Functional NMDA receptors with atypical properties are expressed in podocytes

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N-METHYL-D-ASPARTIC ACID (NMDA) receptors have been extensively studied in the nervous system, where they are known to play a role in the initiation of several forms of plasticity at excitatory synapses (54), in the refinement of topographic maps during development (7), and in neurogenesis (45). A basal level of NMDA receptor activation is required for normal neuronal function and survival (19). However, excessive activation of NMDA receptors by glutamate (5, 19) and by other endogenous ligands of glutamate receptors (29) can also initiate a process known as excitotoxicity, which is thought to contribute to the progression of neurodegeneration initiated by a variety of insults (4, 16, 42, 60).

NMDA receptors, which are named after their prototypical pharmacological agonist (9), comprise a class of cation-selective heterotetrameric ionotropic receptors with a high intrinsic Ca\(^{2+}\) permeability (25, 40). These receptors contain so-called NR2 subunits, which are encoded by four distinct genes (NR2A, NR2B, NR2C, and NR2D) (11). The NR2 subunits contain binding sites for glutamate and other agonists that are responsible for rapid physiological activation of these receptors (14). Functional NMDA receptors also contain NR1 subunits that are encoded by a single gene that can occur in at least eight different splice variants (11). The NR1 subunits contain a positive allosteric site that binds glycine or d-serine (26, 31, 55), and occupation of this site is obligatory for receptor activation by glutamate or NMDA (31, 34). Because of the large number of distinct NMDA receptor NR1 and NR2 subunits, a very large number of stoichiometrically distinct NMDA receptors are possible, and functional differences have been observed in the NMDA receptors of different neuronal populations, in the same neurons at different developmental stages, and even at different locations in a single neuron (32). Only a small subset of the stoichiometrically possible NMDA receptors has been functionally characterized.

NMDA subunits have also been detected in peripheral tissues, including heart (36, 57), parathyroid (48), bone (50), pancreas (21, 43), immune cells (38), erythrocytes (36), and kidney (36, 53, 67). The physiological significance and functional properties of NMDA receptors in peripheral tissues are not as well understood. In the kidney, there is evidence that NMDA receptor subunits play a role in Na\(^+\) reabsorption by proximal tubules and in glomerular filtration rate (10, 67), and NR1 subunits are expressed in glomeruli and in kidney cortex brush border membranes (53, 67). Interestingly, ischemia results in upregulation of NR1 subunits throughout the kidney, and NMDA blockade is reported to improve renal function after ischemia (67) and during gentamicin nephrotoxicity (35). It has been suggested that, as in the brain, basal activation of NMDA receptors may be essential for normal kidney function, but excessive activation of these receptors may trigger a number of pathophysiological processes (10). With respect to glomerular function, it has been suggested that NMDA receptors are expressed in human and rodent glomeruli (53, 67) and that systemic inhibition of these receptors causes a trend toward proteinuria in mice and humans, possibly mediated by a local glutamatergic signaling pathway in podocyte foot processes (17). Many of these conclusions have been drawn from examination of the effects of NMDA antagonists on various physiological measures of kidney function, and there is an underlying assumption in several of the experimental designs that drugs are acting locally within the kidney. However, no reports describing the functional characteristics of NMDA receptors in renal cells have appeared.

In the present study, we demonstrate that functional NMDA receptors are expressed in several different podocyte preparations, including mouse and human immortalized cell lines, and in primary cultures of podocytes from mice. The pharmacological and biophysical properties of these receptors are, in
broad outline, similar to those reported for neurons. Specifically, these receptors are readily activated by NMDA, Ca²⁺-permeable, blocked by typically used competitive and noncompetitive antagonists, and modulated by d-serine, which is known to allosterically regulate neuronal NMDA receptors. These receptors are also activated by endogenously occurring ligands, including t-homocysteate (HCA) and d-aspartate, and they do not desensitize, even with very prolonged applications of NMDA. However, rather surprisingly, the NMDA receptors do not respond to t-glutamate or d-aspartate. In addition, we show that sustained activation of NMDA receptors causes activation of secondary signaling cascades in podocytes, including the protein kinases Erk and Akt and the small GTPase Rho.

