N-methyl-D-aspartate receptors are expressed in rat parathyroid gland and regulate PTH secretion

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Parisi E, Almadén Y, Ibarz M, Panizo S, Cardús A, Rodriguez M, Fernandez E, Valdivielso JM. N-methyl-d-aspartate receptors are expressed in rat parathyroid gland and regulate PTH secretion. Am J Physiol Renal Physiol 296: F1291–F1296, 2009. First published April 8, 2009; doi:10.1152/ajprenal.90557.2008.—N-methyl-d-aspartate receptors (NMDAR) are tetrameric amino acid receptors which act as membrane calcium channels. The presence of the receptor has been detected in the principal organs responsible for calcium homeostasis (kidney and bone), pointing to a possible role in mineral metabolism. In the present work, the presence of the receptor was determined in normal parathyroid glands (PTG) by real-time PCR, immunoprecipitation, and immunohistochemistry. Healthy animals showed a decrease in blood parathyroid hormone (PTH) levels 15 min after the treatment with NMDA. This effect was also observed in animals with high levels of PTH-induced EDTA injection, but not in uremic animals with secondary hyperparathyroidism (2HPT). Normal rat PTG incubated in media with low calcium concentration (0.8 mM CaCl2) showed a decrease in PTH release when NMDA was added to the media. This effect of NMDA was abolished when glands were coincubated with MK801 (a pharmacological blocker of the NMDA channel) or PD98059 (an inhibitor of the ERK-MAPK pathway). Glands obtained from animals with 2HPT showed no effect of NMDA in the in vitro release of PTH, together with a decrease in the expression of NMDAR1. In conclusion, NMDAR receptor is present in PTG and is involved in the regulation of the PTH release. The mechanism by which NMDAR exerts its function is through the activation of the MAPK cascade. In uremic 2HPT animals the receptor expression is downregulated and the treatment with NMDA does not affect PTH secretion.

secondary hyperparathyroidism; ERK-MAPK

THE PARATHYROID GLANDS (PTG) play an essential role in mineral ion homeostasis because of their capacity to recognize and respond to small changes in the extracellular ionized calcium concentration. Calcium changes and parathyroid hormone (PTH) secretion are detected and regulated, respectively, by the cell surface calcium-sensing receptor (CaSR). The activation of CaSR by extracellular calcium results in a Gq/11-mediated activation of phosphatidylinositol-phospholipase C (PI-PLC), leading to intracellular calcium mobilization, protein kinase C (PKC) activation, and a resulting PKC-mediated stimulation of the mitogen-activating protein kinase (MAPK) cascade. Activated MAPK then phosphorylates and activates cPLA2, which releases free arachidonic acid (AA) that is metabolized to biologically active mediators which suppressed PTH secretion (23). This pathway can be modulated by an increase in intracellular calcium that produces an activation of cPLA2 followed by a release of AA (1), confirming the important role of free intracellular calcium as a mediator of the inhibition of PTH secretion.

When serum calcium levels decrease, PTH is released and acts on its target organs, mainly kidney and bone, to bring back calcium levels to normal. Hypocalcemia and the following PTH release stimulate calcitriol production which contributes to the restoration of normocalcemia. Calcitriol also regulates PTH secretion through the vitamin D receptor (VDR) in the PTG. Patients with chronic renal disease with persistent reductions in serum calcitriol and calcium show an increase in PTH synthesis and release. Continued stimulus of the PTG produces hyperplasia and hypertrophy causing secondary hyperparathyroidism (2HPT). In these hypertrophic PTG, the expression of CaSR and VDR is downregulated (5), accounting for the lack of effect of Ca and vitamin D on severe 2HPT.

N-methyl-D-aspartate receptors (NMDAR) are tetrameric amino acid receptors which act as membrane calcium channels. The receptor has two subunits: R1 with the catalytic function and R2 with regulatory properties. There are four types of the subunit R2 (A, B, C, D) and their presence is different depending on the distribution, properties, and regulation of the receptor. The receptor is gated by the binding of L-glutamate and its cofactor L-glycine, allowing calcium to enter the cell. In addition, there is a binding site for polyamines which can act as allosteric activators (16).

