Neuron, Vol. 13, 645-655, September, 1994, Copyright © 1994 by Cell Press

Mechanosensitivity of NMDA Receptors in Cultured Mouse Central Neurons

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Summary

Changes in osmotic and hydrostatic pressure were found to modulate NMDA responses of cultured embryonic mouse neurons recorded in various patch-clamp configurations. In nucleated patches, NMDA currents were potentiated by reductions in external osmolarity and were reduced in hyper-osmotic solutions. These changes, which were greater for low concentrations of NMDA, were not observed for responses to kainate, glycine, or GABA. They could be mimicked by directly changing the pipette pressure in nucleated, outside-out, inside-out, and cell-attached patches. Osmosensitivity of NMDA responses was also observed in the whole-cell mode, but only after prolonged dialysis. Mechanosensitivity of NMDA receptors could have an important role in neuronal regions experiencing changes in membrane tension, such as spines or growth cones.

Introduction

The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is modulated by a variety of small diffusible ions and molecules (like Mg^{2+}, Ca^{2+}, glycine, Zn^{2+}, and H^{+}) acting either extracellularly or intracellularly. Recent findings show that NMDA is also modulated by intracellular proteins such as protein kinases, protein phosphatases, and cytoskeletal elements (see McBain and Mayer, 1994). We now report evidence that NMDA responses can also be modulated by mechanical stimuli. This phenomenon was first suggested by experiments in which osmotic pressure changes were found to alter the NMDA responses recorded in nucleated patches. In this preparation (Sather et al., 1992), the cell nucleus is stuck by suction onto the tip of the recording pipette and then separated from the cell body while remaining surrounded by plasma membrane. Nucleated patches combine the high resolution of outside-out patch recording with the presence of numerous channels and thus resemble in many ways "giant" patches or "macropatches." However, they differ from such preparations inasmuch as they are three compartment systems. Because the exchange between the pipette and the internal (nuclear) compartment is slow, the nucleated patch will swell if exposed to a hypo-osmotic solution and shrink if exposed to a hyper-osmotic solution. The observation that the NMDA responses recorded in nucleated patches were modified by changes in osmotic pressure was thus reminiscent of a large body of reports of conductance changes in cells submitted to osmotic challenges. The most common explanation of this effect involves volume-sensitive conductances, and many patch-clamp experiments have shown that the effects of the osmotic challenges could be mimicked by applying positive or negative pressure through the patch pipette (e.g., Ubl et al., 1988; Sackin, 1989; Doroshenko and Neher, 1992; Langton, 1993; Oliet and Bourque, 1993). Following a similar approach, we examined the sensitivity of the NMDA responses to hydrostatic pressure changes. These changes were applied to nucleated, outside-out, inside-out, and cell-attached patches, and all procedures that were expected to increase membrane tension produced an increase of the NMDA response. Procedures leading to a reduced tension had opposite effects.

These observations suggested that NMDA channels are members of the broad family of mechanosensitive channels. This family comprises the mechanosensitive channels of specialized mechanosensory organs (see Howard et al., 1988; French, 1992) and the stretch-sensitive channels first observed by Guharay and Sachs (1984) on cultured muscle cells and since seen in cells as diverse as epithelial cells, neurons, and bacteria (see Sachs, 1990). The NMDA channels differ from most other stretch-activated channels inasmuch as the mechanical stimulus is not their primary stimulus, since they cannot open in the absence of an agonist. However, as will be seen, they share many properties with the stretch-activated channels, including the fact that their mechanosensitivity is much more visible in patches than in the whole-cell configuration.

Results

Osmosensitivity of the NMDA Response in Nucleated Patches

The osmosensitivity of responses to different agonists mediating fast ionotropic responses was first tested in nucleated patches. In all experiments, patches were first exposed to a standard external solution containing 140 mM NaCl. The external solution was then switched to one in which the NaCl concentration was either reduced to 125 mM (hypo-osmotic solution) or increased to 155 mM (hyper-osmotic solution), so that in both cases there was a 10% change in osmolarity (27 mOsm). After this osmotic challenge, which lasted from 10 s to over 2 min, the patches were reexposed to the control solution, to determine the reversibility of the changes observed.

Nucleated patches were first tested with NMDA, which was applied at two concentrations: 10 μM, which is below the EC_{50} of the peak response (estimated at 57 μM in this preparation by Sather et al. [1992]), and 1 mM, which is saturating. Figure 1 illustrates the effects of a hypo-osmotic solution on the

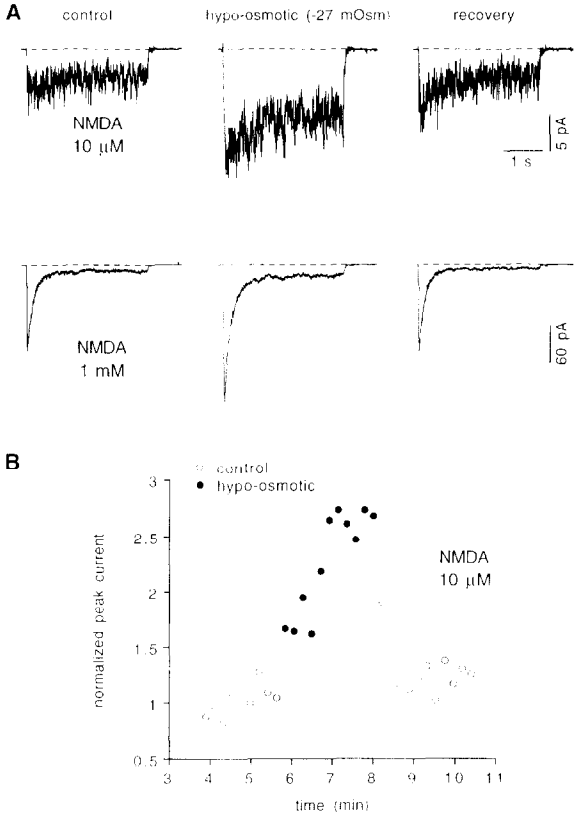

Figure 1. NMDA Responses of Nucleated Patches Are Increased by Superfusion with a Hypo-osmotic Solution

The agonist pulses lasted 3 s each and were repeated every 10 s, ensuring a nearly complete recovery of the receptors from desensitization.

(A) Responses in the control solution (140 mM NaCl; left), in the hypo-osmotic solution (125 mM NaCl; center), and after return to the control solution (right). Each trace is the average of seven successive sweeps. The upper row represents responses to 10 μM NMDA. The peak current was potentiated 2.3-fold. The lower row represents currents evoked by a saturating concentration of NMDA (1 mM). The peak increased by 1.6-fold. All responses were obtained from the same patch.