MATERIALS AND METHODS

Cell culture and isolation. Immortalized mouse and human podocyte cell lines (obtained from Dr. Peter Mundel, University of Miami Miller School of Medicine) were maintained as described in detail previously (28). Cell lines were propagated at 33°C in the presence of mouse interferon-γ (10 U/ml; Sigma). Removal of interferon and elevation of the temperature to 37°C for 2 wk induced differentiation and expression of podocyte markers (28). For preparation of primary cultures of podocytes, 6-wk-old mice were anesthetized with pentobarbital sodium and killed according to procedures approved by the University of Houston Institutional Animal Care and Use Committee, and kidneys were excised and placed in ice cold PBS. The capsule and adhering fat were removed, and the cortex was carefully dissected and chopped into 1-mm³ pieces using a razor blade. A preparation enriched in glomeruli was made using a standard sequential sieving procedure that utilized three stainless steel sieves with mesh sizes of 150, 75, and 53 μm. Glomeruli were then collected by brief centrifugation and incubated at 37°C for 20 min in a saline solution free of divalent cations containing 0.5 mg/ml type II collagenase (Sigma). The glomeruli were transferred to cell culture medium and gently dissociated by 10 passes through a fire-polished Pasteur pipette (0.5 mm tip diameter), and the cell suspension was plated onto poly-lysine-coated glass coverslips. Recordings were made 72 h after plating, at which time glomerular cells were adherent and podocytes could be readily identified by their primary processes extending from the cell body, which were easily seen under Hoffman modulation contrast optics.

Whole cell recordings and data analysis. Whole cell recordings were made at room temperature (21–23°C) using standard techniques. Normal external saline solution contained (mM) 150 NaCl, 5.4 KCl, 0.8 MgCl₂, 5.4 CaCl₂, and 10 HEPES-NaOH (pH 7.4). In experiments designed to examine relative ionic permeability, CaCl₂ was increased to 10 mM in the control solution and nominally decreased to 0 mM in the experimental solution, and NMDA responses were obtained over a range of membrane potentials in both solutions. Recording electrodes were filled with a solution containing (mM) 120 KCl, 10 NaCl, 10 HEPES-KOH, and 10 EGTA (pH 7.2). In some experiments, MgCl₂ was increased to 5 mM in internal or external solutions (in which case EGTA was omitted from the internal solution). The resistance of filled recording electrodes was 4–6 MΩ. In pilot studies, we used recording electrode solutions in which KCl was replaced with CsCl and obtained identical results. Because recordings lasted longer with KCl, we used those solutions for the rest of these studies. It was possible to compensate 80–90% of the series resistance without introducing oscillations into the current output of the clamp amplifier (Axopatch 1D, Molecular Devices, Sunnyvale, CA). Agonists (NMDA, t-glutamate, t-aspartate, d-aspartate, HCA, or glycine) were obtained from Sigma and dissolved in extracellular saline at various concentrations and applied by pressure injection (4 lb/in² = 27.6 kPa for 1–60 s) from a second micropipette (~2 μm tip diameter) positioned 30–40 μm from the cell body of the podocyte. The amplitudes and waveforms of responses to agonists showed some variability from cell to cell, depending in part on injection pipette placement relative to the podocyte cell body and bath perfusion rate (Fig. 1A). However, once the pressure injection pipette was placed, amplitudes and waveforms of responses in individual cells were highly reproducible and could often be maintained for up to 30 min with little variation. With this apparatus, only a single concentration of agonist could be applied to any given cell. The antagonists MK-801 and d-2-aminophosphonovaleric acid (d-APV; Sigma) and L-689560 (Tocris) and allosteric modulators (glycine or d-serine) were dissolved in extracellular saline and applied to cells by bath superfusion. Macroscopic currents were digitized with a Digidata 1322A interface (Molecular Devices) and stored for offline analysis with pCLAMP software (Molecular Devices). Curve fitting was carried out using the nonlinear least-squares algorithms implemented in Origin version 6.0 software (Microcal Software, Northampton, MA).

