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Activation of *N*-Methyl-D-Aspartate Receptor Attenuates Acute Responsiveness of δ-Opioid Receptors

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SUMMARY

Coadministration of antagonists of *N*-methyl-D-aspartate (NMDA) receptor and opioids has been shown to prevent development of opiate tolerance in animal and clinical studies, but its cellular and molecular mechanisms are not understood. In this study, the effect of NMDA on δ-opioid receptor (DOR)-mediated signal transduction was investigated in neuroblastoma × glioma NG108–15 cells that functionally express both DOR and NMDA receptors. Acute incubation of NG108–15 cells with NMDA, a specific agonist of NMDA receptor, significantly attenuated the ability of DOR agonist [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) to inhibit forskolin-stimulated cAMP production. The attenuation caused by NMDA was dose-dependent, and the EC_{50} of DPDPE increased 100-fold (from 4.6 nM to 500 nM) after NMDA treatment. The NMDA effect on responsiveness of δ-opioid receptors to DPDPE could be blocked by ketamine, a NMDA receptor-specific antagonist. This NMDA attenuation effect on DOR activity was also observed in neuronal primary cell cultures from fetal mouse brain but not in the Chinese hamster ovary cell line stably transfected with DOR alone. Interestingly, NMDA pretreatment reduced the cellular response to epinephrine but not to that of prostaglandin E_1 in NG108–15 cells, which suggests differential modulation of NMDA on different G protein-coupled receptors. Pretreatment of NG108–15 cells with ketamine along with DPDPE greatly attenuated DPDPE-induced acute desensitization of DOR. Furthermore, the specific inhibitors of protein kinase C, either chelerythrine chloride or Gö 6979, effectively blocked the NMDA effect, which indicates the involvement of protein kinase C in the process. In conclusion, the activation of NMDA receptors can attenuate acute responsiveness of DOR in neuronal cells, whereas its blockage leads to reduction of DOR desensitization. These results have thus provided an insight into cross-talk between NMDA and opioid signal transduction.

Chronic exposure to an opioid agonist leads to drug tolerance, which is characterized by a decrease in analgesic efficacy *in vivo* and by reduced functions of opioid receptors at the cellular level (1, 2). Significant reduction of opioid responsiveness after acute opioid treatment was also documented and is functionally defined as receptor desensitization (3). Although they are known to involve phosphorylation, internalization, and down-regulation of opioid receptors, uncoupling of the opioid receptor/G protein system, as well as adaptations in the cAMP signal transduction cascade, the molecular mechanisms underlying opioid tolerance and receptor desensitization are not yet fully understood (1–8).

The evidence accumulated in recent years suggests the existence of interactions between the signal transduction systems of excitatory amino acid receptors and opioid receptors. On one hand, opioid peptides functionally and directly interact with the NMDA receptor (9). On the other hand, various antagonists of NMDA receptors profoundly attenuate opioid tolerance in animal experiments, and a NMDA receptor antagonist, ketamine, was found to potentiate morphine's analgesic effect in cancer patients (10–15). The cellular or molecular mechanisms of such an interaction are unclear, but experimental data indicate that the NMDA antagonist does not seem to regulate opioid tolerance by altering the affinity or density of μ-, δ-, κ-1-, and κ-3-opioid receptors or by displacing opioid ligands at their binding sites (11).

Recent reports have revealed the presence of functional NMDA receptors in neuroblastoma × glioma NG108–15 hybrid cells (16), a cellular model system in opioid research. The expression of both NMDA receptors and DOR in NG108–15 cells make them useful in the study of the interaction and cross-talk between opioid receptors and NMDA receptors at the cellular level. Furthermore, it has been suggested recently that the δ-opioid receptor plays a crucial role in morphine tolerance and dependence in mice (17). Therefore, we

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DOR, δ-opioid receptor; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; EPI, epinephrine; α_2AR, α_2-adrenergic receptor; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; CHO, Chinese hamster ovary.

undertook the present study to look into the potential effects of NMDA receptor activation or blockage on the acute responsiveness and desensitization of the δ-opioid receptor.

