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Neurobiology

Activation of the metabotropic glutamate receptor attenuates *N*-methyl-D-aspartate neurotoxicity in cortical cultures

(*trans*-1-aminocyclopentyl-1,3-dicarboxylic acid/carbachol/Alzheimer disease/development/neurodegeneration)

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ABSTRACT **Excitatory amino acid receptor-mediated neurotoxicity (excitotoxicity) has been proposed to contribute to neuronal loss in a wide variety of neurodegenerative conditions. Although considerable evidence has accumulated implicating *N*-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in the processes of excitotoxicity, relatively little research has focused on the ability of other neurotransmitter systems to influence excitotoxic neuronal injury. In the present study, we examined the effects of *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD), a selective agonist for the metabotropic glutamate, or ACPD, receptor, and carbachol, an agonist at the acetylcholine receptor, on neuronal degeneration produced by brief exposure to NMDA in murine cortical cultures. Since excitotoxic neuronal injury is probably caused by increases in intracellular Ca^{2+} concentrations, the two transmitter agonists were of particular interest as both have been shown to mobilize intracellular calcium stores. Contrary to what might be expected, ACPD and, to a lesser degree, carbachol attenuated NMDA neurotoxicity. The neuroprotective effect of ACPD, but not of carbachol, was dependent upon the developmental state of cultures; in older cultures (≥18 days *in vitro*), the protective effect decreased. The neuroprotection by ACPD may be, in part, mediated by protein kinases, since protection is partially reversed by the protein kinase antagonists H-7 and HA-1004. These data suggest that concomitant activation of the ACPD receptor may serve as a protective mechanism against neurotoxicity that could be produced by brief intense NMDA receptor activation during normal or abnormal brain function.**

Excitatory amino acid (EAA) neurotoxicity (excitotoxicity) has been proposed to contribute to neuronal loss in a broad spectrum of neurodegenerative conditions including ischemia, Huntington disease, and Alzheimer disease (AD) (1, 2). As neurodegenerative diseases are known to be associated with a variety of specific pathologies, understanding the factors that can alter EAA neurotoxicity may prove clinically important. For example, β/A4 protein that accumulates in amyloid plaques of AD patients increases the vulnerability of cortical neurons to EAA-mediated neuronal injury (3). Similarly, other neurotransmitter systems, which are often altered anatomically or functionally in various disease states, could affect the susceptibility of neurons to excitotoxic damage. For instance, in AD, there is widespread reduction in cholinergic (4) and somatostatinergic markers (5, 6). The ability of the non-EAA system to affect EAA-mediated injury may reflect transmitter interactions in normal brain, as serotonin has been reported to change electrophysiological responses of neurons to *N*-methyl-D-aspartate (NMDA) (7, 8).

It is now well established that there are at least three general classes of glutamate receptors (9). The NMDA and the non-NMDA receptors (i.e., α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and kainate receptors) are directly associated with the specific ligand-gated ionophores that permit influx of cations upon activation (ionotropic receptor). In contrast, the recently discovered metabotropic glutamate, or *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD), receptor (10–12) activates a phospholipase, via a guanine nucleotide-binding regulatory protein, that hydrolyzes membrane phospholipid to generate the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG, in combination with Ca^{2+}, activates protein kinase C (PKC) (13), whereas IP_3 mobilizes Ca^{2+} from intracellular stores in various cell types (14).