Immunochemoical procedures. For confocal microscopy, podocytes were fixed by 30 min of exposure to 4% paraformaldehyde in PBS, rinsed in PBS, blocked with 10% normal goat serum, and then permeabilized in PBS containing 0.5% Triton X-100. They were then incubated with rabbit anti-nephrin (Santa Cruz Biotechnology) and rhodamine-conjugated phalloidin (GE Healthcare) or anti-NR1 (Santa Cruz Biotechnology) overnight at 4°C. For measurements of nephrin, cells were then treated with Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen), washed in PBS, and mounted using Vectashield (Vector Laboratories, Burlingame, CA). For measurements of NR1, we used Alexa Fluor 594-conjugated anti-rabbit antibody. Processing of the sample without primary antibodies eliminated all fluorescent signals. All images were collected on an Olympus FV-1000 inverted-stage confocal microscope with a Plan Apo N ×60 1.42 numerical aperture oil-immersion objective. Immunoblot analyses of podocyte lysates were carried out as described previously (28) using anti-nephrin and probing for anti-β-actin (Sigma) to control for loading. Akt and Erk1/2 phosphorylation was measured by immunoblot using antibody pairs that recognize phosphorylated (active) and total forms of these enzymes (Cell Signaling Technology, Danvers, MA). GST pull-down assays to analyze Rho activation were carried out with reagents from Cytoskeleton (Denver, CO) according to the manufacturer’s instructions. Coimmunoprecipitation was carried out as described in detail previously (28, 29) using an antibody against synaptopodin (Synpo; Santa Cruz Biotechnology) and anti-NR1. Briefly, podocyte lysates were cleared by centrifugation, and the resulting extracts were incubated in the presence of anti-NR1, anti-Synpo, or IgG for 4 h at 4°C. Protein A/G agarose (Santa Cruz Biotechnology) was added to the lysates, which were incubated for 12 h. Pellets were washed, and proteins were separated by SDS-PAGE on 10% gels and transferred to filters. Cell extracted protein (50–100 μg) was used as a positive input control in each experiment.

RESULTS

Initial experiments were carried out on differentiated cells of an immortalized mouse podocyte cell line. We used confocal immunofluorescence and an antibody against NR1 subunits to establish that these cells express NMDA receptors (Fig. 1A). Using reciprocal coimmunoprecipitation, we also observed that NR1 subunits endogenously expressed in podocytes interact with the podocyte marker Synpo (Fig. 1B). Thus, NR1 could be detected in immunoprecipitates prepared using an antibody against Synpo, whereas Synpo could be detected in immunoprecipitates prepared using an antibody against NR1. No signal was obtained if immunoprecipitates were prepared with IgG.

The NMDA receptors in cultured podocytes are functional. Using whole cell recording, we observed that application of NMDA by pressure injection onto podocytes held at −60 mV
evoked inward currents with amplitudes and waveforms that were highly reproducible with repeated applications to any given cell (Fig. 1C). The main factors determining the rate of rise and the rate of decay of currents were bath flow rate and placement of the pressure injection pipette relative to the cell. Pressure injection of bath saline had no effect in any of 10 cells tested (data not shown). NMDA-evoked currents were observed in every podocyte studied under normal physiological ionic conditions. These currents did not desensitize, even during the course of quite sustained applications of NMDA, such as the 60-s application shown in Fig. 1D. The amplitudes of these currents were concentration-dependent. With our apparatus, we are not able to test more than one concentration of agonist on any single cell. Instead, we determined the mean responses to NMDA concentrations ranging from 1 μM to 1 mM, with each concentration tested in a separate group of four cells. Maximum mean responses were observed with 100 μM NMDA (Fig. 1E). With use of a nonlinear least-squares algorithm, the concentration-response curve could be fitted with the following equation:

\[ I = I_{\text{max}} \left[ 1 + \left( \frac{[\text{NMDA}]}{EC_{50}} \right)^n \right] \]

where \( I \) is the mean current evoked by a given concentration of NMDA, \( I_{\text{max}} \) is the mean response to a saturating concentration of NMDA, \( EC_{50} \) is the concentration of NMDA that yields half-maximal mean responses, and \( n \) is the Hill coefficient, which describes the steepness of the dose-response curve. The fitted curve has an \( EC_{50} \) of 36 μM NMDA and a Hill coefficient of 1.98. These values are similar to NMDA sensitivities measured in cultured telencephalic neurons, which have an estimated \( EC_{50} \) for NMDA of 25–40 μM and Hill coefficients of 1.3–1.4 (49, 51).

NMDA-evoked currents in podocytes typically reversed slightly positive to 0 mV with physiological solutions in the pipette and bath (Fig. 2A and B). To estimate the relative Ca\(^{2+}\) permeability of podocyte NMDA receptors, current-voltage characteristics and reversal potentials were also determined with external solutions containing 10 mM free Ca\(^{2+}\) and in the same cells after bath perfusion with Ca\(^{2+}\)-free external solutions (Fig. 2C and D). No other divalent cations were present in the extracellular solutions. Superfusion of Ca\(^{2+}\)-free external solutions resulted in a marked reduction in inward evoked inward currents with amplitudes and waveforms that were highly reproducible with repeated applications to any given cell (Fig. 1C). The main factors determining the rate of rise and the rate of decay of currents were bath flow rate and placement of the pressure injection pipette relative to the cell. Pressure injection of bath saline had no effect in any of 10 cells tested (data not shown). NMDA-evoked currents were observed in every podocyte studied under normal physiological ionic conditions (n = 86). These currents did not desensitize, even during the course of quite sustained applications of NMDA, such as the 60-s application shown in Fig. 1D. The amplitudes of these currents were concentration-dependent. With our apparatus, we are not able to test more than one concentration of agonist on any single cell. Instead, we determined the mean responses to NMDA concentrations ranging from 1 μM to 1 mM, with each concentration tested in a separate group of four cells. Maximum mean responses were observed with 100 μM NMDA (Fig. 1E). With use of a nonlinear least-squares algorithm, the concentration-response curve could be fitted with the following equation:

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Fig. 1. Responses to N-methyl-D-aspartate (NMDA) in differentiated cells of an immortalized mouse podocyte cell line. A: confocal immunofluorescence images showing expression of NR1 subunit of NMDA receptors in podocyte cell lines. B: reciprocal coimmunoprecipitation of NR1 subunits and the podocyte marker synaptopodin (Synpo). Input lane shows diluted sample of lysate used for preparation of immunoprecipitates. No signals of appropriate molecular weight were seen in immunoprecipitates prepared with IgG. C: whole cell recordings from 2 different podocytes. A micropipette filled with 100 μM NMDA was placed adjacent to the recorded cells and used to deliver NMDA in response to brief pressure pulses (horizontal bars above traces). Once the pipette was placed, responses to NMDA in a given cell were very consistent, as shown by response to 3 separate NMDA applications in each of the 2 cells. V\(_m\), membrane (holding) potential. D: responses to NMDA do not desensitize or fade during prolonged application. Record shows a response to a continuous 60-s application of 100 μM NMDA to a podocyte at a holding potential of −60 mV. E: concentration-response curve for NMDA in podocytes. Data points show mean ± SE of currents evoked by a given NMDA concentration applied to groups of 4 cells. Superimposed curve is a nonlinear least-squares fit to the logistic equation with an \( EC_{50} \) of 36 μM NMDA and a Hill coefficient of 1.98. (R\(^2\) = 0.96413).
currents observed between $-60$ and $-20$ mV as well as an increase in outward rectification at potentials positive to $+20$ mV (Fig. 2C). When currents were measured at several membrane potentials between $-20$ and $+20$ mV, we observed a shift in reversal potential of $-4.1 \pm 0.2$ (SE) mV ($n = 4$ cells) after switching to Ca$^{2+}$-free external saline (Fig. 2D). The mean shift in reversal potential was used to estimate relative ionic permeabilities according to the following equation: $P_{Ca}/P_{M} = M_{o}(1 - \exp[2(\Delta E_{R})(RT/F)])/\exp[2(\Delta E_{R})(RT/F)][4C_{ao}]$, where $P_{Ca}$ is the permeability to Ca$^{2+}$, $P_{M}$ is the permeability to monovalent cations, $M_{o}$ is the total activity of external monovalent cations, $\Delta E_{R}$ is the change in reversal potential after perfusion with Ca$^{2+}$-free solution, $C_{ao}$ denotes the activity of Ca$^{2+}$ used in the control extracellular solution, and $R$, $T$, and $F$ have their usual meanings. Using published activity coefficients for Na$^{+}$ (61) and Ca$^{2+}$ (2), the estimated $P_{Ca}/P_{M}$ of NMDA receptors in podocytes at room temperature is 2.11. This is a quite significant Ca$^{2+}$ permeability, but somewhat less than the $P_{Ca}/P_{M}$ of 4–5 that was measured for NMDA receptors of cultured hippocampal neurons using similar methods (25, 40). The reduction in outward rectification in high external Ca$^{2+}$ also occurs in neuronal NMDA receptors and has been attributed to partial pore blockade by Ca$^{2+}$ (40).

Similar NMDA-evoked currents were observed in differentiated cells of a human immortalized podocyte cell line (data not shown) and also in primary cultures of mouse podocytes examined 72 h after their preparation from isolated glomeruli (Fig. 3). The primary podocytes were quite fragile, possibly because they were examined relatively soon after isolation, and it was not possible to obtain sustained recordings from them, but properties of NMDA-evoked currents were similar, except they were, on average, somewhat smaller in amplitude.