The receptor has been well-described in the nervous system where the entry of calcium produces nitric oxide (NO) synthase activation and NO production. However, little is known about NMDAR in other tissues. Recently, the presence of this receptor has been shown in the kidney (6) where it plays a role in the maintenance of basal arterial tone and in the bone (3, 18) where it contributes to the local regulation of bone cell function. The presence of the receptor in the principal organs responsible for calcium homeostasis led us to think that the NMDAR could be involved in its regulation and also be present in the PTG.

In the present work, we show for the first time the presence of the NMDAR in the PTG and its effect in the regulation of PTH secretion.

MATERIALS AND METHODS

10, about the protection of animals used for experimentation and other scientific finalities. Moreover, the present work was approved by the Ethic Animal Experimentation Committee of the University of Lleida.

The study was performed with 10 Sprague-Dawley rats (200–250 g) per group maintained under standard conditions. Food and water were available ad libitum. Room temperature was at 21°C with a 12:12-h dark-light cycle.

The 5/6 nephrectomized animals were obtained following the method performed by Perez-Ruiz et al. (19): animals were anesthetized with isoflurane and suffered 2/3 nephrectomy in the left kidney by ligation of both poles. After 1 wk, contralateral nephrectomy was performed.

Animals with high levels of PTH were obtained by EDTA-induced hypocalcemia performed following a modified protocol described by Thomas et al. (24): rats received one intramuscular injection of EDTA (300 mg/kg) which resulted in 6 h of sustained hypocalcemia.

To obtain parathyroid tissue, on the day of the experiment, animals (controls and 5/6 nephrectomized) were killed with pentobarbital sodium (50 mg/kg) and PTG were dissected free of the thyroid glands with a dissecting microscope.

**PTG incubation conditions.** PTG were placed in individual wells, containing 1 ml of incubation medium, resting inside a nylon basket; the glands were maintained at 37°C, with constant rocking and shaking motions (1). The incubation medium contained 125 mM NaCl, 5.9 mM KCl, 0.5 mM MgCl₂, 1 mM sodium pyruvate, 4 mM glucose, 10 mM HEPES, 0.1 IU/ml human insulin, 0.1% bovine serum albumin, 100 IU/ml penicillin/GM, 5.9 mM KCl, 0.5 mM MgCl₂, 1 mM sodium pyruvate, 4 mM glucose, 10 mM HEPES, 0.1 IU/ml human insulin, 0.1% bovine serum albumin, 100 IU/ml penicillin/GM.

**Basal media** were collected, and PTH levels were measured and used as a basal level of PTH. To promote PTH release, the following 3-h incubation media contained low Ca concentration (0.8 mM). In addition to low calcium (used as control), PTG were incubated with NMDA (500 μM), low calcium + NMDA (500 μM), low calcium + NMDA + MK801 (100 nM; receptor antagonist), and low calcium + NMDA + PD98059 (10 μM; inhibitor for the MAPK-ERK kinase or MEK).

The glands were stabilized in 1.25 mM calcium, the media were collected, and PTH levels were measured and used as a basal level of PTH. To promote PTH release, the following 3-h incubation media contained low Ca concentration (0.8 mM). In addition to low calcium (used as control), PTG were incubated with NMDA (500 μM), low calcium + NMDA (500 μM), low calcium + NMDA + MK801 (100 nM; receptor antagonist), and low calcium + NMDA + PD98059 (10 μM; inhibitor for the MAPK-ERK kinase or MEK).

The media were changed every hour and collected for the subsequent analysis. At the end of the experiment, some glands were used to determine pre-pro-PTH expression by real-time PCR analysis.

