Transient NMDA Receptor Inactivation Provides Long-Term Protection to Cultured Cortical Neurons from a Variety of Death Signals

Roger Tremblay, Balu Chakravarthi, Kimberley Hewitt, Joseph Tauskela, Paul Morley, Trevor Atkinson, and Jon P. Durkin

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

NMDA receptor antagonists, such as (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), potentely block glutamate-induced neuronal death in myriad in vitro cell models and effectively attenuate ischemic damage in vivo. In this report, a novel role for MK-801 and other NMDA receptor antagonists in preconditioning neurons to withstand a wide range of subsequent lethal insults is described. A brief 30 min exposure to 0.1 μM MK-801, applied up to 96 hr before a "lethal" insult, protected primary cortical neurons from a diverse group of neurotoxic agents, including NMDA, β-amyloid, staurosporine, etoposide, and oxygen–glucose deprivation. This neuroprotective preconditioning by MK-801 arose from transient NMDA receptor inactivation, because the noncompetitive NMDA receptor antagonists memantine and nylindin and the competitive antagonist AP-5 gave similar effects. MK-801 protection was dependent on new protein synthesis during the first 2 hr, but not from 2 to 5 hr, after MK-801 exposure. The MK-801 transient did not alter the ability of NMDA to trigger normally lethal [Ca2+]i influx 48 hr later, but it did block early downstream signaling events coupled to NMDA neurotoxicity, including PKC inactivation and the activation of calpain. Moreover, MK-801 protected neurons from staurosporine-induced apoptosis, although caspase activation in these cells was unimpeeded. It is likely that the stress associated with transient inactivation of NMDA receptors triggered a rapid compensatory survival response that provided long-term protection from a spectrum of insults, inducing apoptotic and nonapoptotic death. The possibility that MK-801 preconditioning blocks an event common to seemingly diverse death mechanisms suggests it will be an important tool for obtaining a clearer understanding of the salient molecular events at work in neuronal death and survival pathways.

Key words: apoptosis; death signals; lethal injury; MK-801; oxygen–glucose deprivation; stress; preconditioning
Heat-inactivated fetal bovine serum (FBS) and l-glutamine were purchased from Life Technologies (Grand Island, NY). For a modification of a previously described method (Durkin et al., 1996), timed-pregnant Sprague-Dawley rats were killed at embryonic day 18 (E18), their brains were removed and placed in ice-cold FBS, and the cortices were dissected. The corticospinal neurons were dissociated by triturating with a 10 ml pipette, and the cells were centrifuged at 250 x g for 5 min at 4°C. The cells were gently resuspended in plating medium consisting of 80% MEM, 10% heat-inactivated horse serum, 10% heat-inactivated FBS, 250 mM sodium pyrophosphate, and 2 mM l-glutamine. For the determination of neurotoxicity and protein kinase C (PKC) activity, the cells were plated in poly-l-lysine-coated 35 mm tissue culture dishes (DuPont-Life Technologies, Burlington, MA) and cultured at 37°C in 5% CO2 and 95% air. At the time of NMDA or staurosporine treatment and was removed along with the cytotoxic. All other additions to cells, whether transient or persistent, were added to the cultures twice with ice-cold PBS, suspended in 0.5 ml of ice-cold hypotonic lysis medium (1 mM NaHCO3, 5 mM MgCl2, and 100 mM phenylmethylsulfonyl fluoride) for 2 min, and lysed by vortexing vigorously for 2 min. All subsequent steps were performed at 4°C. Nuclei and unlysed cells were sedimented at 600 x g for 5 min, and membrane and cytosol fractions were separated by centrifugation at 100,000 x g for 10 min in a Beckman TL-100 ultracentrifuge. PKC activity in isolated membranes was measured by the method described as described previously (Zauscher et al., 1993). The reaction mixture contained peptide substrate, acetyl-CoA, sodium pyrophosphate, and ATP. The PKC activity in the membranes was measured using a peptide substrate, acetyl-CoA, and ATP. After incubation for 10 min at 25°C, the reaction was stopped by adding 10 mM of EDTA. The reaction products were separated by thin layer chromatography and quantitated by liquid scintillation counting. To calculate the amount of radioactivity incorporated specifically into the substrate, the nonspecific binding of [3H]spectrin to the P81 papers was determined as described previously (Tauskela et al., 1999). Oxysterol–glucose depitration was performed by plating culture dishes in BSS buffer [in mM]: NaCl 120, KCl 5.4, MgCl2 0.8, CaCl2 0.8, glucose 15, and 4 mM sodium pyrophosphate, and 2 mM l-glutamine, producing an O2 partial pressure equal to 10–15 mm Hg. After 4 hr, the cultures were washed several times with serum-free medium and maintained at 37°C in 5% CO2 and 95% air. After 24 hr, the cells were subjected to hypoxia–hypoglycemia treatment (10% O2, 5% CO2, and 95% N2,–5% CO2), and the samples were clarified by centrifugation at 10,000 x g for 10 min. The neuroprotective effects of MK-801 on 30 min in CM were assessed 24 hr after the normally lethal insult was applied. Although the extent of kill induced in the above cases, cell viability was assessed 24 hr after the normally lethal insult was applied. Although the extent of kill induced in the above cases, cell viability was assessed.

