Journal of Cellular Biochemistry 90:424–436 (2003)

NMDA Glutamate Receptors Are Expressed by Osteoclast Precursors and Involved in the Regulation of Osteoclastogenesis

Blandine Merle,* Cecile Itzstein, Pierre D. Delmas, and Chantal Chenu

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

Abstract We previously identified functional *N*-methyl-D-aspartate (NMDA) glutamate receptors in mature osteoclasts and demonstrated that they are involved in bone resorption in vitro. In the present work, we studied the expression of NMDA receptors (NMDAR) by osteoclast precursors and their role in osteoclastogenesis using two in vitro models, the murine myelomonocytic RAW 264.7 cell line and mouse bone marrow cells, both of which differentiate into osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF) and Rank ligand (RankL). Using RT-PCR analysis with specific probes, we showed that RAW 264.7 cells and mouse bone marrow cells express mRNA of NMDAR subunits NMDA receptor 1 (NR1) and NMDA receptor 2 (NR2) A, B, and D. These subunits are expressed all along the differentiation sequence from undifferentiated precursors to mature resorbing osteoclasts. Semi-quantitative PCR analysis showed no regulation of the expression of these subunits during the differentiation process. Two specific non competitive antagonists of NMDAR, MK801 and DEP, dose-dependently inhibited osteoclast formation in both models, indicating that osteoclastogenesis requires the activation of NMDAR expressed by osteoclast precursors. MK801 had no effect when added only during the first 2 days of culture, suggesting that NMDAR are rather involved in the late stages of osteoclast formation. Finally, we demonstrated using Western-blotting and immunofluorescence that activation of NMDAR in RAW 264.7 cells by specific agonists induces nuclear translocation of NF-kappa B, a factor required for osteoclast formation. Altogether, our results indicate that osteoclast precursors express NMDAR that are involved in the osteoclast differentiation process through activation of the NF-kappa B pathway. J. Cell. Biochem. 90: 424–436, 2003.

Key words: osteoclast differentiation; RAW 264.7 cells; mouse bone marrow precursors; NMDA glutamate receptors; NF-kappa B

There has been growing evidence during the past years that glutamate, the major neuromediator of the nervous system, contributes to the local regulation of bone cell functions. Glutamate itself and most of the components involved in its signal transduction, glutamate receptors and transporters, were identified in bone [Mason et al., 1997; Chenu et al., 1998; Patton et al., 1998; Bhangu et al., 2001]. Glutamate was detected in the network of nerve fibers running in bone marrow [Serre et al., 1999], as well as in vesicles in osteoblasts where it can be released by exocytosis [Bhangu et al., 2001; Genever and Skerry, 2001]. The expression and putative functions in bone metabolism of the *N*-methyl-D-aspartate receptor (NMDAR) subtype of glutamate receptors were the most characterized. Osteoblasts and osteoclasts express *N*-methyl-D-aspartate (NMDA) receptor 1 (NR1) together with different NMDA receptor 2 (NR2) subunits, supporting a molecular diversity of NMDAR in bone, similar to what was demonstrated in brain [Chenu et al., 1998; Patton et al., 1998; Itzstein et al., 2001]. Electrophysiological studies have demonstrated that NMDAR are functional on mature osteoclasts and osteoblasts with similar electrophysiological and

Abbreviations used: NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; NR1, NMDA receptor subunit 1; NR2, NMDA receptor subunits 2; RankL, Rank ligand; TRAP, tartrate-resistant acid phosphatase; CTR, calcitonin receptor.

Grant sponsor: Institut National de la Santé et de la Recherche Médicale (France); Grant sponsor: Les Fonds de Recherche Hoechst Marion Roussel.

*Correspondence to: Blandine Merle, INSERM Unit 403, Hôpital E. Herriot, Pavillon F, 69437 LYON Cedex 03, France. E-mail: merle@lyon.inserm.fr

Received 22 April 2003; Accepted 24 June 2003

DOI 10.1002/jcb.10625

pharmacological properties to those described in neuronal cells [Espinosa et al., 1999; Laketic-Ljubojevic et al., 1999; Peet et al., 1999; Gu et al., 2002]. While the function of NMDAR has not yet been elucidated in osteoblasts, our group has demonstrated that activation of NMDAR on mature osteoclasts is required for their resorptive function. Using an in vitro model of bone resorption, we showed that specific antagonists of NMDAR prevent osteoclast sealing zone formation required for bone resorption [Chenu et al., 1998; Itzstein et al., 2000]. By contrast, Peet et al. [1999] showed that MK801, an antagonist of NMDAR, inhibits bone resorption by a mechanism predominantly involving osteoclast differentiation rather than activity. In their model, osteoclasts develop in cocultures of bone marrow cells and osteoblasts and it is presently unknown whether MK801 inhibits osteoclastogenesis by acting directly on osteoclast precursors that express NMDAR or indirectly through NMDAR present in osteoblasts.

Osteoclasts are multinucleated cells that differentiate from hematopoietic precursors in presence of Rank ligand (RankL) and macrophage colony-stimulating factor (M-CSF) in vitro [Lacey et al., 1998; Yasuda et al., 1998]. Commitment of mononuclear precursors to mature osteoclasts involves multiple transcription factors including NF-kappa B. Mice deficient in both p50 and p52 subunits of NF-kappa B are osteopetrotic due to the failure of osteoclast differentiation [Franzoso et al., 1997; Iotsova et al., 1997], but this factor also plays a role in osteoclast survival and activity [Jimi et al., 1996; Ozaki et al., 1997]. In brain, NF-kappa B is an essential factor of neuronal development and controls the expression of a wide variety of genes [Rothwarf and Karin, 1999]. It has been shown that glutamate specifically activates NF-kappa B in neuronal cells by binding to NMDAR [Guerrini et al., 1995; Kaltschmidt et al., 1995].

In this article, we analyzed the expression of NMDAR subunits by osteoclast precursors and studied their role in the osteoclastic differentiation process. Using two in vitro models of osteoclast differentiation, the murine myelomonocytic RAW 264.7 cell line and mouse bone marrow cells, we show that osteoclast precursors express mRNA for NR1 and several NR2 subunits. We demonstrate that specific antagonists of NMDAR inhibit osteoclast differentiation in vitro, indicating that activation of NMDAR is required for osteoclastogenesis. Finally, we observed that stimulation of NMDAR by glutamate and NMDA up-regulates NF-kappa B activity, suggesting that the NF-kappa B pathway is involved in glutamate regulation of osteoclast formation through NMDAR.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles's medium (DMEM), α modified essential medium (αMEM), penicillin/streptomycin, L-glutamine, and fungizone were purchased from Gibco (Invitrogen, Cergy-Pontoise, France), and fetal bovine serum (FBS) from Hyclone (Perbio Sciences France, Bezons, France). Sterile culture plasticware were obtained from Falcon Becton Dickinson (Le Pont De Claix, France). Recombinant murine M-CSF was purchased from R&D Systems (London, England). RankL was kindly given by Amgen (Thousands Oaks, CA). Monoclonal antibody anti-NF-kappa B p65 protein was bought from Santa Cruz Biotechnology (Tebu International, Le Perray en Yvelynes, France). Peroxidase-conjugated goat anti-mouse IgG and cyanine-conjugated anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Western lightning chemiluminescence reagent was bought from Perkin Elmer Life Sciences (Courtaboeuf, France). Glutamate receptor agonists NMDA, glutamate, and glutamate receptor antagonists (5R,10S)-(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a, d]cyclohepten-5,10-imine (MK801), 1-(1,2-diphenylethyl) piperidine (DEP), and 2,3-Dioxo-6-nitro-1,2,3,4 tetrahydrobenzo [f] quinoxaline-7-sulphonamide (NBQX) were purchased from Tocris (Fisher Bioblock Scientific, Illkirch, France). Unless specified, all other chemicals were obtained from Sigma (L'Isle d'Abeau Chesnes, France).

