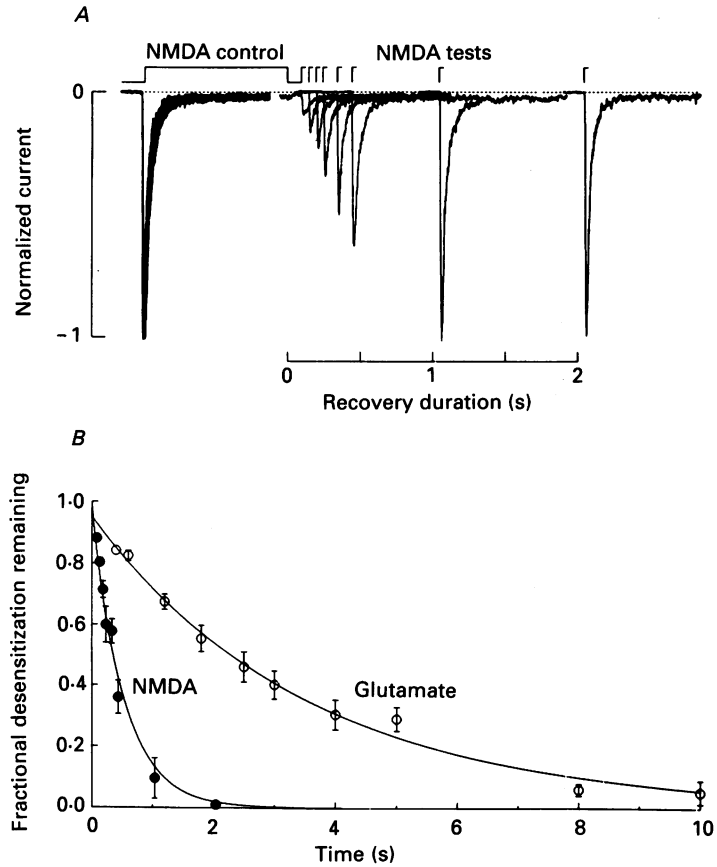


Fig. 11. Recovery from desensitization. *A*, an example of a recovery experiment. Control responses (1 mM-NMDA) are superimposed on the left. Test responses (1 mM-NMDA), which were recorded at various times after the end of the control responses, are superimposed on the right. The periods of application of the agonist are indicated by the rectilinear trace above the current records, and the time scale (shown beneath the records) begins at the end of the control application of NMDA. Onsets of the test applications are indicated by vertical tick marks above the test records. Data are displayed on a normalized scale due to a rundown of control response amplitude towards the end of the experiment. Ensemble mean responses shown were composed of fifteen to fifty-six individual responses. The patch was not exposed to NMDA during the 5 s preceding the control application. Glycine concentration was 10 μM. *B*, time course of the recovery from desensitization for 1 μM-NMDA (●) or 300 μM-L-glutamate (○). The mean data for NMDA were obtained from three patches. The single exponential function fit to the data had a maximum value of 0·98 (time = 0) and a time constant of 0·5 s. The glycine concentration was 10 μM. Patches were not exposed to NMDA during the 5 s preceding the control applications. The mean data for L-glutamate were obtained from three patches. The single exponential fit to the data had a maximum value (time = 0) of 0·95 and a time constant of 3·5 s. The glycine concentration was 20 μM and 2 μM-CNQX was included in all solutions. Patches were not exposed to L-glutamate during the 3–10 s preceding the control applications.

of the test response peak. Data collected and analysed in this way were pooled into two groups, according to the agonist employed (1 mM-NMDA; 300 μM-L-glutamate) and plotted in the lower part of Fig. 11. Single exponential functions were fitted to these mean data, with the values of the functions at time zero set equal to the difference between the peak and steady current values of the control response. Recovery time constants derived from the fitted curves were 0·5 s for NMDA and 3·5 s for L-glutamate.