Considering the evidence that calcium ions may play a critical role in producing excitotoxic neuronal damage (15, 16), each of the three receptors appears capable of contributing to neuronal damage by increasing intraneuronal free calcium concentrations. While activation of the ionotropic receptors is sufficient to produce most of the EAA neurotoxicity (1, 17, 18), probably through a large influx of calcium (15, 16, 19), evidence for involvement of the metabotropic receptor in excitotoxic neuronal damage was not available until recently, largely due to the lack of selective agonists or antagonists. In this regard, certain unusual pharmacological effects of a nonselective agonist such as quisqualate have often been difficult to interpret. For example, Garthwaite and Garthwaite (20) proposed that the 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX)-insensitive induction of CNQX-sensitive neurotoxicity by a pulse exposure to quisqualate may be mediated by the metabotropic receptor. However, a similar quisqualate neurotoxicity in cortical cultures has been shown to be largely attributable to the cellular uptake and subsequent release of quisqualate (21). The recent discovery of ACPD, a selective agonist for the metabotropic receptor (12), now makes it possible to directly examine the role of the ACPD receptor in excitotoxic neuronal damage. We have found that ACPD is not neurotoxic itself nor does it potentiate kainate or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid neurotoxicity in cortical cultures (22). However, effects of ACPD on NMDA receptor-mediated neurotoxicity were not examined.

As a step toward a better understanding of neurotransmitter interactions in neuronal degeneration, the present study examines the effects of ACPD and carbachol, an agonist at the acetylcholine receptor, on neurotoxicity produced by brief pulse exposure to NMDA.

MATERIALS AND METHODS

Cortical Cell Culture. Primary cerebral cortical cultures were prepared from 14- to 15-day-old fetal mice generally as

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Abbreviations: NMDA, *N*-methyl-D-aspartate; EAA, excitatory amino acid; ACPD, *trans*-1-aminocyclopentyl-1,3-dicarboxylic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; DIV, days *in vitro*; LDH, lactate dehydrogenase; PI, phosphatidylinositol; AD, Alzheimer disease; DAG, diacylglycerol; IP_3, inositol trisphosphate; PKC, protein kinase C; CPP, 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid.

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described by Choi *et al.* (23) with minor modifications (3). Dissociated cortical cells were plated in 16-mm multiwell vessels (4×10^5 cells per well) in Eagle's minimal essential medium (with Earle's salts) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM), and glucose (21 mM). After 5–10 days *in vitro* (DIV), nonneuronal cell division was inhibited by exposure to 10 μM cytosine arabinoside for 1–3 days, and the cells were shifted into a serum-free maintenance medium containing ingredients described by Brewer and Cotman (24). Subsequent medium change was carried out twice a week. For neurotoxicity experiments, only cultures of DIV 14 or greater were used, since previous studies showed that younger cortical neurons are resistant to brief glutamate exposure (23).

Exposure to EAAs. Exposure to EAAs was carried out as described (21). All agonist exposure was performed at room temperature in a Hepes-buffered salt solution with the following composition: 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM Hepes (pH 7.4 at 25°C), and 15 mM glucose. After the exposure, serum-free maintenance medium was replaced, and cultures were returned to the 5% CO_2/95% O_2 incubator until the next day, when the evaluation of neuronal degeneration was made. In most experiments, ACPD or carbachol was added to cultures along with NMDA. In some experiments, cultures were exposed for 5 min to ACPD, carbachol, or other indicated glutamate agonists in Hepes-buffered salt solution containing 50 μM 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP) and 50 μM CNQX and then exposed to NMDA for 5 min. Multiple rapid rinses were made to remove all the compounds before the NMDA exposure (calculated dilution > 2000-fold).

Quantitative Estimation of Neuronal Degeneration. Overall neuronal cell injury was estimated by examination of cultures with a phase-bright microscope (Olympus model IMT-2); damaged neurons were easily identifiable as most turned into debris (see Fig. 1*A*) and were stainable with trypan blue (0.4%, 5 min). In most experiments, neuronal cell injury was further quantitated chemically by measurement of the lactate dehydrogenase (LDH) released by damaged cells into the extracellular fluid 1 day after EAA exposure (25), using an automated microplate reader (Thermomax; Molecular Devices, Palo Alto, CA) and kinetics software. Previous control studies have demonstrated that specific LDH release is linearly proportional to the number of neurons damaged (23, 25).