RAW 264.7 Cell Culture and Differentiation

The murine monocytic cell line RAW 264.7 was purchased from the American Type Culture Collection (Manassas, VA) and grown, as indicated by supplier, in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamax, and 0.5% fungizone.

For differentiation into osteoclasts, RAW 264.7 cells were plated on 100 mm culture dishes in αMEM medium containing 10% FBS,

1% penicillin/streptomycin, 1% glutamax, 0.5% fungizone, and supplemented with 30 ng/ml RankL. Cells were cultured for 5 days at 37°C in a humidified atmosphere containing 5% CO_2, with fresh medium and cytokines replaced on day 3. RAW 264.7 cells differentiate into multinucleated osteoclast-like cells expressing specific markers of osteoclasts: tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (CTR), as previously shown [Hsu et al., 1999]. For RT-PCR analysis of NMDAR subunits mRNA expression during RAW 264.7 cell differentiation, cells were collected on each day of culture for RNA extraction after washing with phosphate-buffered saline (PBS) to remove non adherent cells.

Bone Marrow Cell Culture and Differentiation

Bone marrow cells were prepared, as previously described [Raynal et al., 1996]. Briefly, long bones from 8 week-old NMRI male mice were removed and the bone marrow cavity was flushed with αMEM. After centrifugation, this heterogeneous population containing osteoclast precursors was resuspended in αMEM containing 10% FBS, 1% penicillin/streptomycin, 1% glutamax, and seeded into 100 mm culture dishes. To obtain 1-day bone marrow cells for PCR analysis, cultures were maintained in this medium at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h and adherent cells were collected after washing with PBS. For osteoclast differentiation assay, bone marrow cells were seeded in the same medium supplemented with 30 ng/ml M-CSF and 20 ng/ml RankL. Cultures supplemented with fresh medium and cytokines every 2 days were maintained for 6 days at which time, multinucleated mature osteoclasts were formed. For RT-PCR analysis of NMDAR subunits mRNA expression during osteoclastogenesis, bone marrow cells were collected at each day of the differentiation process for RNA extraction after vigorous washing with PBS to remove non adherent cells.

RNA Isolation, RT-PCR, and Southern Analysis

Total RNA obtained from RAW 264.7 cells or bone marrow cells was extracted with RNeasy^{R} minikit (Qiagen SA, Courtaboeuf, France) and reverse-transcribed using omniscript reverse transcriptase from Qiagen SA and oligo$(dT)_{12-18}$ primers (Amersham Pharmacia Biotech, Orsay, France). The cDNAs were amplified by PCR with HotStarTaq^{TM} DNA polymerase (Qiagen SA) with specific primers designed for the different NMDAR subunits, CTR and TRAP (Table I), according to published sequences [Hollmann and Heinemann, 1994; Monyer et al., 1994; Sullivan et al., 1994; Reddy et al., 1995; Inoue et al., 1999]. The identity of the PCR amplified fragments was confirmed by DNA sequence analysis.

For time course analysis of NMDAR subunits expression during osteoclastogenesis, total RNA from each day culture of RAW 264.7 and bone marrow cells was extracted and reverse-transcribed as described above. cDNAs were subjected to semi-quantitative PCR: during the last 12 cycles of the PCR process, 10 μl aliquots of each PCR sample were collected every three cycles and run on 1.5% agarose gels. Amplification products were transferred to Hybond-N nylon membranes (Amersham Biosciences, Saclay, France) and ultra-violet cross-linked. The membranes were then hybridized overnight at 65°C with specific PCR generated cDNA probes (QIAquick^{TM} Spin, Qiagen SA), purified, and labeled with [^{32}P]dCTP using a random priming labeling kit (Life Technologies, Grand Island, NY), as described elsewhere [Church and Gilbert, 1984]. After washing, the radiolabeled membranes were exposed to X-ray films.

TABLE I. Primers Sequences Used in the Polymerase Chain Reaction (PCR)

	Oligonucleotide sequences	
Target cDNA (accession no.)	Sense primer	Antisense primer
Human NR1 (L05666)	5′-gatgtcttccaagtatgcgga-3′	5′-gggaatctccttcttgaccag-3′
Rat NR1 (U08261)	5′-aatgaccccaggctcagaaac-3′	5′-tgaagcctcaaactccagcac-3′
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′
Mouse CTR (U18542)	5′-gtcttgcaactacttctggatgc-3′	5′-aagaagaagttgaccaccagagc-3′
Mouse TRAP (NM007388)	5′-ctctctgaccacctgtgcttcctc-3′	5′-gaacctcttgtcgctggcatcgtg-3′

Osteoclastic Differentiation Assay in the Presence of NMDAR Antagonists

To analyze the effects of NMDAR antagonists on osteoclast differentiation, bone marrow cells or RAW 264.7 cells were seeded in 24-well plates in αMEM supplemented with RankL with or without M-CSF, as described above. Cells were treated with specific NMDAR antagonists: MK801 or DEP at 10, 50, or 100 μM added at the beginning of the culture and all along the differentiation sequence, or for 2–4 days pulses at different stages of the differentiation process. NBQX, an antagonist of AMPA receptors, a second class of ionotropic glutamate receptors, was used as control at the same concentrations. At the end of culture, cells were washed with PBS, fixed, and stained for TRAP. Multinucleated TRAP-positive osteoclasts (>3 nuclei) were scored under a light microscope.

NF-Kappa B Nuclear Translocation Analysis by Immunoblotting

To analyze the role of NMDAR agonists on nuclear translocation of NF-kappa B, RAW 264.7 cells grown in DMEM were starved in PBS for 30 min, then stimulated with 100 μM NMDA in the presence of its coagonist glycine (100 μM) in PBS for 10 or 20 min. Alternatively, cells were pretreated for 10 min with MK801 before stimulation with NMDA and glycine. Stimulation by RankL in the same conditions was used as a positive control [Wei et al., 2001].

Untreated or NMDA-stimulated RAW 264.7 cells were lifted from the dish with citrate buffer (15 mM) and nuclear proteins were extracted for Western-blot analysis. Briefly, cells were resuspended in hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin) and incubated at 4°C for 45 min. Nuclei were pelleted, washed, resuspended in low salt nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 4 μg/ml leupeptin, 4 μg/ml pepstatin) to which was added an equal volume of high salt nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 4 μg/ml leupeptin, 4 μg/ml pepstatin). After a 30 min rotation at 4°C, samples were centrifuged and the supernatants containing nuclear proteins were dialyzed against HEPES buffer (20 mM HEPES, 0.4 mM PMSF, 1 mM DTT) for 1 h at 4°C. After centrifugation, the supernatants were collected and nuclear protein concentrations were determined by the micro BCA™ protein assay (Pierce, Perbio Sciences France, Bezons, France).

