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mRNA from NCB-20 cells encodes the *N*-methyl-D-aspartate/phencyclidine receptor: A *Xenopus* oocyte expression study

(excitatory amino acids/glutamate receptors/oocyte expression system/neuroblastoma clonal cell line)

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ABSTRACT **The mouse neuroblastoma–Chinese hamster brain hybrid cell line NCB-20 is the only clonal cell line in which binding studies indicate the presence of phencyclidine (PCP) receptors. We report here that *Xenopus* oocytes injected with NCB-20 cell poly(A)$^+$ RNA express *N*-methyl-D-aspartate (NMDA)-activated channels and that these channels include the PCP receptor site. In injected oocytes, NMDA application evoked a partially desensitizing inward current that was potentiated by glycine, blocked by the competitive antagonist D-2-amino-5-phosphonovaleric acid, blocked by Mg^{2+} and by Zn^{2+}, and blocked in a use-dependent manner by the PCP receptor ligands PCP and MK-801. There was little or no response to kainate or quisqualate (agonists of the other excitatory amino acid receptors), to γ-aminobutyric acid (an inhibitory transmitter), or to glycine (an inhibitory transmitter as well as an allosteric potentiator of NMDA channels). Thus, NMDA/PCP receptors expressed from NCB-20 cell mRNA exhibit properties similar to those of the neuronal receptors. The absence of expression of other excitatory amino acid receptors in this system makes it particularly useful for study of NMDA-evoked responses without interference from responses mediated by other receptors. Moreover, NCB-20 mRNA may be an appropriate starting material for cloning the cDNA(s) encoding the NMDA/PCP–receptor complex.**

The *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor has been implicated in long-term potentiation, memory, developmental structuring, epilepsy, excitotoxicity, and the psychotomimetic actions of phencyclidine (PCP) (see refs. 1–3). Studies of the actions of PCP receptor ligands on NMDA-activated channels in neurons (4–9) and in *Xenopus* oocytes injected with rat brain mRNA (10, 11) suggest that the PCP receptor is a site within the NMDA channel. PCP receptor ligands appear to be open channel blockers in that they block the actions of NMDA in a use- and voltage-dependent manner. Antagonism of NMDA-evoked responses is stereoselective (10), and drug potencies in modulating NMDA effects correlate well with binding affinities for the PCP receptor in rat brain membranes (3, 12–14) and with behavioral potencies in the drug discriminative stimulus paradigm (15–17). The mouse neuroblastoma–Chinese hamster brain hybrid cell line NCB-20 is the only clonal cell line shown, by binding assays, to have PCP receptors (18). This site was labeled by the potent phencyclidine derivative *N*-[1-(2-thienyl)cyclohexyl]piperidine with a binding affinity (K_d) of 335 nM and a receptor density (B_{max}) of 9300 fmol per mg of protein, which corresponds to ≈60,000 sites per cell. We report here that *Xenopus* oocytes injected with NCB-20 cell poly(A)$^+$ RNA expressed NMDA/PCP receptors with properties similar to those of the neuronal receptor.

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METHODS

The mouse neuroblastoma–Chinese hamster brain hybrid cell line NCB-20 (provided by Richard Miller, University of Chicago) was grown in monolayer in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (GIBCO) and garamycin (50 μg/ml) on 100-mm^2 Falcon tissue culture dishes in a humidified atmosphere of 5% CO_2/95% air. Cells were harvested at confluency, frozen immediately in liquid N_2, and stored at −76°C until use. RNA was extracted from NCB-20 cells by a guanidinium isothiocyanate method (30). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (19) and stored at −76°C. *Xenopus laevis* oocytes were obtained from ovarian lobes, which were dissected from anesthetized frogs and incubated (2 hr at 22°C) in Ca^{2+}-free ND96 medium (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM Hepes-NaOH, pH 7.5) (31) supplemented with sodium pyruvate at 2.5 mmol/liter, to which penicillin (100 units/ml), streptomycin (1 mg/ml), and collagenase (2 mg/ml) (Sigma; type 1A) were added. After transfer to Ca^{2+}-containing ND96, stage V and VI (20) oocytes were manually dissected from ovarian membranes and follicle cells and injected with mRNA (100 ng per cell). Oocytes were maintained for 4–8 days at 16.5°C in Leibovitz's L-15 medium (0.7 strength) (Sigma) supplemented with 5 mM Hepes buffer (pH 7.6), penicillin (100 units/ml), and streptomycin (1 mg/ml). These oocytes were placed in a bath with a vol of ≈0.1 ml and were perfused with Mg^{2+}-free amphibian Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/5 mM Hepes, pH 7.2). All drugs were dissolved and applied in this medium. Cells were voltage clamped at a holding potential of −60 mV with two beveled electrodes filled with 1 M KCl (1–2 MΩ). All compounds were bath-applied with an access time of <0.4 sec; solutions were washed out within 2 sec as shown by visual inspection of dye application.