Phosphatidylinositol (PI) Hydrolysis. Agonist-induced PI hydrolysis was measured as described (26). Briefly, cells were labeled for 24 hr at 37°C with 1 ml of serum-free culture medium containing 2.5 μCi (1 Ci = 37 GBq) of *myo*-[^{3}H]inositol. The cells were washed three times with 1 ml of physiological saline buffer with the following composition: 127 mM NaCl, 2 mM KCl, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 10 mM D-glucose, equilibrated with 95% O_2/5% CO_2. The cells were then preincubated for 15 min at 37°C in 500 μl of physiological saline buffer containing 10 mM LiCl, 500 nM tetrodotoxin, 50 μM CPP, and 50 μM CNQX. *trans*-ACPD was then added to 100 μM, and the incubation was continued for 10 or 30 min. The reaction was terminated by aspiration of the medium, and the cell layer in each well was extracted with 10% perchloric acid and rinsed. The precipitate was removed, and the combined supernatants were analyzed for inositol phosphate content (26).

Chemicals. All glutamate agonists and antagonists were obtained from Tocris Neuramin (Bristol, England). 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfoneamide (HA-1004) were

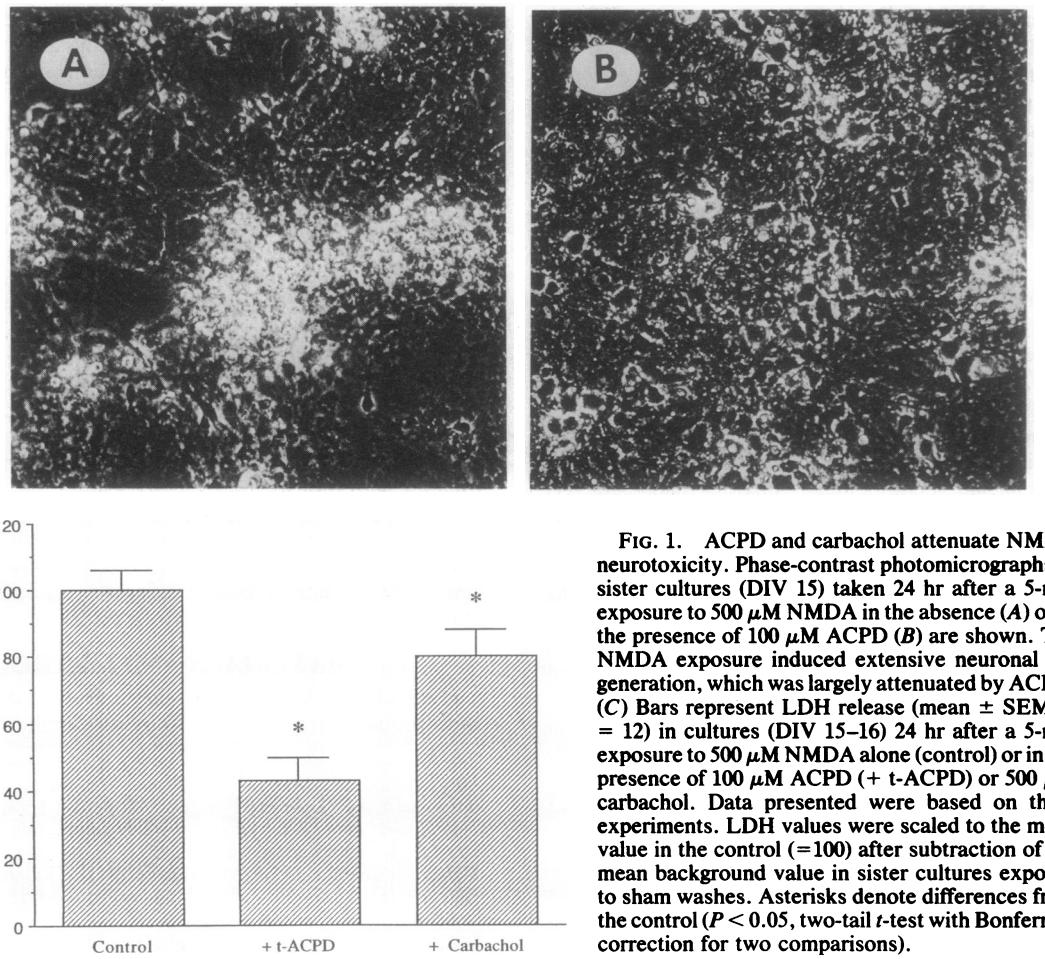

FIG. 1. ACPD and carbachol attenuate NMDA neurotoxicity. Phase-contrast photomicrographs of sister cultures (DIV 15) taken 24 hr after a 5-min exposure to 500 μM NMDA in the absence (*A*) or in the presence of 100 μM ACPD (*B*) are shown. The NMDA exposure induced extensive neuronal degeneration, which was largely attenuated by ACPD. (*C*) Bars represent LDH release (mean ± SEM; n = 12) in cultures (DIV 15–16) 24 hr after a 5-min exposure to 500 μM NMDA alone (control) or in the presence of 100 μM ACPD (+ t-ACPD) or 500 μM carbachol. Data presented were based on three experiments. LDH values were scaled to the mean value in the control (=100) after subtraction of the mean background value in sister cultures exposed to sham washes. Asterisks denote differences from the control ($P < 0.05$, two-tail t-test with Bonferroni correction for two comparisons).

obtained from Seikagaku (Rockville, MD). *myo*-[^{3}H]Inositol was obtained from Amersham. All other chemicals including carbachol were obtained from Sigma.

RESULTS