An aliquot of nuclear proteins was mixed with loading buffer, boiled for 5 min, and subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a nitrocellulose membranes using a semidry blotter (VWR International, Fontenay-sous-Bois, France). Membranes were blocked in Tris-buffered saline (TBS) (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 and 5% non fat dry milk for 1 h to reduce non specific binding. Membranes were then incubated overnight at 4°C with a monoclonal antibody directed against NF-kappa B p65 protein in TBS containing 0.1% Tween 20 and 5% non fat dry milk. After washing, membranes were exposed to peroxidase-conjugated goat anti-mouse IgG for 1 h, washed, and chemiluminescence detection was performed. A picture was made and analyzed with ImageQuant v1.2 software (Amersham Pharmacia Biotech) to quantify bands intensity (integral density of the band).

Immunofluorescence Analysis

NF-kappa B nuclear translocation in RAW 264.7 cells after glutamate treatment was studied by immunofluorescence. RAW 264.7 cells grown on glass coverslips in DMEM were pre-incubated in PBS for 30 min and stimulated by 1 mM glutamate and 1 mM glycine in PBS for the indicated times. Cells were then fixed with 4% paraformaldehyde in 0.1 M PBS for 5 min and permeabilized with methanol at −20°C for 20 min. Non specific immunoreactions were blocked with TBS containing 3% bovine serum albumin for 2 h. Cells were incubated for 1 h at room temperature with a monoclonal antibody directed against NF-kappa B p65 protein or with purified mouse immunoglobulins as control, diluted in PBS with 0.05% bovine serum albumin. After rinsing, the cells were incubated for 1 h at room temperature with a cyanine 3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories). Hoechst 33258 (Aventis, Strasbourg, France), a fluorochrome used to visualize nuclei, was added concomitantly. After washing, coverslips were mounted with Mowiol (Aventis) and analyzed

by epifluorescence with a Leica microscope. Treatment with RankL (100 ng/ml) was used as a positive control. Alternatively, cells were pre-incubated for 30 min in PBS containing 100 μM pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-kappa B activation before treatment with 1 mM glutamate, glycine, and 100 μM PDTC in PBS.

Statistical Analysis

Data were subjected to Mann–Whitney *U*-test, using the Instat software (Graphpad, San Diego, CA).

RESULTS

RAW 264.7 Cells and Bone Marrow Cells Express NR1 Together With Different NR2 Subunits

To determine if osteoclast precursors express NMDAR, we studied by RT-PCR the presence of the different NMDAR subunits in RAW 264.7 cells and mouse bone marrow cells using specific oligonucleotides primers designed for each subunit (Table I). RAW 264.7 cells, a homogenous population of mouse myelomonocytic cells that can differentiate into osteoclast-like cells, express NR1 as well as NR2A, NR2B, and NR2D (Fig. 1). Mouse bone marrow cells cultured for 1 day, which contain osteoclast precursors, express NR1 and the same NR2 subunits as RAW 264.7 cells. No amplification was obtained for these two cell populations with NR2C, which was only present in mouse brain used as a positive control (Fig. 1).

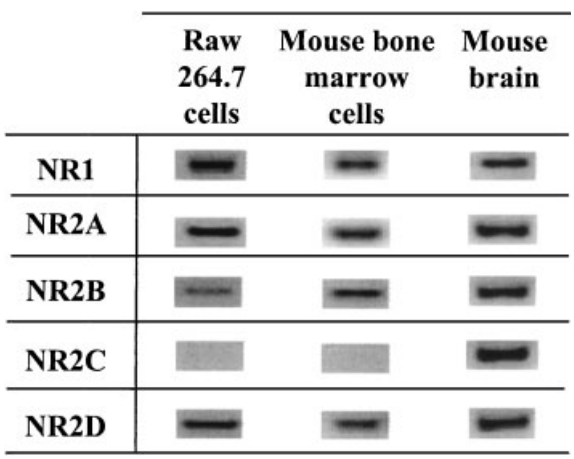

Fig. 1. RAW 264.7 cells and bone marrow cells express NR1 and several NR2 subunits mRNA. cDNAs were synthesized from RNA isolated from RAW 264.7 cells or mouse bone marrow cells cultured for 1 day and amplified by PCR using specific primers designed for *N*-methyl-D-aspartate receptor (NMDAR) subunits. cDNA from mouse brain was used as a positive control.

Time Course of NMDAR Subunits mRNA Expression During Osteoclastogenesis

Since both osteoclast precursors and mature osteoclasts express NMDAR subunits [Itzstein et al., 2001], we studied the expression of these subunits all along osteoclastogenesis using two in vitro models of osteoclastogenesis: the monocyte/macrophagic RAW 264.7 cell line and mouse bone marrow cell cultures. In both models, precursors differentiate into resorbing osteoclasts in the presence of RankL, in 5 days for RAW 264.7 cells and 6 days for bone marrow cells.

Expression of NR1 and NR2 subunit mRNAs was demonstrated at every stage of the differentiation process of RAW 264.7 cells from non differentiated cells, illustrated by the first day of culture in the presence of RankL, to mature osteoclast-like cells on day 5 of the culture (Fig. 2A). Semi-quantitative RT-PCR analysis did not show any regulation of the expression of these NMDAR subunits during differentiation, in contrast to CTR and TRAP mRNA, two markers of the osteoclast lineage, whose expression increased with osteoclast differentiation (Fig. 2A).

Similarly, no regulation of NMDAR subunits mRNA expression throughout the successive stages of osteoclast differentiation was demonstrated for mouse bone marrow cells (Fig. 2B). In this model, CTR mRNA expression was almost constant all along osteoclastogenesis (Fig. 2B), while TRAP mRNA expression increased during osteoclast differentiation.

NMDAR Antagonists Inhibit Osteoclast Differentiation

The presence of NMDAR on osteoclast precursors has led us to study their role in osteoclast differentiation. We showed that specific non competitive channel blockers of NMDAR, MK801, and DEP significantly inhibit, in a dose-dependant manner, the number and the size of the TRAP-positive multinucleated osteoclasts formed by RAW 264.7 cells cultured for 5 days with RankL, compared to control (Fig. 3A). In contrast, NBQX, an antagonist of AMPA receptors, another class of glutamate receptors, had no effect on the number of TRAP-positive osteoclasts formed in this model (Fig. 3A).