RESULTS

Oocytes injected with NCB-20 mRNA expressed NMDA receptors as shown electrophysiologically. In Mg^{2+}-free medium, an inward current was evoked at short latency (≈0.2 sec) by application of NMDA (with 10 μM glycine unless otherwise noted; see below). This current decayed with a time constant of 2–3 sec to a steady level, a response like that elicited by NMDA in oocytes injected with rat brain mRNA (10). Positive responses to NMDA were detected in 36 of 84 (43%) of the oocytes injected with NCB-20 mRNA; four mRNA preparations were sampled and all caused expression of NMDA responses. In 12 batches of oocytes from eight

Abbreviations: PCP, phencyclidine; NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid.

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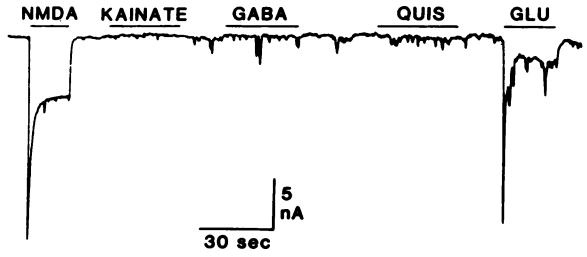

FIG. 1. Expression of neurotransmitter receptors in *Xenopus* oocytes injected with NCB-20 cell poly(A)$^+$ mRNA. Records from an oocyte clamped at −60 mV injected with mRNA 6 days previously. Horizontal bars indicate duration of drug application. Inward currents were obtained in response to application of NMDA (100 μM in the presence of 10 μM glycine) and glutamate (GLU) (100 μM) application. Kainate (500 μM), GABA (100 μM), and quisqualate (QUIS) (10 μM) had no effect. The small brief inward currents were due to endogenous Ca^{2+}-activated chloride channels; these currents disappeared in these oocytes after injection of EGTA.

different donors, expression of NMDA receptors ranged from 14% to 88%. Uninjected (n = 40) and water-injected (n = 20) oocytes did not respond to NMDA as we previously reported (10) nor did 10 additional water-injected oocytes respond in this study. Moreover, no response to NMDA was detected in oocytes ($n \geq 6$ for each case) injected with an mRNA preparation from each of the following: rat neuroblastoma cell line B103C16, rat kidney, rat liver, and rat cardiac muscle. These results indicate that the NCB-20 cells contain the precursor mRNA necessary to encode NMDA receptors.

In contrast to responses elicited by NMDA, no currents were evoked in oocytes injected with NCB-20 mRNA upon application of kainate (500 μM; 34 cells), quisqualate (10 μM; 10 cells) or γ-aminobutyric acid (GABA) (100 μM; 6 cells) (Fig. 1). Application of glycine (100 μM; 12 cells) alone or of serotonin (50 nM; 12 cells) produced no detectable current (data not shown). Application of glutamate elicited an inward current that resembled the current elicited in the same oocytes by NMDA (Fig. 1). All these compounds except glycine evoke large currents when applied to oocytes injected with rat brain mRNA (10). Thus, mRNA from NCB-20 cells encodes NMDA receptors but not other excitatory or inhibitory amino acid receptors.