The time constant measured for NMDA appears somewhat smaller than the one (0·99 s) calculated in our previous study (Sather *et al.* 1990*a*) using the whole-cell recording mode, 100 μM-NMDA and 10 μM-glycine and is close to that obtained in that study (0·43 s) using a lower (0·3 μM) glycine concentration. We have not attempted to establish the significance of this discrepancy, except for the fact that it does not appear to be due to the use of a different NMDA concentration since, in two experiments using 30 μM-NMDA, 10 μM-glycine and nucleated patches, the time constant of recovery was 0·3 s. The time constant of recovery also did not appear to depend on the duration of the agonist application, since, in three experiments in which recovery was measured after a 0·1 s application, the rate of recovery was similar to that observed after a 3 s application. In any case, two points appear very clear, which are that (i) the time constant for NMDA recovery is much smaller than the time constant for the L-glutamate recovery and (ii) for both agonists the time constants of recovery are much longer than the time constants of the off-relaxations.

DISCUSSION

The results presented above allow a 'semi-quantitative' characterization of the interaction of NMDA and L-glutamate with the NMDA receptor as observed in a 'stabilized' outside-out patch. In the first part of the following discussion we shall try to show that, by making a few plausible assumptions, it is possible to describe the behaviour of the responses by a 'cyclic model' derived from the one first used by Katz & Thesleff (1957) in describing the desensitization of the nicotinic acetylcholine (ACh) receptor. We shall then turn to the analysis of the change in desensitization which occurs after the excision of the patch, and shall try to establish that some of the conclusions valid for the stabilized patch are also valid for the initial (physiological) situation. Finally we shall examine if the evolution described here for the NMDA receptor may not also occur in the study of other receptors.

The cyclic model of desensitization

Concentration–response relations

In attempting to account for the data by the simplest model, we have first searched for a model relating satisfactorily the three series of concentration–response curves: (i) the relation between the peak response and the agonist concentration, for which the EC_{50} was denoted K_{app}; (ii) the relation between the steady-state response and the agonist concentration, for which the EC_{50} was denoted K_{ss}; (iii) the relation between the predesensitization of a maximal response and the agonist concentration, for which the IC_{50} was denoted K_{pre}.

We required the model to account for the co-operativity of the concen-

tration–response relations, and for the amplitude and the speed of the desensitization observed with a saturating concentration of agonist. The amplitude of this 'maximal' desensitization was measured as the ratio of the maximum peak amplitude to the maximum steady-state current and was denoted $A = I_{p,max}/I_{ss,max}$. The speed was characterized by the inverse of the time constant of the decay of the response, τ_D.

TABLE 2. Mean measured parameters of activation and desensitization

	K_{app} (μM) (Fig. 7)	K_{ss} (μM) (Fig. 7)	K_{pre} (μM) (Fig. 9)	A	τ_D (ms)
NMDA	57	5·7	1·7	44 ± 13 ($n = 16$)	31 ± 6 ($n = 4$)
L-Glutamate	16	1·1	0·3	26 ± 8 ($n = 10$)	35 ± 4 ($n = 4$)

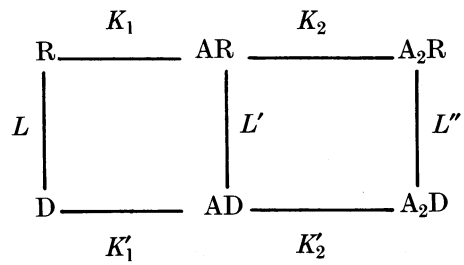
The first three columns of this table summarize the EC_{50}s and IC_{50}s derived from the concentration–response curves illustrated in Figs 7 and 9. K_{app} is the EC_{50} of the relation between the peak response and the agonist concentration, and K_{ss} is the corresponding EC_{50} for the steady-state response. K_{pre} is the IC_{50} obtained in predesensitization experiments.

In the last two columns are given the values characterizing the amplitude of the desensitization (measured by A, the ratio of the peak current over the steady-state current) and the time constant of the desensitization, τ_D. The value of A was deduced from a large series of experiments, and the values obtained for NMDA and for L-glutamate were not all obtained on the same cell. The values of τ_D, on the other hand, correspond to four experiments in which NMDA and L-glutamate were applied alternatively on the same patches. On those patches the A values were in the range 20–40.

Table 2 summarizes the values of K_{app}, K_{ss}, K_{pre}, A and τ_D for NMDA and L-glutamate.