(B) Time course of the effects of the hypo-osmotic solution. Same patch as in (A). The open circles correspond to responses recorded in the control solution, and the closed circles correspond to responses recorded in the hypo-osmotic solution. The nine control responses showed a slight tendency to increase over time. The size of each successive response was evaluated as the ratio of the response to the mean value of the nine control responses recorded prior to perfusion with the hypo-osmotic solution. The holding potential was −50 mV. Glycine (10 μM) was present in all solutions. The pipette solution was CsF (10 mM EGTA).

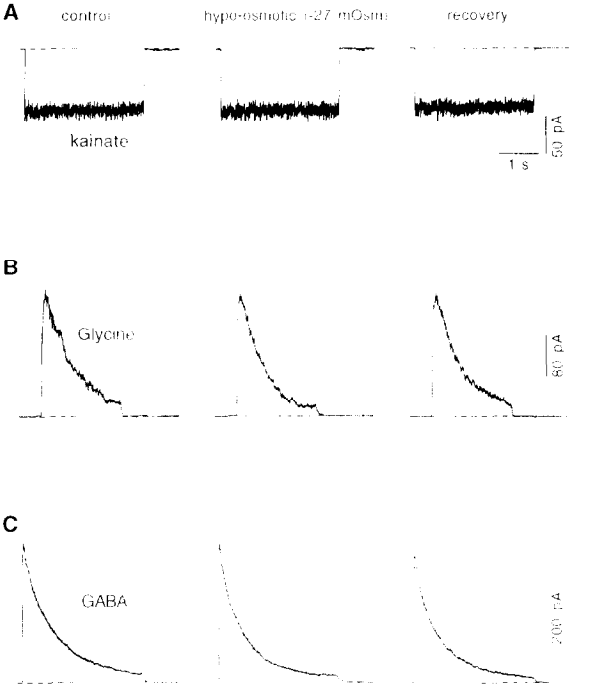

Figure 2. Responses to Kainate, Glycine, and GABA Are Not Sensitive to Hypo-Osmotic Challenges

The agonist pulses lasted 3 s for kainate and GABA and 2 s for glycine. They were applied at 10 s intervals. Responses in the control solution (140 mM NaCl; left), in the hypo-osmotic solution (125 mM NaCl, center), and after return to the control solution (right). (A), (B), and (C) are from different patches. The pipette solution was CsF (10 mM EGTA).

(A) Responses to 50 μM kainate. Individual traces. Holding potential was −50 mV.

(B) Responses to 50 μM glycine. Individual traces. Holding potential was 0 mV.

(C) Responses to 50 μM GABA. Each trace is the average of two sweeps. Holding potential was 0 mV.

two NMDA responses. The response to 10 μM NMDA increased immediately after the change in solution and had more than doubled after 1 min. During the same period, the response to 1 mM NMDA also increased but significantly less; the maximum potentiation was about 1.7, for both the peak and the plateau (Figure 1A). The recovery upon returning to the control solution was quite fast; as illustrated in Figure 1B, it was largely complete after 1 min. Symmetrical effects were observed with hyper-osmotic solutions (data not shown).

One possible explanation of the observed effects could be the occurrence of a change in the surface area of membrane exposed to the agonist. To test this hypothesis, the effects of the same osmotic challenges were tested on the conductances elicited by kainate, glycine, and γ-aminobutyric acid (GABA). In the cells under study, kainate induces a cationic current, whereas GABA and glycine induce Cl^- currents. The three compounds were applied at 50 μM; this concentration is close to the EC_{50} reported for most glycine and GABA inhibitory currents (e.g., Fatima-Shad and Barry, 1992) and below the EC_{50} of kainate-induced currents (Patneau and Mayer, 1990). The GABA and glycine responses showed rapid desensitization during the applications. The three responses were subject to some run-down as a consequence of the dialysis of the cytoplasm by the pipette solution. Nevertheless, the run-down was usually slow, and over a period of a few minutes, the peak amplitude of the responses remained sufficiently stable to per-

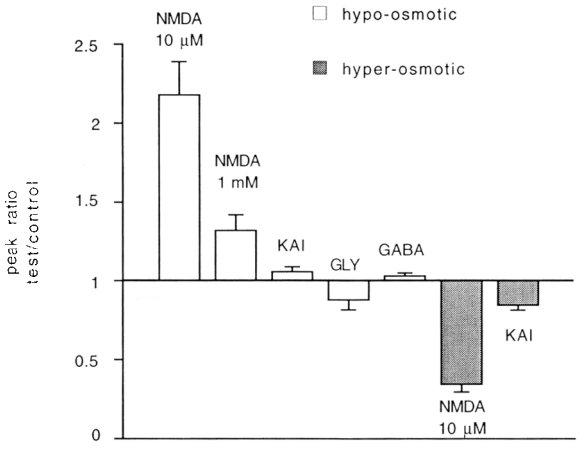

Figure 3. Modulation by Changes in External Osmolarity Is Most Marked for the NMDA Receptor and Depends on NMDA Concentration

The results are expressed as the ratio of the average peak current in the test solution to the average peak current in the control solution. Changes in osmolarity were −27 mOsm with the hypo-osmotic solution (125 mM NaCl) and +27 mOsm with the hyper-osmotic solution (155 mM NaCl). Kainate, glycine, and GABA were applied at 50 μM. The holding potential was −50 mV for NMDA and kainate and 0 mV for glycine and GABA. The pipette solution was CsF (10 mM EGTA). Error bars represent SEM.

mit an evaluation of the effect of a brief change in osmotic pressure. As illustrated in Figure 2, the responses to the three agonists did not share the sensitivity of the NMDA responses to osmotic pressure.

Figure 3 summarizes the results obtained with the four agonists (NMDA, kainate, glycine, and GABA) when nucleated patches were exposed either to the hypo-osmotic or hyper-osmotic solution. All analyses were performed on the peak currents. For the hypo-osmotic challenge, the ratios of the peak current in the test solution to the peak current in the control solution were 2.18 ± 0.21 (10 μM NMDA; n = 7), 1.32 ± 0.1 (1 mM NMDA; n = 6), 1.06 ± 0.03 (50 μM kainate; n = 5), 0.88 ± 0.06 (50 μM glycine; n = 6), and 1.03 ± 0.02 (50 μM GABA; n = 4). The potentiation of the response to 1 mM NMDA was significant ($p < .05$; Student's t test) but significantly smaller ($p < .05$; Tukey's test modifed for unequal sample sizes) than the potentiation of the response to 10 μM NMDA. This suggests that the hypo-osmotic solutions simultaneously increase the affinity of the receptor and the maximal response. Hyper-osmotic challenges were tested only on the responses to NMDA (10 μM) and kainate (50 μM), and the ratios of the test response to the control were, respectively, 0.34 ± 0.05 (n = 3) and 0.84 ± 0.03 (n = 3).

