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Molecular Identification of NMDA Glutamate Receptors Expressed in Bone Cells

Cécile Itzstein, Hervé Cheynel, Brigitte Burt-Pichat, Blandine Merle, Léon Espinosa, Pierre D. Delmas, and Chantal Chenu*

INSERM Unit 403, Hôpital E. HERRIOT, Pavillon F, 69437 LYON Cedex 03, France

Abstract The N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor has recently been identified in bone, but the molecular composition of this receptor expressed by bone cells is unknown. NMDA receptor (NMDAR) is a hetero-oligomeric protein composed of two classes of subunits, the essential subunit NR1 and NR2A to D subunits that do not by themselves produce functional channels but potentiate NR1 activity and confer functional variability to the receptor. These subunits coassemble in different combinations to form functionally distinct NMDAR. In this study, we have investigated the molecular composition of NMDAR expressed by osteoblasts and osteoclasts in culture, using RT-PCR analysis, in situ hybridization and immunocytochemistry. Specific probes were designed for the different subunits of the NMDAR, and we showed by RT-PCR analysis that mammalian osteoclasts expressed NR2B and NR2D subunits mRNAs but not NR2A and NR2C mRNAs. Rat calvaria and MG63 osteoblastic cells also expressed several NR2 subunits mRNAs, namely NR2A, NR2B, and NR2D. In situ hybridization on isolated rabbit osteoclasts and MG63 cells has confirmed the localization of NR1, NR2B, and NR2D transcripts in osteoclasts and NR1, NR2A, NR2B, and NR2D transcripts in MG63 cells. The expression of NR2D protein by bone cells was shown by immunofluorescence. These results demonstrate for the first time that osteoblasts and osteoclasts express several NR2 subunits, suggesting a molecular diversity of NMDAR channels similar to what was shown for brain. The presence of distinct functional NMDAR on bone cells may be associated with various states of bone cell differentiation and function. J. Cell. Biochem. 82: 134–144, 2001.

Key words: osteoclasts; osteoblasts; NMDA glutamate receptor subunits; RT-PCR analysis; in situ hybridization

The N-methyl-D-aspartate (NMDA) subtype of the glutamate (Glu) receptor is essential for various physiological functions such as brain development, learning, and memory [Mayer and Westbrook, 1987; Ozawa et al., 1998]. NMDA receptor (NMDAR) is a hetero-oligomeric protein composed of two and possibly three classes of subunits, namely NR1, NR2, and NR3 [Das et al., 1998; Sun et al., 1998; Hollmann, 1999]. NR1 is an essential subunit, while NR2A to D subunits do not by themselves produce functional channels but potentiate NR1 activity and confer functional variability to the NMDAR [Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993]. NR3 is also a regulatory subunit that decreases the NMDAR channel activity [Das et al., 1998]. These subunits coassemble in different combinations to form functionally distinct NMDAR, but highly active NMDAR channels are produced only when the NR1 subunit is expressed together with one of the four NR2 subunits [Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993]. However, NMDAR subunit stoichiometry is still not very clear. Functional studies indicate that the binding of at least two molecules of both Glu and glycine is required for NMDAR activation, suggesting that a minimum of four subunits must coassemble to allow binding and activation of the receptor by agonists and coagonists. The common assumption is that NMDAR is an heteromeric protein composed of two NR1 subunits and three NR2 subunits that coassemble to form a pentamer [Hawkins et al., 1999], although other data support a tetramer [Rosenmund et al., 1998]. The C-terminus region of the NR1 subunit is anchored to the actin cytoskeleton by its direct interaction with α-actinin 2

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*Correspondence to: Dr. Chantal Chenu, INSERM Unit 403, Hôpital E. HERRIOT, Pavillon F, 69 437 Lyon Cedex 03, France. E-mail: chenu@lyon151.inserm.fr

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[Wyszynski et al., 1997]. NR2 subunit C-terminal tail interacts with the PDZ domain-containing protein, PSD-95 [Kornau et al., 1995]. This interaction may be important for the clustering of NMDA receptors in the membrane. Several NMDAR subtypes with distinct properties and functions have been identified in brain, that consist of different combinations of NR1 with a variety of NR2 subunits [Yamakura and Shimoji, 1999].

The presence of NMDAR in bone cells has been recently documented, suggesting a new role for Glu in the regulation of bone remodeling [Chenu et al., 1998; Patton et al., 1998]. NMDAR are expressed by both osteoblasts and osteoclasts. We have demonstrated that NMDAR present on osteoclasts are functional and that their electrophysiological and pharmacological properties in these cells are similar to those documented for neuronal cells [Espinosa et al., 1999]. Furthermore, we have shown that specific antagonists of NMDAR inhibit bone resorption in vitro by preventing osteoclast sealing zone formation, suggesting that activation of NMDAR is required for the resorptive function of these cells [Chenu et al., 1998; Itzstein et al., 2000]. Others have demonstrated that NMDAR are active in osteoblastic cells [Laketic-Ljubojevic et al., 1999], but it is not presently known what is the function of these receptors in these cells.

The composition of NMDAR expressed by bone cells is unknown. NR1 subunit mRNA and protein were detected in osteoblasts and osteoclasts, but the nature of NR2 subunits associated with NR1 to form functional receptors in these cells has not been determined [Chenu et al., 1998; Patton et al., 1998]. In this study, we have investigated the molecular composition of NMDA receptors expressed by bone cells, using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) analysis, in situ hybridization and immunocytochemistry. We showed for the first time that osteoblasts and osteoclasts express several NR2 subunits, suggesting a molecular diversity of NMDAR channels in bone as demonstrated for brain.