NMDA-evoked currents in mouse podocyte cell lines were inhibited by superfusion of external saline containing elevated (5 mM) Mg$^{2+}$ (Fig. 4A). Current-voltage plots show that the effects of elevated external Mg$^{2+}$ are largest at negative membrane potentials around $-40$ mV (Fig. 4B), and there was no significant blockade at positive holding potentials. NMDA-evoked currents were not detectable in any of six cells tested using recording pipettes filled with a solution containing 5 mM Mg$^{2+}$ that was otherwise identical to control normal pipette solutions (Fig. 4C). The magnitude of the inhibition by external Mg$^{2+}$ at $-60$ mV in podocytes was similar to that observed for neuronal NMDA receptors (40, 41). NMDA-evoked currents in

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**Fig. 2.** Reversal potentials of NMDA-evoked currents in mouse podocyte cell lines. A: NMDA-evoked currents in a podocyte at holding potentials shown at left of each trace. B: current-voltage (I-V) plot of data in A. In this and subsequent plots, lines represent spline fits to data and have no theoretical significance. C: I-V plot for NMDA-evoked currents in podocytes in 10 mM external Ca$^{2+}$ and in the same cells after perfusion with nominally Ca$^{2+}$-free external solution. Note decrease in inward currents in Ca$^{2+}$-free solutions at holding potentials negative to $-20$ mV and increase in outward currents at positive holding potentials. D: I-V plot showing results from an experiment similar to that described in C in a different group of cells. NMDA currents were evoked at membrane potentials that more closely bracketed the reversal potentials. Note negative shift in interpolated reversal potential after switch to Ca$^{2+}$-free external saline solution. Data in C and D are means ± SE from 4 cells.

**Fig. 3.** NMDA-evoked currents from podocytes in primary cell culture. Whole cell recordings of cells with visible primary processes were made 72 h after isolation from a 6-wk-old mouse. A: NMDA-evoked currents from 2 different cells held at $-60$ mV. B: I-V plot constructed from mean ± SE of responses in 4 different primary podocytes. Plot was constructed using methods described in Fig. 2A legend.
Podocytes were also inhibited by the voltage-dependent uncompetitive inhibitor MK-801 (10 μM; Fig. 5, A and C) and by the competitive antagonist D-APV (10 μM; Fig. 5, B and D). The effect of D-APV was at least partially reversible over a period of 10–20 min (Fig. 5B), but we did not observe recovery from MK-801 inhibition even after 30 min of washout (data not shown).

Neuronal NMDA receptors have an allosteric binding site for glycine or D-serine (26, 31, 55), and occupation of this site is required for NMDA-evoked currents (31, 34). We observed that superfusion of up to 10 mM glycine had no significant effect on the amplitude of NMDA-evoked currents in podocytes (Fig. 6, A and B) and application of glycine by pressure injection even at 10 mM did not evoke macroscopic currents in these cells (Fig. 6C). However, bath superfusion of 1 mM D-serine caused a marked and statistically significant increase in the amplitude of NMDA responses in podocytes (Fig. 7, A and B). Moreover, the effect of D-serine was almost completely blocked after application of 100 nM L-689560, a potent inhibitor of the NR1 allosteric site (18) (Fig. 7C). Indeed, even in the continued presence of D-serine, L-689560 caused a 90% inhibition of responses to NMDA, suggesting that, as in neurons, occupation of this site by an allosteric modulator is required for receptor activation.

Podocyte NMDA receptors have a highly atypical agonist activity profile. Specifically, we were unable to detect robust responses to L-glutamate, even at concentrations as high as 10 mM, in any of 30 cells tested. In a few cells, we observed

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**Fig. 4.** Responses to NMDA in podocytes are blocked by external Mg²⁺ (Mg⁰) or internal Mg²⁺. A: NMDA-evoked current in an immortalized podocyte before and after application of a solution containing 5 mM Mg²⁺. Holding potential was −60 mV. B: I-V plot of mean NMDA-evoked responses to NMDA in normal saline solution (●) and in the same 4 cells after application of external saline solution containing 5 mM MgCl₂ (■). Error bars represent SE. C: NMDA did not evoke detectable currents in recordings made using pipettes filled with a solution containing 5 mM Mg²⁺ at a holding potential of −60 mV.