Changing the NaCl concentration from 140 to 125 mM reduced the single-channel current at −50 mV by 8% and induced a slight negative shift of the reversal potential of the NMDA responses (from 0 to −3 mV). No attempt was made to correct for these effects or for the opposite changes observed when superfusing with the hyper-osmotic solution. As a result, the numbers given in Figure 3 correspond to a slight underestimation of the changes of the open probability produced by the osmotic challenges.

Changes in osmolarity have been found to induce changes in both intracellular Ca^{2+} (e.g., Yamaguchi et al., 1989; Nitschke et al., 1993) and intracellular pH (e.g., Muallem et al., 1992). In the experiments of Figures 1–3, the pipette solution contained 10 mM EGTA and 10 mM HEPES, which should have been sufficient to buffer the changes in intracellular Ca^{2+} and H^+. However, since EGTA is a relatively weak buffer, we repeated the experiments after replacing EGTA with BAPTA (10 mM), which is much less sensitive to pH and more efficient than EGTA (Tsien, 1980). This substitution did not modify the effect of hypo-osmotic solutions on the NMDA responses (2.14 ± 0.39 peak potentiation with 10 μM NMDA; n = 4; see Figure 7C). This suggests that the osmosensitivity of the NMDA response is mediated directly by a membrane deformation rather than a change in intracellular pH or pCa. To evaluate this hypothesis further, we examined the sensitivity of the NMDA response to membrane deformations produced by changes in the pipette pressure.

NMDA Response in Nucleated Patches Is Sensitive to the Pipette Pressure

During the excision of a nucleated patch from the parent cell, negative pressure is applied in the recording pipette. After the excision, it is possible to release the suction. We found that this generally did not affect the quality of the recording (although it appeared to lead to a diminution of the lifetime of the nucleated patch), but it provoked a marked change in the NMDA responses.

Figure 4A (upper and center parts) shows the effect of releasing the pipette suction on responses induced by two concentrations of NMDA (10 μM and 1 mM) alternately applied to the same nucleated patch. The lower part shows the response to kainate (50 μM), obtained from another patch subjected to the same protocol. The left panels illustrate the responses obtained with the nucleated patches under negative pipette pressure (suction on). The right panels show the corresponding responses after the pipette suction had been suddenly released (suction off). Releasing the pipette suction induced a large potentiation of the NMDA responses, whereas the kainate response was affected less. Moreover, as shown for the osmosensitivity of the NMDA response (Figure 3), the amplitude of the effect depended on the agonist concentration. In this example, the response to 10 μM NMDA was potentiated 6-fold upon release of the suction, whereas the peak response to 1 mM NMDA showed only a 2-fold increase. Figure 4B summarizes the results of many similar experiments. As in the case of hypo-osmotic challenges, the potentiation was significantly larger for the responses to 10 μM NMDA than

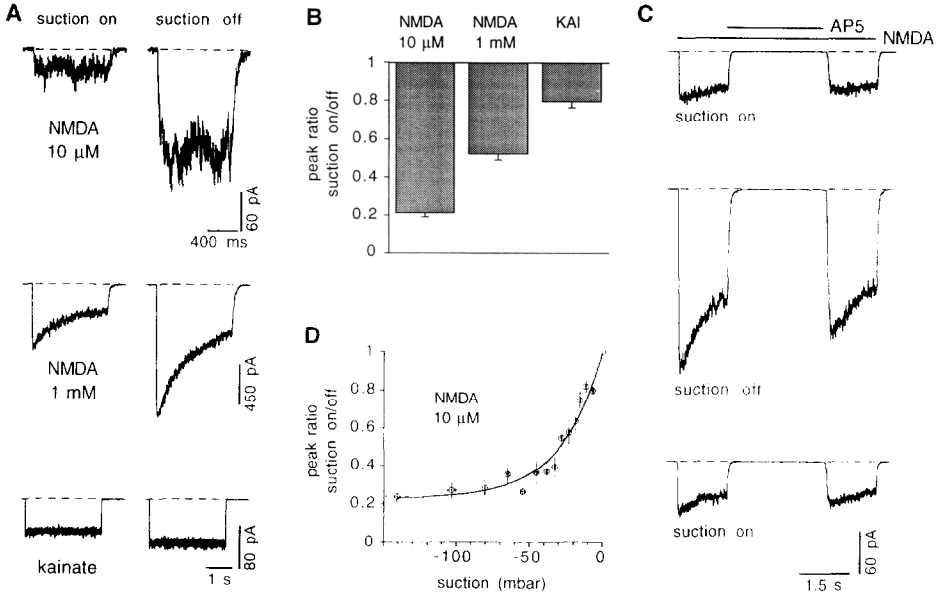

Figure 4. Hydrostatic Pressure Changes Modulate the NMDA Response in Nucleated Patches

(A) NMDA was applied (for 1 s every 5 s) alternately at 10 μM (top) and 1 mM (center) to a nucleated patch. The suction used to excise the nucleated patch (see Experimental Procedures) was maintained at the beginning of the recording and then suddenly released. On the left are shown individual responses of the patch subjected to −150 mbar (suction on); on the right are the responses after release of the suction (suction off). The maximal current amplitude was increased 6-fold with 10 μM NMDA compared with a 2-fold potentiation with 1 mM. The lower panel shows the response of another nucleated patch induced by 50 μM kainate recorded during the same protocol as described above. Left trace, suction on (−70 mbar); right trace, suction off. The current increased 1.3-fold.

(B) Pooled data of several experiments done in the same conditions as in (A). Pipette pressure varied between −200 and −65 mbar. NMDA was used at 10 μM (n = 16) or 1 mM (n = 10), and kainate was used at 5, 30, or 50 μM (n = 12; with no noticeable differences between the effects at these concentrations).

(C) D-AP5 was applied at 100 μM for 3 s in the middle of a 6 s pulse of 30 μM NMDA. Each trace is the average of ten traces. A suction of −150 mbar was applied in the patch pipette (top), the suction was released (center), and the initial suction was reapplied (bottom).

(D) Pressure sensitivity of the NMDA response. The curve was constructed from 11 different patches. NMDA (10 μM) was used in all experiments. The data represent the peaks of each individual response normalized to the peak current obtained after releasing the suction. The data are fitted with an exponential curve given by $R = (1 - 0.22)[\exp(-s/28)] + 0.22$, where R is the peak ratio and s is the applied suction. The pipette solution was CsF (10 mM EGTA). Holding potential was −50 mV. Error bars represent SEM.

for the responses to 1 mM NMDA ($p < .05$; Tukey's test modifed for unequal sample sizes).