MATERIALS AND METHODS

Materials

Medium 199 (M199), RPMI medium, αMinimal Essential Medium (αMEM), penicillin, streptomycin, L-glutamine, HEPES buffer, and Fetal Calf Serum (FCS) were purchased from Life Technologies. Sterile culture plasticware were obtained from Falcon Becton Dickinson (Le Pont de Claix, France). Pronase E and toluidine blue were bought from Sigma Chemical Co. (Saint Quentin Fallavier, France). Agonists and antagonists of Glu receptors were purchased from Tocris Cookson (Bristol, UK). The polyclonal antibody directed against NR2D was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). RNA isolated from human giant cell tumors of bone was a gift from Pr GD Roodman (San Antonio, TX). Cell suspensions containing giant osteoclast-like cells were isolated from several giant cell tumors of bone, as previously described [Ohsaki et al., 1992]. The cells released from the tumors were incubated with the 23C6 monoclonal antibody directed against the vitronectin receptor, and the 23C6-positive cell fraction containing the giant cells was used for RNA extraction. Giant osteoclast-like cells isolated from these tumors expressed an osteoclast phenotype, as shown by the high level expression of tartrate resistant acid phosphatase activity and the formation of resorption lacunae on calcified matrices [Ohsaki et al., 1992]. PC12 cells were kindly given by Dr BB Rudkin (Ecole Normale Supérieure, Lyon, France).

All biochemicals and ^{35}S were obtained from NEN Life Science Products (Boston, MA).

Cell Cultures

Human MG63 and rat UMR106.01 osteosarcoma cells were cultured in RPMI medium containing 10% FCS, 2 mM L-glutamine, 100 UI penicillin/streptomycin.

The rat PC12 neuronal cell line was grown in Dulbecco's Modified Eagle's Medium (Bio Media SA, Boussens, France) containing 7% FCS and 7% horse serum, 2 mM L-glutamine, and antibiotics.

Rat Calvaria Cell Isolation

Osteoblastic cells were enzymatically isolated from the calvaria of 21-day-old Wistar rat fetuses by sequential digestion with collagenase as previously described [Bellows et al., 1986]. The populations obtained from each digestion step (I to V) were plated into 75 cm^2 tissue culture flasks in αMEM containing 15% FCS and antibiotics. After 24 h at 37°C, the cells were released with 0.01% trypsin in citrate-saline,

counted, and a pool of cells corresponding to digests II to V was plated at 3,000 cells/cm^2 in 100-mm dishes and grown in αMEM containing 10% FCS, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and antibiotics. Total RNA was extracted from cultures stopped at Day 20, a stage at which the cultures contain mature osteoblast colonies.

Osteoclast Isolation and Purification

Osteoclasts were isolated from long bones of 1-day-old New Zealand rabbits, as previously described [Raynal et al., 1996]. Bones were removed, cleaned of soft tissues and bone marrow, split, and scraped into M199, 10% FCS, 20 mM HEPES, 100 UI penicillin/streptomycin. Cells were centrifuged at low speed and this heterogeneous cell population containing osteoclasts was seeded onto glass coverslips, sylanized slides, or 100-mm culture dishes for 90 min at 37°C, in a 5% CO_2 atmosphere. Cultures were then washed to remove nonadherent cells, and the remaining cells were incubated for 24 h in αMEM containing 2% FCS, 100 UI penicillin/streptomycin, at pH 7.2. This cell population was used for immunofluorescence, in situ hybridization, and electrophysiological studies. To obtain purified osteoclasts for RT-PCR analysis, the cell culture was subsequently treated with 0.001% pronase E and 0.02% EDTA during 4 min at 37°C. Nonadherent cells were then washed off and the osteoclast population remaining on the dishes was more than 98% pure, as visualized by light microscopy. Using RT-PCR analysis, we showed that this cell population expressed all the specific osteoclast markers.

RT-PCR

Total RNA from rabbit osteoclasts was extracted using RNAeasy mini kit (Qiagen, Hilden, Germany). Total RNA from calvaria cells, MG63 and UMR 106.01 cells, rabbit and rat brains was isolated using the RNAxel kit (Eurobio, Les Ulis, France). First strand cDNA was synthesized from 1 μg of RNA using Avian Myeloblastisis Virus (AMV) reverse transcriptase (Boehringer Mannhein, Mannheim, Germany). Polymerase chain reaction (PCR) amplification primers were designed from published sequences from rat NR2A to NR2D subunits [Hollmann and Heinemann, 1994; Monyer et al., 1994; Sullivan et al., 1994], and postsynaptic density-95 (PSD-95) [Cho et al., 1992]. Each NR2 subunit was amplified with two different pairs of primers, both designed in sequences highly conserved between species. The validity of all primers was checked on total RNA isolated from rabbit and rat brains as positive controls, and similar amplification of cDNAs was obtained for both pairs of primers. The sequences of primers corresponding to the data presented in this paper are given in Table I.