Materials and Methods

Cell cultures. Neuroblastoma × glioma NG108–15 hybrid cells were cultured as previously described (3). Dissociated brain neurons obtained from mouse embryos (at embryonic day 18) were cultured in poly-D-lysine (Sigma, St. Louis, MO)-coated plates with basal Eagle's medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (GIBCO BRL) and 10% calf serum (GIBCO BRL). CHO cells were transfected with mouse DOR in pcDNA3 (6) by calcium phosphate precipitation. Clones of DOR transfectants were selected using medium containing 1 mg/ml Geneticin (GIBCO BRL). Expression levels of DOR were measured by using saturation binding (6) with [^{3}H]diprenorphine (Amersham, Arlington Heights, IL), and a clone (DOR-CHO) that expressed DOR at approximately 0.6 pmol/mg total membrane protein was used in this study. The DOR-CHO clone was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.1 mg/ml Geneticin.

cAMP assay. The cells were challenged with control medium or medium containing NMDA (Sigma) at different concentrations at 37° for 5 min. Then cells were further treated with different concentrations of DPDPE (Sigma) in the presence of 1 μM forskolin (Sigma) and 500 μM 1-methyl-3-isobutylxanthine (Sigma) at 37° for 10 min. The reactions were terminated, and the cAMP levels of each sample were measured using radioimmunoassay as previously described (3). The values presented represent the means ± standard error of at least three experiments, calculated as 100 × [cAMP(For + D) − cAMP(basal)]/[cAMP(For) − cAMP(basal)] where cAMP(For + D) is cAMP accumulation in the presence of forskolin and DPDPE, cAMP-(basal) is cAMP in the absence of forskolin and DPDPE, and cAMP-(For) is cAMP in the presence of forskolin alone. In the DOR desensitization experiments, cells were pretreated with 10 μM DPDPE in the absence or presence of the NMDA-specific antagonist ketamine (Sigma) at 37° for 10 min. After being washed with phosphate-buffered saline, the cells were challenged with 1 μM DPDPE, and the cAMP levels were measured as described above.

Inhibition of protein kinases. To inhibit PKC, a specific PKC inhibitor, either chelerythrine chloride (20 μM, K_i = 0.66 μM; Calbiochem, San Diego, CA) or Gö 6979 (0.2 μM, K_i = 0.008 μM; Calbiochem), was applied to NG108–15 cells at 37° for 5 min before NMDA pretreatment. Separately, the specific inhibitor of cAMP-dependent protein, kinase (PKA) H-89 (1.2 μM, K_i = 0.048 μM; Calbiochem), was also used to treat NG108–15 cells to inhibit PKA in the same way. The subsequent DPDPE-stimulated reduction of cAMP accumulation was measured as described above.

Phosphorylation of DOR. DOR cDNA with the influenza hemagglutinin epitope at the amino terminus (6) was transiently transfected in NG108–15 cells using LipofectAMINE (GIBCO BRL) according to the manufacture's instructions. Forty-eight hours after transfection with DOR expression levels at 1–2 pmol/mg protein, the cells were labeled with 100 μCi/ml [^{32}P]orthophosphate (DuPont-New England Nuclear, Boston, MA) for 60 min. Labeled cells were then stimulated with 5 μM NMDA, 5 μM DPDPE, or 5 μM DPDPE and 5 μM NMDA for 10 min at 37°. After stimulation, DOR was immunoprecipitated with 12CA5 monoclonal antibody and resolved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described by Pei *et al.* (6). DOR phosphorylation was visualized and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Data were analyzed using Student's *t* test for comparison of independent means with pooled estimates of common variances (6).