In our initial search of a model relating these values in a satisfactory way, we followed Katz & Thesleff (1957) in trying first simple models like the 'sequential' and 'parallel' models (see also Rang & Ritter, 1970; Adams, 1987). It rapidly became evident that these models were inadequate, in particular because they did not predict the marked differences measured between K_{ss} and K_{pre}. We therefore turned towards the cyclic model, and from the start used it in a form assuming two agonist binding sites for both the 'activatable' and 'desensitized' states of the receptor. This choice was based on the values of the Hill coefficient ($1 < n_H < 2$) obtained for both the peak and the steady-state response–concentration relations, both in our studies and in those of other authors (e.g. Verdoorn & Dingledine, 1988; Patneau & Mayer, 1990; Clements & Westbrook, 1991).

The model can be written



Model (1)

where L, L' and L'' are the equilibrium constants between the R and D states, with $[D] = L[R]$, $[AD] = L'[AR]$, $[A_2D] = L''[A_2R]$. K_1 and K_2 are the macroscopic dissociation constants of the two reactions $A + R \leftrightarrow AR$ and $AR + A \leftrightarrow A_2R$, K'_1 and K'_2 are the macroscopic dissociation constants of the two reactions $A + D \leftrightarrow AD$ and $AD + A \leftrightarrow A_2D$, and A_2R corresponds to the open state. In a first attempt to account for the values summarized in Table 2 we made the simplifying assumption of Katz & Thesleff (1957), according to which the reactions leading to the opening of the channel ($A + R \leftrightarrow AR$; $AR + A \leftrightarrow A_2R$) are much faster than the reaction leading to equilibration between the R states and the D states ($R \leftrightarrow D$, $AR \leftrightarrow AD$, $A_2R \leftrightarrow A_2D$). In this case the peak response is readily defined by the equilibrium between R, AR and A_2R, whereas the steady-state response involves an equilibrium between the six possible states. Three equations can be derived ((A1), (A2), (A3), see Appendix) which allow the calculation of K_1 and K_2 if one knows K_{app}, K_{pre}, K_{ss} and Λ.

It turned out, however, that the calculated values of K_1 and K_2 were so widely separated (e.g. $K_1 = 0{\cdot}9$ μM and $K_2 = 14{\cdot}6$ μM for L-glutamate) that one could not account for the co-operativity of the various concentration–response curves. More generally, the fact that, when the concentration of agonist is around K_{app}, the onset of the response is not much faster than the onset of desensitization at saturating concentration, led us to conclude that the peak of the response, whether directly measured or extrapolated as described in Fig. 2, depends not only on K_1 and K_2 but also on the desensitization processes. In such a case, one cannot use the value of K_{app} to evaluate K_1 and K without characterizing the onset of the response.

On the other hand, this characterization is not necessary in defining K_{ss}, and may be neglected in the evaluation of K_{pre} if at saturating concentrations the kinetic limiting step of the onset does not involve binding of agonist. Since the rising phase of the response to the test pulse after predesensitization has the same shape as the control one (Fig. 8) the ratio of the two responses should give an accurate evaluation of the number of receptors which have shifted to AR, AD and A_2D during predesensitization.

We therefore tried to fit our data without using the values of K_{app}. The two equations relating K_{ss}, K_{pre} and Λ ((A2) and (A3), see Appendix) contained three unknown parameters and could not lead to a unique solution. We therefore made an additional assumption, which is that the binding sites for L-glutamate (or NMDA) on the receptor are equivalent, and that this is valid both for the activatable states (R, AR) and for the desensitized states (D, AD). The hypothesis of equivalent sites has been used by Patneau & Mayer (1990) and Benveniste & Mayer (1991) and shown in both cases to lead to good fits of both concentration–response curves and relaxations following concentration jumps. This is equivalent to assuming a ratio of 4 between the macroscopic dissociation constants, or, if we define K_R and K_D as the microscopic equilibrium constants of the binding reactions to the R and D states, this is equivalent to

$$K_R = 2K_1 = \tfrac{1}{2}K_2, \tag{1}$$

and

$$K_D = 2K'_1 = \tfrac{1}{2}K'_2, \tag{2}$$

which leads to

$$L'^2 = LL''. \tag{3}$$

This simplification, combined with equations (A2) and (A3) of the Appendix, leads to the following two equations.