PCR amplification of all sequences was performed for 35 cycles of 94°C 1 min, 55°C 30 sec, and 72°C 1 min, using Taq polymerase (Appligene, Illkirch, France). After amplification, PCR samples were run on 1.5 % agarose gels and the products were visualized by ethidium bromide staining. Each PCR-amplified fragment of the predicted size was submitted to restrictive enzymatic digestion, and fragments of the appropriate sizes were produced (results not shown). The nucleotide sequences of the PCR products were confirmed by automated sequence analysis (Genaxis Biotechnology, Montigny le Bretonneux, France). RT-PCR reactions performed in the absence of reverse transcripase or in the absence of template served as negative controls for possible amplification of genomic DNA or contaminating RNA. Each RNA sample was also subjected to RT-PCR amplification using specific primers designed from the ribosomal protein L32, as an internal control for RNA amounts (results not shown).

TABLE I. Oligonucleotides Used in the Amplification and Detection of RT-PCR Products

	Oligonucleotide sequences	
Target cDNA (Accession number)	Sense primer	Antisense primer
Rat NR2A (AF001423)	5′-tatagagggtaaatgttgga-3′	5′agaaactgtgaggcatttct-3′
Rat NR2B (U11419)	5′-actgtgacaacccacccttc-3′	5′-cggaactggtccaggtagaa-3′
Rat NR2C (08259)	5′tgtgtcaggccttagtgaca-3′	5′ccacactgtctccagcttct-3′
Rat NR2D (L31612)	5′aagaagatcgatggcgtctg-3′	5′ggatttcccaatggtgaagg-3′
Rat PSD-95 (M96853)	5′tcaaagaggcaggttccatc-3′	5′-cattgtccaggtgctgagaa-3′

In Situ Hybridization

MG63 osteoblastic cells, rabbit osteoclasts and PC12 cells cultured on sylanized slides (Dako, Copenhagen, Denmark), were fixed 10 min with 4% paraformaldehyde in phosphate buffer 0.1 M, washed in phosphate buffer, and dehydrated through a graded ethanol series. Antisense oligonucleotides specific probes were constructed against cDNA sequences of human NR1 [Planells-cases et al., 1993] and human and rat NR2A to NR2D [Hollmann et al., 1993; Hollmann and Heinemann, 1994; Monyer et al., 1994; Sullivan et al., 1994]. Two different probes, chosen in highly conserved sequences between rat and human, were tested for each NMDAR subunit. Identical results were obtained for both probes. Sequences and accession numbers of the probes corresponding to the data illustrated in this paper are given in Table II. Sense probes were used as controls. Probes were 3′-end labeled using terminal deoxynucleotidyl transferase and (^{35}S)-dATP. Hybridization was carried overnight at 37°C in a humidified box, in 50% formamide, 3X SSC (Sodium Cloride-Sodium Citrate) pH 7, 10% dextran sulfate, 1X Denhardt's solution, 10 mM DTT, 100 μg/μl salmon sperm DNA, 125 μg/μl yeast tRNA, and 100 pg/μl of radiolabeled probe. Slides were then washed with SSC containing 10 mM DTT, using concentrations of increasing stringency (2, 1, and 0.5X), dehydrated in graded alcohol concentrations and air dried. In the dark, slides were dipped in K5 emulsion (Ilford, Saint Priest, France), dried and stored in dark boxes for two weeks at 4°C. Following development, the slides were counterstained with 1% toluidine blue for cell observation.

Immunofluorescence Studies

MG63 cells, rat calvaria cells, and rabbit osteoclasts cultured on glass coverslips were fixed 10 min in 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Non–specific immunoreactions were blocked with Tris Buffer Saline (TBS) containing 3% Bovine Serum Albumin (BSA) for 2 h. The cells were incubated for 1 h at room temperature with primary antibody (NR2D: 10 μg/ml) diluted in TBS 0.05% BSA. Control coverslips were incubated with purified goat IgGs. Coverslips were washed in TBS and incubated with Cyanine 3-conjugated mouse anti-goat IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After rinsing with TBS, the coverslips were mounted in Mowiol (Hoechst, Frankfurt, Germany) and observations were performed by epifluorescence in a Leica microscope.

Electrophysiology Experiments

Electrophysiological recordings were performed on spread rabbit osteoclasts cultured on glass coverslips during 24 h, as previously described [Espinosa et al., 1999]. The conventional whole-cell patch-clamp configuration was used, and currents were recorded with RK-400 amplifier (Bio-Logic, Grenoble, France). Current–voltage (I–V) relationships were obtained using a voltage ramp protocol defined by a 15 ms pulse from −30 mV holding potential (H_p) to −130 mV, followed by a ramp from −130 mV to +60 mV over 150 ms. Each protocol was applied every 2 sec and experiments were performed at room temperature. Na^+ external control solution contained in mM: NaCl 145, CsCl 5, $CaCl_2$ 2, glucose 5, HEPES 10, pH = 7.4 adjusted with NaOH. The pipette filling solution contained in mM: CsCl 15, NaCl 5, Aspartic acid 100, EDTA 5, HEPES 20, pH = 7.2 adjusted with CsOH. Glu was added to external solution at 100 μM with 20 μM glycine.