As previously reported (21), mature (≥DIV 14) cortical cultures exposed to 500 μM NMDA for 5 min showed signs of extensive neuronal degeneration over the following day (Fig. 1*A*). Immediately after the exposure, neuronal cell bodies looked swollen, neurites appeared fragmented, and, by the next day, most neurons had turned into debris. Addition of 100 μM ACPD to the NMDA exposure solution, however, often (four of six experiments) attenuated the NMDA neurotoxicity in sister cultures (Fig. 1*B*). The addition of 500 μM carbachol also reduced the NMDA neurotoxicity but to a much lesser degree. These morphological findings were confirmed by measuring LDH activity released into the bathing medium from the damaged neurons (Fig. 1*C*).

The relationship between the protective effects and the developmental stage of cultures was also examined (Fig. 2*A*). Sister cultures of different ages (DIV 14–18) were exposed to 500 μM NMDA for 5 min in the absence or presence of 100 μM ACPD or 500 μM carbachol. In 14- and 16-day-old cultures, ACPD attenuated the NMDA neurotoxicity significantly ($P < 0.05$, two-tail *t*-test with Bonferroni correction for two comparisons). However, in DIV 18 sister cultures, no protection was observed with ACPD ($P > 0.1$). In contrast, carbachol reduced the NMDA neurotoxicity by about 20% in cultures throughout the ages tested (DIV 14–18). As both ACPD and carbachol are known to induce membrane PI hydrolysis in neurons, we examined the possibility that carbachol shows less protective effect than ACPD, simply because the former is a weaker agonist for PI hydrolysis. We found no correlation between the amount of PI hydrolysis and the degree of neuroprotection; carbachol-induced PI hydrolysis was consistently larger than ACPD-induced PI hydrolysis at all ages (DIV 13–21) (Fig. 2*B*), although the protective effect was usually greater with ACPD. Furthermore, ACPD-stimulated PI hydrolysis was virtually identical at all culture ages tested, indicating that the age-dependent protection by ACPD is not entirely due to age-dependent changes in PI hydrolysis.

The dose–response relationship of ACPD required to protect against NMDA neurotoxicity was examined (Fig. 3*A*). Cultures (DIV 14–15) were exposed to 500 μM NMDA for 5 min in the presence of various concentrations of ACPD. All LDH values were represented as relative to the mean value in sister cultures exposed to NMDA alone. Some reduction in NMDA neurotoxicity was seen with 30 μM ACPD. The IC_{50} of ACPD that protected against neurotoxicity produced by a 5-min NMDA exposure was between 30 and 100 μM. To examine whether ACPD protection was dependent on NMDA concentrations, cultures (DIV 14) were exposed for 5 min to various concentrations of NMDA, either alone or in the presence of 100 μM ACPD. The protective effect of ACPD was not overcome by increasing NMDA concentrations up to 3 mM, a finding that suggests a noncompetitive mode of inhibition (Fig. 3*B*).

