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Secondary Activation of a Cation Conductance Is Responsible for NMDA Toxicity in Acutely Isolated Hippocampal Neurons

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One of the key questions concerning glutamate toxicity is how a transient NMDA exposure can lead to a delayed death of neurons. To address this issue, we performed whole-cell recording on acutely isolated hippocampal CA1 neurons to monitor the membrane response after NMDA exposure. Transient NMDA exposure (100 μM, 10 min) induced an inward current (postexposure current; I_{pe}) which was associated with a Ca^{2+}- and Na^{+}-permeable cation conductance. I_{pe} continuously increased (in the absence of NMDA) until death of the neuron occurred. Application of NMDA in the absence of extracellular calcium failed to trigger I_{pe} and neuronal death. Postexposure suppression of I_{pe} protected against NMDA toxicity. These results indicate that a cation current, which is induced by an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and is itself partly carried by Ca^{2+}, links the initial NMDA exposure to neuronal death.

Key words: NMDA; neurotoxicity; postexposure current; I_{pe}; excitotoxicity; calcium; hippocampus; glutamate; toxicity; cell death; neuronal death

Excess levels of the excitatory neurotransmitter glutamate can induce neuronal degeneration, primarily through NMDA receptor channel-mediated Ca^{2+} influx (Choi, 1988). Neuronal death associated with ischemia, hypoglycemia, trauma, and epilepsy can be reduced with antagonists of the NMDA type of glutamate receptor, suggesting the involvement of NMDA toxicity in these clinical conditions (Simon et al., 1984; Wieloch, 1985; Faden et al., 1989; Meldrum, 1994). However, the clinical utility of NMDA receptor antagonists faces a key impediment: the timing of treatment. The neuroprotective effect of NMDA receptor antagonists is significant only when administered before the insult (ischemia or transient NMDA exposure) or within a short window after the insult (Hartley and Choi, 1989; Graham et al., 1993). This short therapeutic window of NMDA receptor antagonists limits their usefulness and prompts the study of downstream mechanisms in the postexposure phase, the period between the initial transient NMDA exposure (exposure phase) and the death of neurons. During the postexposure phase, there is a secondary rise in intracellular calcium concentration ($[Ca^{2+}]_i$), which is not reversed by NMDA antagonists (Randall and Thayer, 1992; Tymianski et al., 1993). This secondary rise in $[Ca^{2+}]_i$ is correlated with subsequent cell death (Randall and Thayer, 1992; Tymianski et al., 1993). Neither the secondary rise in $[Ca^{2+}]_i$ nor the cell death occurs if the NMDA is applied in Ca^{2+}-free solution (Randall and Thayer, 1992), suggesting that Ca^{2+} influx through NMDA receptor channels somehow triggers a process that causes the secondary rise in $[Ca^{2+}]_i$ and subsequent cell death. Suggested mechanisms for the secondary rise in $[Ca^{2+}]_i$ include an increase in Ca^{2+} conductance, derangement of Ca^{2+} transport, and release of Ca^{2+} from intracellular stores (de Erausquin et al., 1990; Randall and Thayer, 1992; Tymianski et al., 1993). In this paper, we have done electrophysiological recordings to monitor the membrane response after NMDA exposure and have determined that Ca^{2+} influx through NMDA receptors leads to the activation of a persistent Ca^{2+}- and Na^{+}-permeable cation conductance that is responsible for the subsequent neuronal death.

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We are saddened by the passing of our colleague, Dr. Qiang X. Chen, and dedicate this article to his memory.

MATERIALS AND METHODS

Whole-cell voltage-clamp recording was performed on acutely isolated hippocampal CA1 neurons. Neurons were prepared according to the Kay and Wong (1986) method, with the following modifications to increase the harvest of healthy neurons and preserve NMDA responses. Hippocampal slices were prepared by vibratome instead of tissue chopper, the $[Ca^{2+}]$ in the incubation solution was reduced to 0.5 mM and the $[Mg^{2+}]$ was increased to 7 mM, and 12–15 μM CPP (Tocris Cookson, St. Louis) was added during trypsin incubation of tissue slices to preserve the NMDA response (Chen and Wong, 1995a). Healthy neurons were selected for our experiments by choosing those that were uniformly bright under phase-contrast microscopy. These neurons have a normal (approximately −60 mV) and stable resting potential within the first hour of recording, have an ability to fire action potentials, and show reversible receptor-channel modulation by second-messenger systems (Chen et al., 1990; Chen and Wong, 1995a,b).