**Fig. 5.** Inhibition of NMDA-evoked currents in podocytes by prototypical antagonists. A: NMDA-evoked currents in an immortalized podocyte were reduced after application of MK-801. Currents were evoked at a holding potential of −60 mV. MK-801 inhibition did not recover after 30-min wash in control solution. B: inhibition of NMDA-evoked currents by D-2-aminophosphonovaleric acid (D-APV). Inhibition was at least partially reversible with a 20-min wash. C and D: results (means ± SE) of several repetitions of experiments described in A and B. *P < 0.0001 (Student’s paired t-test).
responses of ~10–20 pA, much smaller than responses to NMDA (Fig. 8A), but in other cells we observed no evoked currents. This was also seen in primary cultures of podocytes (data not shown). L-Aspartate was as weak as L-glutamate (Fig. 8A). By contrast, we observed consistent inward currents in response to D-aspartate (Fig. 8A), typically about half the amplitudes of those evoked by NMDA, and we observed an even more robust response to HCA (Fig. 8A and C). The inward currents evoked by HCA in podocytes were comparable in amplitude to those evoked by NMDA and were markedly reduced by 10 μM D-APV, indicating that HCA acts through NMDA receptors, although in several cells there was a component of inward current with a slower onset that persisted after 10 μM D-APV (Fig. 8B). It is important to emphasize again that podocytes invariably exhibit robust responses to NMDA under recording conditions in which we failed to observe responses to L-glutamate or L-aspartate. Although this result was extremely unexpected, it was obtained repeatedly, even when we included 1 mM D-serine in the bath solution and when we looked for currents over a wide range of membrane potentials. In an effort to address possible artifacts, we examined a wide range of L-glutamate concentrations without effect, and we tested multiple lots of L-glutamate. In some cases, after failing to observe a response to L-glutamate, we would make a recording from an adjacent podocyte in the same preparation and observe a large response to NMDA (sometimes >300 pA

Fig. 6. Bath application of glycine does not increase NMDA-evoked currents in immortalized podocytes. A: NMDA-evoked currents before and after 5 min of exposure to 1 mM glycine. Holding potential was −60 mV. B: results (means ± SE) of 6 repetitions of experiment described in A. Mean currents are not significantly different after application of 1 mM glycine. C: application of 10 mM glycine by pressure injection did not evoke membrane currents in podocytes.

Fig. 7. Bath application of D-serine potentiates NMDA-evoked currents in immortalized podocytes. A: currents before and 5 min after bath perfusion of 1 mM D-serine at a holding potential of −60 mV. *P < 0.0001 (Student’s paired t-test). B: results (means ± SE) of 6 repetitions of experiment described in A. C: inhibition of NMDA-evoked currents by an inhibitor of the D-serine allosteric site of NMDA receptors. Examples of NMDA-evoked currents are shown before and after application of 1 mM D-serine, which caused its usual potentiation of current. After exposure of the same cell to external solutions containing 1 mM D-serine and 100 nM L-689560, NMDA responses were nearly completely inhibited.
in amplitude at holding potentials of +60 mV). Ultimately, we were convinced that L-glutamate is not an effective agonist in the podocyte preparations. Is L-glutamate an antagonist or a high-affinity weak partial agonist? To test this, we examined responses to NMDA before and after application of 1 mM L-glutamate. However, we did not see any change in responses to NMDA as a result of this treatment (Fig. 8D), indicating that L-glutamate is not an antagonist of podocyte NMDA receptors.

In neuronal systems, activation of NMDA receptors can activate many different secondary signaling cascades that can lead to changes in synaptic strength and, over time, to changes in gene expression (8). We examined three such signaling systems in mouse podocyte cell lines and found that all three could be activated by sustained exposure to NMDA, depending on the concentrations used. Thus, 2 h of exposure to 10 mM NMDA caused increases in the amount of phosphorylated Erk (Fig. 9, A and B) and Akt (Fig. 9, C and D) detected by immunoblot, suggesting activation of these cascades (Fig. 9, A and B). By contrast, GST pull-down assays indicated that 10 mM NMDA did not cause activation of the small GTPase RhoA, but this system was strongly activated by 2 h of exposure to 50 mM NMDA (Fig. 9E). All three of these secondary responses could be blocked by pretreatment of cells with 10 mM MK-801 (Fig. 9, B, D, and F), indicating that they are specific effects of NMDA. Each of these signaling systems has been shown to be important for various aspects of podocyte function (6). Because NMDA can evoke toxic responses in neurons, we also examined whether NMDA could cause cell death in podocytes, as it does in neurons. We observed that 50 µM NMDA did not result in a loss of cultured podocytes, even when it was present continuously for 24 h. However, 6 h of exposure to 50 µM NMDA resulted in a marked decrease in nephrin expression in these cells that could be seen with confocal microscopy and by immunoblot analysis (Fig. 10).