RESULTS

Analysis by RT-PCR of the Presence of the Different NMDAR Subunits

To determine the composition of NMDA receptors in bone cells, we have studied by RT-PCR the presence of the different NR2 subunits

TABLE II. Antisense Oligonucleotides Used for In Situ Hybridization Experiments

Target cDNA (Accession number)	Antisense oligonucleotide
Human NR1 (L05666)	5′-ctcctcctcctcctcgctgttcaccttgaaccggccgaaggg-3′
Human NR2A (U09002)	5′-agaaggcccgtgggagctttccctttggctaagtttc-3′
Human NR2B (U88963)	5′-caagtcgtcgtggccactgtagcggtcgctcttgaaggaggaa-3′
Human NR2C (M91563)	5′-tgcagcatcttcagcacattggcctgggct-3′
Rat NR2D (U08260)	5′- cgtggccaggcttcggttatagcccacaggactgaggt-3′

of the receptor in these cells. We designed specific oligonucleotides primers based on rat sequences of NR2 subunits. PCR products of the predicted sizes were detected with all these primers in the rat brain (Fig. 1A, lane 1) and rabbit brain (lane 5) positive controls, with the exception of NR2A which was not amplified from rabbit brain. As shown in Figure 1A (lanes 4 and 6), osteoclasts isolated from rabbit long bones or human osteoclast-like cells derived from giant cell tumors of bone expressed NR2B and NR2D subunits mRNAs of NMDAR, but not NR2A and NR2C mRNAs. Rat calvaria osteoblasts (lane 2) and MG63 human osteoblastic cells (lane 3) also expressed several NR2 subunits mRNAs, namely NR2A, NR2B, and NR2D, but NR2C was absent from these cells. For each NR2 subunit, PCR products of the appropriate sizes were submitted to restrictive enzymatic digestion and DNA sequence analysis to verify their identity. All the sequences of these fragments amplified from bone cells were 91–98% identical to the published sequences of the corresponding human and rat NR2 subunits. As demonstrated on Figure 1B, bone cells also possessed PSD-95, a postsynaptic density protein known to interact in neuronal cells with the C-terminus of the NR2 subunits of NMDAR. Products of the predicted size were identified in rat brain positive control (lane 1), in rat UMR106.01 and human MG63 osteoblastic cells (lanes 3 and 4), as well as in human osteoclast-like cells (lane 5). The expression of PSD-95 mRNA was very weak in rabbit osteoclasts (lane 6) and absent from rat calvaria cells (lane 2).

In Situ Hybrdization

The expression of mRNAs coding for NR1 and several NR2 subunits was confirmed by in situ hybridization on MG63 osteoblastic cells and rabbit osteoclasts in culture. Antisense and sense radiolabeled probes were selected for each NMDA subunit mRNA and the validity of all antisense probes was checked on PC12 neuronal

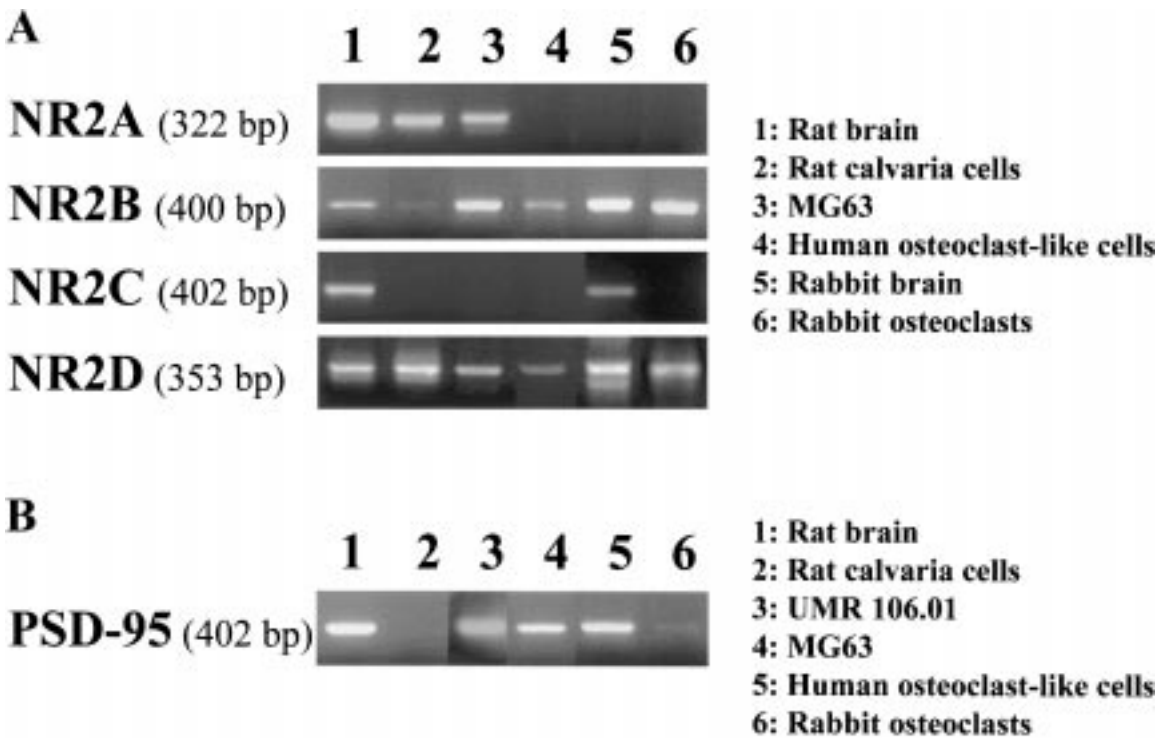

Fig. 1. RT-PCR analysis of NMDAR2 subtypes and PSD-95 expression in bone cells. cDNAs were synthesized from RNA isolated from purified rabbit osteoclasts, human osteoclast-like cells issued from giant cell tumors of bone, human MG63, and rat UMR106.01 osteoblastic cell lines, and rat calvaria cells. They were amplified by PCR using primers specific for NMDAR2A to D subunits and PSD-95, all designed in highly conserved sequences between species. RNA isolated from rat and rabbit brains were used as positive controls to verify the validity of amplification by designed oligonucleotide primers. **A:** RT-PCR analysis revealed NR2B and NR2D expression in MG63 cells, rat calvaria cells, purified rabbit osteoclasts and human osteoclast-like cells, whereas NR2A expression was only demonstrated in osteoblastic cells. **B:** PSD-95 mRNA was identified in osteoblastic cell lines, in purified rabbit osteoclasts and human osteoclast-like cells. RT-PCR analysis was performed on two RNA preparations independently isolated from each tissue and cell culture.