Measurement of calpain activation by spectrin hydrolysis in intact neurons. A calpain-specific anti-brain-a-spectrin antibody was prepared as described previously (Roberts-Lewis et al., 1994) that selectively detects the 155 kDa spectrin breakdown product generated by calpain. A modification of a previously described method (Durkin et al., 1996), timed-pregnant Sprague-Dawley rats were killed at embryonic day 18 (E18), their brains were removed and placed in ice-cold FBS, and the cortices were dissected. The corticospinal neurons were dissociated by triturating with a 10 ml pipette, and the cells were centrifuged at 250 x g for 5 min at 4°C. The cells were gently resuspended in plating medium and washes were performed by gently stirring for 10 min. The radioactivity bound to the washed papers was determined by liquid scintillation counting. To calculate the amount of radioactivity incorporated specifically into the substrate, the nonspecific binding of [3H]spectrin to the P81 papers was determined as described previously (Tauskela et al., 1999). Oxysterol–glucose depitration was performed by plating culture dishes in BSS buffer [in mM]: NaCl 120, KCl 5.4, MgCl2 0.8, CaCl2 0.8, glucose 15, and 4 mM sodium pyrophosphate, and 2 mM l-glutamine, producing an O2 partial pressure equal to 10–15 mm Hg. After 4 hr, the cultures were washed several times with serum-free medium and maintained at 37°C in 5% CO2 and 95% air. After 24 hr, the cells were subjected to hypoxia–hypoglycemia treatment (10% O2, 5% CO2, and 95% N2,–5% CO2), and the samples were clarified by centrifugation at 10,000 x g for 10 min. The neuroprotective effects of MK-801 on 30 min in CM were assessed 24 hr after the normally lethal insult was applied. Although the extent of kill induced in the above cases, cell viability was assessed 24 hr after the normally lethal insult was applied. Although the extent of kill induced in the above cases, cell viability was assessed.