MK801 and DEP showed a more drastic inhibitory effect on TRAP-positive osteoclast formation from bone marrow precursors with

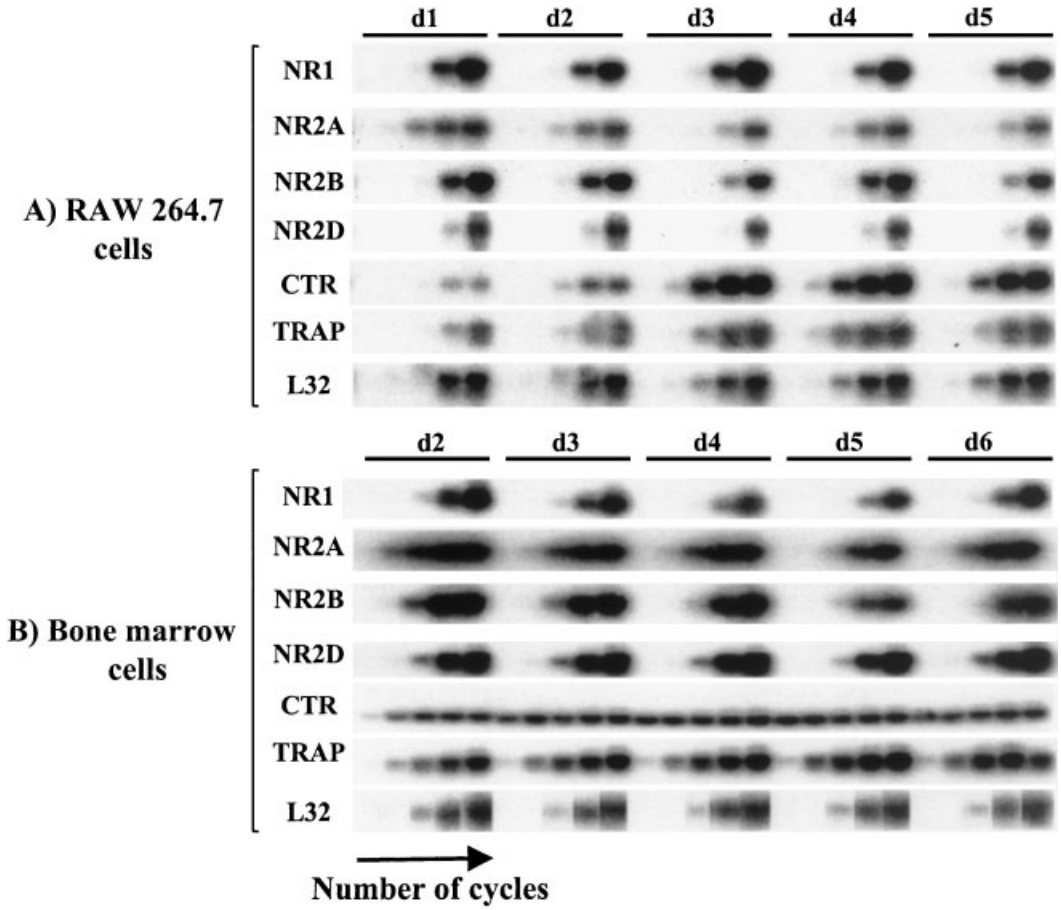

Fig. 2. Time course of NMDAR subunits mRNA expression during osteoclastogenesis. RAW 264.7 cells (**A**) and bone marrow cells (**B**) were grown in the presence of Rank ligand (RankL) for 5 or 6 days, respectively. Total RNA was collected every day and subjected to RT-PCR analysis using specific primers of NMDAR subunits, CTR and TRAP. L32 was used as an internal control. Aliquots of PCR products were collected every three cycles of amplification and subjected to Southern-blot detection as described in ''Materials and Methods.''

more than 88% inhibition at 50 μM (Fig. 3B). NBQX at 100 μM had a slight inhibitory effect on osteoclast differentiation from marrow precursors. In both models, DEP was the most powerful antagonist.

MK801 Preferentially Inhibits the Late Stages of Osteoclast Differentiation

To determine at which stage of the differentiation sequence NMDAR antagonists were inhibitory, they were added to the cultures for only 2 or 4 days (d). Addition of MK801 (100 μM) during the last 2 days of RAW 264.7 cell differentiation cultures (d3–d5) led to a significantly greater inhibition of TRAP-positive osteoclast formation (41%) than when MK801 was present only during the first 3 days of culture (d0–d3, 27% inhibition) (Fig. 4A). Similarly to Figure 3A, the inhibitory effect of MK801 added during the entire culture was around 80%.

A similar observation was made during mouse bone marrow cell differentiation. MK801 treatment (100 μM), restricted to the first 2 days of culture (d0–d2), had no significant effect on osteoclast formation (11% inhibition) while inhibition was almost total when MK801 was added from day 2, for 2 or 4 days (Fig. 4B). MK801 present for a 4 day pulse (d0–d4 or d2–d6) led to a very strong reduction of TRAP-positive multinucleated osteoclast number and size (more than 98% inhibition), as observed when it is present during the entire differentiation sequence (d0–d6). An identical inhibition was demonstrated when MK801 was added only for 2 days (d2–d4 or d4–d6) (Fig. 4B). Altogether, these results indicate that the inhibitory effect of MK801 is maximal in the late stages of osteoclagenesis, suggesting that NMDAR may play a role in more differentiated osteoclast precursors.