cells (data not shown). Transcript levels of NR1 subunit gene were prominent in MG63 cells and osteoclasts (Fig. 2, lanes A and C), probably reflecting the requirement for the NR1 subunit as a constituent of all NMDA receptor channels. Hybridization of the antisense NR2B and NR2D probes was seen in osteoclasts, in agreement with the RT-PCR results (Fig. 2, lane C). Transcription of these subunit genes was detected at high levels in osteoclasts. Specific labelling was observed on MG63 cells for NR2A, NR2B, and NR2D subunits (lane A), and transcript levels of these three subunits genes were similar in these cells. NR2C mRNA was not detected in bone cells (lanes A and C). No labelling was observed for each of these subunits using sense probes (lanes B and D). All MG63 cells present on the slides were similarly labelled with the positive probes, while osteoclast labelling was less homogenous and very few multinucleated osteoclasts on each slide were unlabelled.

Immunofluorescence Study

Intense, cytoplasmic perinuclear labelling of MG63 cells was observed with the NMDAR2D antibody (Fig. 3A). In mature primary rat calvaria cell cultures, labelling with the NMDAR2D antibody was restricted to osteoblastic colonies in which fully differentiated

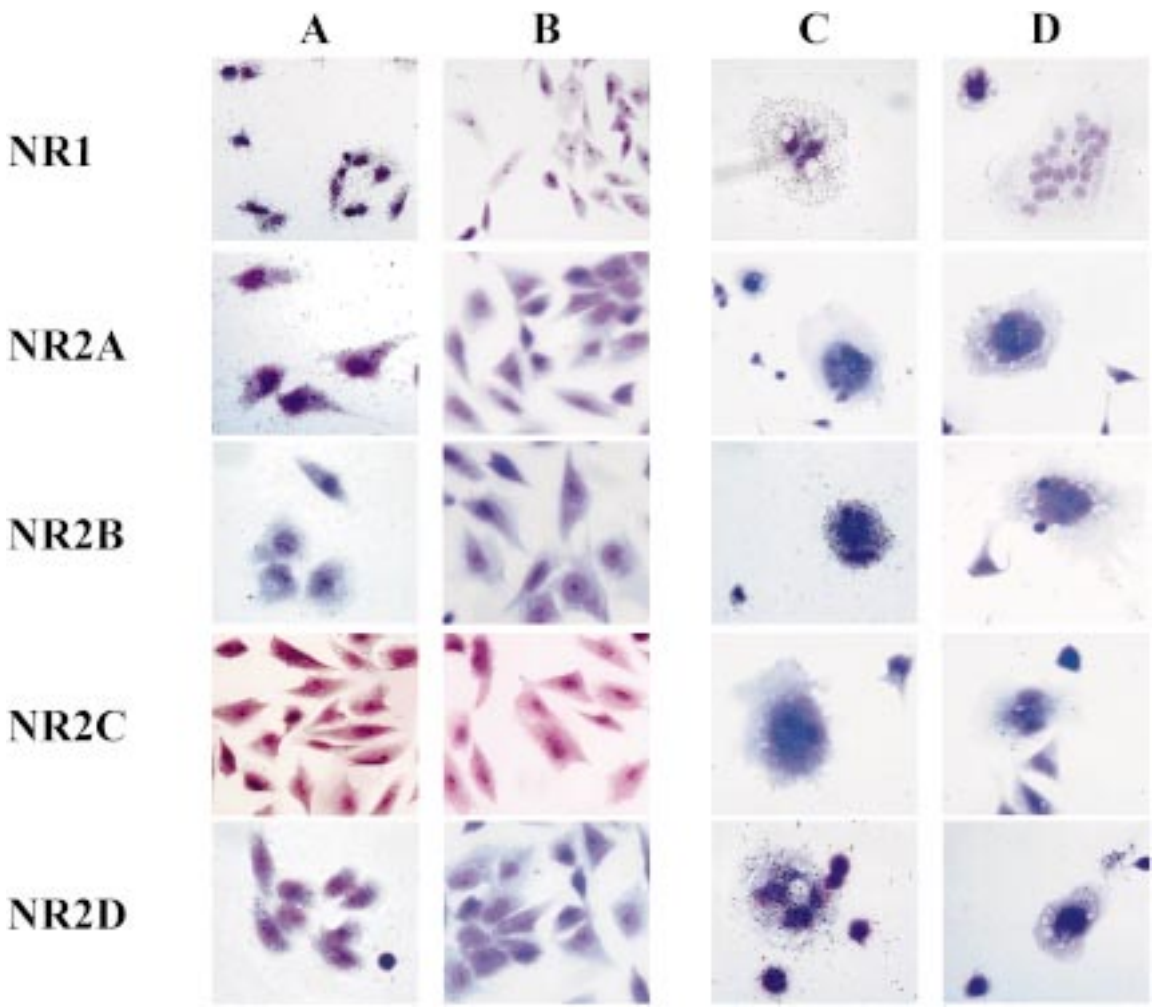