RESULTS

Primary cortical neurons were pretreated with 1 mM MK-801 for 30 min in CM, washed, and returned to drug-free CM for up to 96 hr before being subjected to a number of cytotoxic conditions. This brief MK-801 preconditioning was found to evoke a powerful, long-lasting and broad-based neuroprotective response in cortical cells. Thus, cultures transiently exposed to MK-801 were protected from the excitotoxic effects of NMDA applied 48 hr later (Fig. 1A). Preconditioning with MK-801 offered complete protection against subsequent NMDA, even when the excitotoxin was added 96 hr after transient MK-801 application (data not shown). Significantly, cortical neurons were also protected from the excitotoxic effects of AMPA, a 6 hr treatment with staurosporine or etoposide, or from Aβ (1–40) when subjected to a 30 min MK-801 treatment 48 hr before insult (Fig. 1A). The neuroprotective effects of MK-801 also extended to cortical cells exposed to 2 hr of OGD 48 hr after transient MK-801 application (Fig. 1B). In contrast, the addition of BDNF, under conditions that strongly protect cortical cultures from excitotoxicity ( Tremblay et al., 1999), had no ability to protect cells from hypoxia-induced damage (Fig. 1B). In all of the above cases, cell viability was assessed 24 hr after the normally lethal insult was applied. Although the extent of kill induced in cortical cells varied with the specific cytotoxic agent–condition used (i.e., ~85% for NMDA to ~40% for etoposide), MK-801 pretreatment consistently reduced the degree of kill in these cultures to ~20% (Fig. 1A). Moreover, MK-801 pretreatment protected neurons from concentrations of NMDA and staurosporine that were, respectively, at least fourfold and tenfold higher than

Statistical analysis. Statistical analyses were performed with the software package Statistica, using ANOVA or t tests where appropriate.
A

from at least three independent experiments. In phase-contrast microscopy and PI-labeled nuclei (dead cells) under fluorescence microscopy. Viability was calculated as the ratio of phase-bright cells to

returned to the cultures. Dishes were incubated an additional 24 hr before cell viability was assessed by counting phase-bright cells (live cells) under phase-bright cells to

cultures were exposed continuously to 100 ng/ml neurotrophin [concentrations and conditions shown to effectively block NMDA injury (Durkin et al., 1996) for 24 hr before leakage. In all cases after neurototoxic treatment, dishes were washed once in CSS buffer, and the saved conditioned medium was returned to the cultures. Dishes were incubated an additional 24 hr before cell viability was assessed by counting phase-bright nuclei (live cells) under phase-contrast microscopy and PI-labeled nuclei (dead cells) under fluorescence microscopy. Viability was calculated as the ratio of phase-bright cells to

and then returned to drug-free CM for 48 hr. Cultures were then exposed to the indicated concentrations of NMDA for 5 min, staurosporine or etoposide for 6 hr, or Aβ (1–40) for 24 hr, all presented to cells in CM. Control cultures were treated with vehicle under identical conditions. For BDNF addition, cultures were exposed continuously to 100 ng/ml neurotrophin [concentrations and conditions shown to effectively block NMDA injury (Durkin et al., 1996) for 24 hr before leakage. In all cases after neurototoxic treatment, dishes were washed once in CSS buffer, and the saved conditioned medium was returned to the cultures. Dishes were incubated an additional 24 hr before cell viability was assessed by counting phase-bright cells (live cells) under phase-contrast microscopy and PI-labeled nuclei (dead cells) under fluorescence microscopy. Viability was calculated as the ratio of phase-bright cells to

 protective endothelial cells used as controls, were then washed once in CSS buffer, and saved conditioned medium was returned to the cultures for 24 hr, at which time cell viability was assessed by PI exclusion described in the legend to Figure 1. Results are presented as percent viable cell counts in quadruplicate dishes. In A, values are means ± SEM of at least four separate determinations, and values in B are of two separate experiments. *p < 0.01 compared with non-MK-801-treated conditions.

normally needed to inflict substantial cell death (Fig. 2), indicating that protection was not the result of small changes in the effective lethal dose of these cytotoxins. MK-801 protected neurons from NMDA and staurosporine for at least 3 d after insult (data not shown), suggesting that the effects of transient MK-801 were long-lasting and not just delaying cell death as may well be the case with other protectants, such as caspase inhibitors (Lemaire et al., 1998; Grabb and Choi, 1999).