$$\frac{K_R^2}{K_{ss}^2}+\frac{2K_R}{K_{ss}}A'-A=0, \tag{4}$$

$$\frac{K_R^2}{K_{pre}^2}+\frac{2K_R}{K_{pre}}(2-A')+A=0, \tag{5}$$

with

$$A=\frac{I_{peak}}{I_{ss}}=\frac{1+L''}{1+L}, \tag{6}$$

$$A'=\frac{1+L'}{1+L}. \tag{7}$$

The equations (3)–(7) can be solved for K_R, K_D, L, L' and L'' and one obtains the results of Table 3.

TABLE 3. Calculated values of the parameters of Model (1) obtained by introducing the experimental values of Table 2 into eqns (3)–(7)

	K_R (μM)	K_D (μM)	L	L'	L''
NMDA	18·1	2·0	1·2	11	98
L-Glutamate	2·7	0·4	2·7	16	95

The calculated value of L, the equilibrium constant between the non-liganded R and D states, should be independent of whether one uses NMDA or L-glutamate to evaluate it. The discrepancy between the two values in Table 3 (1·2, 2·7) appears to be a minor one. Indeed the value of 2·7 for L-glutamate can be reduced to 1·2 (the value calculated for NMDA) by using for K_{pre} a value of 0·35 μM instead of 0·3 μM. It therefore seems reasonable to conclude that, even if the value of L cannot be defined with great precision, it is likely to be in the range of 1–3. This is actually a surprisingly high value if compared with similar evaluations made in other systems (like the nicotinic ACh receptor) and it suggests that at least half of the receptors are in the D state in the absence of agonist.

Kinetics of desensitization

The time constant of the onset of desensitization has a mean value of 30–35 ms with saturating concentrations of agonists, for which one can assume that the pathway $R \leftrightarrow AR \leftrightarrow A_2R$ is much faster than the pathways leading to the desensitized states, and that the speed of desensitization will be controlled by the reaction $A_2R \leftrightarrow A_2D$. The time constant of the onset τ_D should be equal to $\tau_D = (\alpha''+\beta'')^{-1}$, where α'' and β'' are the forward and backward rate constants of the equilibrium between A_2R and A_2D. Knowing the value of τ_D and the value of $L'' = \alpha''/\beta''$, we can calculate α'' and β'' for NMDA- and L-glutamate-liganded receptors. We find values of $\alpha'' = 32\ s^{-1}$, $\beta'' = 0{\cdot}25\ s^{-1}$ for NMDA, and $\alpha'' = 28\ s^{-1}$, $\beta'' = 0{\cdot}29\ s^{-1}$ for L-glutamate.

When returning to control after a long desensitizing pulse the responses' decay should follow a double exponential, as seen in Fig. 10. The time constants (τ_f, τ_s) of these exponentials are in the general case dependent on α'' and β'' as well as on k_- and k'_-, the unbinding rates of the agonist from the A_2R and A_2D states. If we assume that α'' and k_- are much larger than k'_- and β'', and that $k'_- \gg \beta''$, then the time constant of the first exponential, τ_f, will be close to $1/(k_- + \alpha'')$ and the time constant of the second one, τ_s, close to $1/k'_-$. Numerical values of k_- and k'_- can be calculated using the values K_R and K_D listed in Table 3 by making an assumption on the values of the forward binding constants k_+ and k'_+. If we take $k_+ = k'_+ = 5 \times 10^6$ M^{-1} s^{-1}, one calculates $k_- = 13$ s^{-1} and $k'_- = 2$ s^{-1} and then predicts $\tau_f = 24$ ms and $\tau_s = 500$ ms. These values are in the range of the measured values.