Fig. 2. In situ hybridization of NMDAR subunit mRNAs in bone cells. MG63 osteoblastic cell line and rabbit osteoclast preparations were cultured on sylanized slides and fixed with 4% paraformaldehyde. Two independent preparations were performed for both cell types. Antisense oligonucleotide probes specific for NR1 and NR2A to D subunits were labelled with ^{35}S-dATP and terminaldeoxynucleotidyl transferase. All probes were chosen in sequences highly conserved between rat and human. NR1, NR2A, NR2B, and NR2D transcripts were present in MG63 cells (**lane A**), while only NR1, NR2B, and NR2D transcripts were demonstrated in multinucleated osteoclasts (**lane C**). No labelling was observed for any of these subunits using sense probes (**lanes B** and **D**).

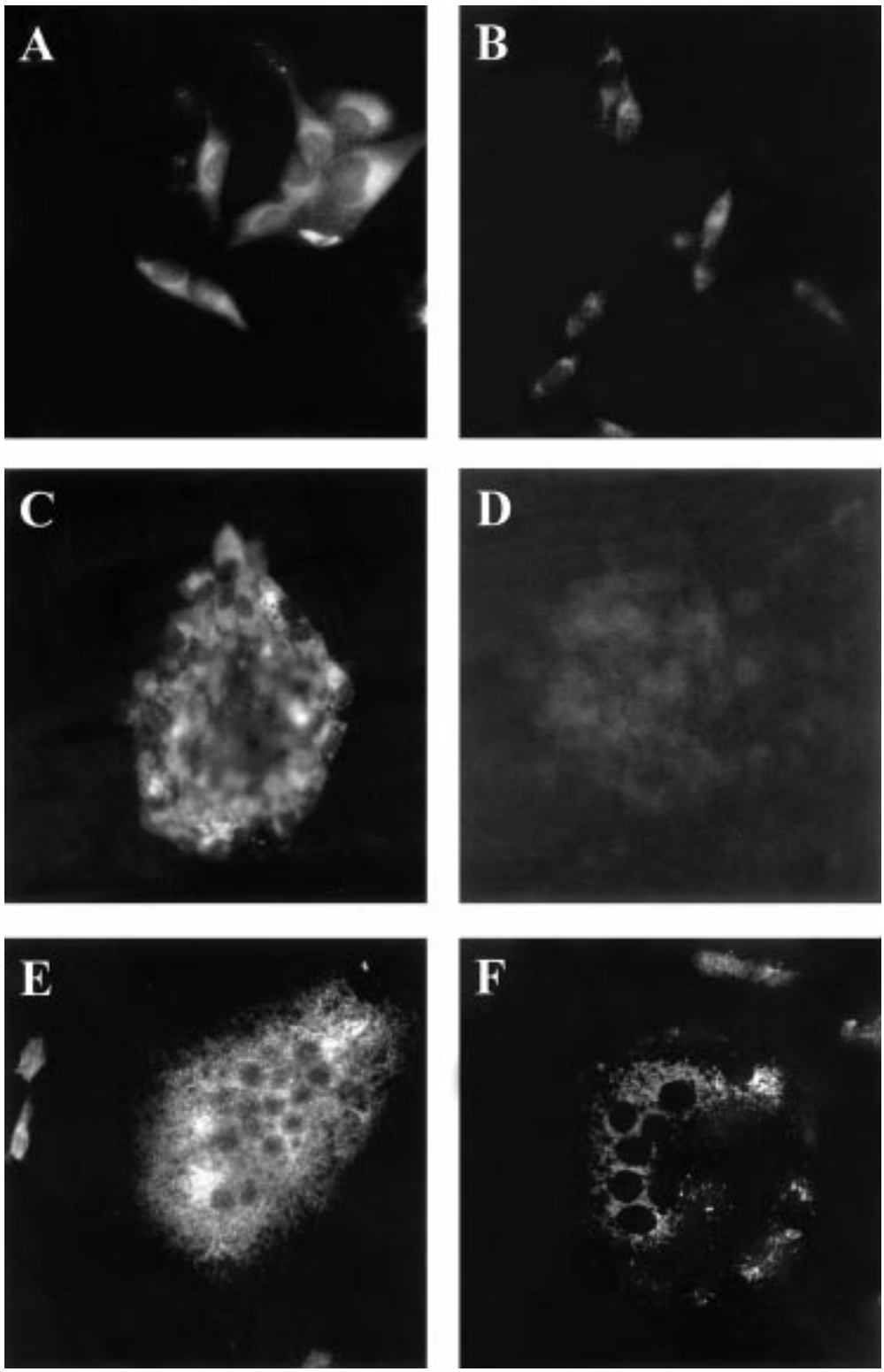

Fig. 3. Expression of NMDAR2D subunit by MG63 cells, rat calvaria cells and rabbit osteoclasts using immunofluorescence. **A:** All MG63 cells were immunostained with NMDAR2D antibody. The staining was cytoplasmic, concentrated at the periphery of the nuclei (original magnification ×400). **C:** Osteoblastic colonies obtained in rat calvaria cell cultures were immunostained with NMDAR2D antibody. Differentiated osteoblasts showed a cytoplasmic staining concentrated around the perinuclear area (original magnification ×200). **E:** A similar cytoplasmic staining was also observed in osteoclasts isolated from rabbit long bones using this antibody (original magnification ×400). **B**, **D**, **F:** No Cell was labelled with the purified goat IgGs. Three independent experiments were performed for each cell culture.