Several lines of evidence indicated that MK-801 preconditioning was mediated by its interaction with, and inhibition of, NMDA receptors: (1) the neuroprotective effect of MK-801 was mimicked by the structurally unrelated noncompetitive NMDA channel blocker memantine (Fig. 3A), the ifenprodil–halperidol site antagonist nylidrin, and the competitive antagonist AP-5 (Fig. 3B); (2) MK-801 protection was not observed in staurosporine- or etoposide-treated human neuroblastoma cells (SH-SY5Y) or primary rat astrocytes (data not shown), cells that do not possess functional NMDA receptors (Backus et al., 1989; Jensen and Chiu, 1990; Porter and McCarthy, 1997); and (3) MK-801 preconditioning protection was not observed at any concentration range (i.e., 0.01–0.1 μM) that the acute administration of MK-801 effectively blocked NMDA toxicity, that is when the drug was added 5 min before and was present during a 5 min NMDA exposure (Fig. 3C). In contrast, cortical cells exposed to the AMPA receptor antagonist CNQX

under similar conditions were not protected from either NMDA or AMPA applied 48 hr later (Fig. 3D). Interestingly, the enzymatic removal of basal glutamate present in the conditioned culture medium of cortical cultures by a 2 hr exposure to 10 U/ml glutamate-pyruvate transaminase somewhat mimicked the effects of NMDA receptor inactivation in that the cells were partially protected from lethal NMDA applied 48 hr later (Fig. 3B). Increasing the concentration of GPT in the medium beyond 10 U/ml was unable to enhance the neuroprotective effects (data not shown). Thus, briefly blocking NMDA receptor activity by any of a number of NMDA receptor antagonists or conditions caused a protracted cellular response that protected cells from diverse cytotoxic agents.

Neuroprotection by MK-801 and other NMDA receptor antagonists arose from long-lasting changes induced in cells during and shortly after their application rather than to any effects on NMDA receptors arising from residual drug present at the time of lethal injury. First, long-term neuroprotection afforded by transient NMDA receptor blockade occurred with a spectrum of NMDA receptor antagonists, including agents that would be expected to have rapid washout rates [i.e., the competitive antagonist AP-5 (Fig. 3B)]. In addition, MK-801 preconditioning had no discernible effects on subsequent Ca2+ influx via the NMDA receptor. Control and MK-801 preconditioned neurons were loaded with fura-2 AM 48 hr later and subsequently challenged with NMDA. As shown in Figure 4A, NMDA-induced Ca2+ influx was not discernibly different in control and MK-801 pretreated cells, a result inconsistent with the neuroprotective effects of MK-801 arising from residual drug present at the time of the excitotoxic insult. Moreover, although MK-801 preconditioning blocked NMDA-induced cell death, it did not prevent a rapid and transient NMDA-mediated cell swelling (Fig. 4C), a well described early marker of glutamate–NMDA-induced injury (Basavappa and Ellory, 1996; Churchwell et al., 1996; Sakaguchi et al., 1999). More compelling evidence came from the observation that, as reported previously (Prehn et al., 1997), staurosporine-induced neuronal death is independent of glutamate receptors. As shown in Figure 4A, adding MK-801 to cortical cultures immediately before a 6 hr staurosporine exposure had no ameliorative effects on cell death, indicating that NMDA receptor activation was not coupled to staurosporine neurotoxicity in cortical cells. Despite this observation, MK-801 protection became apparent in cultures that were transiently (i.e., 30 min) pretreated with the drug 1–2 hr, or longer, before exposure to staurosporine (Fig. 5A). Thus, the transient suppression of NMDA receptor function evoked a neuroprotective response that was not immediate but took time (albeit a relatively short period).
to develop and mature. Attempts to perform similar “timing” experiments with NMDA-treated cells were not possible, because unlike staurosporine, the acute addition of MK-801 predictably blocked NMDA toxicity (Fig. 3C). However, it was possible to perform such experiments with AP-5 that, as a competitive antagonist, would be expected to exhibit rapid off rates upon its removal from the culture medium. NMDA toxicity was effectively blocked by 100 μM AP-5 present during the 10 min exposure to lethal NMDA (Fig. 5B, compare lanes b, c). In contrast, AP-5 added transiently for 30 min 1 hr before NMDA exposure was not protective (Fig. 5B, lane d), indicating the drug indeed was effectively removed by washing. Nevertheless, almost complete protection was observed in cultures exposed for 2 hr to AP-5 between 150 and 300 min before NMDA (Fig. 5B, lane e). This finding demonstrated that, like the MK-801–staurosporine combination in Figure 5A, blocking NMDA receptor function transiently ~2 hr before a lethal death signal was sufficient to generate the neuroprotective response. However, once triggered in neurons, this neuroprotective mechanism exhibited remarkable longevity. As shown in Figure 5C, a 2 hr exposure to AP-5 protected cells completely from lethal NMDA added up to 48 hr later. Unlike MK-801, which was still maximally protective in cortical neurons challenged with NMDA 96 hr later (data not shown), the neuroprotective effects of AP-5 gradually declined beyond the 48 hr point (Fig. 5C).