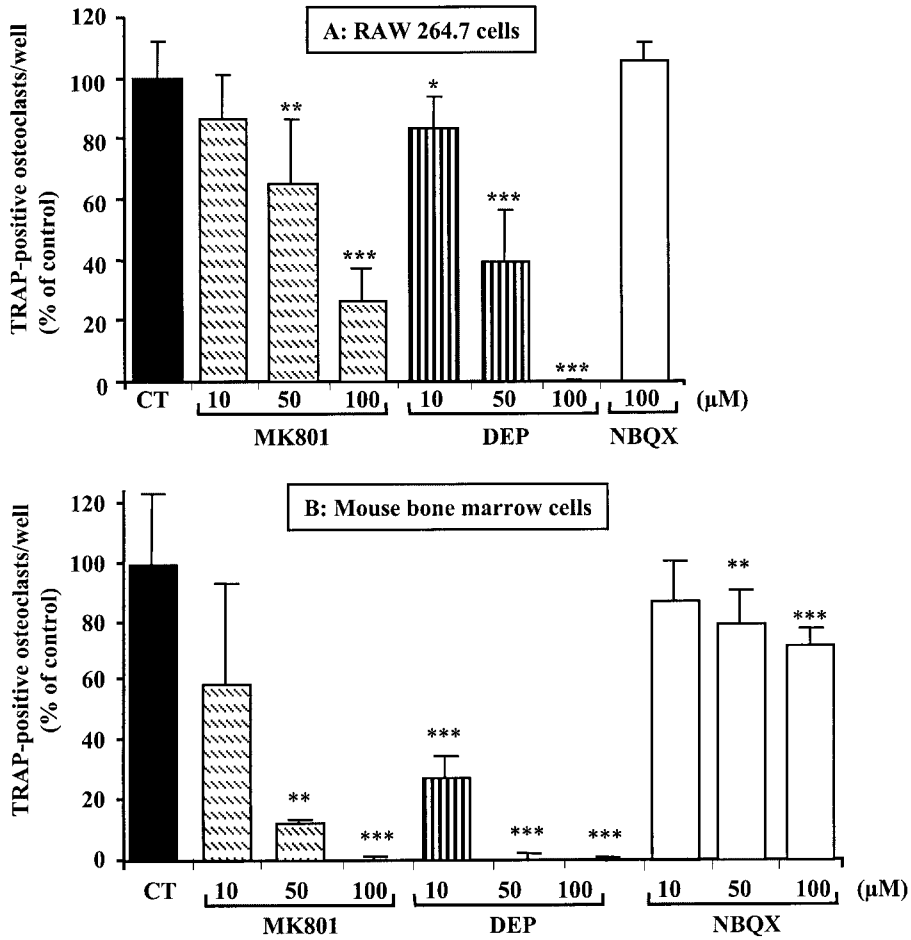

Fig. 3. NMDAR antagonists inhibit osteoclast differentiation. RAW 264.7 cells (**A**) and mouse bone marrow cells (**B**) were grown in αMEM in the presence of RankL with or without specific non competitive antagonists of NMDAR, MK801 and DEP at 10, 50, or 100 μM. NBQX, an antagonist of AMPA receptors, was used as a control. At the end of the differentiation process, osteoclasts were stained for TRAP and the number of TRAP-positive multinucleated osteoclasts (>3 nuclei) was counted for each condition. Results, expressed as percentage of control, are mean ± SD of two independent experiments, each performed in quadruplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (vs. untreated cells, by Mann–Whitney *U*-test).

NMDAR Activation Induces Nuclear Translocation of NF-Kappa B

Since commitment of mononuclear precursors to mature osteoclasts involves transcription factors such as NF-kappa B and glutamate activates this transcription factor in neuronal cells, we investigated whether NF-kappa B is activated by glutamate or NMDA in osteoclast precursors [Guerrini et al., 1995; Kaltschmidt et al., 1995; Franzoso et al., 1997; Iotsova et al., 1997]. Using a specific antibody against NF-kappa B p65 subunit, we showed by Western-blotting that addition of exogenous NMDA during 10 or 20 min to RAW 264.7 cells greatly enhances nuclear translocation of NF-kappa B (174 and 341% of control, respectively) (Fig. 5A,B). When RAW 264.7 cells were previously treated with MK801, nuclear translocation of NF-kappa B induced by a 10 or 20 min NMDA treatment was strongly inhibited (83 and 21% of inhibition, respectively) (Fig. 5A,B). RankL, a known activator of NF-kappa B used as a positive control, caused nearly five times stimulation of nuclear translocation of p65 (Fig. 5A,B).

To confirm these results, we carried out immunofluorescence experiments in RAW 264.7 cells stimulated with glutamate using the same antibody against p65 protein labeled with cyanine. In untreated control cells (Fig. 6), p65 protein expression revealed by the red labeling was mainly observed in the cytoplasm, although some labeling was also shown in the nucleus,

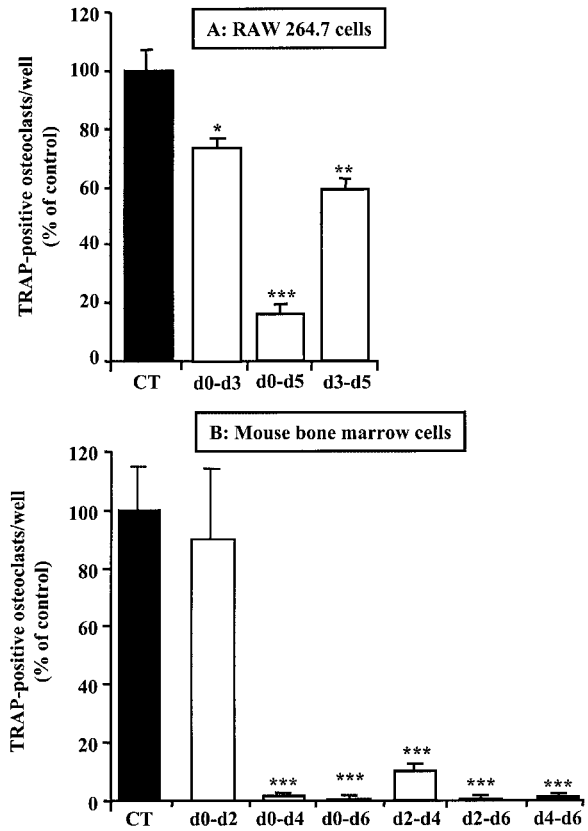

Fig. 4. MK801 preferentially inhibits the late stages of osteoclast differentiation. RAW 264.7 cells (**A**) and mouse bone marrow cells (**B**) were grown in αMEM in the presence of RankL, with or without MK801 added all along the differentiation process (d0–d6) or for 2–4 days pulses at different stages of cell differentiation. At the end of the differentiation sequence, osteoclasts were stained for TRAP and the number of TRAP-positive multinucleated osteoclasts (>3 nuclei) was counted in each condition. Results, expressed as percentage of control, are mean ± SEM of two independent experiments, each performed in quadruplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (vs. untreated cells, by Mann–Whitney U test)

confirming the presence of NF-kappa B activity in nuclei of untreated cells as observed using Western-blotting (Fig. 5A,B). Treatment with glutamate (1 mM) for 10 min increased translocation of p65 immunoreactivity to the nucleus, while cytoplasmic p65 labeling was greatly reduced or absent. p65 labeling was still concentrated in the nuclei after 20, 30, and 60 min of glutamate treatment with a progressive return of p65 to the cytosol after 60 min (Fig. 6). Treatment of RAW 264.7 cells with RankL (100 ng/ml), used as a positive control, induced a stimulation of nuclear labeling of p65 (Fig. 6) while no labeling was observed with non specific control immunoglobulins (data not shown).

DISCUSSION

The present study shows for the first time that osteoclast precursors, like mature osteoclasts, express NMDAR. We used RAW 264.7 cells and mouse bone marrow cells to investigate NMDAR expression during osteoclast differentiation. RAW 264.7 cell line is an homogenous population of mouse macrophage/monocytes equivalent to mononuclear osteoclast precursors that can be induced with RankL to form osteoclast-like cells that express a number of osteoclast-specific markers [Battaglino et al., 2002]. This is confirmed in our study by an increased expression with RAW 264.7 cell differentiation of CTR and TRAP mRNA, two markers of the osteoclast lineage. By contrast, bone marrow contains many different cell types, including in its adherent fraction precursors of the monocytic/macrophagic lineage from which osteoclasts originate. The adherent cell population cultured for 2 days with RankL already expressed TRAP and CTR mRNA, confirming the presence of the osteoclast lineage in this population of cells that are more mature than RAW 264.7 cells. We showed that RAW 264.7 cells and bone marrow cells express NR1 together with NR2A, 2B, and 2D mRNA. Although we cannot exclude in our heterogenous bone marrow cultures a possible amplification of cDNA from cells different from the osteoclast lineage, the fact that RAW 264.7 cells express identical NR2 subunits to bone marrow cells supports the specificity of these results. Expression of NR2A might refer to a species specificity since, in contrast to mature rabbit osteoclasts and human osteoclast-like cells [Itzstein et al., 2001], this subunit is present in osteoclasts differentiated from RAW and mouse bone marrow cells. We did not find expression of NR2C subunit mRNA in the osteoclast lineage while it is present in osteoblasts [Gu and Publicover, 2000]. The presence of mRNA for several NR2 subunits suggests that different subtypes of NMDAR composed of NR1/NR2A, NR1/NR2B, or NR1/NR2D might be expressed by the osteoclast lineage, as demonstrated for neuronal cells. It is also possible that osteoclast precursors express heteromeric NMDAR composed of NR1 associated with more than one type of NR2 subunit or coexpress different subtypes of NMDAR, as previously reported for neuronal cells [Chazot and Stephenson, 1997; Plant et al., 1997; Dunah et al., 1998].