ACPD or carbachol also appeared to protect neurons against NMDA neurotoxicity when cultures were preexposed to these compounds. Cultures (DIV 14–15) were exposed to 100 μM ACPD or 500 μM carbachol in the presence of 50 μM CNQX and 50 μM CPP. Under this condition, neither the NMDA nor the non-NMDA receptors should be activated. Control cultures were treated the same but without ACPD or carbachol. After a 5-min exposure, multiple rinses were quickly made (<1 min) to effectively remove all of the compounds, and cultures were then exposed to 500 μM NMDA for 5 min. Preincubation in ACPD or carbachol substantially decreased NMDA neurotoxicity (Fig. 4). It should also be noted that the protective effect of carbachol was completely reversed by the coapplication of the muscarinic antagonist atropine (10 μM). The similar protective effects against NMDA neurotoxicity were also observed when cultures were preexposed for 5 min to 10 μM quisqualate in the presence of 50 μM CPP and 50 μM CNQX (Fig. 4).

Since activation of receptors linked to PI hydrolysis generates the PKC activator DAG, in addition to IP_3, we examined whether PKC or other protein kinases may mediate the protective effect of ACPD. To test this hypothesis, we used the protein kinase antagonists H-7 and HA-1004. The former is regarded as the more specific antagonist of PKC, but substantial overlap exists in concentration ranges of both H-7 and HA-1004 that act at several different protein kinases (27). A concentration of 200 μM was chosen, as similar concentrations of H-7 have previously been used and shown effective in slice preparations (28, 29). We found that both protein kinase antagonists partially reversed the protective effect of ACPD (Fig. 5), suggesting that the effect, in part, may be mediated by protein kinase(s).

DISCUSSION

The present study demonstrates that activation of the ACPD receptor substantially attenuates neurotoxicity produced by