Neuroprotective preconditioning afforded by MK-801 was effectively blocked by the protein synthesis inhibitor CHX present during and briefly after MK-801 treatment. Cells were exposed to CHX (10 μg/ml) during and for 90 min after a 30 min MK-801 treatment, or alternatively, from 2 to 5 hr after MK-801 withdrawal. Cultures treated in this manner were subsequently challenged with lethal NMDA (50 μM) 48 hr later. As shown in Figure 6, MK-801 neuroprotection was reduced by >50% when CHX was applied during the first 2 hr after MK-801. In contrast, delaying the addition of CHX until 2 hr after the MK-801 transient was far less effective in reversing the preconditioning effect (Fig. 6). At the concentration of CHX used (i.e., 10 μg/ml), the steady-state level of protein synthesis in cortical cultures was inhibited by ~50% within 30 min (data not shown). These results suggest that MK-801 preconditioning was dependent on new protein synthesis, and likely on new gene expression, during the first few hours after the MK-801 transient.

Perhaps the most significant aspect of MK-801 preconditioning was its ability to prevent the death of cortical cells by diverse agents acting by seemingly different routes. Thus, both staurosporine and etoposide kill cells, including cortical neurons, by apoptotic mechanisms (Bertrand et al., 1994; Koh et al., 1995; Solovy et al., 1998; Tronov et al., 1999). In contrast, hypoxia and NMDA can trigger cortical death by necrosis or what has been described as “nonclassical” apoptosis (Ankarcrona et al., 1995; Lesort et al., 1997; Ankarcrona, 1998; Sohn et al., 1998). In our hands, stauro-
sporine, but not NMDA, caused condensed chromatin and cell morphology characteristic of “classical” apoptosis (Fig. 7A). This distinction was also evident by changes in apoptosis-associated caspases after such insults. Whereas lethal concentrations of staurosporine and etoposide caused activation of caspase 3 in cortical cells, NMDA did not (Fig. 7B). In the case of NMDA, caspase activity was measured 6 hr after the NMDA transient, a point at which ~30% of the cells were already dead as measured by propidium iodide uptake. Interestingly, caspase 3 activity increased in MK-801 preconditioned cortical cells challenged with staurosporine, despite the fact MK-801 protected the cells from death (Fig. 7B). This staurosporine-induced increase in caspase activation in MK-801 protected cortical cultures was not likely attributable to activation of the protease in glial cells present in the mixed cortical culture because staurosporine did not stimulate caspase in glial-enriched cultures in which the neuronal population was previously removed by chronic NMDA exposure (data not shown). This finding is consistent with a previous study (MacManus et al., 1997) showing that 1 μM staurosporine is not toxic to glial cells present in mixed cortical cultures. These data suggest that the neuroprotective response evoked by MK-801 acts either downstream, or is independent, of caspases in staurosporine-injured neurons.