In most cases, the release of the suction neither changed the leak current nor activated endogenous stretch-activated channels: channel openings were seen only during the agonist applications and thus, in the case of NMDA responses, must have involved NMDA-activated channels. Further support for this claim is given by the fact that the NMDA antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5), applied at a concentration of 100 μM, completely blocked the NMDA-induced current, whether suction was on or off. This is illustrated in Figure 4C and was confirmed in three experiments. Figure 4C also illustrates that the potentiation produced by the release of the suction can be reversed by reapplying suction through the pipette.

The mechanical stability of the nucleated patches enabled the determination of the relation between the applied pipette suction and the peak NMDA-induced current. Thus, we progressively decreased the pipette suction from the initial values (up to −150 mbar) used for the excision of the patch. The data of Figure 4D (n = 11) show that the response to 10 μM NMDA is particulary sensitive to small transmembrane pressure gradients (up to −50 mbar). A similar relation was obtained using 1 mM NMDA (n = 3; data not shown), except that the maximal potentiation was on average 2-fold rather than 5-fold, as for 10 μM NMDA. In contrast, the curve obtained with kainate showed little sensitivity to suction over this pressure range (<1.3-fold modulation; n = 4; data not shown).

In a few experiments, we could apply positive pressures and observed an increase in the responses triggered by NMDA (data not shown). Thus, like the osmosensitivity previously described, the modulations produced by the hydrostatic pressure changes are inverted by an inversion of the pressure.

Mechanosensitivity in Standard Outside-Out and Inside-Out Patches

If hypo-osmotic solutions act on nucleated patches by increasing the membrane tension, one would expect NMDA responses of standard outside-out patches not to be affected by hypo-osmotic solutions. The amount of water flowing through the patch is negligible rel-

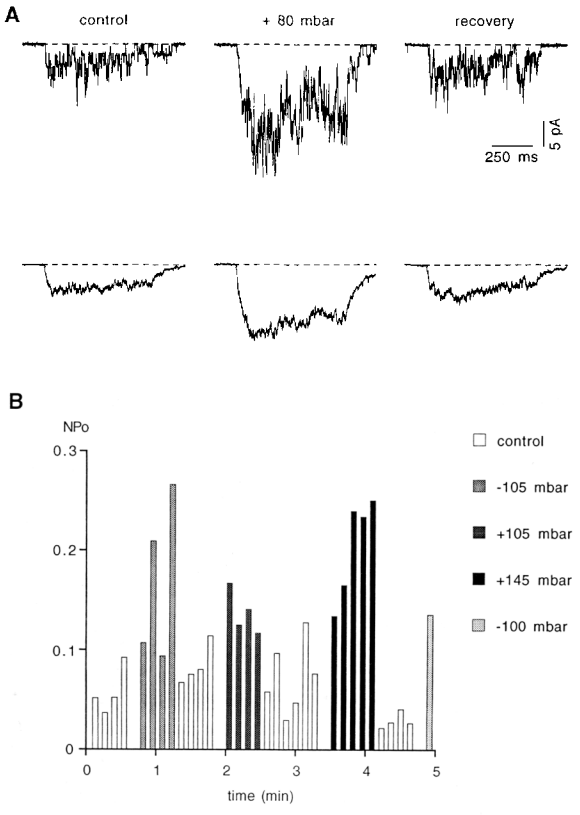

Figure 5. Stretch Sensitivity of the NMDA Response in Excised Patches

(A) An outside-out patch. The upper panel shows individual traces from an outside-out patch before, during, and after applying positive pressure to the patch pipette (+80 mbar). NMDA (10 μM) was applied for 600 ms every 5 s. The lower panel shows the corresponding averaged responses. Eight traces were used for each average. In this patch, the average peak current at +80 mbar showed a 2.3-fold increase compared with the peak current in the absence of applied pressure. Currents were sampled at 2 kHz and filtered at 1 kHz (−3 dB). The pipette solution was CsF (10 mM EGTA). Holding potential was -50 mV.

(B) An inside-out patch. NMDA channel activity recorded in the inside-out configuration was increased by applying either positive or negative pressure to the patch pipette. Each bar represents the channel activity recorded for 8.2 s. The activity recorded during the time necessary to reach the indicated pressures (about 5 s) was deliberately omitted from this histogram (as shown by the successive blanks). NP_o in each condition was 0.06 (control), 0.17 (−105 mbar), 0.08 (control), 0.14 (+105 mbar), 0.07 (control), 0.2 (+145 mbar), 0.03 (control), and 0.14 (−100 mbar). The pipette solution contained 10 μM NMDA and 10 μM glycine. EGTA (10 mM) was added to both the pipette solution and the solution facing the cytoplasmic side of the membrane. Pipette potential was +50 mV.

ative to the volume of the pipette. Thus, no additional hydrostatic pressure can develop in the pipette, and no deformation of the patch is expected. In three experiments in which hypo-osmotic solutions were applied on outside-out patches, the only change observed in the NMDA response was a slight reduction, which could be entirely explained by the (8%) reduction of the single-channel currents (data not shown).

In contrast, changes of the hydrostatic pressure modulated the response of the patches to 10 μM NMDA. In the example illustrated in Figure 5A, the average peak current increased 2.3-fold when a positive pressure of 80 mbar was applied inside the pipette. The effect was almost fully reversible and could be repeated many times on a given outside-out patch. Although the NMDA response was potentiated invariably when positive pressure was applied to the pipette, its amplitude for a given pressure differed considerably between patches. For pipette pressures of 79 ± 5 mbar, the potentiation of the peak NMDA currents ranged from 2.1- to 7-fold (mean = 3.3 ± 0.5; n = 5). This large variability could reflect variability of the resistance of the tip of the pipette or could reflect variations in the patch size. A large pipette means improved transmission to the patch of the pressure applied to the pipette, but it also means an increased patch size. If the patch curvature decreases when the patch diameter increases and if tension is the effective stimulus (see Discussion), Laplace's law predicts that the tension change produced by a given pressure change will increase with the patch diameter (see Sokabe et al., 1991).

In two experiments, kainate and NMDA were alternately applied to standard outside-out patches that were submitted to the same hydrostatic challenge. In the first patch, applying a pressure of +60 mbar to the patch pipette increased the NMDA response 2.5-fold, whereas the kainate response showed only a 1.1-fold potentiation. In the second patch, upon applying a pressure of +85 mbar, the NMDA response increased 5.6-fold, whereas the kainate response was potentiated 1.6-fold. These results provide further evidence for a marked effect of stretch in modulating NMDA channel activity.

NMDA channel activity was also found to be modified by pressure changes in the inside-out configuration (10/13 patches). The pressures required to detect clear effects were usually higher than those in outside-out patches (see above) or cell-attached patches (see below), and the variability of the effects for a given pressure was important. However, inside-out patches turned out to be the only configuration in which both positive and negative pressure could be applied without damage. As shown in Figure 5B (and verified in 3 other patches), both pressures increased the single-channel activity in a reversible manner. The size of the unit current was unaffected by such a treatment (data not shown).