Results

NMDA attenuates acute responsiveness of DOR in NG108–15 cells. DOR is functionally coupled to the inhibitory G protein (G_i) and, thus, negatively regulates adenylyl cyclase in NG108–15 cells. After the cells were treated with various concentrations (10^{-9} to 10^{-5} M) of NMDA, a specific agonist of the NMDA receptor, for 5 min, the basal cellular cAMP level or forskolin-stimulated cAMP production in the treated cells did not change (data not shown), which indicates that NMDA does not have a significant effect on adenylyl cyclase under these conditions. However, the ability of DPDPE, a specific DOR agonist, to inhibit forskolin-stimulated cAMP production was greatly attenuated (more than 50% of its maximum) by the NMDA pretreatment (Fig. 1A). The

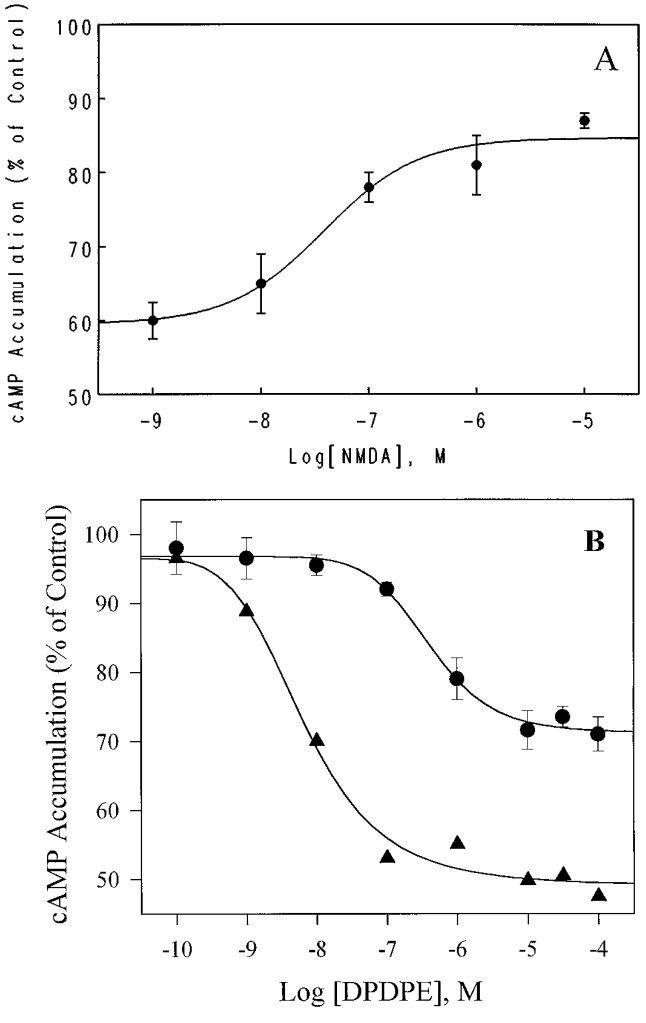

Fig. 1. A, Dose-response curve of NMDA on DPDPE inhibition of cAMP accumulation in NG108–15 cells. Cells were first incubated with different concentrations of NMDA at 37° for 5 min, then cells were treated at 37° for 10 min with 1 μM forskolin and 500 μM 1-methyl-3-isobutylxanthine with or without 1 μM DPDPE. The cAMP level of each sample was measured using radioimmunoassay. *Plotted values,* averages of at least three experiments. B, Effect of NMDA on DPDPE-induced inhibition of cAMP accumulation in NG108–15 cells. Cells were pretreated without ([triaf]) or with (●) 1 μM NMDA at 37° for 5 min. The percentage of forskolin-stimulated cAMP production in the presence of different concentrations of DPDPE was measured and calculated as described above. The EC_{50} values of DPDPE to inhibit cAMP accumulation in naive and NMDA-prechallenged cells were 4.6 nM and 500 nM, respectively.

effect of NMDA on the opioid-dependent attenuation of cAMP accumulation was dose-dependent, with an EC_{50} of approximately 40 nM. After incubation of NG108–15 cells with 1 μM NMDA, the DPDPE dose-response curve shifted to the right, and the EC_{50} of DPDPE increased from 4.6 nM to 500 nM (Fig. 1B). The attenuation of acute responsiveness of DOR by NMDA was unlikely mediated through direct NMDA interference with DPDPE binding to DOR because NMDA did not reduce the maximal [^{3}H]DPDPE or [^{3}H]diprenorphine binding in NG108–15 cells (data not shown).