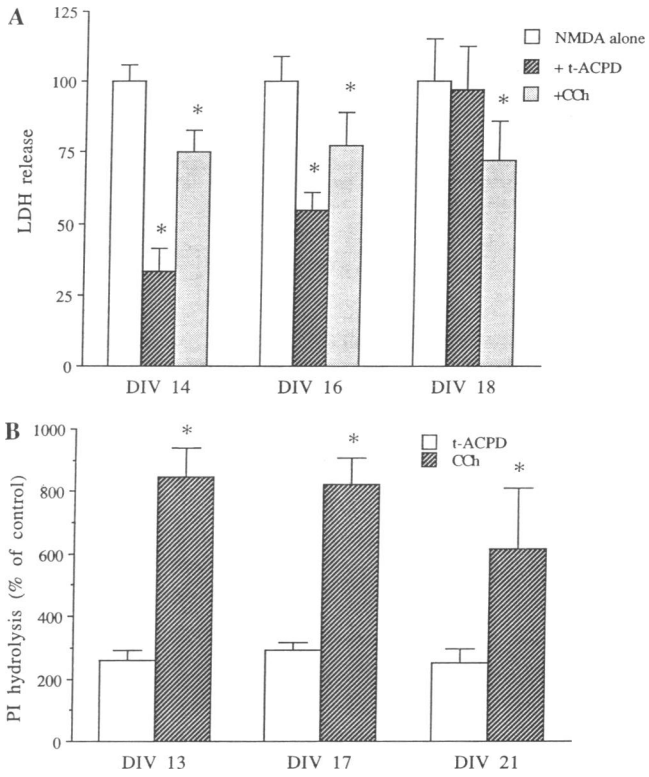

FIG. 2. (*A*) Age dependence of the ACPD effect. Data represent LDH release (mean ± SEM; $n = 7$ or 8) in cultures at different ages (DIV 14, 16, and 18) 24 hr after a 5-min exposure to 500 μM NMDA alone or in the presence of 100 μM ACPD (+t-ACPD) or 500 μM carbachol (+CCh). LDH values were scaled to the mean value in age-matched sister cultures exposed to NMDA alone (blank bars, =100). Asterisks denote differences from the control ($P < 0.05$, two-tail *t*-test with Bonferroni correction for two comparisons). (*B*) Age dependence of PI hydrolysis induced by ACPD and carbachol. Bars show levels of PI hydrolysis (percent of the control; mean ± SEM, $n = 4$) in sister cultures at different ages, after a 10-min exposure to 100 μM ACPD or 500 μM carbachol, in the presence of 50 μM CPP and 50 μM CNQX. At all ages, carbachol induced more PI hydrolysis than ACPD did (*, $P < 0.01$; two-tail *t*-test with Bonferroni correction for three comparisons). Unlike the protective effect of ACPD that decreased with culture age, PI hydrolysis by ACPD remained relatively constant throughout the culture ages tested.

a 5-min exposure to NMDA in cortical cultures. A weaker protective effect was also observed with carbachol, an agonist of the cholinergic receptor.

The protective effect of ACPD appeared to be dependent on the age of cultures in such a way that little or no protection was observed in cultures older than DIV 18. In contrast to ACPD, carbachol reduced NMDA neurotoxicity throughout the range of culture ages tested (DIV 14–18). The protective effect of carbachol observed in the present study apparently is in contrast to a previous report that acetylcholine potentiates glutamate neurotoxicity in hippocampal cultures (30). However, the pharmacology of glutamate neurotoxicity appears to be fundamentally different between the two culture systems; in the hippocampal cultures of Mattson *et al.* (31), glutamate neurotoxicity develops slowly and is largely mediated by the non-NMDA receptors, whereas in the present cortical cultures, glutamate neurotoxicity develops more rapidly (in minutes) and is largely produced by activation of the NMDA receptor (17).

As both ACPD and carbachol increase membrane PI hydrolysis in neurons (32), the protective effects may be produced by intracellular mobilization of calcium. Of note, ACPD or other agonists such as carbachol that activate PI hydrolysis often induce oscillatory increases in intracellular free calcium concentrations (22, 33, 34). It may be that the oscillatory increases in intracellular calcium render neurons more able to tolerate a subsequent calcium influx. However, a simple quantitative relationship between the level of PI hydrolysis and the degree of protection could not be found in the present study, as carbachol induced more PI hydrolysis than ACPD but provided less protection. It appears that some downstream event in the signaling cascade is not responsive to the activation of PI hydrolysis at later developmental periods.