Mechanosensitivity Can Be Observed in Cell-Attached Patches

The cell-attached patch-clamp configuration, unlike either the nucleated patch or the standard outside-out and inside-out configurations, is obtained without membrane excision and can thus provide a model closer to native membrane than excised patches (but see Discussion). Moreover, it preserves an intact cytoplasm. To observe single-channel currents, a low con-

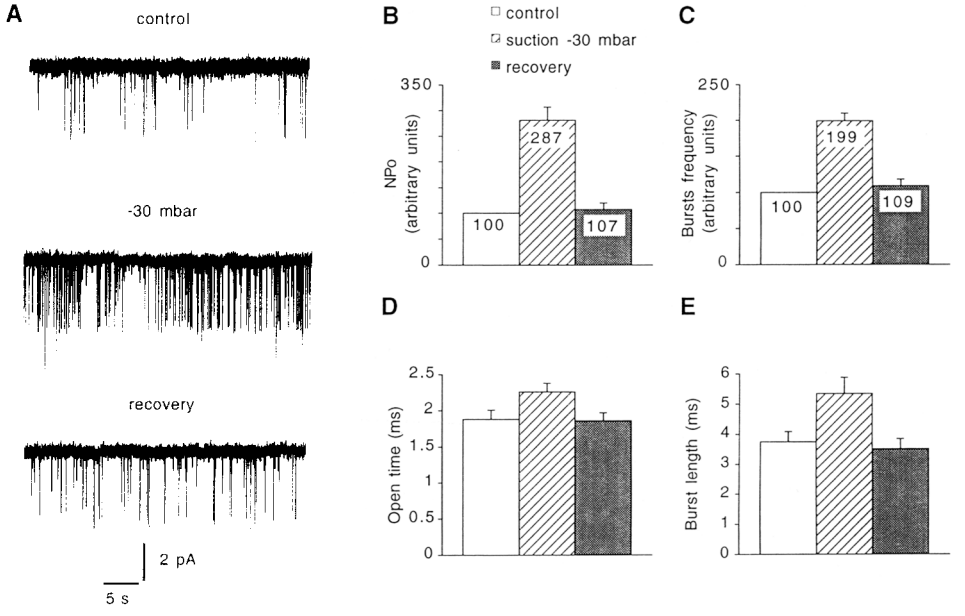

Figure 6. Stretch Sensitivity of NMDA Channel Currrents in Cell-Attached Patches

(A) Single-channel currents were recorded from cell-attached patches with 1 μM NMDA and 10 μM glycine added to the CsF pipette solution (see Experimental Procedures). Downward deflections correspond to currents flowing from the pipette to the cell interior. The left panels show, on a slow time base (each segment corresponds to 40 s of recording), the effect of applying a negative pressure (−30 mbar) to the patch pipette. The open probability for this patch was 3.6×10^{-3} when no suction was applied, 8.3×10^{-3} at −30 mbar, and 4.2×10^{-3} after releasing the suction. The pipette potential was 0 mV. These data were filtered at 1 kHz (−3 dB).

(B–E) The histograms in the right panels summarize the results from 14 different cell-attached patches. The applied suction was −30 mbar in each case. In each condition (control, suction, and recovery), 51 s of data were analyzed. Error bars represent SEM. For (B) and (C), the mean values, normalized to the control values, are indicated in the histograms. (B) Open probability. (C) Bursts frequency. (D) Mean open time. (E) Mean burst length.

centration of NMDA (1 μM) and 10 μM glycine were added to the pipette solution. After a control recording period, patches were transiently subjected to a negative pipette pressure, usually fixed at −30 mbar. The NMDA channel activity for one such trial is shown in Figure 6A, which clearly indicates that channel activity was enhanced when negative pressure was applied. In this example, the open probability increased from 3.6×10^{-3} in the absence of applied pressure to 8.3×10^{-3} at −30 mbar. After releasing the suction, the channel activity recovered almost completely, to an open probability of 4.2×10^{-3}. Similar results were obtained in 14 cell-attached patches (Figure 6B). In all experiments, changes in channel activity, induced by either applying or releasing suction, were instantaneous (at least on the second time scale), as would be expected for a direct mechanical effect.

No difference could be detected in the unitary current between control and suction (−30 mbar) conditions (<2% change). The analysis of dwell time parameters indicated that the mean open time was affected only slightly by pipette pressure. The mean open times were 1.88 ± 0.13 ms in control conditions, 2.26 ± 0.12 ms with suction (−30 mbar), and 1.86 ± 0.11 ms after recovery (Figure 6D). The mean burst length was increased by suction (3.75 ± 0.34 ms, 5.34 ± 0.54 ms, and 3.51 ± 0.34 ms, respectively; Figure 6E), but the major change concerned the burst frequency (closures > 2.5 ms), which almost doubled when suction was applied (Figure 6C). In contrast, the mean closed time within the bursts (closures ≤ 2.5 ms) was unaffected (0.91 ± 0.03 ms, 0.89 ± 0.03 ms, and 0.88 ± 0.04 ms, respectively). Hence, these data strongly suggest that mechanical membrane deformation modulates NMDA channel activity primarily by modifying the frequency of openings.

We could not extend this study to the longer components (clusters and superclusters) of NMDA channel activity because this would have required long recordings (tens of minutes) and therefore prolonged mechanical deformations, which often damaged the patches. Another way to evaluate the slow kinetic components of the NMDA channel is to measure the decay of currents at the end of brief pulses of L-glutamate (see Lester et al., 1990). We used this approach on nucleated patches. L-Glutamate was applied for 40 ms at 100 μM (in the presence of 5 μM 6-cyano-7-dinitroquinoxaline-2,3-dione). Deformation of the membrane was achieved by releasing the suction initially applied in the pipette (see Figure 4). This produced the expected increase of the current amplitude but also a change of time course. The rise time (10%–90%) was unaffected, but the relaxation of the current after the end of the pulse was slowed. In the three