DISCUSSION

Previous immunochemical studies provide evidence that at least some of the subunits of NMDA receptors are expressed in rodent and human glomeruli (53, 67), with a distribution that suggests expression in podocytes (53) and proximal tubules (67). There are also reports that inhibition of NMDA receptors can cause changes in renal function, including changes in glomerular filtration rate (10, 67) and, in some cases, proteinuria (17). Nearly all the studies on renal function relied primarily on NMDA antagonists and did not address the nature of the signaling pathways in the kidney that these drugs are proposed to inhibit. In the present study, we have shown that functional NMDA receptors are expressed in immortalized podocyte cell lines derived from mice and humans, as well as in primary cultures of mouse podocytes examined 72 h after dissociation. Podocyte NMDA receptors have several pharmacological and biophysical properties similar to those of neurons. However, they differ from neuronal NMDA receptors in a physiologically crucial way: they do not respond robustly to L-glutamate or L-aspartate, which are the most important agonists of these receptors in neuronal systems. In addition, we have also shown that sustained activation of NMDA receptors can activate secondary signaling cascades known to be important for podocyte cell physiology (6).

It has been suggested that podocytes have an intrinsic and local glutamatergic signaling system similar to that of neurons that operates in foot processes (17, 53). This proposal is based on expression of synaptic vesicle proteins and exocytotic release of glutamate following application of neurotoxins to cultured podocytes (53), as well as immunogold studies of podocyte membrane vesicles that are supposed to contain glutamate (53). Given that podocytes are not excitable and do not express voltage-activated Ca2+ channels (20), the stimuli that would cause secretion of glutamate from podocytes are a matter of speculation. An important implication of the present study is that if such a system exists, it is unlikely to be signaling through the NMDA receptors of podocytes, which do not respond effectively to L-glutamate or L-aspartate, even at high concentrations. It is possible that locally released glutamate signals through metabotropic glutamate receptors that have also been observed in podocytes (53). If this is the case,
the responses through these pathways were not detected under the recording conditions used here. It is possible that metabotropic glutamate responses in podocytes are highly sensitive to intracellular perfusion during whole cell recording or, alternatively, their signals may not lead to changes in ionic currents.

On the other hand, the NMDA receptors in podocytes are readily activated and modulated by ligands that circulate systemically and are normally present in the kidney, in certain conditions at relatively high concentrations. Of particular interest is HCA, which is the main metabolic product of L-homocysteine (HC) (62). We observed that HCA evokes inward currents comparable in amplitude to NMDA, as well as a second inward current of slower onset that is resistant to blockade by NMDA receptors. Laboratory measurements of circulating HC levels generally summate free HC, the HC that is bound to proteins, and HCA (62). Total HC levels in normal subjects are 9–15 μM depending on age and sex (22). However, in patients with hyperhomocysteinemia, total HC can be as high as 400 μM (27, 62), a level that implies HCA concentrations capable of causing sustained activation of podocyte NMDA receptors. In this regard, in rats, dietary manipulations that cause elevations in HC can, over a period of weeks, cause glomerulosclerosis and podocyte foot process effacement (69). This is also seen in cystathionine-β-synthetase-deficient mice, which have profound hyperhomocysteinemia (58). Moreover, there is a well-established clinical correlation between serum total HC (and, thus, circulating HCA levels) and proteinuria (13, 24, 46, 59).

Another potential class of endogenous ligands for podocyte NMDA receptors are D-amino acids produced by the pyridoxal 5'-phosphate-requiring enzymes aspartate racemase (30) and serine racemase (64), which catalyze production of D-aspartate and D-serine, respectively. D-Aspartate produced through this pathway in the brain has been proposed to play important functional roles in neurogenesis (30). It is not known if aspartate racemase is expressed in the kidney, but D-aspartate is detectable in human blood at micromolar concentrations (12). Endogenous D-serine produced by serine racemase and secreted by astrocytes has been shown to play a role in modulation of central nervous system NMDA receptors through a process known as gliotransmission (44, 47, 68). Serine racemase is expressed in several peripheral tissues, especially in kidney proximal tubules (65). As a result, serum D-serine in humans occurs at ~2 μM, and the D-isomer represents >1% of total circulating serine (66). This amino acid is therefore in a position to modulate NMDA receptors throughout the kidney. In this regard, D-serine has long been known to be nephrotoxic, resulting in glucosuria, aminoaciduria, proteinuria, and polyuria (3, 15). While this is generally thought to be caused by proximal tubule necrosis, it is possible that D-serine also affects glomerular cells by potentiating NMDA-mediated cell signaling.
For completeness, we note that while L-glutamate is not an effective agonist of NMDA receptors in podocytes, nor is it an effective antagonist. We tested this because such an observation might have implied a role for a local glutamatergic signaling system in suppressing tonic activation of NMDA receptors by HCA or other circulating factors. It is not clear why L-glutamate and L-aspartate are ineffective NMDA agonists in podocytes, but it is possible that these amino acids do not have access to the ligand-binding pockets on podocyte NR2 subunits, as opposed to binding within the pocket but failing to induce channel activation (39). Similarly, we note that glycine was not able to evoke any membrane currents by itself in podocytes, even at high concentrations.