Whole-cell voltage-clamp recording was performed using the procedure described by Hamill et al. (1981) with the use of a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany) and pClamp software (Axon Instruments, Foster City, CA). Access resistances were ~10 MΩ and were compensated only in the experiments shown in Figure 2. Liquid junction potentials (V_{lj}) were measured using the procedure of Neher (1992). All potentials have been corrected for V_{lj}, which ranged from 9 to 14 mV. Good recordings were ensured by discarding cells that had seal resistances <20 GΩ, that took more than three gentle sucks to break the membrane, or that did not maintain a stable input resistance during the first 5 min of recording before NMDA exposure. The holding potential was −55 mV.

In one set of experiments, the intracellular perfusion technique was used to switch from control intracellular solution to high-calcium intracellular solution while recording from a single cell. For details of the intracellular perfusion method, see Chen et al. (1990).

Cells were in a 1 ml bath that was perfused continuously with extracellular solution at a rate of 1–2 ml/min. Extracellular control solution contained (in mM): 140 NaCl, 2 KCl, 2 $CaCl_2$, 10 HEPES, 0.01 glycine, 25

glucose, pH adjusted to 7.4 with NaOH (which raised the $[Na^+]$ to 145 mM). Zero-calcium solution was the same as control solution, except with 0.5 BAPTA and no $CaCl_2$. Application of NMDA and changes of ionic concentration around the recorded cells were achieved by a seven-barrel flow tube (Celentano and Wong, 1994). NMDA (100 μM) was dissolved in control solution or zero-calcium solution as indicated. The 20 mM NaCl, 20 mM KCl, and 10 mM $CaCl_2$ solutions contained only the indicated ions, 10 mM HEPES, 0.01 mM glycine, 25 mM glucose, and sucrose added to preserve osmolarity. Intracellular control solution contained (in mM): 20 CsCl, 100 CsOH, 0.5 BAPTA, 10 HEPES, pH adjusted to 7.2 with methane sulfonic acid. High-calcium intracellular solution was made by adding 1 mM Ca^{2+} to the control intracellular solution. The estimate of free Ca^{2+} in the high-calcium intracellular solution is 0.5 mM (Chen et al., 1990). The actual $[Ca^{2+}]_i$ reached because of perfusion of this solution, although likely to be <0.5 mM, is unknown. Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Ionic activities were used for the calculation of relative permeabilities. To simplify the calculation of relative permeabilities, we assumed no significant effect from the surface charge on the permeation of cations. We also assumed that both the ionic composition around the intracellular side of the membrane and the relative permeability of the membrane were unchanged during the experiment. We used cells in which the I_{pe} was less than −1 nA in obtaining data for the evaluation of the relative permeability of the I_{pe} channel, because as I_{pe} increased in amplitude, extracellular ions accumulated around the intracellular side of the membrane as indicated by the gradual negative shift in the reversal potential when the amplitude of I_{pe} exceeded −1 nA.

Trypan blue stain was used to assess cell death. Staining was performed by a 5 min bath perfusion of dye solution (0.4% final concentration, made up in extracellular control solution). Neurons were examined for stain under bright-field microscope 5 min after changing back to control bath perfusion.

In some experiments, cell death was assessed in nonrecorded cells. For these experiments, cells were isolated from a single animal and put in several dishes called "sister dishes." Dishes were prescored into square fields with a 5 mm × 5 mm grid. For each dish, one or two fields, each containing 10–30 healthy neurons, were selected before treatment, and the healthy neurons in the selected fields were identified. Neurons were perfused continuously with extracellular solution at a rate of 1–2 ml/min. Trypan blue stain was used to assess neuronal death after treatment.