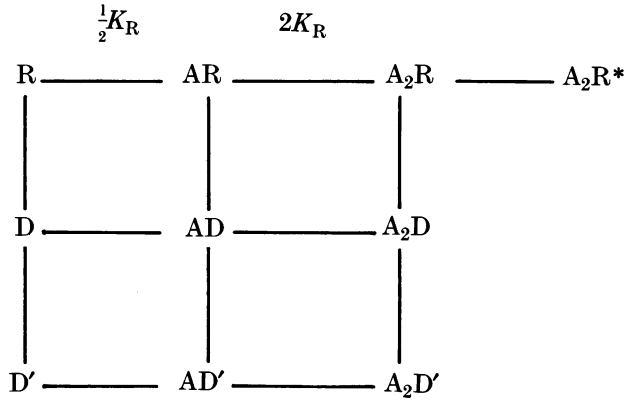
The model presented is, however, clearly insufficient in three respects:

(1) It predicts a very fast rise of the response at high agonist concentrations (if one makes plausible assumptions on the binding rates of the agonists) and this is clearly not what is observed (see Fig. 2 and Lester *et al.* 1990).

(2) It neglects the fact that, at saturating concentrations, NMDA and L-glutamate do not induce the same maximal current (Table 1), a difference in efficacy which may require that an explicit distinction be made between the closed and open forms of the doubly liganded receptor.

(3) It does neither account for the slowness of the recovery nor for the difference in the rates of recovery for L-glutamate and NMDA. Both features suggest that the recovery from desensitization is in part limited by the dissociation of the agonists from higher affinity states than those regulating the off-relaxation. It is probable that, as in the case of the ACh nicotinic receptor (Sakmann, Patlak & Neher, 1980; Feltz & Trautmann, 1982; Neubig, Boyd & Cohen, 1982; Heidmann, Bernhardt, Neumann & Changeux, 1983), the complete description of desensitization will require two desensitized states. A similar suggestion was recently made for a non-NMDA L-glutamate receptor by Patneau & Mayer (1991).

A model which can account for the data presented above is Model (2), which separates the open state from the closed states and assumes the existence of two desensitized states.



Model (2)

Although this model formally resembles the models used for other receptors, and in particular the nicotinic receptor, it is worth noting that in the case of the NMDA

receptor one usually cannot neglect the speed of the binding and unbinding reactions relative to that of the desensitization transitions. As a result, the peak of the response is controlled both by the concentration of agonist and by desensitization. Another difference with the nicotinic ACh receptor is that the speed of the recovery from desensitization depends on the agonist used, which in turn implies that the limiting step in recovery cannot be the $D' \rightarrow D \rightarrow R$ reaction for both agonists. Finally, our results suggest that a rather high proportion of receptors occupies the D and D′ states in the absence of agonist.

The evolution of the receptor kinetics after formation of the patch

Approximate constancy of the activation pathway

The peak response to high concentrations of NMDA and L-glutamate, which in a first approximation involves only the activation pathway ($R \leftrightarrow AR \leftrightarrow A_2R$), showed a variable behaviour from one experiment to another: sometimes it remained stable throughout the experiment, but more often it decreased with time. This variability contrasts with the fact that in all patches the speed and the amount of desensitization increased with time towards a final 'stabilized' state which was very similar for all patches. This suggests that the evolution of desensitization does not much alter the activation pathway and that, in some cases at least, this pathway has the same kinetics in an intact cell and in an outside-out patch.

If this were true, the EC_{50}s measured in 'intact' cells and in outside-out patches should be similar. This comparison cannot make use of the values of K_{app} measured in experiments like those of Fig. 7 which we know to be overestimates, and must use the values of K_R calculated in Table 3. If one assumes that these values were valid for intact cells, the corresponding EC_{50}s would be $(1+\sqrt{2})\,K_R$ (see text eqn (1) and Appendix equation (A1)) i.e. 43·7 μM for NMDA and 6·0 μM for L-glutamate. These values are somewhat higher than those found by other authors in intact cells (Verdoorn & Dingledine, 1988, NMDA 31 μM, L-glutamate 2·3 μM; Lerma, Kushner, Spray, Bennett & Zukin, 1989, NMDA 21·6 μM; Patneau & Mayer, 1990, NMDA 34·1 μM, L-glutamate 2·3 μM). This difference could be inherent to the differences in the neurones used, but they could also indicate that, after formation of the patch, the increase of desensitization is accompanied by a slight decrease of the affinity in the activation pathway.