Despite the unusual agonist specificity, many of the other general properties of podocyte NMDA receptors were similar to those of neurons. Specifically, they have a similar sensitivity to NMDA, were blocked by supraphysiological concentrations of internal or external Mg$^{2+}$, and were inhibited by the prototypical antagonists MK-801 and D-APV. They have a marked permeability to Ca$^{2+}$ relative to monovalent cations, although the observed $P_{Ca}/P_M$ was somewhat less than has been reported for neuronal NMDA receptors using similar experimental designs (25, 40), which may explain why podocyte NMDA receptors show little tendency to desensitize (33).

Nevertheless, the Ca$^{2+}$ influx through podocyte NMDA receptors probably accounts for activation of secondary signaling cascades by NMDA. Thus we observed an increase in Erk and Akt phosphorylation at low NMDA concentrations (10 $\mu$M) and activation of RhoA at somewhat higher concentrations (50 $\mu$M) that are still less than maximal in electrophysiological experiments. However, continuous activation of NMDA receptors for several hours did not cause a noticeable loss of podocytes in these cultures, even though NMDA at similar concentrations and durations causes destruction of cultured cortical neurons through a process known as excitotoxicity (5). It is possible that the somewhat reduced Ca$^{2+}$ permeability of podocyte NMDA receptors compared with neuronal receptors allows podocytes to resist the acute toxicity of NMDA receptor agonist responses. In addition, podocytes do not express voltage-activated Ca$^{2+}$ channels (20), which amplify Ca$^{2+}$ signals evoked by NMDA and, thereby, contribute to excitotoxicity in neuronal cells (1).

However, sustained exposure to 50 $\mu$M NMDA caused a reduction in the expression of nephrin in podocyte cell lines, which can be considered a more subtle toxic response. This phenomenon could be observed in confocal microscopy and with immunoblot analysis. Nephrin is an essential component of glomerular slit diaphragms, and mutations that compromise nephrin function result in profound nephrotic syndromes (52). Therefore, hyperactivation of NMDA receptors could be deleterious for the process of glomerular filtration through disruption of slit-diaphragm complexes. In this regard, gain-of-function mutations in Ca$^{2+}$-permeable type 6 canonical transient receptor potential channels (TRPC6) ultimately lead to glomerular diseases and proteinuria, but in most cases these conditions manifest relatively late in life (56, 63). It is possible that moderate increases in Ca$^{2+}$ influx through TRPC6 sensitize podocytes to other insults that ultimately result in glomerular disease. In a similar way, it is possible that moderate hyperactivation of NMDA receptors may also cause subtle dysfunction or an increased sensitivity to other insults that, over time, could lead to podocyte disease. Hyperactivation could occur as a result of an increase in circulating or locally produced NMDA receptor agonists. It may also occur second-

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Fig. 10. Exposure to 50 $\mu$M NMDA for 6 h causes a fall in nephrin expression in immortalized podocytes, as assessed by confocal microscopy (A) or immunoblot analysis (B). In A, expression of actin was imaged using rhodamine-tagged phalloidin, which stains F-actin filaments.
ary to ischemia, which is reported to increase expression of NMDA receptor subunits throughout the kidney (67). At the same time, basal activation of NMDA receptors may also be essential for normal glomerular function, as blockade of these receptors can produce changes in renal function (10, 17). Thus, as in brain (19), normal renal function may require NMDA receptor activation that lies within a range, and deviations on either side of that range may be deleterious.

In summary, we have shown that functional Ca\(^{2+}\)-permeable NMDA receptors are expressed in immortalized podocyte cell lines and in primary cultures of podocytes. The receptors are unusual, in that they do not exhibit robust responses to L-glutamate, but they can be readily activated by ligands that circulate and that are likely to occur in the glomerular microenvironment. Activation of these receptors causes activation of secondary signaling cascades known to be important for podocyte function, and excessive activation of these receptors over time may lead to loss of proteins essential for the normal function of slit diaphragms.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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