Data are reported as mean ± SD.

RESULTS

Prolonged NMDA application triggers I_{pe} and cell death

We have shown that with a short (2–8 sec, 100 μM) NMDA application, the holding current returns to baseline after termination of the NMDA application (Chen and Wong, 1995a,b). Figure 1*A* shows the result of a long (10 min, 100 μM) NMDA application (exposure). The NMDA response changed kinetics at 5.6 ± 1.4 min ($n = 8$) of NMDA application, with the appearance of a superimposing and continuously increasing inward current accompanied by an increase in membrane conductance. This inward current, which we call the postexposure current (I_{pe}), persisted after termination of NMDA application and increased at a rate of ~62 pA/min throughout the recordings, which ended at 30–40 min postexposure ($n = 8$ cells). The input resistance and holding current at −55 mV were 2.9 ± 1.1 GΩ and −22.5 ± 10.3 pA before NMDA exposure and 23 ± 3 MΩ and −2.4 ± 0.3 nA ($n = 8$) at 30 min after exposure. Cell death was assessed at 30 min postexposure; all eight cells showed positive trypan blue stain. In four of these eight cells, trypan blue stain was also performed at 0, 10, and 20 min postexposure; only one neuron showed positive stain at 20 min postexposure.

A rise in $[Ca^{2+}]_i$ induces I_{pe}

When cells were bathed in zero-calcium solution during NMDA exposure, no continuously increasing holding current was observed during the postexposure phase (Fig. 1*B*). The input resistance and holding current were 2.8 ± 1.2 GΩ and −24 ± 12 pA before exposure and 2.5 ± 1 GΩ and −29 ± 19 pA ($n = 6$) after exposure. None of the six neurons stained with trypan blue at 30 min postexposure. The possibility that a rise in $[Ca^{2+}]_i$ triggers I_{pe} was tested further by using the intracellular perfusion technique. Figure 1*C* shows that an increase in $[Ca^{2+}]_i$ caused by an 18 min intracellular perfusion of high-calcium intracellular solution induced an inward current ($n = 7$). This inward current was indistinguishable from I_{pe} in its reversal potential and ionic selectivity (see below).