Measurements of the K_D of L-glutamate binding to purified membranes have led to values ranging from 120 to 620 nM (see Grimwood, Foster & Kemp, 1991). This is in the range expected if the NMDA receptor in purified membranes is in a state comparable to that it adopts in 'stabilized' outside-out patches, in which the K_D of binding would reflect predominantly binding to the desensitized states.

The correlation between the amplitude and the speed of desensitization

During the hour following the formation of the patch the speed and the amplitude of desensitization, measured by $(\tau_D)^{-1}$ and A, appear to evolve in parallel. The relation between the two values (Fig. 5) is a straight line with a slope of about 0·6 s^{-1} for both NMDA and L-glutamate.

As described above, at high concentrations of agonist, one can assume that $(\tau_D)^{-1} =$

$\alpha'' + \beta''$ (where α'' and β'' are the rates of equilibration between A_2R and A_2D) while Λ has been defined as $\Lambda = (1+L'')/(1+L)$.

From there we deduce

$$\frac{(\tau_D)^{-1}}{\Lambda} = \beta''(1+L).$$

The relation should go through the origin of the co-ordinate axes and it does not. The (small) deviation may be due to the fact that τ_D was calculated by fitting a single exponential on the decay of the response. As mentioned before, this is satisfactory for the 'stabilized' patch, but the fit is less adequate during the early period of the experiment.

The approximate constancy of the ratio $(\tau_D)^{-1}/\Lambda$ suggests that $\beta''(1+L)$ is constant after excision of the patch, and by extension it is tempting to assume that neither β'' nor L change significantly. This claim certainly deserves to be further explored but if it were substantiated it would lead to two significant conclusions.

(a) The constancy of L implies that even before the formation of the patch a substantial number of receptors are in a desensitized state;

(b) The acceleration and the deepening of desensitization would result from a progressive increase of α'', the rate of the transition from A_2R to A_2D, with little change of β'', the rate of the reverse transition.

It may be worth noting that if after formation of the patch there is a progressive acceleration of α'' while k_- and k'_- remain constant, one should observe a progressive acceleration of the fast component of the off-relaxations. Similarly, if the decay of the NMDA synaptic current corresponds to an off-relaxation (Lester *et al.* 1990) it should accelerate as the cell is perfused for prolonged periods.

We have no indication on the nature of the events leading to a progressive increase of desensitization in patches. Since the evolution occurs when the pipette solution contains CsF as the main salt but also when it contains KCl (Fig. 5) or KCl and an ATP-regenerating solution (Sather *et al.* 1990*a*) it appears unlikely that the evolution involves a phosphorylation-dependent process, despite the fact that such processes do influence the amplitude of the response (MacDonald, Mody & Salter, 1989). In another example where the properties of the receptor channels in a patch were found to change with time after excision (that of the nicotinic receptor channel; Covarrubias & Steinbach, 1990), changes of phosphorylation were also found an unlikely explanation and the suggestion was made that there was a 'slow loss of a non-covalently associated cytoplasmic component'. We are tempted to follow that suggestion (see also Trautmann & Siegelbaum, 1983, and Benveniste, Clements, Vyklický & Mayer, 1990) and to add that in the nucleated patch configuration the presence of the nucleus at the tip of the pipette may slow the loss of the cytoplasmic component.

Three types of desensitization of NMDA responses

Three types of desensitization have been described for NMDA responses: a Ca^{2+}-sensitive one, a glycine-sensitive one and a Ca^{2+}-insensitive and glycine-insensitive one.

A Ca^{2+}-sensitive desensitization was first considered by Mayer & Westbrook (1985) and later substantiated by the observations of Mayer, MacDermott, Westbrook,