**Real-time PCR.** Total RNA was extracted from tissue using the TRizol method. Reverse transcription was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche) followed by a Taqman real-time PCR amplification with gene-specific primers for NMDAR or pre-pro-PTH, purchased specifically at Applied Biosystems (Branchburg, NJ) and human GAPDH as a reference. Forty cycles at 95°C for 15 s and 60°C for 1 min were performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Triplicate readings were taken and the average was calculated. The results are in relationship to a control sample randomly selected that we consider as value = 1.

**Immunoprecipitation.** Protein extracts from parathyroid tissue were obtained as described previously. We performed immunoprecipitation because the low level of the NMDAR protein in the PTG precludes us from detecting a band in a normal Western blot. We immunoprecipitated 300 μg of parathyroid tissue and 60 μg of brain tissue (used as a positive control). After pre-clearing with G protein sepharose beads, protein extract was incubated overnight with 1 μg of specific antibody (Affinity Bioreagents, Golden, CO) and G protein sepharose beads (Amersham Biosciences, Uppsala, Sweden) at 4°C on rocking platform. After incubation, the mixture was placed 5 min at 95°C to separate the protein from the sepharose beads. We loaded the whole immunoprecipitate in an 8% polyacrylamide- SDS gel. After running and transfer to PDVF membranes (Immobilon-P, Millipore, Bedford, MA), blots were incubated in 3% nonfat milk in TBST for 1 h. Then, primary antibody for NMDAR1 (1:100) was added and incubation was performed overnight at 4°C. After being washed with TBST, horseradish peroxidase-conjugated secondary antibody (1:12,500; Amersham Biosciences) was added for an extra hour. Binding was detected with the ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and visualized at the VersaDoc Imaging system model 4000 (Bio-Rad Laboratories, GmbH, München, Germany).

**Immunohistochemistry.** PTG were fixed with 4% paraformaldehyde, included in paraffin, and sliced in 4-μm sections. After being deparaffinized, antigen retrieval was performed with 0.003% hydrogen peroxide in 0.1 M Tris-HCl buffer (pH 7.6) for 5–10 min, under visual control.

**In vivo experiments.** For the in vivo experiments, blood samples were obtained before and 15 and 60 min after intraperitoneal injection with NMDA (10 mg/kg). After learning that the peak of inhibition was at 15 min in the animals with 2HPT and EDTA-induced hypocalcemia, we only assessed basal and 15 min.

**Analytical determinations.** PTH concentration in the PTG incubation medium and in animal serum was measured using a Rat Intact PTH Elisa Kit (Immunotopics, San Clemente, CA).

Blood levels of total calcium were analyzed by the Biochemical Unit of the Hospital Universitari Arnau de Vilanova de Lleida in the Roche/Hitachi modular analytic system.

**Statistical analysis.** Differences between single groups were assessed by paired Student’s t-test. Multiple groups were analyzed by ANOVA followed by Dunnett’s post hoc test. A P < 0.05 was considered statistically significant.

**RESULTS**

In Fig. 1A, we show the expression of all the NMDAR subunits, R1, R2A, R2B, R2C, and R2D, in the PTG, compared with levels present in kidney (taken as reference) assessed by RT-PCR. The main subunits present in PTG are NMDAR1 and NMDAR2A.
Moreover, we also detected the presence of the NMDAR1 protein by immunoprecipitation techniques (Fig. 1B) using brain tissue as a positive control.

Immunohistochemistry analysis using specific antibody showed that most of the cells in the PTG are positive for NMDAR1 (see Fig. 5A).

To investigate the effect of NMDAR activation on PTH release, we first used an ex vivo model. In Fig. 2A we can see that rat PTG incubated with low Ca (0.8 mM CaCl\(_2\)) showed an increase in PTH release that was inhibited when NMDA was added to the incubation media. Moreover, in Fig. 2C we show that the effect of NMDA is dose dependent. This inhibitory effect was abolished by coinubcation with a NMDAR antagonist (MK801), suggesting that the effect observed is mediated by an increase in Ca influx through the activated channel (Fig. 3).