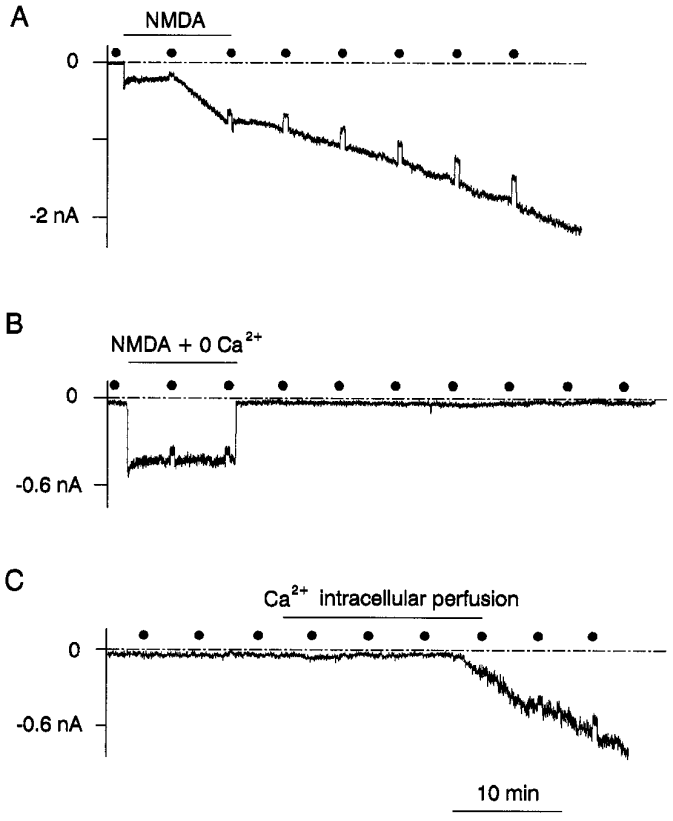

Figure 1. NMDA exposure and rises in $[Ca^{2+}]_i$ trigger I_{pe}. *A*, Response to NMDA exposure (10 min, 100 μM) in the presence of Ca^{2+}_o. *B*, Response to NMDA exposure (10 min, 100 μM) in zero-calcium extracellular solution. *C*, Response to intracellular perfusion of high-Ca^{2+}solution. To measure input resistance, voltage steps (+10 mV, 30 sec) were applied once every 5 min at the times indicated by the *circles*. These voltage steps have no effect on the development of I_{pe}. *Horizontal lines* on *top* of each current trace indicate periods of solution application.

I_{pe} is a cation current

To test the ionic basis of I_{pe}, we performed several ion substitution experiments. In the first, replacing 120 mM extracellular Cl^- with the presumably less-permeant anion gluconate (120 mM) changed neither the reversal potential of nor the conductance associated with I_{pe} (Fig. 2*B*). In another set of experiments, we created a significant salt gradient across the membrane by applying one of three extracellular isotonic low-salt solutions (20 mM NaCl, 20 mM KCl, or 10 mM $CaCl_2$). Reducing the extracellular salt concentration caused a reduction in the amplitude of the inward I_{pe} (Fig. 2*A*) and a negative shift in the reversal potential (Fig. 2*C*), which is the opposite result of what would be expected if I_{pe} were an anion current, indicating that the contribution of anion effluxes to I_{pe} is negligible. The inward I_{pe} could be obtained