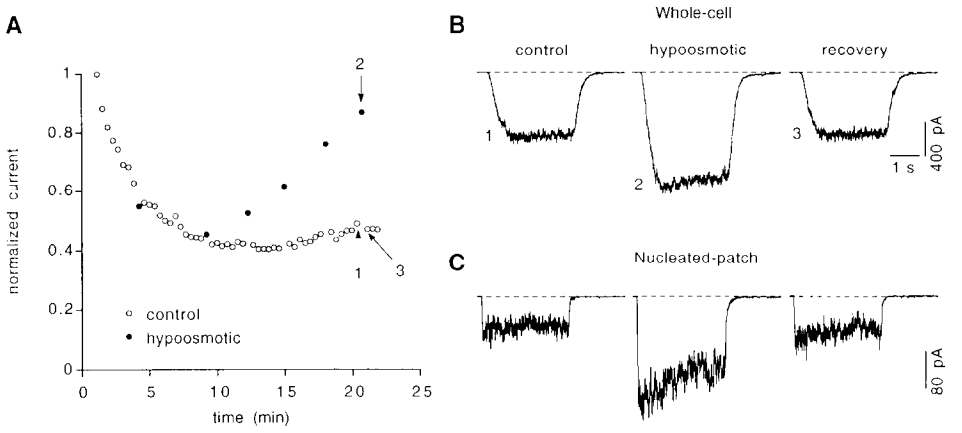

Figure 7. Delayed Appearance of the Stretch Sensitivity of the NMDA Response in the Whole-Cell Mode

(A) Evolution of the peak NMDA currents. Each agonist pulse lasted 3 s and was repeated every 20 s. The cell was perfused with the control solution (140 mM NaCl) and periodically challenged with a hypo-osmotic solution (125 mM NaCl). The hypo-osmotic solution was applied for 20 s before NMDA was applied.

(B) Individual traces before (1), during (2), and after (3) exposure to the hypo-osmotic solution. The numbers refer to (A). The peak potentiation was 1.8-fold.

(C) A nucleated patch was excised from the same cell after a 22 min recording in the whole-cell configuration. The NMDA response was recorded before, during, and after exposure of the patch to the same hypo-osmotic change as in (A) and (B). The peak potentiation was 2.7-fold. NMDA at 10 μM; glycine at 10 μM. The pipette solution was $CsMeSO_3$ (10 mM BAPTA). Holding potential was −50 mV.

experiments analyzed, in which the peak response increased by an average of 2.5-fold, the short and long time constants of the decay increased from 37 ± 3 ms to 70 ± 11 ms and from 240 ± 25 ms to 420 ± 15 ms, respectively. Inasmuch as the off relaxation of the current mimicks the decay of the synaptic current (Lester et al., 1990) and provided that the effects of tension on the NMDA responses are present at the postsynaptic membrane, these results predict that an increased tension will increase and prolong the NMDA component of the synaptic current.

Osmosensitivity in the Whole-Cell Mode

When we analyzed the effect of hypo-osmotic solutions in the whole-cell mode, we found that the potentiation was nearly undetectable during the first few minutes following rupture of the membrane patch but developed progressively within a period that varied from 10 to more than 30 min. This duration is in the same range as that of the process labeled run-down by various authors (MacDonald et al., 1989; Rosenmund and Westbrook, 1993; Vyklicky, 1993; Wang et al., 1993). In all cases, the osmosensitivity developed only after the current amplitude had reached a quasi steady level, i.e., once the run-down was nearly complete. This is illustrated in Figure 7A. Shortly after the beginning of the perfusion, switching from a standard external solution (140 mM NaCl) to a diluted solution (125 mM NaCl) did not potentiate the response but actually induced a slight reduction of the current (expected from the small changes in elementary current). During the next 10 min, the response to NMDA progressively decreased, until it stabilized at about 40% of its initial amplitude. At about the same time, exposure to the same hypo-osmotic solution started to overcome the effect of the decrease in elementary current. Soon after, a net potentiation was visible, which then increased with time. As shown in Figure 7B, this potentiation was fully reversible. In this example, the largest increase in the peak current was 1.8-fold, but this was probably not the maximum possible increase. The experiment was terminated by excision of a nucleated patch, the response of which showed a 2.7-fold potentiation in response to the same osmotic challenge (Figure 7C). The mean ratio of the hypo-osmotic current to the control current was 0.93 ± 0.01 ($n = 12$), during the first few minutes of recording ($t < 6$ min), whereas the mean potentiation observed at later times (20 min $\leq t \leq$ 62 min) was 1.3 ± 0.08 ($n = 12$). Thus, a clear effect could be observed, but it was never as large as that observed in nucleated patches.

Kainate-induced (50 μM) whole-cell currents challenged with the same hypo-osmotic changes were examined in parallel with NMDA currents (10 μM) in five cells. Both responses ran down. However, even after prolonged intracellular dialysis and the concomitant development of the osmosensitivity of the NMDA response (≥1.3-fold potentiation), kainate currents always showed a slight decrease (data not shown).

Discussion

Our results indicate that, under certain experimental conditions, NMDA responses are modulated by changes in osmotic pressure and by changes in hydrostatic pressure. We will first discuss the evidence suggesting that the common factor linking these effects

is a mechanical deformation modifying the tension of the plasma membrane. We will then discuss the nature of the structural elements transmitting the deformation (lipid bilayer and/or cytoskeleton). Finally, we will discuss the possible functional implications of the mechanosensitivity of NMDA channels.

Mechanical Stress Is the Most Likely Common Stimulus in All the Experiments

Our first observations were made on nucleated patches submitted to changes in the extracellular osmotic pressure. The resulting changes in the NMDA responses could be attributed to a number of factors other than the mechanical deformation associated with swelling or shrinkage. One could invoke, for example, the changes in extracellular Na^+ concentrations or the osmotic flow of water through ionic channels, with its possible effects on ion fluxes through the channels. However, such explanations are ruled out by the fact that the effects of the osmotic challenges observed on nucleated patches were not observed when the experiments were repeated on standard outside-out patches, which experience the same extracellular Na^+ concentration change and in which the transmembrane osmotic gradient must actually be higher than that of nucleated patch (where part of the osmotic pressure difference must dissipate between the pipette and the nuclear compartment). A second type of explanation of the effect of the hypo-osmotic challenge could be that water entry dilutes some intracellular messenger. Changes of Ca^{2+}, pH, or phosphorylations are unlikely, since the experiments were performed in the presence of internal pH and pCa buffers, without ATP or GTP, and in the presence of internal F^- (which blocks most phosphatases), but one could still invoke the dilution of unknown regulatory signals. This interpretation, however, does not account for the effects observed by applying hydrostatic pressure to nucleated, outside-out, inside-out, or cell-attached patches. In nucleated patches, the NMDA-induced activity was potentiated by releasing the suction or applying positive pressure to the pipette. This could be explained by a displacement of the nucleus unblocking the pipette, allowing the Ca^{2+} buffer to enter the nuclear compartment, and releaving some Ca^{2+}-dependent inactivation of the NMDA channels (Legendre et al., 1993). However, one does not expect such a process to show the reversibility illustrated in Figure 4C. Furthermore, hydrostatic pressure changes were also effective in excised and cell-attached patches. Under these conditions, changes in channel activity followed very rapidly the changes in pressure and could be observed under conditions (inside-out configuration; Figure 5B) in which no Ca^{2+} accumulation was likely to occur. Thus, all our observations can be interpreted by assuming that mechanical deformation is the cause of the effects observed following either pressure or osmotic changes.