Furthermore, the inhibition mediated by NMDA was also abolished by a pharmacological inhibitor of the MEK (PD98059), suggesting that the MAPK pathway is involved in the NMDA-mediated inhibition of PTH secretion (Fig. 3). The incubation with only MK801 or PD98059 did not modify PTH secretion [0.8 mM CaCl\(_2\) = 277.98% over the basal; MK801 = 243.11%; PD98059 = 221.81%; \(P\) not significant (n.s.)].

Real-time PCR of the glands at the end of the incubation period showed that the effect of NMDAR activation on PTH secretion was not mediated by a decrease in pre-pro-PTH gene expression (Fig. 2B).

Table 1 shows the effect of a single intraperitoneal administration of NMDA (10 mg/kg) on blood PTH, Ca, and phosphorus levels in different animal models. In normal animals, PTH levels showed a significant decrease 15 min after the administration of the drug. Sixty minutes after the administration of NMDA, PTH levels returned back to normal. Those changes on PTH levels were achieved with no significant changes in blood total calcium.

We repeated the same approach with animals with 2HPT. Glands obtained from animals with 2HPT did not respond to incubation with NMDA (Fig. 4A). Furthermore, 2HPT animals injected with NMDA did not show decreases in blood PTH levels after 15 min (Table 1) contrary to what was observed in normal animals. When we analyzed NMDAR expression in the...
glands obtained from 2HPT rats, we found that mRNA amount of most of the NMDAR subunits was significantly decreased with respect to the glands of normal animals (Fig. 4B). Only levels of NMDAR2D were increased, but basal expression of that particular subunit was very low, suggesting that changes in its expression are not likely to play a significant role in receptor functionality. Immunohistochemistry for NMDAR1 also showed a decrease in staining in glands obtained from animals with 2HPT (Fig. 5B).

In a third approach, we administered NMDA to rats with high levels of PTH but without 2HPT. Thus, animals with EDTA-induced hypocalcemia showed a decrease in blood PTH levels 15 min after the administration of NMDA (Table 1). This decrease in PTH levels was obtained even after the persistency of EDTA-induced hypocalcemia.

DISCUSSION

NMDAR is a potent calcium channel of the central nervous system gated by the binding of glutamate and glycine (16). Glutamate is one of the most common amino acids found in nature and is the main component of many proteins and peptides. The role of NMDAR has been widely studied in the central nervous system, and its presence has been detected in other tissues like adrenal medulla, lung, pancreatic islets, heart, aorta, and ileum (11, 15, 20, 21, 25). Recent studies described the presence of NMDAR in the kidney (6) where it plays a role in the maintenance of basal arterial tone and in the bone where it stimulates bone resorption (3, 18). Thus, the presence and role of this receptor outside the nervous system are a new field to study.

The presence of NMDAR in organs that control calcium homeostasis (bone and kidney) indicates that the receptor could be implicated in mineral metabolism regulation. In this context, our results demonstrate for the first time that PTG express NMDAR. Functional NMDAR require the presence of both NMDA R1 and NMDA R2 subunits. In our experiments, we identify mRNA for both NMDA R1 and all the subunits of NMDA R2 (mainly NMDA R2A) in parathyroid tissue, suggesting the presence of fully functional NMDAR and a new role for neuroexcitatory amino acids in PTG function.

In the present work, functionality of the NMDAR in PTG has been proved both ex vivo and in vivo. In PTG incubated in low calcium, the addition of NMDA blunted the increase in PTH secretion. Furthermore, this effect could be blocked by the addition of MK801, a specific antagonist of NMDAR suggesting that the intracellular Ca increase induced by NMDAR activation inhibits PTH secretion. Our results agree with previously published reports, which described that activation of Ca channels in the PTG can inhibit PTH release (7, 8, 17). In addition, raising intracellular calcium with thapsigargin also blocks PTH release (1, 22). The mechanism of blocking PTH secretion by increases in intracellular Ca has been extensively studied. Kifor et al. (13) showed that increases in intracellular Ca elicited a rapid phosphorylation of ERK1/2. Thus, activated ERK1/2 can phosphorylate its cytosolic protein substrates [e.g., phospholipase A2 (PLA2)] (10, 14), resulting in increased production of AA which has been directly implicated in the regulation of PTH release (1, 2). Thus, the inhibition of ERK1/2 activity has been proven to inhibit the activation of ERK1/2 induced by increases in intracellular Ca (13) and therefore, the decrease in PTH secret-