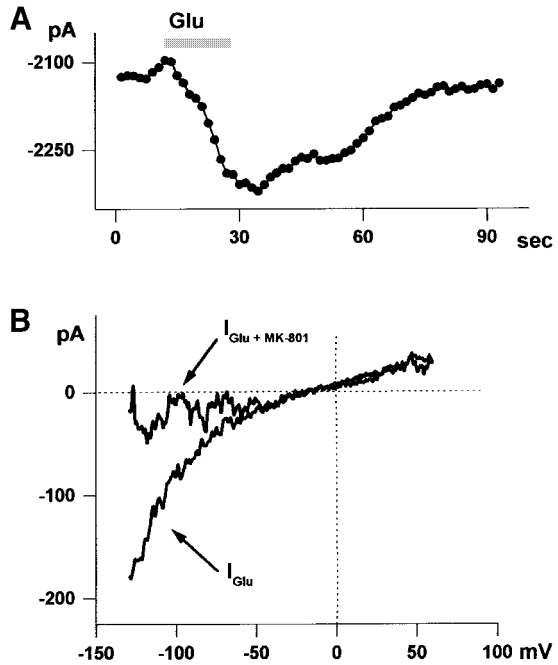

Fig. 4. Glu activates a transient current in rabbit osteoclasts. **A:** This figure illustrates the whole-cell current (expressed in pA) measured every 2 sec at −120 mV during a voltage ramp protocol. Glu (100 μM + Glycine 20 μM) was applied during the time indicated by the bar. It induced an inward current at −120 mV. The current reached a peak in ≈15 seconds and was reversible in ≈60 sec after complete washout of the agonists. **B:** traces represent I–V relationships of Glu-induced current, recorded in the absence (I_{Glu}) or presence of the specific channel blocker MK-801 ($I_{Glu+NK-801}$). I_{Glu} was calculated by subtracting the control current from the current at the peak of response.

osteoblasts presented a cytoplasmic staining intense in golgi (Fig. 3C). Osteoclasts isolated from rabbit long bones showed similar positive staining with the NMDAR2D antibody (Fig. 3E), and ~70% of multinucleated osteoclasts were labelled with this antibody. The staining was cytoplasmic, concentrated in the vicinity of the nuclei. No cell was labelled with the purified goat IgGs (Fig. 3B, D, and F).

Electrophysiology Study

To further identify the NR2 subunits determining functional NMDAR in isolated rabbit osteoclasts, we analyzed the features of Glu-induced whole cell currents recorded in these cells using the patch-clamp technique. It has previously been shown that NMDA Glu receptors containing NR1/NR2D subunits give rise to receptors with unique properties, including unusually very slow deactivation kinetics [Monyer et al., 1994]. Application of Glu (100 μM) and glycine (20 μM) activated a small transient inward current in osteoclasts at −120 mV (Fig. 4A). The current reached a peak many seconds after Glu application, was reversible after complete washout of the agonists, and displayed very slow deactivation kinetic. The I–V relationships of Glu-induced current, recorded in the absence or presence of the specific NMDA channel blocker MK801, confirmed a non-selective cationic current characteristic of NMDAR (Fig. 4B).

DISCUSSION

The present study has identified multiple NR2 subunits in bone cells, suggesting the existence of different functional NMDAR in these cells, as described for neuronal cells. Specific functional properties of NMDAR are attributed to NR2 subunit composition, that specifies kinetic properties and single-channel characteristics, voltage-dependent blockade by Mg^{2+}, and sensitivity to agonists, antagonists, and cofactors [Nakanishi, 1992; Yamakura and Shimoji, 1999].

Our RT-PCR analysis and in situ hybridization studies indicate that, in addition to the previously demonstrated NR1 subunit, osteoclasts express mRNA for NR2B and NR2D subunits. Identical results were obtained with purified osteoclasts freshly isolated from rabbit long bones and purified giant osteoclast-like cells from human bone tumors indicating that expression of these subunit is a characteristic of the mature osteoclast phenotype. These osteoclast preparations were both highly purified and the majority of non-osteoclastic cells were removed. Although we cannot totally exclude a possible amplification of cDNA from small numbers of contaminating cells, our results showed identical NR2 expression by osteoclasts isolated from two different models that are unlikely to contain the same contaminating cells. Furthermore, these data were confirmed by in situ hybridization on well-identified multinucleated rabbit osteoclasts. In situ hybridization experiments on these cells confirmed that the majority of multinucleated osteoclasts present on the cell preparation express mRNA for NR1, NR2B, and NR2D. In contrast, we could not detect mRNA transcripts for the NR2A and NR2C subunits in all osteoclast preparations using RT-PCR analysis and in situ hybridiza-

tion, suggesting that these subunits are not expressed by mature osteoclasts. However, evidence for the expression of NR2A transcript in rabbit brain was not obtained, and it is possible that our primer sequences and/or conditions of amplification for this transcript were not suitable for this species. Nevertheless, in situ hybridization data are consistent with RT-PCR results and human osteoclasts do not either express NR2A, strongly supporting that this subunit is not transcribed in mature mammalian osteoclasts.