Since the first description of stretch-sensitive channels by Guharay and Sachs (1984), it has been assumed that the effective stimulus is membrane tension. This hypothesis is supported by the fact that stretch-activated channels will open with either positive or negative pressure (see Sachs, 1990). The experiments of Sokabe et al. (1991), in which the shape of the membrane could be monitored during the changes in pressure, supported this hypothesis by showing that the lowest level of activity is observed when the patch is flat, and that a similar response is observed when the patch is deformed in opposite directions by pressure or suction. The observation that an increase in NMDA channel activity is seen with either positive or negative pressure (in the inside-out configuration) is in good agreement with these findings.

In nucleated patches, positive and negative pressure changes do not produce similar effects, but opposite ones. Hypo-osmotic and hyper-osmotic solutions produce inverse effects of comparable size (Figure 3; see also Langton, 1993; Oliet and Bourque, 1993). Suction reduces the size of the response (Figure 4D), whereas blowing increases it. These observations can be reconciled with those made on inside-out patches by assuming that, in nucleated patches, the membrane is under tension in the absence of an applied pressure. A stimulus that increases the pressure (osmotic or hydrostatic) increases this tension; a stimulus that decreases the pressure reduces but does not abolish it. The resting tension could be due to the fact that, despite the identity of osmotic pressures in the pipette and bath, the presence of proteins and the nucleus inside the intermediate compartment may create a resting osmotic pressure difference inducing swelling.

Lipid Bilayer or Cytoskeleton?

Hypotheses concerning the structures mediating the mechanosensitivity of stretch-activated channels have usually considered either the lipid bilayer or cytoskeletal proteins. The suggestion that a local change in the lipid bilayer modifies the tension exerted on a channel has received support from experiments indicating that stretch sensitivity persists in channels reincorporated in lipid bilayers (Sukharev et al., 1993; Opsahl and Webb, 1994). The effects of stretch can be mimicked by applying amphipathic molecules, an observation explained by assuming that these molecules incorporate preferentially in one side of the bilayer and modify its curvature (Martinac et al., 1990; Sukharev et al., 1993). If this type of mechanism were involved in the case of NMDA responses, it could link the potentiations produced by stretch to those produced by arachidonic acid (Miller et al., 1992) and docosahexaenoic acid (Nishikawa et al., 1994). In the case of docosahexaenoic acid, the potentiation involves both an increase of apparent affinity and an increase of the maximal response (Nishikawa et al., 1994), two effects also suggested by the lower sensitivity to osmotic challenges (Figure 3) and pressure changes (Figure 4) of the responses to high concentrations of NMDA.

The submembrane cytoskeleton is the second structure often considered when trying to explain how membrane tension can alter the function of a channel (see Sachs, 1990). In the case of NMDA channels, its involvment is supported by the observations of Rosenmund and Westbrook (1993), who showed that cytochalasins induce a run-down of NMDA currents and suggested that NMDA receptors are linked to actin filaments.

The lipid bilayer and the cytoskeleton may both be involved in mechanosensitivity, and this duality may explain why the mechanosensitivity seen in patches is difficult to demonstrate in the whole-cell mode. This observation has been the cause for considerable doubt about the physiological role of stretch-activated channels (see Morris and Horn, 1991). It seems possible that in physiological conditions, in an intact cell, any tension exerted through the lipid bilayer will be dampened by the stiffness of the cytoskeletal fibers. The large effects observed in patches (and in the whole-cell mode after a prolonged perfusion) may be visible only because of damage inflicted to the cytoskeleton by the absence of ATP, by the excision (nucleated, outside-out, or inside-out patches), or simply by the suction applied to seal the pipette against the cell membrane (cell-attached patches; Milton and Caldwell, 1990). Progressive decoupling of the membrane from the cytoskeleton, induced by membrane stress, has also been suggested by the experiments of Hamill and McBride (1992) on stretch-activated channels in Xenopus oocytes. We have observed that, in cell-attached patches, successive suction challenges produce progressively larger effects (unpublished data), which may imply that the NMDA sensitivity to stretch could be increased by destroying the cytoskeleton.

What Roles Could Stretch Sensitivity of NMDA Responses Play in Physiological or Pathological Situations?

There are several situations in which neuronal membranes may be submitted to mechanical deformation. One of the first situations that comes to mind, in view of the effects of osmotic challenges, is the neuronal swelling observed in conditions of excitotoxic damage (Choi, 1988). Dendritic swelling often involves the appearance of focal varicosities (beading), which follows cytoskeletal damage (Goldberg and Bateman, 1993, Soc. Neurosci., abstract). Thus, local conditions are found (existence of a stress and presence of NMDA receptors) that could give a role to the stretch sensitivity of the NMDA receptors. If high glutamate levels induce cell swelling and if cell swelling in turn increases NMDA responses, the mechanosensitivity of the NMDA channels will add a positive feedback loop to the numerous such loops involved in neurotoxicity (Choi, 1988).

If, however, one looks for a physiological role of NMDA receptor mechanosensitivity, osmotic effects are unlikely to be significant, particularly in view of their failure to act in the whole-cell mode. A more promising lead may be obtained by identifying regions of the neurons where NMDA receptors are present and where the abundance of cytoskeletal elements could indicate the possible existence of strong *local* membrane tensions. A link between NMDA receptors and these cytoskeletal elements is suggested by the data indicating that the NMDA channel activity may be modulated by the actin polymerization state (Rosenmund and Westbrook, 1993) and by the data indicating that NMDA receptor activity may modulate cytoskeletal proteins (Halpain and Greengard, 1990). Therefore, it is possible to imagine that local changes in geometry produced by the cytoskeleton modify the NMDA receptor sensitivity. Alternatively, as noted by Howard et al. (1988), if the stretch sensitivity results from the fact that opening of the channels is associated with an increased area, the change in membrane area produced by channel openings will give rise to an increase in membrane compliance. Thus, the activation of the NMDA receptor could modify the compliance locally and make the membrane more easily deformed by a given cytoskeleton-induced tension.