Properties of NMDA receptors expressed by NCB-20 message were typical of neuronal NMDA receptors (21) and of those expressed in oocytes following injection of rat brain mRNA (10, 22). The relationship between the steady level of inward current and NMDA concentration indicated a single component response with an apparent affinity (K_d) of 22 μM and a Hill coefficient of 1.3 (Fig. 2*B*). Application of NMDA at a near-saturating concentration resulted in a relatively larger initial peak than did NMDA at lower concentrations, presumably due to a greater degree of desensitization; the steady level of response was smaller for 300 μM than it was for 100 μM NMDA.

As in neurons (23) and oocytes injected with rat brain mRNA (10, 24), glycine markedly potentiated the response to NMDA. The relationship between the steady level of inward current elicited by 50 μM NMDA and glycine concentration indicated a single component response with an apparent affinity (K_d) of 0.2 μM and a Hill coefficient of 1.3 (Fig. 2*A*). This value was not, however, corrected for a possible background level of glycine. In the presence of 0 or 10 nM added glycine, a transient inward current was observed at the onset of drug application. One interpretation of these results is that desensitization is more prominent at low glycine levels. Similar transient responses were obtained when glutamate was applied without added glycine (data not shown). To see the transient response elicited by either drug in the absence of glycine, the oocyte had to be optimally oriented. Because NMDA receptors are expressed at highest density at the animal pole (25), the best responses were obtained when the oocyte was positioned with its animal pole facing the upstream side of the bath application trough. Because of its potentiating action, glycine (10 μM) was routinely included in the NMDA solutions.

The NMDA-activated currents in oocytes injected with NCB-20 mRNA exhibited several other pharmacological properties characteristic of neuronal NMDA receptors (21, 26, 27) and of NMDA receptors expressed in oocytes injected with rat brain mRNA (10, 11, 22). The responses were blocked by the selective NMDA antagonist D-(2)-amino-5-phosphonovaleric acid. At 50 μM NMDA, the concentration that elicited half-maximal inhibition (IC_{50}) was 2.5 μM and the Hill coefficient was 1.3 (Fig. 2*C*). NMDA currents were also blocked by Mg^{2+} at inside negative potentials, and the Mg^{2+} block was relieved by depolarization (Fig. 3). Whereas the amplitude of the NMDA response was a nearly linear function of voltage in the absence of extracellular Mg^{2+}, the current–voltage relationship exhibited a negative slope in the presence of Mg^{2+} (100 μM) and the inward current was maximal at about −40 mV. Zn^{2+} also inhibited