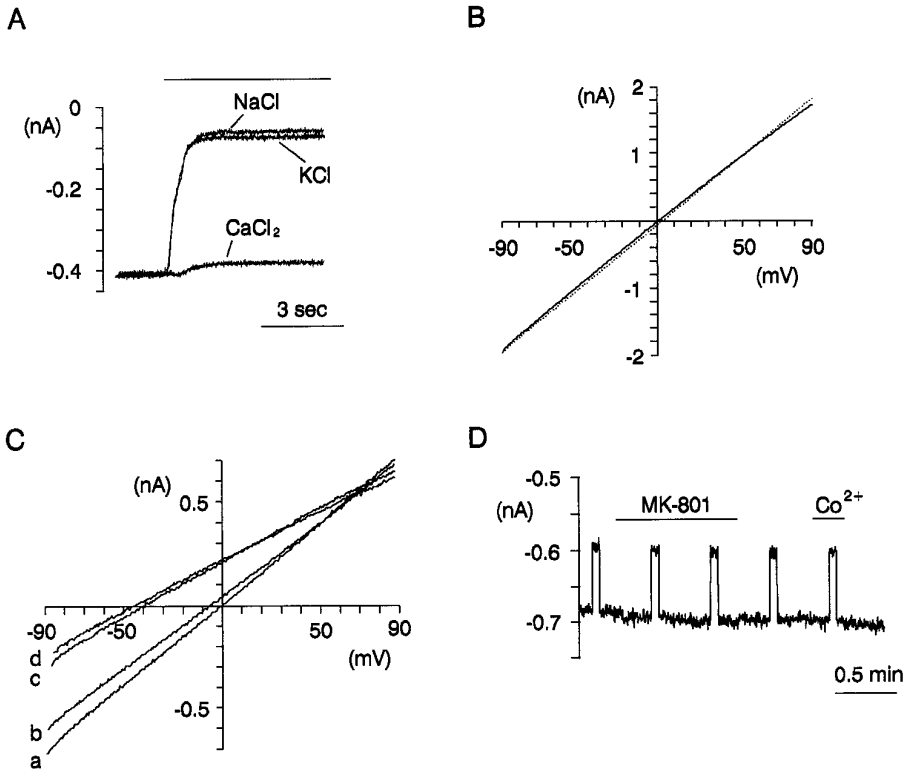

Figure 2. I_{pe} is carried by Ca^{2+} and Na^+. *A–D*, I_{pe} was triggered by NMDA exposure (10 min, 100 μM) or intracellular perfusion of high-calcium solution. *A*, Responses of I_{pe} to changes of extracellular ionic composition. *Line above* current traces indicates period of perfusion of 20 mM NaCl solution, 20 mM KCl solution, or 10 mM $CaCl_2$ solution. Changes of I_{pe} reached a plateau (indication of complete solution change around cell) within 2 sec. These responses are not from the same cell; because I_{pe} grows over time, we chose instead to show responses in which the I_{pe} amplitudes (directly before the change to test solution) matched. *B*, *C*, Voltage ramps (−90 mV to +90 mV, 600 msec duration) performed 3–6 sec after changing to the indicated solutions. *B*, I_{pe} during the voltage ramp in the presence of extracellular control solution (*continuous trace*) and low-Cl^- solution (*dotted trace*). (Traces essentially overlap.) The reversal potentials of I_{pe} were 0.8 ± 0.6 mV (control solution) and 0.8 ± 0.7 mV (low-Cl^- solution, mean ± SD, $n = 5$). *C*, I_{pe} during the voltage ramp in the presence of extracellular control (*a*), 10 mM $CaCl_2$ (*b*), 20 mM KCl (*c*), and 20 mM NaCl (*d*) solutions. During the voltage ramps, TTX (5 μM) and Co^{2+} (200 μM) were applied to block voltage-dependent Na^+ and Ca^{2+} currents. Baseline leak current before NMDA exposure was not subtracted from current traces shown in *B* and *C*, because the subtraction made no significant difference in the reversal potential or conductance measured. *D*, Neither MK-801 (20 μM) nor Co^{2+} (200 μM) suppressed I_{pe}. Voltage steps (+10 mV) were applied every 25 sec. Cells examined had an I_{pe} smaller than −1 nA.

with any single cation tested (Na^+, K^+, or Ca^{2+}) alone in the extracellular solution (Fig. 2*A*,*C*), indicating that Na^+, K^+, and Ca^{2+} can all carry the inward I_{pe}. With Cs^+(120 mM) on the intracellular side of the membrane and either Na^+ (20 mM), K^+ (20 mM), or Ca^{2+} (10 mM) on the extracellular side of the membrane, the reversal potentials of I_{pe} were −45 ± 0.6, −43 ± 0.8, and −5 ± 0.7 mV, respectively (mean ± SD, $n = 5$). With these data, we estimated the relative permeabilities $P_{Cs}/P_{Na}/P_K/P_{Ca}$ to be 1:0.9:0.9:6.3. The high-Ca^{2+} permeability and the lack of discrimination among different monovalent cations are comparable with the properties of the NMDA receptor channel (Mayer and Westbrook, 1987). However, blockers for the NMDA receptor channel, MK-801 (20 μM, 1 min exposure) and ketamine (20 μM, 1 min exposure), had no effect on I_{pe} ($n = 4$; Fig. 2*D*). In addition, voltage-dependent Ca^{2+} channel blockers Co^{2+} (200 μM, 20 sec exposure; Fig. 2*D*) and nimodipine 10 μM (20 sec exposure), and the Na^+ channel blocker tetrodotoxin (5 μM, 10 sec exposure) all failed to affect the I_{pe} ($n = 4$).

I_{pe} is responsible for NMDA toxicity

We have demonstrated that I_{pe} was carried by Ca^{2+} and Na^+ (Fig. 2) and persisted and increased continuously in amplitude during the postexposure phase (Fig. 1*A*). These properties give I_{pe} the potential of causing substantial accumulation of Na^+ and Ca^{2+} in the intracellular space. Accumulation of both ions, particularly Ca^{2+}, inside a neuron is cytotoxic (Choi, 1988). As presented above, a normally toxic exposure to NMDA does not kill the cells when I_{pe} is not activated (zero-calcium extracellular solution; Fig. 1*B*). To further address the causal connection between I_{pe} and cell death, we tested whether postexposure suppression of I_{pe} would reduce neuronal death. Without a specific blocker for I_{pe}, we took advantage of the fact that I_{pe} can be suppressed by the 20 mM NaCl solution (see Fig. 2*A*). Extracellular perfusion with the 20 mM NaCl solution after the NMDA exposure caused an immediate suppression of I_{pe} of 86 ± 1% ($n = 8$; Fig. 3*A*). In addition, perfusion with the 20 mM NaCl solution during the postexposure phase prevented the growth of I_{pe} (Fig. 3*A*); the change in I_{pe} amplitude during the 30 min postexposure phase was ≤18% ($n = 8$) compared with a ≥100% change in I_{pe} amplitude in control extracellular solution ($n = 8$). None of the cells exposed to the 20 mM NaCl solution during the postexposure phase stained with trypan blue at 30 min postexposure ($n = 8$), indicating that suppression of I_{pe} in the postexposure phase was neuroprotective.