Another protease linked to death mechanisms in neurons is the Ca2+-dependent, neutral protease calpain (Siman and Noszek, 1988; Wang and Yuen, 1994; Brorson et al., 1995; Rami et al., 1997). We have demonstrated previously that calpain activation is selectively coupled to the influx of Ca2+ via the NMDA receptor. Ca2+ influx triggered by other routes, such as voltage-dependent calcium channels or ionomycin, failed to induce calpain activation or cell death in cortical cells (Hewitt et al., 1998). The activation of calpain appears to be a critical event in the mechanism by which NMDA effects cortical death because concentrations of the calpain inhibitor calpeptin, which effectively block NMDA-induced hydrolysis of endogenous spectrin [a marker of calpain activation (He- witt et al., 1998)], were also effective in preventing neuronal death by the excitotoxin (Fig. 8A). As shown in Figure 8B, a 30 min MK-801 transient applied either 48 hr before or concomitant with NMDA strongly ablated calpain activation and cell death effected by the excitotoxin. These results are consistent with those presented in Figure 4 in which the rapid inactivation of PKC activity, one of the cardinal Ca2+-dependent signaling events coupled to NMDA receptor overstimulation and toxicity (Durkin et al., 1996, 1997), was also blocked by MK-801 pretreatment 48 hr earlier (Fig. 4B). Thus, both the inactivation of PKC and calpain activation were attenuated in MK-801 protected neurons, although Ca2+ influx via hyperactivated NMDA receptors appeared unaffected (Fig. 4). Collectively, these results suggest that the protracted protection afforded by MK-801 against NMDA may arise at least in part from its ability to block cellular events normally coupling the lethal influx of Ca2+ to a rapid PKC inactivation and calpain
activation. However, this possible mechanism for MK-801 neuroprotection under excitotoxic conditions appeared not to translate to cortical neurons challenged with staurosporine. Unlike with NMDA, staurosporine-challenged neurons did not respond by activating calpain (Fig. 8C). In addition, whereas the selective calpain inhibitor calpastatin blocked NMDA neurotoxicity, it had no protective effects in cortical cultures treated with lethal staurosporine (Fig. 8C).

**DISCUSSION**

Neuroprotective preconditioning evoked by MK-801 in cortical cells was directly mediated by transient inhibition of the NMDA receptor because the effect was also observed with a series of other NMDA receptor antagonists. Because MK-801 and memantine are use-dependent, open-channel NMDA receptor antagonists (Parsons et al., 1995; Palmer et al., 1997), it is likely that transient blockade of basal NMDA receptor activity is responsible for initiating the resultant neuroprotective response. The fact that AP-5, an antagonist that competes with glutamate for its binding site, is as neuroprotective as MK-801 is strong evidence that preconditioning arises from transient blockade of basal NMDA receptor activity. In the mixed cortical cultures used, the levels of glutamate secreted by both glial cells and neurons is likely sufficient to maintain basal glutamate receptor activation. Thus, one explanation for MK-801 neuroprotection is that selective blockade of NMDA receptor function by transient MK-801, in the presence of active AMPA and metabotropic receptors, exerts a nonlethal stress on neuronal cells. The finding that brief exposure to GPT partially mimicked the neuroprotective effects of MK-801 against lethal NMDA 48 hr later is consistent with this concept.

It has been shown recently that a continuous 48 hr exposure to MK-801 is lethal to cortical neurons (Hwang et al., 1999), indicating that long-term inhibition of NMDA receptor activity constitutes a lethal insult. As such, the 30 min MK-801 exposure used in the present study constitutes a "sublethal" stress that is consistent with conventional thinking related to preconditioning mechanisms. As with other preconditioning paradigms (Ferdinandy et al., 1998; de Zeeuw et al., 1999), this imposed stress can stimulate compensatory survival mechanisms that protect neurons from lethal insults at later times. However, to the best of our knowledge, no reported preconditioning agent or condition exhibits such long-lasting protection against such a diverse group of neurotoxic agents as does MK-801. This is in stark contrast to OGD preconditioning, which although effective against subsequent “lethal” OGD, has...
little capacity to protect neurons from staurosporine or excitotoxins (Grabb and Choi, 1999).