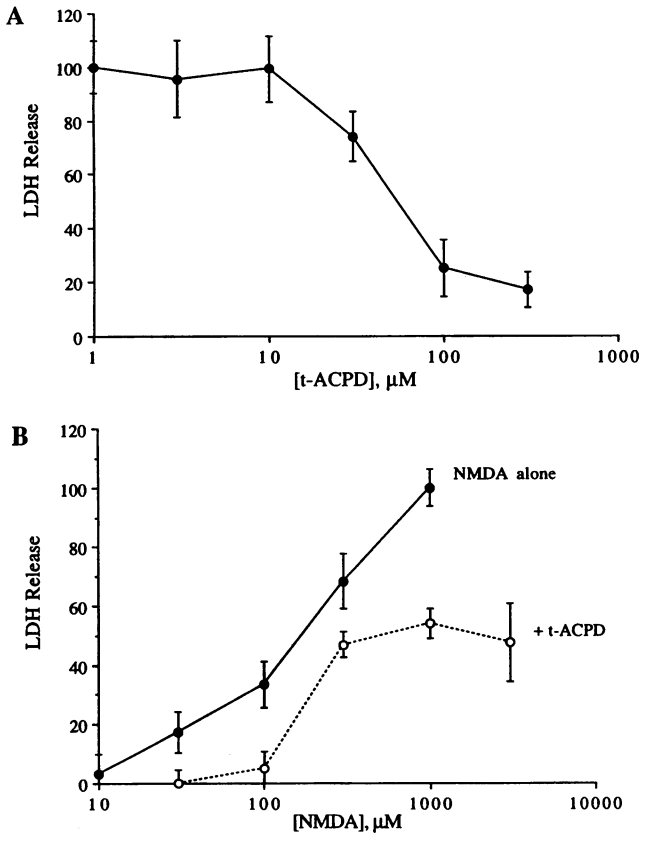

FIG. 3. (*A*) Concentration–protection relationship of ACPD. Data represent LDH release (mean ± SEM; n = 4–12) in cultures (DIV 14–15) 24 hr after a 5-min exposure to 500 μM NMDA in the presence of various concentrations of ACPD (t-ACPD). The protective effect of ACPD was observed at concentrations between 30 and 300 μM. (*B*) Noncompetitive mode of protection by ACPD against the NMDA neurotoxicity. Sister cultures (DIV 14) were exposed for 5 min to various concentrations of NMDA alone or in the presence of 100 μM ACPD. Data represent LDH release (mean ± SEM; n = 4) in the bathing media 24 hr after the exposure. The protective effect of ACPD remained unchanged with NMDA concentrations between 300 and 3000 μM. LDH values were scaled to the mean value in sister cultures exposed to 1 mM NMDA (=100) after subtraction of mean background values.

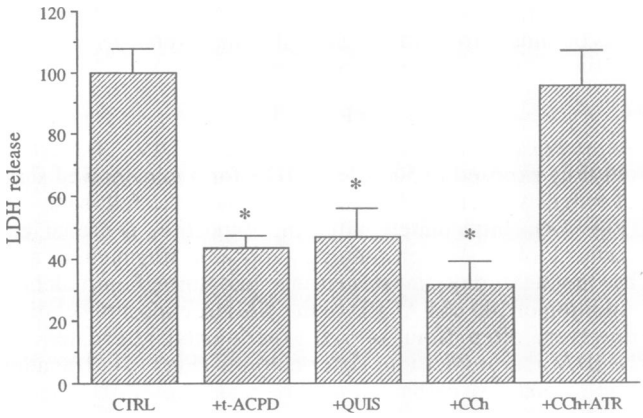

FIG. 4. Preincubation in ACPD or carbachol also reduces subsequent NMDA neurotoxicity in the presence of CNQX and CPP. Cultures (DIV 14–15) were exposed for 5 min to 100 μM ACPD (+t-ACPD), 10 μM quisqualate (+QUIS), or 500 μM carbachol (+CCh) [alone or with 10 μM atropine (+ATR)] in the presence of 50 μM CPP and 50 μM CNQX to block both NMDA and non-NMDA receptors. Immediately after a 5-min preexposure, cultures were washed several times to remove preexposure chemicals and exposed to 500 μM NMDA for 5 min. Control cultures were preexposed to CPP and CNQX alone and subsequently exposed to NMDA (CTRL). Bars represent LDH release (mean ± SEM) 24 hr after the NMDA exposure. Asterisks denote differences from the control ($P < 0.05$, two-tail t-test with Bonferroni correction for five comparisons).

The concentration range of ACPD that attenuates the NMDA neurotoxicity (30–300 μM) is consistent with the range in which ACPD increases PI hydrolysis in hippocampal cultures. Interestingly, but not unexpectedly, the mode of protection appears