To determine whether Ca^{2+} and/or Na^+ influx led to the growth of I_{pe} and the death of neurons, we removed only Ca^{2+} from the control extracellular solution during the postexposure phase. Extracellular perfusion with the zero-Ca^{2+} solution during the postexposure phase prevented the continuous increase in I_{pe} amplitude (Fig. 3*B*; compare Fig. 1*A*). In addition, we observed an immediate 32 ± 6% ($n = 6$) increase in I_{pe}, possibly attributable to removing the suppressive effect of $Ca^{2+}{}_o$ on Na^+ influx, as observed for other Ca^{2+}-permeable conductances (Mayer and Westbrook, 1987; Lux et al., 1990). Only neurons with I_{pe} amplitudes larger than −1 nA immediately after Ca^{2+} removal (2 of 6 neurons tested) stained with trypan blue at 30 min postexposure.

Because whole-cell recording disturbs the metabolism of the recorded cell, we tested to make sure that whole-cell recording itself is not necessary for the activation of I_{pe} and the resulting cell death. Nonrecorded cells (both NMDA-exposed and nonexposed cells in sister dishes) were examined by short-duration whole-cell recording (point recording) performed at different times on dif-

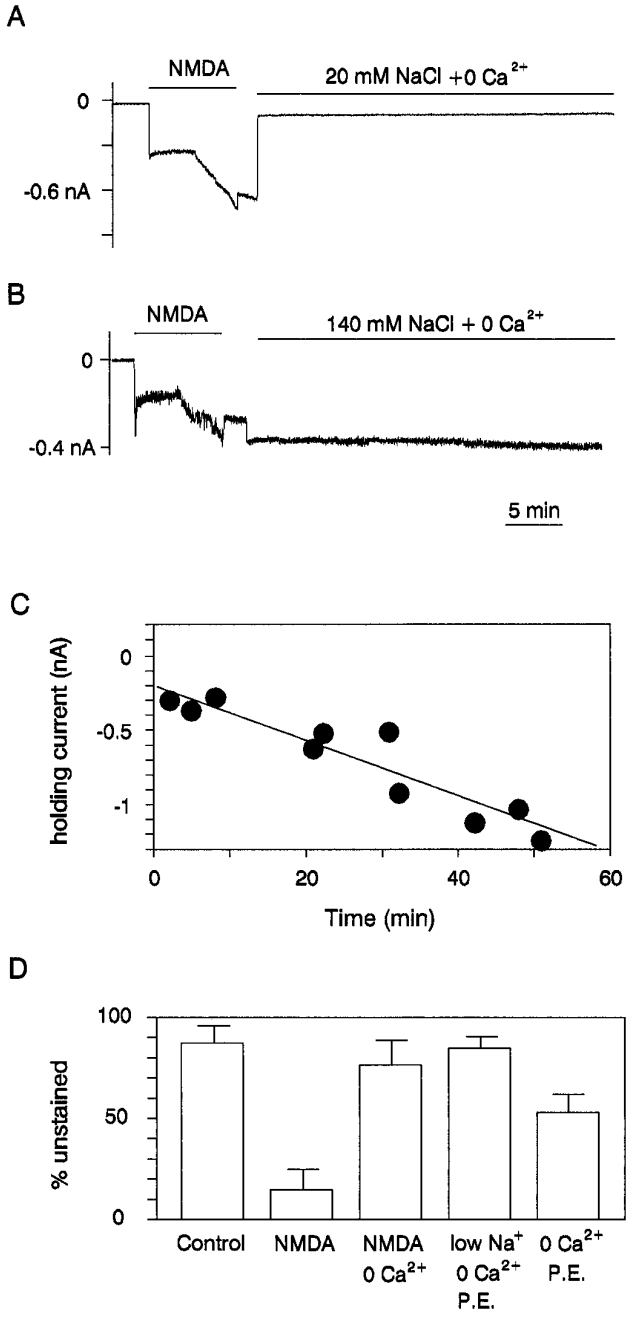

Figure 3. I_{pe}-mediated Ca^{2+} and Na^+ influx leads to the death of NMDA-exposed neurons. *A–D*, I_{pe} was triggered by NMDA application (10 min, 100 μM). *A*, Response of I_{pe} to simultaneous removal of $Ca^{2+}{}_o$ and reduction of $[Na^+]_o$ for the postexposure phase (started 1 min postexposure). *B*, Response of I_{pe} to removal of $Ca^{2+}{}_o$ for the postexposure phase. *C*, NMDA exposure triggers a gradually developing I_{pe} in nonrecorded cells. Healthy pyramidal cells within a field were identified before NMDA exposure and randomly selected at different times postexposure for point recording. Zero time indicates beginning of postexposure phase. Each *circle* represents a point recording from a different cell in the dish. *D*, Percentage of survival of neurons in sister dishes with the same treatment as those applied to recorded cells. NMDA application and manipulation of extracellular ionic composition were performed by bath perfusion. Healthy neurons in two fields per dish were identified before treatment. To evaluate the percentage of neuronal death after different treatments, trypan blue staining was performed at 60 min postexposure. The five *bars* represent average survival values obtained from (*left* to *right*) the control group (not exposed to NMDA), the