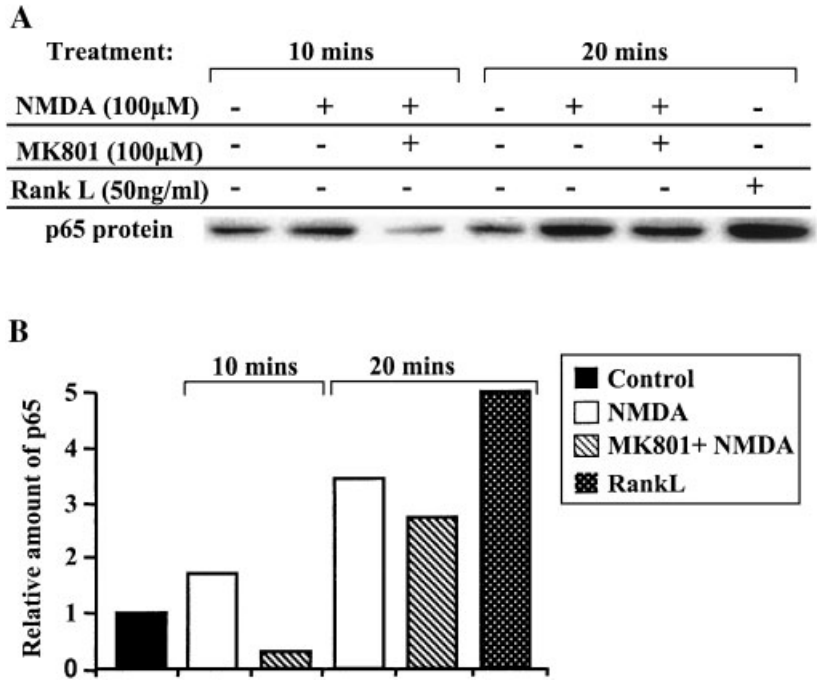

Fig. 5. NMDAR activation induces nuclear translocation of NF-kappa B in RAW 264.7 cells. **A**: RAW cells were stimulated with *N*-methyl-D-aspartate (NMDA) (100 μM) and glycine (100 μM) for 10 or 20 min, with or without a 10 min pretreatment with MK801 (100 μM). Nuclear extracts were prepared, electrophoresed, and analyzed by Western-blotting with an antibody directed against the p65 subunit of NF-kappa B. Stimulation by RankL (50 ng/ml) was used as a positive control. **B**: Graph showing the relative amount of NF-kappa B expression, obtained from the immunoreactive bands of **panel A** using ImageQuant v1.2 software analysis. The data illustrate one representative experiment.

The functional properties of these different NMDA receptor channels vary as they are related to the nature of the NR2 subunits involved in the complex [Cull-Candy et al., 2001].

We demonstrated that mRNA for NR1 and NR2 subunits are expressed all along the differentiation process from the non differentiated precursors to mature osteoclasts. Semi-quantitative RT-PCR analysis did not show any regulation of these subunits expression during the differentiation sequence. However, this absence of NMDAR subunit mRNA regulation does not exclude a modulation at the protein expression level. In brain, control of NMDAR number and composition has been demonstrated both at transcriptional and translational levels [Monyer et al., 1994; Huh and Wenthold, 1999; Cull-Candy et al., 2001]. Unfortunately, we could not investigate expression of NR1 and NR2 subunit proteins during osteoclastogenesis due to a lack of good specific antibodies directed against those subunits. Rapid modulation of NMDAR cell surface expression may also be regulated by the turnover rate of NMDAR subunits [Huh and Wenthold, 1999].

The demonstration of NMDAR on osteoclast precursors suggests that, besides its effect on bone resorption [Chenu et al., 1998; Itzstein et al., 2000], glutamate may also have a direct effect on osteoclastic differentiation. This is supported by our results that clearly showed that two specific NMDAR channel blockers inhibit osteoclastic differentiation from RAW 264.7 cells and bone marrow precursors. This is not the first demonstration that NMDA glutamate receptor function is important for osteoclast development since it has been previously shown that MK801 prevents bone resorption by inhibiting osteoclastic differentiation in a coculture model of bone marrow cells and mouse osteoblasts [Peet et al., 1999]. In this study, the authors have shown evidence that NMDAR function is critical during osteoclastogenesis while less important in regulating mature osteoclast activity. However, they did not discriminate between direct effects of MK801 mediated by receptors on osteoclast precursors or indirect ones through receptors expressed by osteoblasts that are present in their cultures and necessary to induce osteoclastogenesis. Our results, which clearly demonstrate that