Smith & Barker (1987), Mayer, Vyklický & Clements (1989), Lerma, Zukin & Bennett (1990), Clark, Clifford & Zorumski (1990) and Vyklický, Benveniste & Mayer (1990). Its mechanism is not yet clear, since intracellular chelation of calcium gave somewhat conflicting results. This desensitization does not seem to occur on outside-out patches (Sather *et al.* 1990*a*). A glycine-sensitive desensitization has been analysed by Mayer *et al.* (1989) and more recently by Vyklický *et al.* (1990) and Lerma *et al.* (1990). This desensitization decreases when the concentration of glycine is increased, and at saturating concentrations of glycine it disappears. In neurones, it is only seen in the whole-cell mode and during the first minutes of the recording. Thereafter one observes a progressive change towards a glycine-insensitive, Ca^{2+}-insensitive desensitization. This last type of desensitization has been described by many authors for cells perfused for a long period of time or for outside-out patches (Benveniste *et al.* 1990; Shirasaki, Nakagawa, Wakamori, Tateishi, Fukuda, Murase & Akaike, 1990; Sather *et al.* 1990*a*; Chizhmakov, Kiskin, Tsyndrenko & Krishtal, 1990; Traynelis & Cull-Candy, 1991).

The glycine-insensitive desensitization described by Shirasaki *et al.* (1990) differs from the one that we have studied by the fact that these authors found values for K_{app} and K_{ss} (the EC_{50}s of the peak and of the plateau) that were similar to one another while we find a more than tenfold difference between these two values. The fact that in the study of Shirasaki *et al.* (1990) the plateau response was not maximal at concentrations of NMDA as high as 100 μM is puzzling in view of the fact that the same authors observed a nearly complete 'predesensitization' at NMDA concentrations as low as 0·1 μM.

Chizhmakov *et al.* (1990) have argued that the (glycine-insensitive) desensitization 'does not proceed in the presence of kynurenate'. However, their experiments are actually 'predesensitization' experiments, in which partial predesensitization is produced by adding a high concentration of agonist in the nominal absence of glycine. There are many reasons to assume that a glycine contamination is present (see e.g. Henderson, Johnson & Ascher, 1990) and that this allowed some activation and predesensitization of NMDA receptors. Adding kynurenate is equivalent to suppressing the glycine contamination, and therefore the predesensitization. In our interpretation, the experiments of Chizhmakov *et al.* (1990) only indicate that desensitization, like activation (Kleckner & Dingledine, 1988), has an absolute requirement for occupation of the glycine site by a glycine agonist.

For the complete description of the evolution occurring in an outside-out patch after its formation (but also in the whole-cell mode as the internal perfusion proceeds) it will ultimately be necessary to explain not only the progressive acceleration of the glycine-insensitive desensitization, but also the disappearance of the glycine-sensitive desensitization and the fact that this disappearance is not accompanied by the disappearance of the glycine sensitivity of the NMDA response (Johnson & Ascher, 1987; Sather *et al.* 1990*a*). Furthermore, the rates of binding and unbinding of glycine do not appear to be substantially different when measured in the whole-cell mode and in outside-out patches (J. W. Johnson & P. Ascher, in preparation), and the affinity of glycine does not seem affected by desensitization (Shirasaki *et al.* 1990; Sather *et al.* 1990*a*).

Whatever the explanations given for these numerous changes, it seems clear that the various differences in NMDA receptor desensitization described in the literature

are probably not linked with the cell type used, as is sometimes assumed (Shirasaki *et al.* 1990; Chizhmakov *et al.* 1990), but rather to the experimental conditions and, in large measure, to the degree of exchange of the cytoplasm with the pipette solution.

Does desensitization of other receptors change?

One may wonder how many of the above observations can be extended to other receptors for which the values of desensitization onset and desensitization amplitude appear to show spectacular differences from one preparation to the next.

In the case of the vertebrate non-NMDA L-glutamate receptor, the fastest rates of desensitization found in the whole-cell mode (Tang, Dichter & Morad, 1989; Patneau & Mayer, 1991) were in the range of 100 s^{-1}, only slightly lower than those measured in outside-out patches by Trussell & Fischbach (1989), Tang *et al.* (1989) and Colquhoun, Jonas & Sakmann (1991). In contrast, in the case of the L-glutamate receptor of arthropod muscle, measurements on outside-out patches have given desensitization time constants in the millisecond range, whereas time constants recorded in the whole-cell mode are in the second range (see Dudel, Franke & Hatt, 1990). In another well-studied example, that of the nicotinic ACh receptor, a similar discrepancy exists between the shortest time constants in outside-out patches (15 ms, Maconochie & Knight, 1989; 50 ms, Dilger & Brett, 1990; 20 ms, Franke, Hatt & Dudel, 1991) and the smallest values found in the whole-cell mode (300 ms, Siara, Ruppersberg & Rüdel, 1990; most values for the endplate are in the second range; see Cachelin & Colquhoun, 1989). Furthermore, in the case of the nicotinic ACh receptor of adrenal chromaffin cells, Inoue & Kuriyama (1991) have reported a progressive increase of desensitization with time resembling that seen with NMDA in the whole-cell mode (Sather, MacDonald & Ascher, 1991).