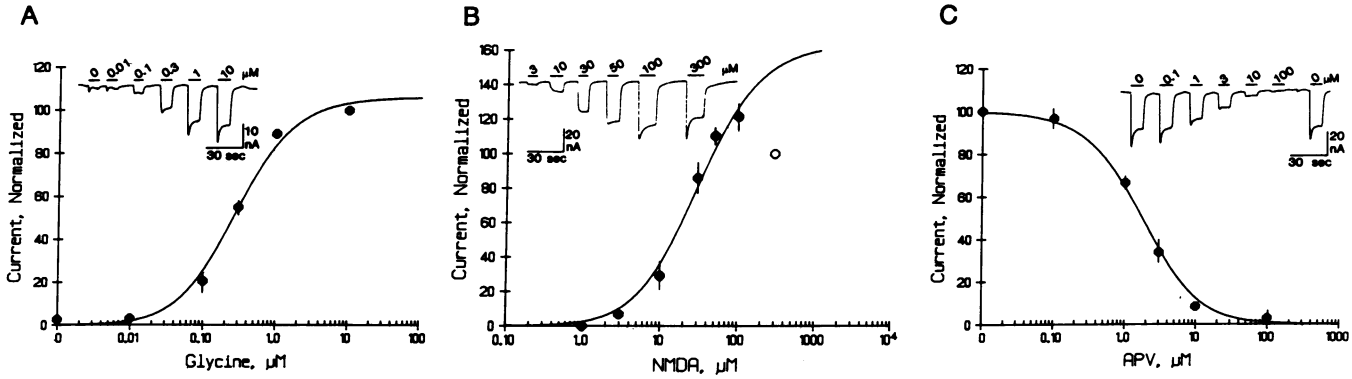

FIG. 2. Dose–response curves for glycine, NMDA, and aminophosphonovaleric acid (APV). (*Insets*) Typical response series that were repeated several times for each oocyte. Points are means ± SD of averaged values for a minimum of two oocytes. Curves are the best fits determined by the least-squares method. (*A*) Glycine potentiation of responses to 50 μM NMDA normalized with respect to the response at 10 μM glycine. The apparent K_d for glycine was 0.20 ± 0.08 μM, and the Hill coefficient was 1.3 ± 0.4. (*B*) Responses to increasing NMDA concentrations in the presence of 10 μM glycine. Responses were normalized with respect to current induced by 300 μM NMDA (○), but those values were omitted for curve fitting because of desensitization. Estimated K_d and Hill coefficient for NMDA were 21.6 ± 4.1 μM and 1.3 ± 0.1, respectively. (*C*) Dose–response curve for APV antagonism of NMDA (50 μM)-induced currents. IC_{50}, 2.5 ± 0.9 μM; Hill coefficient, 1.3 ± 0.3. Sensitivities were comparable to those found for oocytes injected with rat brain mRNA (10).

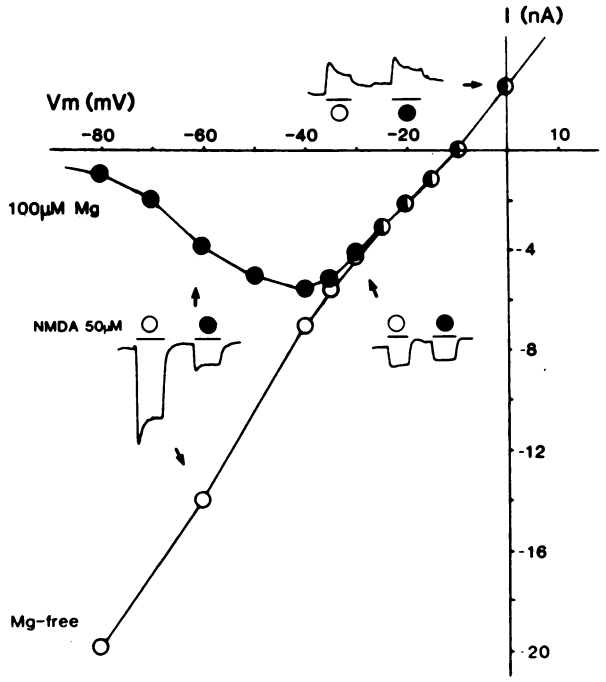

FIG. 3. Current–voltage relationship. Currents evoked in an oocyte injected with NCB-20 cell poly(A)$^+$ mRNA by perfusion of 50 μM NMDA (plus 10 μM glycine) in the absence (○) and presence (●) of 100 μM Mg^{2+} were plotted as a function of membrane potential (Vm). (*Insets*) Sample records. As for NMDA receptors of neurons, block by Mg^{2+} is marked at inside negative potentials and relieved by depolarization.

the response to NMDA in a dose-dependent manner (≈90% inhibition at 100 μM Zn^{2+}; data not shown).

The PCP receptor ligands, PCP (0.1 μM) and MK-801 (0.1 μM), largely blocked the NMDA-evoked currents (about 70% and 90%, respectively). Application of PCP together with NMDA resulted in an initial peak of inward current that was little different from the response to NMDA alone; the PCP block developed slowly (Fig. 4). Application of PCP alone for up to several minutes had no effect on subsequent NMDA-evoked responses (data not shown). Recovery from PCP block occurred over tens of seconds in the presence of agonist (Fig. 4) but very slowly in the absence of agonist (data not shown). These data suggest that the NMDA channel must be open for PCP receptor ligands to enter or exit from it. Thus, the PCP receptor encoded by NCB-20 mRNA appears to be within the NMDA channel.