![Fig. 4.](image-url) A: PTH levels in the incubation media of glands obtained from animals with secondary hyperparathyroidism (2HPT) and stimulated with low Ca (0.8 mM). Black lines: glands incubated in low Ca media. Gray lines: glands incubated in low Ca media plus NMDA (500 μM). Data are means ± SE. B: percentage of decrease in mRNA levels of the different NMDAR subunits from PTG of animals with 2HPT with respect to PTG of normal animals. Data are means ± SE.

![Fig. 5.](image-url) Immunostaining of parathyroid tissue for NMDAR1 showing the presence of the receptor in parathyroid cells of normal animals (A) or animals with 2HPT (B).
tion (4). Indeed, our results show that the decrease in PTH release induced by NMDA can be blocked by addition of PD98059, an ERK1/2 phosphorylation inhibitor, pointing to a role of MAPK in the effect of NMDAR activation in PTH secretion, probably mediated by an increase in intracellular Ca after activation of the channel. Furthermore, the decrease of PTH release was not due to a decline in its synthesis because pre-pro-PTH expression was not inhibited, but to an inhibition of its release.

The role of NMDAR on the regulation of PTH release was also demonstrated in our rat model in vivo, where animals were treated with NMDA. Administration of NMDA to normal animals resulted in a decrease in blood PTH levels at 15 min, which returned to basal levels at 60 min. Furthermore, all the changes in PTH levels were observed without changes in blood calcium levels, suggesting again a direct effect of NMDA on the PTG.

Renal patients often suffer extrarenal complications that aggravate their overall health condition. One of them is 2HPT, which is characterized by high turnover bone disease and the continuous secretion of excess PTH and driven by a decrease in Ca and calcitriol resulting from decreases in kidney parenchyma (5). In later stages of 2HPT, the levels of CaSR and VDR in the PTG decrease, rendering the gland unresponsive to Ca and calcitriol modulation (9, 12). In our laboratory, we use the 5/6 nephrectomized rats as a model to study 2HPT. These animals have only 1/6 of renal tissue and develop 2HPT in several months. Contrary to the results obtained in the control group, when we treated uremic rats with NMDA, as well as when we incubated the PTG of these animals with it, we did not see effects on PTH release. This difference could be explained by the fact that the PTG obtained from animals with 2HPT showed a marked decrease in the expression of both of the NMDAR subunits that are mainly expressed in the gland. These results suggest that NMDAR show a similar behavior to VDR and CaSR, which are also found to be downregulated in 2HPT. Thus, the gland could be unresponsive to NMDA treatment, like it is to Ca or calcitriol.

In a final approach, we tried to demonstrate whether the activation of NMDAR could reduce PTH secretion in a model of increased PTH release other than 2HPT. We chose a model in which we can render the animal hypocalcemic by injection of EDTA. Thus, the EDTA-induced hypocalcemia will stimulate an increase in PTH release by a decrease of the signaling through the CaSR. In this case, and contrary to the 2HPT, the gland does not show hypertrophy and conserves the same level of receptors. Treatment of these animals with NMDA also produced a decrease in PTH levels, even after the persistence of hypercalcemia, proving that NMDAR activation can modulate PTH secretion independently of Ca levels and thus, of CaSR activation.

In conclusion, our results demonstrate that the NMDAR are present in PTG and take part in the regulation of the PTG function, inhibiting PTH release. The mechanism by which NMDAR exert their function is through allowing the entrance of calcium in the parathyroid cell with the following activation of the PLC and MAPK cascade. In uredmic conditions, the receptor expression is downregulated and consistently the treatment with NMDA does not affect PTH secretion.

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