In most of the above examples, the difference could be linked with the cell type used or could be due to the fact that it was not possible to apply the agonist with sufficient speed and synchrony in the whole-cell mode. But a third possibility is that, as in the case of the NMDA receptor, intracellular perfusion and formation of an outside-out patch alter desensitization.

APPENDIX

In the first variation of the cyclic model (Model 1) it is possible to calculate K_1 and K_2 knowing K_{app}, K_{ss} and K_{pre} provided that one makes some specific assumptions on the relative speeds of the activation reactions ($A \leftrightarrow R \leftrightarrow A_2R$) and the speeds of the isomerizations between the R and D states.

(1) The value of the peak current (which is proportional to A_2R) can only be calculated easily if one assumes that the equilibria between A, AR and A_2R are much faster than the isomerizations between the R and D states.

In this case, the value of K_{app} (the agonist concentration at which the number of channels opened is half the number of channels opened by a saturating agonist concentration) is linked to K_1 and K_2 by

$$\frac{K_1K_2}{K_{app}^2}+\frac{K_2}{K_{app}}-1=0. \qquad (A\ 1)$$

(2) The relation between K_1, K_2 and K_{ss} does not require any specific assumption concerning the relative speed of the various reactions. If $[A_2R]_{ss}/[R_T]$ is the ratio between the number of receptors in the state A_2R at steady state and the total number of receptors $[R_T]$, then, when $[A] \rightarrow \infty$, $[A_2R]_{ss}/[R_T] \rightarrow 1/1+L''$. The agonist concentration K_{ss} at which $[A_2R]_{ss}/[R_T] = 1/2(1+L'')$ is given by

$$\frac{K_1K_2}{K_{ss}^2}+\frac{K_2}{K_{ss}}\Lambda'-\Lambda = 0, \tag{A 2}$$

where $$\Lambda' = \frac{1+L'}{1+L} \text{ and } \Lambda = \frac{1+L''}{1+L}.$$

(3) A simple relation between K_1, K_2 and K_{pre} can be written without assuming that the activation reactions are always much faster than the isomerizations between the R and D states, provided that this condition is satisfied when saturating agonist concentrations are used to test the level of desensitization. In such a case, K_{pre} is defined as the agonist concentration at which, after desensitization has reached its steady state, all the receptors available for rapid activation by a saturating concentration of agonist represent a fraction of the total number of receptors which satisfies the following equation

$$\frac{[R]+[AR]+[A_2R]}{[R_T]} = \frac{1}{2(1+L)}.$$

This leads to

$$\frac{K_1K_2}{K_{pre}^2}+\frac{K_2}{K_{pre}}(2-\Lambda')+(2-\Lambda) = 0. \tag{A 3}$$

(4) In the case where the activation reactions are much faster than the isomerizations at all agonist concentrations, eqns (A1), A2) and A3) can be combined and the values of K_1, K_2 and Λ' deduced if one knows K_{app}, K_{pre}, K_{ss} and Λ.

If the above condition does not apply, eqn (A1) cannot be used. However, if one makes the assumption that the two binding sites are equivalent, and that the system satisfies the conditions under which eqn (A3) was derived, eqns (A2) and (A3) can be transformed into text eqns (4) and (5) (see Discussion) and these two equations can be combined to give

$$K_R^2(K_{pre}+K_{ss})+4K_RK_{ss}K_{pre}-K_{ss}K_{pre}(\Lambda(K_{ss}+K_{pre})-2K_{pre}) = 0. \tag{A 4}$$

Knowing K_{pre}, K_{ss} and Λ allows calculation of K_R, the microscopic equilibrium constant of the agonist binding to the activatable states.

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