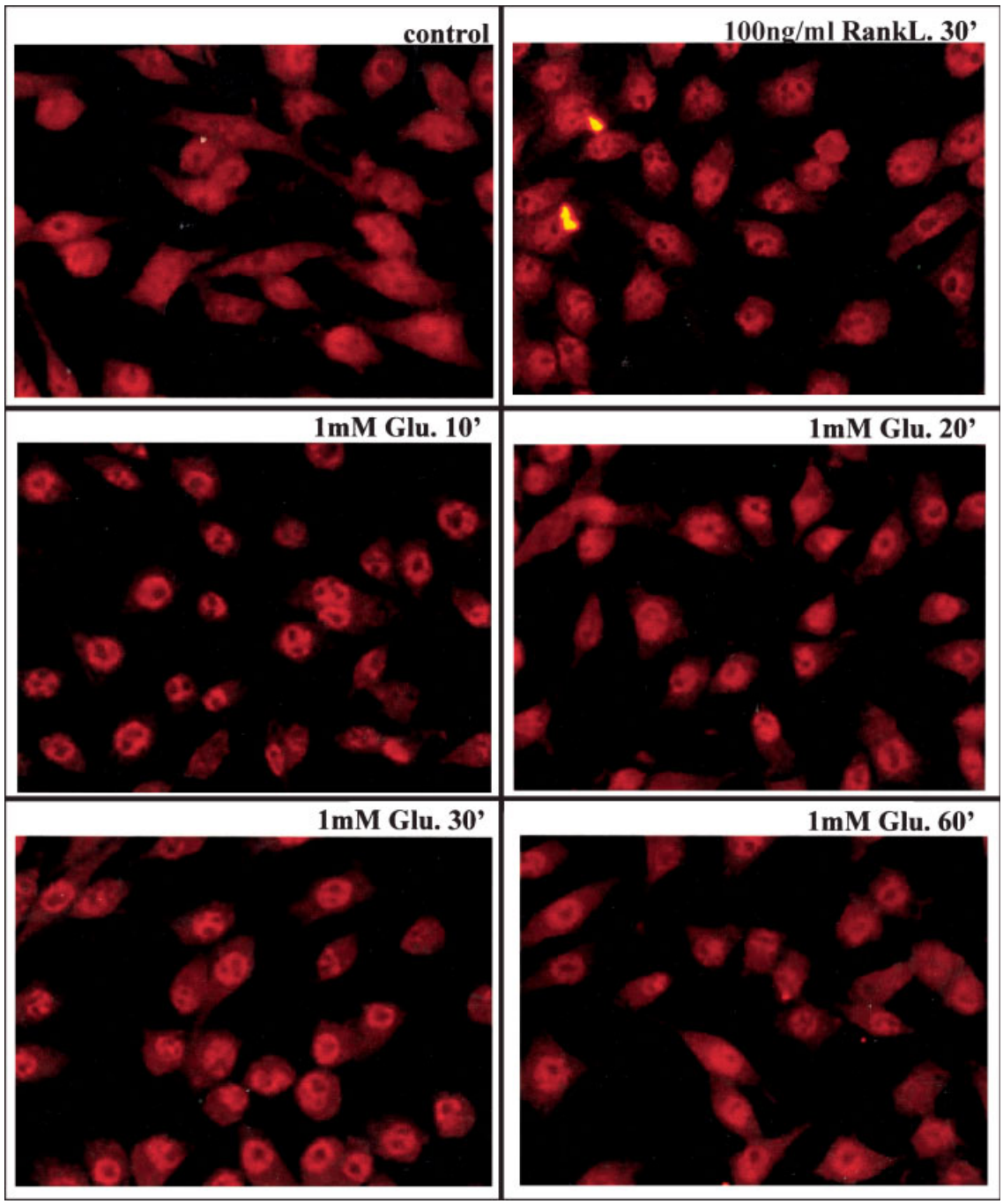

Fig. 6. Glutamate induces nuclear translocation of NF-kappa B in RAW 264.7 cells. RAW 264.7 cells were immunostained with an antibody against NF-kappa B p65 protein and detected using a secondary antibody labeled with cyanine 3 (red fluorescence). In control conditions, p65 expression was mainly localized in the cytoplasm. A 10 min (10′) treatment with 1 mM glutamate increased nuclear translocation of p65. Nuclear expression of p65 was still concentrated in the nuclei after 20, 30, or 60 min of glutamate treatment with a progressive return to the cytosol after 60′. Treatment with RankL (100 ng/ml, 30 min) was used as a positive control to illustrate nuclear translocation of p65.

osteoclast precursors express NMDAR and that MK801 inhibits osteoclastogenesis in RAW 264.7 cells, are in favor of a direct effect of glutamate on osteoclast precursors. Indeed, if we cannot exclude in bone marrow cells, the presence of other cells expressing NMDAR [Genever et al., 1999], it is not true for the homogenous RAW cells, therefore our results a direct action of antagonists on osteoclastic precursors. We showed that antagonists of

NMDAR inhibit osteoclastogenesis only when added from day 2 of 5 or 6 days cultures, suggesting that NMDAR are involved in the late stages of the differentiation process, maybe in the fusion of mononucleated precursors in multinucleated mature osteoclasts. By contrast, in their coculture model, Peet et al. [1999] showed that MK801 inhibits osteoclast formation even when added only for the first 2 days of the 11-day culture. These discrepancies might be explained by the presence in their coculture model of other cells that express NMDAR, which could indirectly regulate osteoclast formation. In our models, NBQX an AMPA receptor antagonist had no effect on RAW 264.7 cell differentiation while it showed a slight inhibitory effect on bone marrow cell differentiation at high concentrations. We cannot exclude an involvement of AMPA receptor in osteoclastogenesis since expression of these receptors was previously demonstrated in bone cells [Chenu et al., 1998; Taylor et al., 2000].

Our present data demonstrate for the first time that NMDAR activation involved in osteoclast formation stimulates NF-kappa B in osteoclast precursors, as demonstrated for neuronal cells [Guerrini et al., 1995; Kaltschmidt et al., 1995]. This transcription factor has been shown to play an essential role in osteoclastogenesis as mice deficient in both p50 and p52 subunits of NF-kappa B are osteopetrotic due to impaired osteoclast differentiation [Franzoso et al., 1997; Iotsova et al., 1997]. Furthermore, RankL-induced osteoclastogenesis is mediated, at least in part, by NF-kappa B signaling [Wei et al., 2001]. Using immunoblotting and immunofluorescence, we showed that NMDA and glutamate, both agonists of NMDAR, lead to a rapid nuclear translocation of NF-kappa B in RAW 264.7 cells, which is inhibited by MK801. The fact that PDTC, a selective and potent inhibitor of NF-kappa B activation, inhibited nuclear translocation of this factor induced by glutamate (data not shown) further supports a role for NF-kappa B in glutamate-mediated signal. Our data do not exclude, however, the involvement of other transcription factors or kinases known to be involved in osteoclastogenesis, such as PU.1, cFos, or c-jun N-terminal kinase [Wang et al., 1992; Tondravi et al., 1997; Hsu et al., 1999]. c-jun N-terminal kinase and cFos were indeed shown to be activated by NMDAR in neuronal cells [Herdegen and Leah, 1998; Ko et al., 1998]. NF-kappa B regulates the expression of numerous genes involved in osteoclastic differentiation, including cytokines [Boyce et al., 1999]. The target genes of NF-kappa B induced by NMDAR activation in osteoclast precursors remain to be determined. It is likely that activation of NF-kappa B translocation induced by NMDAR agonists is Ca^{2+}-dependent, as shown in brain [Ko et al., 1998].

In addition to the previously described role of NMDAR in osteoclast activity, our results provide evidence for a control of osteoclastogenesis by NMDAR, indicating a major role for glutamate in the bone resorption process. This work supports the increasing number of studies published during the past years, which demonstrate that glutamate signaling regulates bone cell differentiation and function. Further work is nevertheless needed to assess the relevance of glutamate-mediated processes in bone in vivo and to consider them as possible targets for the development of new therapeutics to treat bone pathologies.

ACKNOWLEDGMENTS

We thank Amgen (Thousands Oaks, CA) for the kind gift of RankL and Dr. L. Malaval for helpful comments on this project.

REFERENCES

Battaglino R, Kim D, Fu J, Vaage B, Fu XY, Stashenko P. 2002. c-myc is required for osteoclast differentiation. J Bone Miner Res 17:763–773.

Bhangu PS, Genever PG, Spencer GJ, Grewal TS, Skerry TM. 2001. Evidence for targeted vesicular glutamate exocytosis in osteoblasts. Bone 29:16–23.

Boyce BF, Xing L, Franzoso G, Siebenlist U. 1999. Required and nonessential functions of nuclear factor-kappa B in bone cells. Bone 25:137–139.

Chazot PL, Stephenson FA. 1997. Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: Direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. J Neurochem 69:2138–2144.

Chenu C, Serre CM, Raynal C, Burt-Pichat B, Delmas PD. 1998. Glutamate receptors are expressed by bone cells and are involved in bone resorption. Bone 22:295–299.

Church GM, Gilbert W. 1984. Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995.

Cull-Candy S, Brickley S, Farrant M. 2001. NMDA receptor subunits: Diversity, development, and disease. Curr Opin Neurobiol 11:327–335.