Two neuronal regions in which NMDA receptors may be subject to local tensions are dendritic spines and growth cones. Dendritic spines, which are known to bear numerous NMDA receptors, contain a very dense network of cytoskeletal elements, including actin, tubulin, fodrin, microtubule-associated protein 2, etc. (Gulley and Reese, 1981; Matus et al., 1982; Morales and Fifková, 1989a, 1989b; Kennedy, 1993), and many reports suggest that activity-dependent enhancement of synaptic transmission is associated with morphological changes of the spines (for review, see Fifková and Morales, 1992). However, evidence for local membrane tension has remained circumstantial. In contrast, there is a wide consensus that the membranes of growth cones experience large changes in tension during their extension and retraction (see Condeelis, 1993; a direct correlation of growth cone advance and increase in membrane tension was observed by Lamoureux et al. [1989]), but a role for NMDA receptors in these processes has remained, until recently, much less clear. There is evidence supporting a role for NMDA receptors in neurite extension and formation (Cambray-Deakin and Burgoyne, 1992; Rashid and Cambray-Deakin, 1992) and in cell migration (Komuro and Rakic, 1993), but the localization of the NMDA receptors involved in these processes has remained vague. However, M. Herkert and C.-M. Becker (personal communication), using an antibody against the NR2B subunit of the NMDA receptor, have recently revealed a very strong staining in growth cones of cultured hippocampal neurons. This could be of special interest concerning the functional relevance of the mechanosensitivity of the NMDA receptors, since tension changes experienced during migration could affect intracellular Ca^{2+} via a modification of NMDA channel activity and thereby regulate neuritic growth. Glutamate may thus function as a

guidance cue of growth cones. In contrast to its synaptic role, it would act here as a more diffuse messenger and in a lower concentration range in which the mechanosensitivity of the NMDA receptors is maximal.

Experimental Procedures

Cultures

Cortical and diencephalic neurons taken from 15- to 16-day-old mouse embryos were cultured for 2–5 weeks as previously described (Ascher et al., 1988).

Recording Conditions

Experiments were performed in four usual patch-clamp configurations (whole-cell, outside-out, inside-out, and cell-attached; Hamill et al., 1981) as well as in nucleated patches. Nucleated patch formation starts like that of conventional outside-out patches (Hamill et al., 1981): after obtaining the whole-cell recording configuration, the patch pipette is carefully withdrawn from the cell, while maintaining the pipette suction (see Sather et al., 1992). Soft glass patch pipettes of 4–6 MΩ were filled with a solution containing 120 mM CsF, 10 mM CsCl, 10 mM EGTA (or 10 mM BAPTA), and 10 mM HEPES (CsF solution); CsOH was used to adjust the pH to 7.2. The osmolarity was 270 mOsm/kg. In all whole-cell experiments, the major intracellular anion was methanesulfonate ($CsMeSO_3$ solution) instead of F^-. All whole-cell pipette solutions contained BAPTA (10 mM) as a Ca^{2+} chelator. The intracellular free Ca^{2+} concentration was usually 50 nM.

The standard external solution (control solution) contained 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES; NaOH was used to adjust the pH to 7.3. The osmolarity was 270 mOsm/kg. Hypo-osmotic external solution was obtained by reducing external NaCl (from 140 to 125 mM) while keeping constant other ionic concentrations. Hyper-osmolarity was obtained by increasing NaCl to 155 mM. The osmolarities of the solutions were systematically checked on a vapor pressure osmometer (Wescor 5500). The osmolarity difference between the control solution and the hypo-osmotic and hyper-osmotic solutions was ±27 mOsm/kg (± 5%). Drugs were applied to the patch by means of a four barrel fast perfusion system (Sather et al., 1992). A saturating concentration of glycine (10 μM) was added to all solutions when NMDA responses were studied. Solutions flowed continuously by gravity from all barrels. All whole-cell experiments were done in the presence of tetrodotoxin (200 nM) to inhibit spontaneous activity. For single-channel recordings in cell-attached and inside-out patches, NMDA (1 μM for cell-attached and 10 μM for inside-out) and glycine (10 μM) were added to the Ca^{2+}-free (10 mM EGTA) CsF pipette solution defined above. All experiments were conducted at room temperature (18°C–25°C).

Glycine and GABA were obtained from Sigma; NMDA, kainate, and D-AP5 from Tocris; tetrodotoxin from Janssen.

Recording and Data Analysis

Whole-cell and single-channel currents (Hamill et al., 1981) were recorded using a List EPC-7 amplifier and a Racal FM tape recorder. The voltage-clamp current was filtered (8-pole Bessel) with the corner frequency (250 Hz for nucleated patch and whole-cell recordings; 1 or 2 kHz for outside-out, inside-out, and cell-attached recordings) half that of the sampling frequency, digitized, and later analyzed using Strathclyde Electrophysiology Software on a PC computer. The pipette pressure was monitored by a pressure transducer (Sensyn). For cell-attached patches, NMDA channels were identified by their characteristic conductance levels and their flickering, voltage-dependent behavior when 10 μM Mg^{2+} was present in the pipette solution. To estimate the main unitary NMDA channel conductance, the pipette potential was varied until the reversal potential could be evaluated, and the conductance could be derived from the slope of the current–voltage relation, which was linear between −50 and 0 mV. The value was around 70 pS, as expected for cell-attached recordings with a Ca^{2+}-free pipette solution (Gibb et al., 1993, J. Physiol., abstract). The holding potential was usually around −60 mV (0 mV in the pipette; membrane potential determined by assuming the reversal potential was equal to 0 mV). Inside-out patches were obtained by excising the patch in Ca^{2+}-free solutions (10 mM EGTA, no added Ca^{2+}), and NMDA channels were characterized by their conductance (which showed inward rectification [Gibb et al., 1993, J. Physiol., abstract] with low extracellular Ca^{2+}) and their sensitivity to Mg^{2+} applied to the cytoplasmic face of the membrane (Johnson and Ascher, 1990). Events were detected using a 50% threshold criterion, and NP_o (with N being the number of channels available and P_o being the absolute open probability) was determined as the fraction of the time spent in the open state. To evaluate the changes in the open probability of the channels recorded in inside-out and cell-attached patches, NMDA activity was analyzed over periods ranging from tens of seconds to a few minutes, since NMDA channel activity in patches shows spontaneous fluctuations with time (Gibb and Colquhoun, 1992). Bursts of openings were defined as groups of openings separated by closures of a duration less than a critical gap length, fixed at 2.5 ms (evaluated from the briefest shut time component). Analysis of nucleated patch data was complicated by the evolution of the NMDA receptor behavior during prolonged dialysis (Sather et al., 1992). Currents did not always recover fully from the membrane deformation protocols, necessitating extrapolation of the data for quantitation.

For whole-cell recordings, the series resistance (5–12 MΩ) was compensated partially (50%–90%) and controlled throughout the experiment. Osmotic changes did not modify the series resistance in a measurable way. All mean values have been reported together with standard errors of the mean.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique (URA 295), by a grant from the Human Frontier Research Program, and by the Université Pierre et Marie Curie. We thank Boris Barbour, Dominique Chesnoy-Marchais, Anne Joly, JacSue Kehoe, Alain Marty, and Jacques Neyton for comments on the manuscript, Xavier LeRoux for help with statistical analysis, and Dan Lévy for culturing the neurons.

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Received April 7, 1994; revised June 13, 1994.

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