Modulation of DOR activity by NMDA is mediated through the NMDA receptor in NG108–15 cells. To test whether the effect of NMDA on DOR activity is mediated by activation of NMDA receptor, ketamine, a noncompetitive antagonist of the NMDA receptor, was coadministered with NMDA. As shown in Fig. 2A, NMDA-induced attenuation of DOR activity was completely blocked by ketamine in NG108–15 cells. Another competitive antagonist of NMDA receptor, 2-amino-5-phosphonovaleric acid, was also capable to abolish the NMDA effect under the same conditions (data not shown). In addition, a CHO cell line that was stably transfected with DOR cDNA alone and that expressed functional DOR was used in our control experiments to further verify the role of the NMDA receptor in the modulation of DOR activity. The result showed that NMDA did not affect the ability of DOR to inhibit forskolin-induced cAMP accumulation in the DOR-CHO cells (Fig. 2B). Taken together, our data suggest that the modulation of acute responsiveness of DOR by NMDA in NG108–15 cells was mediated through the NMDA receptor.

Modulation of DOR by NMDA in primary cultured neurons and α2AR in NG108–15 cells. We went on to examine whether the cross-talk between the NMDA receptor and the opioid receptor could occur in a more physiologically relevant system. Using primarily cultured neuronal cells, our results showed that NMDA treatment significantly reduced the inhibitory ability of DOR in a manner similar to its action in NG108–15 cells. The reduction of DOR function by NMDA in primary neuronal cells seemed even greater than in NG108–15 cells (Fig. 3A).

Beside DOR, there are other G protein-coupled receptors expressed in NG108–15 cells (18). It is reasonable to speculate that NMDA may exert its modulating effect on those receptors. To test our hypothesis, NG108–15 cells with or without NMDA pretreatment were challenged with either EPI, an agonist of α_2AR that is also a G_i-coupled receptor, or prostaglandin E_1, which activates the stimulatory G protein (G_s) to elevate the cAMP level. Interestingly, the ability of EPI to inhibit forskolin-stimulated cAMP accumulation was significantly attenuated by NMDA treatment (Fig. 3B), which indicates that there is a similarity in molecular mechanism for NMDA signal to cross-talk with opioid and adrenergic receptors through the G_i-coupled receptor signal pathway. However, no apparent NMDA effect on the cAMP level was observed after prostaglandin E_1 stimulation (data not shown), which is suggestive of differential modulation of NMDA on the different G protein-coupled receptors.

Ketamine attenuates DOR desensitization induced by DPDPE pretreatment in NG108–15 cells. To interpret the *in vivo* function of the NMDA receptor antagonist at the cellular level, NG108–15 cells were pretreated with DPDPE in the absence or presence of the antagonist of the NMDA receptor, ketamine. The DOR responsiveness to DPDPE was profoundly desensitized by DPDPE pretreatment in the absence of ketamine, which is a typical phenomenon of receptor-homologous desensitization (3). In contrast, the presence of ketamine during DPDPE pretreatment significantly reduced the extent of DOR desensitization (Fig. 4). Similarly, another NMDA receptor antagonist, 2-amino-5-phosphono-