Dunah AW, Luo J, Wang YH, Yasuda RP, Wolfe BB. 1998. Subunit composition of *N*-methyl-D-aspartate receptors in the central nervous system that contain the NR2D subunit. Mol Pharmacol 53:429–437.

Espinosa L, Itzstein C, Cheynel H, Delmas PD, Chenu C. 1999. Active NMDA glutamate receptors are expressed by mammalian osteoclasts. J Physiol 518:47–53.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U. 1997. Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 11:3482–3496.

Genever PG, Skerry TM. 2001. Regulation of spontaneous glutamate release activity in osteoblastic cells and its role in differentiation and survival: Evidence for intrinsic glutamatergic signalling in bone. Faseb J 15:1586–1588.

Genever PG, Wilkinson DJ, Patton AJ, Peet NM, Hong Y, Mathur A, Erusalimsky JD, Skerry TM. 1999. Expression of a functional *N*-methyl-D-aspartate-type glutamate receptor by bone marrow megakaryocytes. Blood 93: 2876–2883.

Gu Y, Publicover SJ. 2000. Expression of functional metabotropic glutamate receptors in primary cultured rat osteoblasts. Cross-talk with *N*-methyl-D-aspartate receptors. J Biol Chem 275:34252–34259.

Gu Y, Genever PG, Skerry TM, Publicover SJ. 2002. The NMDA type glutamate receptors expressed by primary rat osteoblasts have the same electrophysiological characteristic as neuronal cells. Calcif Tissue Int 70:194–203.

Guerrini L, Blasi F, Denis-Donini S. 1995. Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons in vitro. Proc Natl Acad Sci USA 92: 9077–9081.

Herdegen T, Leah JD. 1998. Inducible and constitutive transcription factors in the mammalian nervous system: Control of gene expression by Jun, Fos and, Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28:370–490.

Hollmann M, Heinemann S. 1994. Cloned glutamate receptors. Annu Rev Neurosci 17:31–108.

Hsu H, Lacey D, Dunstan CR. 1999. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 96:3540–3545.

Huh KH, Wenthold RJ. 1999. Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the *N*-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. J Biol Chem 274:151–157.

Inoue D, Shih C, Galson DL, Goldring SR, Horne WC, Baron R. 1999. Calcitonin-dependent down-regulation of the mouse C1a calcitonin receptor in cells of the osteoclast lineage involves a transcriptional mechanism. Endocrinology 140:1060–1068.

Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. 1997. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 3:1285–1289.

Itzstein C, Espinosa L, Delmas PD, Chenu C. 2000. Specific antagonists of NMDA receptors prevent osteoclast sealing zone formation required for bone resorption. Biochem Biophys Res Commun 268:201–209.

Itzstein C, Cheynel H, Burt-Pichat B, Merle B, Espinosa L, Delmas PD, Chenu C. 2001. Molecular identification of NMDA glutamate receptors expressed in bone cells. J Cell Biochem 82:134–144.

Jimi E, Ikebe T, Takahashi N, Hirata M, Suda T, Koga T. 1996. Interleukin-1 alpha activates an NF-kappaB-like factor in osteoclast-like cells. J Biol Chem 271:4605–4608.

Kaltschmidt C, Kaltschmidt B, Baeuerle PA. 1995. Stimulation of ionotropic glutamate receptors activates transcription factor NF-kappa B in primary neurons. Proc Natl Acad Sci USA 92:9618–9622.

Ko HW, Park KY, Kim H, Han PL, Kim YU, Gwag BJ, Choi EJ. 1998. Ca2+-mediated activation of c-Jun N-terminal kinase and nuclear factor kappa B by NMDA in cortical cell cultures. J Neurochem 71:1390–1395.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess R, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Dalaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Laketic-Ljubojevic I, Suva LJ, Maathuis FJ, Sanders D, Skerry TM. 1999. Functional characterization of *N*-methyl-D-aspartic acid-gated channels in bone cells. Bone 25:631–637.

Mason DJ, Suva LJ, Genever PG, Patton AJ, Steuckle S, Hillam RA, Skerry TM. 1997. Mechanically regulated expression of a neural glutamate transporter in bone: A role for excitatory amino acids as osteotropic agents? Bone 20:199–205.

Monyer H, Burnashev N, Laurie DJ, Sakman B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neurons 12:529–540.

Ozaki K, Takeda H, Iwahashi H, Kitano S, Hanazawa S. 1997. NF-kappaB inhibitors stimulate apoptosis of rabbit mature osteoclasts and inhibit bone resorption by these cells. FEBS Lett 410:297–300.

Patton AJ, Genever PG, Birch MA, Suva LJ, Skerry TM. 1998. Expression of *N*-methyl-D-aspartate-type receptor by human and rat osteoblasts and osteoclasts suggests a novel glutamate signaling pathway in bone. Bone 22: 645–649.

Peet NM, Grabowski PS, Laketic-Ljubojevic I, Skerry TM. 1999. The glutamate receptor antagonist MK801 modulates bone resorption in vitro by a mechanism predominantly involving osteoclast differentiation. FASEB J 13:2179–2185.

Plant T, Schirra C, Garaschuk O, Rossier J, Konnerth A. 1997. Molecular determinants of NMDA receptor function in GABAergic neurones of rat forebrain. J Physiol 499:47–63.

Raynal C, Delmas PD, Chenu C. 1996. Bone sialoprotein stimulates in vitro bone resorption. Endocrinology 137: 2347–2354.

Reddy SV, Hundley JE, Windle JJ, Alcantara O, Linn R, Leach RJ, Boldt DH, Roodman GD. 1995. Characterization of the mouse tartrate-resistant acid phosphatase (TRAP) gene promoter. J Bone Miner Res 10:601–606.

Rothwarf DM, Karin M. 1999. The NF-kappaB activation pathway: A paradigm in information transfer from membrane to nucleus. Sci STKE 5:621–663.

Serre CM, Farley D, Delmas PD, Chenu C. 1999. Evidence for a dense and intimate innervation of the bone tissue, including glutamate-containing fibers. Bone 25:623–629.

Sullivan JM, Traynelis SF, Chen HS, Escobar W, Heinemann SF, Lipton SA. 1994. Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. Neuron 13:929–996.

Taylor AF, Brabbs AC, Peet NM, Laketic-Ljubojevic I, Dobson KR, Skerry TM. 2000. Bone formation/resorption and osteoblast/adipocyte plasticity mediated by AMPA/Kainate receptors in vitro and in vivo. J Bone Miner Res 15(Suppl 1):S275.

Tondravi MM, McKercher SR, Anderson K, Erdman JM, Quiroz M, Maki R, Teitelbaum SL. 1997. Osteopetrosis in mice lacking hematopoietic transcription factor PU. 1. Nature 386:81–84.

Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, Wagner EF. 1992. Bone and hematopoietic defects in mice lacking c-fos. Nature 360:741–745.

Wei S, Teitelbaum SL, Wang MW, Ross FP. 2001. Receptor activator of nuclear factor-kappa B ligand activates nuclear factor-kappa b in osteoclast precursors. Endocrinology 142:1290–1295.

Yasuda H, Shima N, Nakagawa K, Yamaguchi K, Kinosaki M, Moshizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602.