CHX effectively blocked neuroprotection when present during the first 2 hr after the 30 min MK-801 transient but not when added 2 hr after MK-801 withdrawal. Thus, the preconditioning effects of transient NMDA receptor inactivation depends on either new protein synthesis within the initial hours of NMDA receptor blockade or maintaining existing proteins with a high turnover rate. During this critical period, the broad-based neuroprotective response generated by MK-801 remains responsive for at least 48 hr and for 96 hr or longer in the case of NMDA-induced cortical death (data not shown). These findings indicate that a brief 1–2 hr recovery period was sufficient, and necessary, for a MK-801 transient to protect cortical cells from staurosporine (Fig. 5). The finding that a 2 hr exposure to AP-5 removed 30 min before NMDA application was highly neuroprotective supports this contention. Parenthetically, the fact that AP-5 exposure times of <2 hr gave progressively less protection from subsequent NMDA suggests that the degree of neuroprotection seen with a 30 min MK-801 exposure (i.e., Fig. 1) was attributable to slow washout rates, an understatement of the actual time required to exact maximal neuroprotection. Collectively, the data are consistent with neuroprotection arising from an MK-801-induced cortical death (data not shown). These findings indicate that a brief 1–2 hr recovery period was sufficient, and necessary, for a MK-801 transient to protect cortical cells from staurosporine (Fig. 5). The

It is well known that NGF and BDNF protect neurons from injury under various in vivo and in vitro scenarios (Kirschner et al., 1999).
NMDA-mediated calpain activation. Cortical cells were pretreated with 1 mM NMDA for 5 min and then (1) returned to CM for 2 hr before cell lysates were prepared and calpain activity was estimated in Western blots by the extent of spectrin hydrolysis, as described in Materials and Methods or (2) returned to drug-free CM for 48 hr before being subjected to 50 μM NMDA for 5 min. Cultures were then subjected to Western blot or cell viability analyses as described above. The calpain inhibitor calpastatin was added to cultures 1 hr before the cytotoxic agents. Cell viability was determined in parallel cultures exposed, and calpain activation was determined 3 hr later by the extent of spectrin hydrolysis measured in lysates subjected to Western blot analysis as described above. The calpain inhibitor calpastatin was added to cultures 1 hr before the cytotoxic agents. Cell viability was determined in parallel cultures 24 hr later as described in the legend to Figure 1. *p < 0.01, significantly different from NMDA alone.

Figure 8. MK-801 preconditioning prevents calpain activation in NMDA-injured cortical neurons. A. The ability of the calpain inhibitor calpeptin to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. Cortical cultures were treated with the indicated concentrations of calpeptin for 1 hr before being exposed to 50 μM NMDA for 5 min and then (1) returned to CM for 2 hr before cell lysates were prepared and calpain activity was estimated in Western blots by the extent of spectrin hydrolysis, as described in Materials and Methods or (2) returned to CM for 24 hr before cell viability was determined by PI exclusion as described in the legend to Figure 1. Bars represent the mean ± SEM and are typical of at least in part, from MK801-mediated BDNF production. In fact, the inability of MK-801 preconditioning to affect NMDA-induced Ca2+ influx, but to block the downstream inactivation of PKC that normally arises from lethal Ca2+ through the NMDA receptor (i.e., Fig. 4), was similar to that reported for neuroprotection of cortical cells from NMDA by BDNF applied 24 hr before insult (Tremblay et al., 1999). However, several lines of evidence argue against MK-801 preconditioning being mediated solely by BDNF. First, whereas MK-801 protected cortical cells from OGD, BDNF did not (Fig. 1B). Although BDNF protected cortical cells from both NMDA and staurosporine (Tremblay et al., 1999), unlike MK-801, it was unable to protect from Aβ toxicity (our unpublished observations). Moreover, MK-801 protects cortical neurons from staurosporine without blocking caspase 3 activation (Fig. 7B), whereas BDNF blocked staurosporine-mediated caspase 3 activation under similar conditions (data not shown). Thus, MK-801 neuroprotection cannot be accounted for solely by the upregulation of BDNF in neurons or in supporting glia.