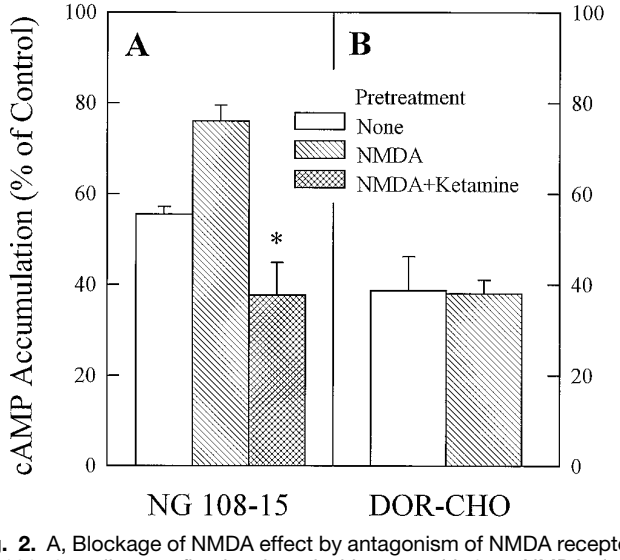

Fig. 2. A, Blockage of NMDA effect by antagonism of NMDA receptor. NG108–15 cells were first incubated without or with 1 μM NMDA alone or 1 μM NMDA and 1.8 mM ketamine at 37° for 5 min, then challenged with 1 μM DPDPE. cAMP levels were measured as described in Fig. 1. Results are means ± standard error of three separate experiments and are expressed as percentages of forskolin-stimulated values in the absence of agonists. *, $p < 0.01$ compared with cells pretreated with NMDA. B, Effect of NMDA on the responsiveness of DOR to DPDPE in a CHO cell line stably expressing mouse DOR (*DOR-CHO*).

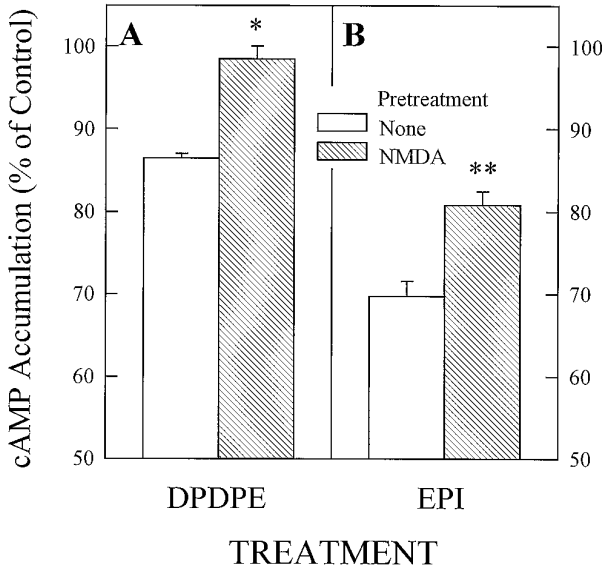

Fig. 3. A, Effect of NMDA on DPDPE-induced cAMP accumulation in neuronal cells. Primarily cultured mouse neuronal cells were exposed to no or 1 μM NMDA at 37° for 5 min. The percentage of forskolin-stimulated cAMP production in the presence of 1 μM DPDPE was measured as described in Fig. 1. *, $p < 0.05$ compared with control. B, NMDA effect on α_2AR in NG108–15 cells. EPI (100 μM) was applied to naive or NMDA-pretreated cells, and cAMP levels were determined. **, $p < 0.01$ compared with the naive cells.