NMDA group (exposed to NMDA), the NMDA + 0 Ca^{2+} group (exposed to NMDA in the presence of zero $Ca^{2+}{}_o$), the low-Na^+ + 0 Ca^{2+} postexposure group (exposed to NMDA and then bathed in the 20 mM NaCl solution during postexposure), and the 0 Ca^{2+} postexposure group (exposed to NMDA and then bathed in zero-calcium solution during postexposure). Consistent with data obtained from recorded cells, postexposure removal of $Ca^{2+}{}_o$ reduced neuronal death caused by NMDA exposure (0 Ca^{2+} postexposure group vs NMDA group, $p < 0.01$). Postexposure reduction of $[Na^+]_o$ in addition to removing $Ca^{2+}{}_o$ provided additional protection (low-Na^+ + 0 Ca^{2+} postexposure group vs 0 Ca^{2+} postexposure group, $p < 0.01$) and was not significantly different from control ($p > 0.05$). Error bars represent SD ($n = 12$ fields, 2 fields from each of 6 animals). Student's two-tailed t test was used to obtain p values.

ferent cells in the dish during the postexposure phase. Point recording showed that NMDA-exposed cells had a smaller input resistance and a larger inward current immediately after disruption of the patch membrane than nonexposed cells. This inward current in NMDA-exposed cells was indistinguishable from I_{pe}. In agreement with data from recorded cells, point recordings also revealed that I_{pe} grew over time during the postexposure phase in nonrecorded cells (Fig. 3*C*). The seal resistance of point recordings was the same regardless of whether I_{pe} was present, indicating that the development of I_{pe} after NMDA exposure in recorded cells (see Fig. 1*A*) is not attributable to a gradual deterioration of the seal. Figure 3*D* shows results from trypan blue staining performed at 60 min postexposure on sister dishes of nonrecorded cells that were exposed to the same treatment as recorded cells. Consistent with the results in recorded cells, only protocols shown previously to trigger I_{pe} caused cell death (i.e., NMDA exposure but not NMDA exposure in zero-calcium solution). In addition, protocols shown to suppress I_{pe} postexposure (i.e., postexposure removal of $Ca^{2+}{}_o$ and simultaneous reduction in $Na^+{}_o$) or the growth of I_{pe} during the postexposure (i.e., postexposure removal of $Ca^{2+}{}_o$) caused a significant reduction in the percentage of dead cells. The solution containing zero-calcium and low-Na^+ (the 20 mM NaCl) solution provided the greatest postexposure protection, producing a survival rate indistinguishable from control (Fig. 3*D*).