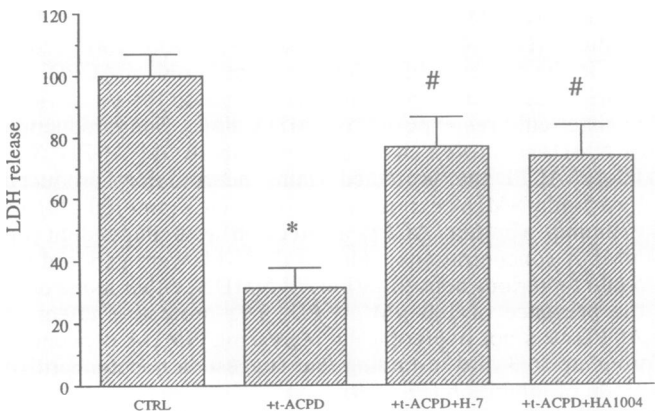

FIG. 5. Antagonists of protein kinases partially reverse the protective effect of ACPD on the NMDA neurotoxicity. As shown above, cultures (DIV 14) exposed to ACPD and NMDA (+t-ACPD) showed less neuronal degeneration and less LDH release than cultures exposed to NMDA alone (CTRL). However, the protective effect of ACPD appears to be largely diminished when H-7 (200 μM) or HA-1004 (200 μM) was applied to cultures 5 min before the exposure; antagonists were available throughout the NMDA exposure. Bars depict mean LDH release (± SEM; n = 4). An asterisk denotes a difference from the control; a pound sign denotes a difference from both the control and the cultures exposed to ACPD and NMDA ($P < 0.05$, two-tail t-test).

to be noncompetitive. Even at saturating concentrations of NMDA, ACPD was effective in protecting neurons.

Of interest, the protective effect produced by ACPD or carbachol was seen even when one of these compounds was applied before the NMDA exposure, suggesting that the cellular changes induced by these agonists may last at least a few minutes following the washout. Also, the fact that the protection by ACPD or quisqualate was observed even in the presence of CPP and CNQX further suggests that these effects are not likely produced by indirect activation of the ionotropic glutamate receptors.

In addition to IP_3, DAG, an activator of PKC, would be produced by ACPD or carbachol. The partial reversal of the protection by H-7 and HA-1004 suggests that protein kinases may be involved, in part, in the protective cascade, although it is difficult to determine whether PKC alone is specifically involved, due to the fact that the actual concentrations achieved in the neuronal membrane are unknown. At high concentrations, biochemical assays have shown that H-7 and HA-1004 can block multiple protein kinases (27). More detailed pharmacological studies are needed to identify the kinases or other events involved.

It is possible that negative modulatory interactions exist between the ACPD receptor and the NMDA receptor. In fact, our previous study has shown that activation of NMDA receptors inhibits ACPD-induced PI hydrolysis in hippocampal slices (26). The present study suggests that the reverse may also exist—i.e., that ACPD receptor activation may reduce responses mediated by the NMDA receptor.

During development, the appearance of the ACPD receptor exhibits a transient peak that coincides with synaptogenesis. For example, PI hydrolysis by activation of the ACPD receptor peaks during 6–12 days after birth in rat hippocampal slices (35). The functional significance of this transient increase is currently unknown, but it has been suggested to play a role in synaptogenesis (35, 36). The NMDA receptor shows similar up-regulation during development (37). Consistent with the transient up-regulation of the NMDA receptor, it has been reported that in young animals NMDA produces more excitotoxic neuronal damage compared to that in adult animals (38). This NMDA receptor up-regulation may normally serve an important function, such as mediating developmental synaptic plasticity in visual cortex (39, 40). The present finding that ACPD attenuates NMDA neurotoxicity in cortical cultures in an age-dependent manner suggests that the transient increase in ACPD receptor *in vivo* also might serve as a necessary protective mechanism against brief, intensive NMDA receptor activation, necessary for normal synaptic plasticity during central nervous system development.

In summary, the present study demonstrates that neurotransmitter interactions may be able to affect the expression of excitotoxic neuronal damage. It may prove clinically important to understand all the factors that can alter excitotoxicity, in light of substantial evidence linking this type of neurotoxicity to a wide variety of neurodegenerative conditions (1, 2). For example, in AD, a marked reduction in cholinergic neurotransmission is probably one of the early abnormalities (4, 41). In addition to the direct functional consequences, the present data suggest that the cholinergic deficit may also subsequently increase the risk of target neurons to excitotoxic injury. Since it is known that central neurons containing neurotransmitters such as somatostatin (5, 6), serotonin (42), and norepinephrine (43) are also affected in neurodegenerative diseases, it may be interesting to see if these neurotransmitters can modulate excitotoxicity as well.

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