DISCUSSION

This study demonstrates the expression of NMDA-activated channels in *Xenopus* oocytes injected with mRNA from NCB-20 cells and indicates that NCB-20 cells contain the precursor mRNA necessary for expression of NMDA receptors. As in neurons and in oocytes injected with rat brain mRNA, the NMDA-evoked responses were potentiated by glycine; inhibited by aminophosphonovaleric acid; and blocked by Mg^{2+}, Zn^{2+}, and PCP–receptor ligands. The agonist dependence of onset and recovery from block are consistent with location of the PCP receptor within the channel, at a site accessible only when the channel is open (8, 9, 11). Thus, PCP receptors, as a site within NMDA-activated channels, are coexpressed with NMDA receptors. Although in earlier experiments NCB-20 cells appeared to exhibit electrophysiological responses to NMDA (18), recent studies failed to show these responses. However, high-affinity stereoselective PCP binding was observed in all cultures tested,

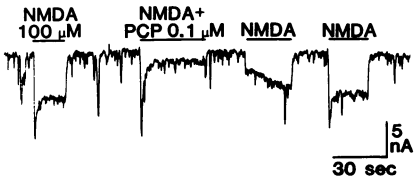

FIG. 4. Block by PCP of NMDA responses in an oocyte injected with NCB-20 mRNA. The small inward currents are due to endogenous Ca^{2+}-activated chloride channels. The initial peak of the response to NMDA with PCP was comparable to that in response to NMDA alone; but the steady level was markedly reduced; block was use dependent. The response recovered slowly during the subsequent application of NMDA alone; recovery was also use dependent.

and mRNA isolated from cells lacking functional NMDA channels did cause their expression in oocytes. The presence of PCP binding sites in homogenates of cells that in an intact state do not respond to NMDA suggests that there may be some defect in posttranslational processing or insertion of the receptors into the surface membrane. We are not aware of other cases in which exogenous mRNAs are expressed in oocytes but not in the cells of origin.

In contrast to rat brain mRNA, which causes expression of NMDA, kainate, quisqualate, GABA, and serotonin receptors, NCB-20 mRNA causes only expression of NMDA receptors. For this reason, NCB-20 mRNA should prove useful for further study of this receptor–channel complex. As an example, kainate can be shown to act as a weak antagonist at NMDA receptors expressed by oocytes injected with NCB-20 mRNA (Fig. 5). NMDA applied in the presence of kainate produced a smaller response than did NMDA alone, whereas kainate alone produced no response at all. This result provides an important piece of information relevant to a study of summation of responses to kainate and NMDA in oocytes injected with rat brain mRNA, which express both kinds of receptors (28). In those oocytes, the response to the two agonists together was less than the sum of the responses to each applied separately, a result that might be taken as supporting the concept of channel sharing by the agonists (29). We showed in those oocytes that NMDA acted as a weak antagonist at kainate receptors, but the effect was too small to explain the entire deficit from linear summation. The antagonist action of kainate at NMDA receptors in oocytes expressing NCB-20 mRNA accounts quantitatively for the rest of the deficit in summation of responses. Together, these data indicate that the agonists activate separate channels. A further potential use for NCB-20 mRNA is in cloning of the cDNAs encoding the NMDA/PCP receptor–channel complex.

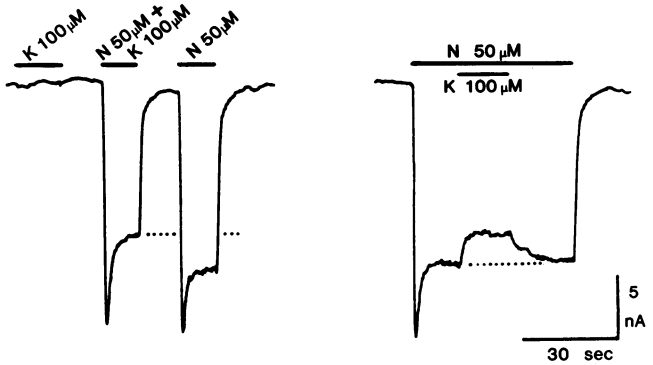

FIG. 5. Antagonist action of kainate at NMDA receptors in an oocyte injected with NCB-20 mRNA. (*Left*) There was no response to kainate (100 μM) alone. The response to kainate plus NMDA was less than the response to NMDA alone. (*Right*) Kainate applied during a response to NMDA application decreased the response.

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