The presence of NR2D protein in osteoclasts was confirmed by immunofluorescence, while expression of other subunits was not investigated with this technique, due to a lack of good specific antibodies directed against those subunits. Labelling for NR2D was intracellular, as already described for NMDA receptor subunits using conventional immunocytochemistry techniques, and it is therefore difficult to know if the majority of NMDAR are expressed on the cell surface [Petralia et al., 1994]. Our electrophysiological studies of NMDAR expressed by mammalian osteoclasts indicate that they are functional [Espinosa et al., 1999]. Very slow deactivation kinetics displayed by NMDAR containing the NR2D subunit are features that allow the identification of this subtype of receptor [Misra et al., 2000]. NMDAR-mediated currents elicited in osteoclasts by application of Glu displayed this property, indicating a major NR2D subunit composition in these cells.

The presence of two subtypes of NR2 subunits in osteoclasts suggests that heteromeric NMDAR composed of NR1/NR2B and NR1/NR2D might be expressed by these cells, although the presence of triplet NMDAR composed of NR1/NR2B/NR2D is not totally excluded [Wafford et al., 1993; Sheng et al., 1994]. The role of two different functional NMDAR in these cells remains to be investigated. Previous in vitro studies by our group demonstrated that NMDAR activation is important for bone resorption, but the identity of NMDAR involved in this process is unknown [Chenu et al., 1998; Itzstein et al., 2000]. While the NR1 subunit is expressed ubiquitously in the brain, NR2 subunits show a distinct spatial and temporal expression pattern [Monyer et al., 1994]. NR2B subunit mRNA is widely distributed compared to NR2D subunit mRNA, but their temporal patterns of expression are similar [Monyer et al., 1994; Ozawa et al., 1998]. Targeted disruptions of NMDAR subunits genes suggest that the role of NR2D subunit in the development is compensated by NR2B, but the skeletal phenotypes of these animals have not been investigated [Ikeda et al., 1995]. Coexpression of heteromeric receptors NR1/NR2D and NR1/NR2B has already been demonstrated for GABAergic neurons [Plant et al., 1997], raising the possibility that both receptors may also be simultaneously expressed by osteoclasts during the resorption process. Another possibility may be a selective change in surface expression of NMDAR depending on cell activation state, as shown in synapses [Huh and Wenthold, 1999].

Osteoblastic cells also expressed NR1 together with several NR2 subunits, suggesting the existence of different NMDAR in these cells. In addition to mRNA for NR2B and NR2D subunits, osteoblasts highly expressed mRNA for NR2A, one of the most common subunit in neuronal cells. RT-PCR analysis showed that rat calvaria cell cultures and MG63 osteosarcoma cell line have a similar expression of NR2A and NR2D subunits, but NR2B mRNA was weakly expressed in rat calvaria cells compared to MG63 cells. Total RNA isolated from calvaria cell cultures was derived from a heterogeneous cell population [Bellows et al., 1986], including among other lineages osteoblastic progenitors which undergo progressive differentiation [Malaval et al., 1999]; it is possible that NR2B mRNA is only expressed by osteoblasts at a very specific cell stage, underrepresented in mature cultures. In situ hybridization experiments confirmed the expression of NR2A, NR2B, and NR2D subunits mRNA by MG63 cells. All cells in the preparation were similarly labelled with the probes, as expected for a homogeneous cell population. The significance of several subtypes of NMDAR on osteoblastic cells has to be determined. Functional NMDA receptors have been described in MG63 cells, and their electrophysiological characteristics were more representative of NR1/NR2D heteromeric receptors [Laketic-Ljubojevic et al., 1999]. We confirmed in this study the expression of NR2D protein by MG63 and primary rat osteoblasts. However, no role for NMDAR in osteoblast differentiation and function has yet been attributed. Outside neuronal cells, pancreatic cells have been shown to express NR1 and several NR2 subunits, but mediation of insulin secretion by NMDA Glu receptors was not assigned to a

preferential expression of a particular type of heteromeric NMDA receptor [Gonoi et al., 1994].

NMDA receptor subunits have a common transmembrane topology with three membrane spanning regions and a C-terminal intracellular tail of variable length that is essential for their function in vivo [Sprengel et al., 1998]. The C-termini of subunits NR2 are known to interact with anchoring proteins containing PDZ domains such as PSD-95 [Cho et al., 1992; Kornau et al., 1995]. This interaction may be important for the clustering of NMDA receptors in the postsynaptic membrane and the connection of NMDAR with cytoplasmic signaling proteins, as PSD-95 also binds to cytoskeletal proteins, protein tyrosine kinases and NO synthase [Sala and Sheng, 1999]. Our RT-PCR analysis showed that MG63 and UMR106.01 osteoblastic cell lines, osteoclasts isolated from rabbit long bones and human osteoclast-like cells express mRNA for PSD-95. These results suggest that the organization of NMDAR in bone cells may be similar to neuronal cells, and that protein interactions of PSD-95 may be important for NMDAR activation and intracellular signaling in these cells. In contrast to osteoblastic cell lines, PSD-95 was not transcribed in mature rat calvaria cell cultures, may be because it is mainly expressed by less differentiated osteoblasts. Further experiments need to be performed to study the expression of PSD-95 in this model throughout the time course of osteogenesis.

The great diversity of NMDAR subunits expressed by bone cells supports a physiological role for Glu receptors in bone. Our results strongly suggest the existence of different functional NMDA channels in bone with distinct properties, and the analysis of their association with bone cell differentiation and activity is required to assess the biological significance of NMDA Glu receptor heterogeneity in bone.

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