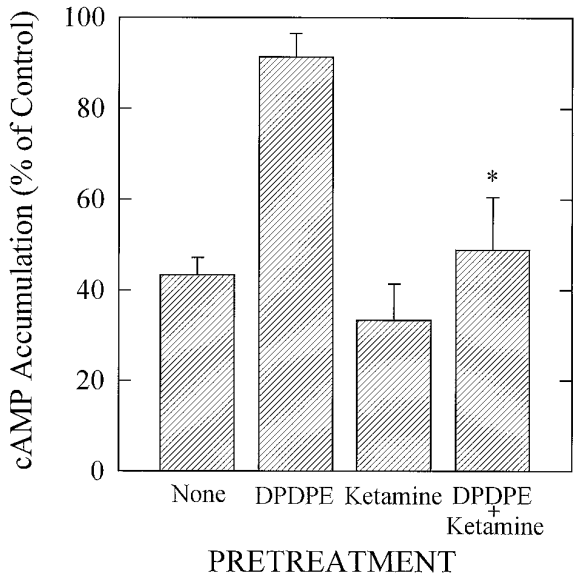

Fig. 4. Attenuation of DPDPE-induced desensitization by specific NMDA antagonist ketamine in NG108–15 cells. After pretreatment without or with 10 μM DPDPE in the absence or presence of 1.8 mM ketamine at 37° for 10 min and washing with phosphate-buffered saline (10 mM sodium phosphate, pH 7.5, 138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$), the NG108–15 cells were rechallenged with 1 μM DPDPE at 37° for 10 min. *, $p < 0.01$ compared with cells pretreated with DPDPE alone.

valeric acid, was also able to decrease DPDPE-induced desensitization of DOR (data not shown).

PKC but not PKA inhibitors block the NMDA effect. As shown in Fig. 5, when NG108–15 cells were pretreated with a specific PKC inhibitor, either chelerythrine chloride (20 μM) or Gö 6979 (0.2 μM), the ability of DPDPE to inhibit the cAMP accumulation was not affected. However, the NMDA effect on acute responsiveness of DOR was effectively blocked by the pretreatment of those PKC inhibitors under the same conditions (Fig. 5). In contrast, the application of a PKA-specific inhibitor, H-89, did not diminish the effect of NMDA (Fig. 5).

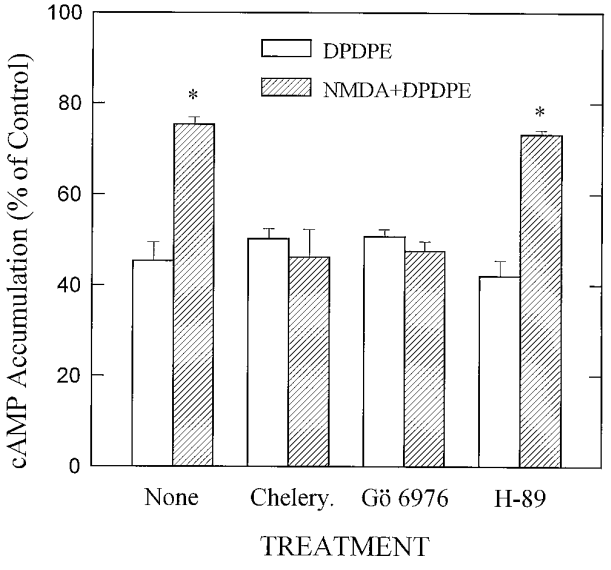

Fig. 5. Blockage of the NMDA effect by the PKC but not the PKA inhibitor. A specific PKC inhibitor (20 μM chelerythrine chloride or 0.2 μM Gö 6979) or a specific PKA inhibitor (1.2 μM H-89) was applied to NG108–15 cells at 37° for 5 min before NMDA (1 μM) pretreatment. The subsequent DPDPE-stimulated reduction of cAMP accumulation was then measured under the conditions described in Fig. 1. *, $p < 0.01$ compared with cells stimulated with DPDPE alone.

NMDA does not increase phosphorylation of DOR. DOR exogenously introduced in NG108–15 cells was phosphorylated upon stimulation with the agonist DPDPE and showed a broad band (due to glycosylation of the receptors) of 60–70 kDa as demonstrated in *lane 4* of Fig. 6. Treatment of NG108–15 cells with NMDA, however, did not increase either basal (Fig. 6, *lane 3*) or DPDPE-stimulated (Fig. 6, *lane 5*) phosphorylation of DOR.