DISCUSSION

I_{pe}

NMDA application caused the appearance of the cation current I_{pe}, which persisted and increased in size after the removal of NMDA. The strict cation selectivity and differential permeability of I_{pe} indicate that I_{pe} is not simply the result of a breakdown of membrane integrity, but is rather associated with a well-behaved membrane conductance. The fact that induction of I_{pe} required the presence of extracellular Ca^{2+} during NMDA exposure indicates that I_{pe} is triggered by Ca^{2+} entry through NMDA receptor channels, a conclusion that is supported by the experiments that showed that intracellular perfusion with Ca^{2+} could also lead to the activation of I_{pe}. Because I_{pe} is itself partly carried by Ca^{2+}, Ca^{2+} entry associated with I_{pe} could induce additional I_{pe}. In fact, removing the Ca^{2+} from the extracellular solution during postexposure prevented the gradual growth in I_{pe} (Fig. 3*A*,*B*), indicating that continued I_{pe}-mediated Ca^{2+} influx is responsible for the continuous increase in the size of I_{pe}. On the other hand, a large I_{pe} persisted (but did not increase in amplitude) when zero-Ca^{2+} solution was perfused during the postexposure phase (Fig. 3*B*), indicating that continued Ca^{2+} influx is not required for the maintenance of I_{pe}. The activation requirements and properties of I_{pe} indicate that it is probably responsible for the secondary $[Ca^{2+}]_i$ increase which has been recorded in response to gluta-

mate exposure (Randall and Thayer, 1992; Tymianski et al., 1993). Additional studies are needed to address the microscopic conductance and modulatory mechanisms associated with I_{pe}.

I_{pe} and neuronal death

Earlier investigations in cultured neurons have shown that cell death from NMDA application is markedly reduced if the NMDA is applied in zero-calcium solution (Choi, 1987). In addition, earlier studies in cultured neurons have shown that removal of extracellular calcium ($Ca^{2+}{}_o$) in the postexposure phase reduces neuronal death (Hartley and Choi, 1989; Manev et al., 1989), and that simultaneous removal of $Ca^{2+}{}_o$ and $Na^{+}{}_o$ during the postexposure phase produces essentially complete blockade of NMDA neurotoxicity (Hartley and Choi, 1989). Our study has replicated these results in a new system and, more importantly, has shown that these two procedures block NMDA toxicity in acutely isolated hippocampal cells, because the first prevents the induction of I_{pe} and the second suppresses I_{pe} in the postexposure phase. Our results indicate that NMDA exposure causes an initial Ca^{2+} influx that triggers the onset of I_{pe} and that it is the large and persistent secondary influx of Ca^{2+} and Na^{+} underlying the I_{pe} that is the downstream event associated with cell death.

Previous studies (Choi, 1987) have measured "delayed" cell death, in which cultured neurons exposed to glutamate or NMDA die several hours later. Our experiments cannot be said to measure delayed cell death, because the NMDA-exposed cells died within 1 hr; in fact, acutely isolated hippocampal cells will die in 2–4 hr without NMDA exposure. Regardless, it is possible that I_{pe} may contribute to NMDA-induced delayed cell death in other systems. The existence of an I_{pe} in other systems could account for the NMDA antagonist-insensitive sustained increase in $[Ca^{2+}]_i$, which has been correlated with cell death (de Erausquin et al., 1990; Randall and Thayer, 1992; Tymianski et al., 1993) and for the protection of neurons seen with the simultaneous removal of Na^{+} and Ca^{2+} in the postexposure phase (Hartley and Choi, 1989). Because the Na^{+} and Ca^{2+} influx associated with I_{pe} may be the downstream event that causes glutamate neurotoxicity, pharmacological agents that could suppress I_{pe} or retard its growth may reduce cell death.

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