The broad-based neuroprotective response evoked by brief MK-801 exposure could have arisen from upregulation of a pleotropic neuroprotective “cassette”-containing elements capable of intervening at pivotal points along the death pathways triggered by NMDA, staurosporine, etoposide, hypoxia, Aβ, and other neurotoxins. Alternatively, the neuroprotective mechanism elicited by MK-801 may be focused on a single event at, or downstream of, a point at which all death pathways converge. Clearly, if the latter were the case, identifying such a “convergence point” would reveal potentially valuable therapeutic targets for blocking neuronal death by myriad processes. However, one line of evidence suggests this may not be the case. MK-801 blocked both the NMDA-induced inactivation of PKC and the activation of calpain, events critical for the excitotoxic process (Durkin et al., 1996, 1997; Hewitt et al., 1998; Tremblay et al., 1999). In fact, the inactivation of PKC appears to be indirectly responsible for calpain activation under these conditions (our unpublished observations). These early steps in the death mechanism take place within the first hour of NMDA addition, well before the first signs of irreversible cell damage. Because calpain inhibitors also protect cortical neurons from NMDA (Fig. 8A,C), it is reasonable to suggest that MK-801 protection arises from its ability, direct or indirect, to prevent activation of this protease. However, calpain activation appears not to play a critical role in the death process imparted by staurosporine in these same cells. Staurosporine failed to induce calpain-mediated spectrin hydrolysis, nor was calpain inhibition able to ablate cell death at the hands of staurosporine. Hence, MK-801 appears to interfere with at least two different “death” events that can occur in cortical cells. These data are consistent with transient NMDA receptor inactivation eliciting a broad-based neuroprotective response capable of interceding at different points along the death pathways initiated by various neurotoxic agents.

On the other hand, the effects of MK-801 preconditioning on PKC activity may partially account for the pleotropic neuroprotective effects of the drug. We and others have reported that PKC activity is dramatically reduced in neuronal cells exposed to glutamate (Durkin et al., 1996, 1997) and stroke (Cardell and Wieck, 1993), and a recent study indicates that Aβ evokes a rapid loss of PKCα in human fibroblasts (Favit et al., 1998). Similarly, staurosporine is a broad-spectrum kinase inhibitor with a clear ability to block PKC activity in cortical cells. Thus, inactivation of specific PKC isoforms may be a crucial and common step in the death paradigms by which MK-801 preconditioning is efficacious. The data in Figures 4 and 8 indicate that the ability of a MK-801 transient to block NMDA-induced PKC inactivation 48 hr later, as well as the activation of calpain coupled to PKC inhibition, is not attributable to the drug affecting NMDA-induced Ca2+ influx. Because both PKC inactivation and calpain activation are fully dependent on Ca2+ influx through NMDA receptors, MK-801 preconditioning likely prevents signaling steps downstream of Ca2+ influx responsible for inactivating PKC and activating calpain. On a broader scope, these data suggest that a seminal neuroprotective response evoked by the brief MK-801 transient is to provide a long-term suppression of cellular processes responsible for inactivating specific PKC pools that are part of multiple death pathways in neurons.
In summary, we have demonstrated that transient inhibition of basal NMDA receptor activity in cortical neurons causes the upregulation of a powerful and long-lasting survival pathway(s) in cortical neurons that is able to intersect and block death mechanisms coupled to a variety of insults leading to apoptotic and nonapoptotic death. Whether MK-801 protection arises from a multifaceted, broad-spectrum neuroprotective cassette or from a specific response acting at or downstream of a point of convergence for multiple death pathways is not clear. The nature of the signaling events during the first few hours of transient NMDA receptor inactivation that initiate and maintain the protective state within cortical cells is clearly a critical issue that is presently being addressed. As such, MK-801 and other NMDA receptor antagonists should be particularly useful in garnering a clearer understanding of the salient molecular events at work in neuronal survival pathways, as well as providing information as to how this neuroprotective mechanism impacts and blocks death pathways evoked by a diverse and broad group of neurotoxic agents.

REFERENCES