Discussion

Although *in vivo* studies in recent years have solidly established that an interaction or cross-talk between NMDA and opioid receptor signal transduction pathways exists, the underlying cellular and molecular mechanisms remain poorly understood. The present study, starting with a model cellular system, has attempted to answer the interesting question directly from the point of NMDA effect on activity of DOR. Our data clearly demonstrate that NMDA mediated through its receptor negatively modulates the acute responsiveness of DOR in neuronal cells. The NMDA modulation was dose-dependent, and it could be blocked by the NMDA receptor antagonist. Moreover, NMDA receptor antagonists effectively attenuated the DPDPE-induced desensitization of DOR. The results of this study provide a cellular basis for the antiopioid tolerance effect of NMDA receptor antagonists and a potentially useful tool in searching for more potent and selective addiction-preventing agents. It is necessary, however, to further investigate the possible effect of NMDA on other opioid receptors such as μ and κ receptors, which also play important roles in analgesia and drug tolerance.

It is rational to speculate that the molecular mechanisms of the modulatory effects of the agonist or antagonist of NMDA receptor mediated by the NMDA receptor, as shown

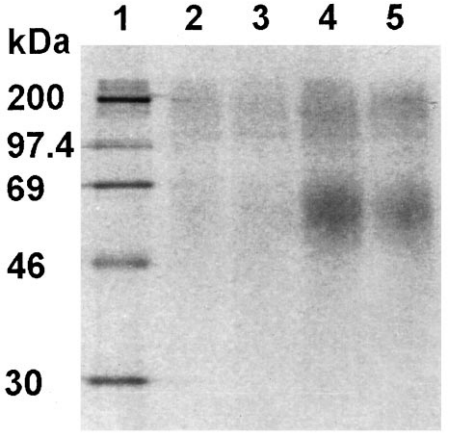

Fig. 6. Phosphorylation of DOR after NMDA pretreatment. NG108–15 cells transfected with the epitope-tagged DOR were labeled with $^{32}P_i$ for 60 min and stimulated without (*lane 2*) or with 5 μM NMDA (*lane 3*), 5 μM DPDPE (*lane 4*), or 5 μM DPDPE plus 5 μM NMDA (*lane 5*) for 10 min at 37°. After stimulation, DOR was immunoprecipitated with 12CA5 monoclonal antibody and resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. DOR phosphorylation was visualized and analyzed with a PhosphorImager. *Lane 1,* molecular weight markers of ^{14}C-methylated proteins (Amersham).

above, are somehow related to the functions of the NMDA receptor. At sites throughout the brain and spinal cord, the NMDA receptor is one type of ion channel permeable to Ca^{2+} as well as Na^+ and K^+ (19). Activation of the NMDA receptor by its specific agonist induces Ca^{2+} influx and thus increases cytoplasmic Ca^{2+} concentration (20, 16). The elevation of Ca^{2+} concentration stimulated by NMDA consequently activates a family of PKC (21), and the activation of PKC could lead to the attenuation of opioid receptor activity, as in the case of direct activation of PKC by phorbol esters (23, 24). Our finding that PKC-specific inhibitors could block the NMDA modulatory effect strongly suggests the involvement of PKC in the process. Activated PKC has been shown to be able to phosphorylate three important components on the DOR signaling pathway: the opioid receptor (6), the α subunit of G_i (25), and adenylate cyclase (26). The results from this study show that NMDA does not affect basal or forskolin-stimulated cAMP accumulation, which indicates that adenylate cyclase may not be the target of PKC in this case. Our data further suggest that DOR is an unlikely candidate for PKC phosphorylation after NMDA treatment. The possible target of PKC therefore could be the α subunit of G_i, because its phosphorylation by PKC has been shown to impair the coupling of the G protein to the receptor (25). Further studies of this subject are under way in our laboratory.

It is interesting to learn from this study that NMDA differentially modulates different G protein-coupled receptors. Among the receptors examined, NMDA apparently attenuates the function of DOR and α_2AR in the G_i receptor family but not that of the prostaglandin E_1 receptor in the G_s receptor family. This is presumably because of the differential regulation of desensitization of the G protein-coupled receptor by different protein kinases: regulation of the G_i receptor family occurs mainly via PKC (3, 5–7, 25), whereas that of the G_s receptor family mostly occurs through cAMP-dependent PKA, as in the case of β_2-adrenergic receptor (27). However, more receptors need to be investigated before any precise conclusion can be drawn.

Acknowledgments

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