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Specific Antagonists of NMDA Receptors Prevent Osteoclast Sealing Zone Formation Required for Bone Resorption

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***N*-Methyl-D-aspartate (NMDA) glutamate receptors, widely distributed in the nervous system, have recently been identified in bone. They are expressed and are functional in osteoclasts. In the present work, we have studied the effects of specific antagonists of NMDA receptors on osteoclast activation and bone resorption. Using an *in vitro* assay of bone resorption, we showed that several antagonists of NMDA receptors binding to different sites of the receptor inhibit bone resorption. Osteoclast activation requires adhesion to the bone surface, cytoskeletal reorganization and survival. We demonstrated by autoradiography that the specific NMDA receptor channel blocker, MK 801, binds to osteoclasts. This antagonist had no effect on osteoclast attachment to bone and did not induce osteoclast apoptosis. In contrast, MK 801 rapidly decreased the percentage of osteoclasts with actin ring structures that are associated with actively resorbing osteoclasts. These results suggest that NMDA receptors expressed by osteoclasts may be involved in adhesion-induced formation of the sealing zone required for bone resorption.** © 2000 Academic Press

Glutamate (Glu) is the predominant excitatory amino acid in the central and peripheral nervous system. This neurotransmitter participates in neuronal plasticity, memory, learning and motricity through its action on two types of membrane receptors, ionotropic receptors which are gated ion channels and metabotropic receptors coupled to protein G (1, 2). Three subtypes of ionotropic glutamate receptors (GluR) have been classified according to their activation by specific agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (2, 3).

Several studies have documented the expression of different subtypes of GluR and Glu transporters by bone cells (4–6). Furthermore, we have recently demonstrated that NMDA receptors (NMDAR) present on osteoclasts are active (7) and that blocking their function leads to an inhibition of bone resorption *in vitro* (4), suggesting a new mechanism for regulating osteoclast activity.

Osteoclasts are multinucleated and polarized cells responsible for bone resorption (8). Osteoclast activation is dependent on their adhesion to bone surface and their subsequent cytoskeletal reorganization required for bone resorption (9–12). Upon activation, the osteoclast cytoskeleton structures called podosomes become concentrated at the periphery of the cell, leading to the formation of an actin ring structure which is a prerequisite for the formation of the sealing zone (13). This specialized cell-extracellular matrix attachment domain that seals off the osteoclastic bone-resorbing compartment is only present in osteoclasts during their resorption phase and actin ring formation is considered to be a marker of osteoclast activation. Survival and apoptosis of osteoclasts represent another mechanism by which bone resorption could be regulated (14–20).

In this study, we wanted to clarify the role of NMDAR in osteoclast activation and bone resorption. Using an *in vitro* model for isolating functional rabbit osteoclasts, we have tested several specific NMDAR antagonists on bone resorption and studied the effects of one of them, MK 801, on the different steps leading to osteoclast activation: adhesion to bone, cytoskeletal reorganization and survival. We showed that MK 801, which inhibits bone resorption *in vitro,* has no effect on osteoclast adhesion and does not induce osteoclast apoptosis, while it rapidly decreases the percentage of osteoclasts with actin ring. These results suggest that NMDAR expressed by osteoclasts may be involved in adhesion-induced formation of the sealing zone required for bone resorption.

MATERIALS AND METHODS

Materials. Medium 199 (M199), α Minimal Essential Medium (αMEM), penicillin, streptomycin and HEPES buffer were purchased from Life Technologies (Grand Island, NY). Sterile culture plastic-

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ware were obtained from Falcon Becton Dickinson (Le Pont de Claix, France). Acid hematoxylin, toluidine blue, sodium borate, the acid phosphatase kit (procedure 386), Bovine Serum Albumin (BSA) and the polyclonal antibody directed against actin were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France). 3(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from ICN Biomedicals Inc. (Aurora, OH). Elephant ivory was kindly donated by the Musée Guimet (Lyon, France). Antagonists of NMDA GluR were purchased from Tocris Cookson (Bristol, UK) and the [^{3}H]-MK 801 was obtained from NEN Life Science products (Boston, MA). Alendronate was given by MSD (West Point, PA).

Osteoclast isolation and resorption assay. Bovine cortical bone slices or ivory slices were cut with an Isomet low speed saw (Buehler, Lake Bluff, IL), cleaned by ultrasonication, and incubated during 2 h before the experiment in M199 containing 10% heat-inactivated FCS.

Osteoclasts were isolated from long bones of 1-day-old New Zealand rabbits. Bones were removed, cleaned of soft tissues and bone marrow, split and scraped into M199, 10% FCS, 20 mM HEPES, 100 IU penicillin/streptomycin. Cells were centrifuged at low speed and this heterogenous cell population containing the osteoclasts was seeded onto cortical bone slices in 96-well plates for 1 h 30 at 37°C, 5% CO_2. After this adhesion period, nonadherent cells were removed, cultures were washed, and the remaining cells were incubated for 24 h with antagonists of NMDAR [D-AP5, MK 801, 1-(1,2-diphenylethyl)piperidine (DEP), L-689,560, Arcaine sulphate] in αMEM (as supplied contains 0.5 mM glutamate and 0.66 mM glycine), 2% FCS, 100 IU penicillin/streptomycin at pH 7.2. At the end of the culture, cells were removed with 1 M NaOH, and the number of resorption pits was scored under a light microscope after staining of the bone slices with acid hematoxylin for 3 min and then with 1% (wt/vol) toluidine blue in 1% (wt/vol) sodium borate for 30 s.

Autoradiography of [^{3}H]-MK 801 binding. The cell population containing the osteoclasts was plated onto glass coverslips for one night. Nonadherent cells were removed and the remaining cells were treated with 10^{-7} M [^{3}H]-MK 801, with or without an excess of 100 times of unlabeled MK 801, in αMEM containing 0.1% BSA during 1 h at room temperature. The cells were then washed four times with cold αMEM and fixed for 10 min in 0.1 M sodium cacodylate buffer (pH 7,4) containing 1% formaldehyde and 1% glutaraldehyde. Coverslips were washed with water, allowed to dry, mounted onto glass microscope slides and stored at 4°C for a night. In the dark, coverslips were dipped in K5 emulsion (Ilford, Saint Priest, France) diluted (50:50) in water, dried and stored in dark boxes at 4°C for 6 weeks. Following development, coverslips were counterstained with hematoxylin for cell observation. To evaluate the percentage of labelled osteoclasts, the number of silver grains was counted in more than 10 osteoclasts and 10 mononucleated cells for each coverslip. Cell-bound radioactivity was determined after substraction of the background radioactivity.

Osteoclast attachment to bone. Cells isolated from newborn rabbit long bones, as described above, were plated onto ivory slices and cultured during 24 h with or without 10^{-4} M MK 801. At the end of the culture, cells were washed with Phosphate Buffer Saline (PBS), fixed and stained for tartrate-resistant acid phosphatase (TRAP). Multinucleated TRAP-positive osteoclasts were scored under a light microscope.

Cell viability assay. Cell viability was determined by a colorimetric MTT assay, based on the conversion by a mitochondrial dehydrogenase of the tetrazolium salt to a formazan product, measured at an absorbance of 570 nm (21). Briefly, the cell population containing the osteoclasts was cultured during 24 h with or without 10^{-4} or 5×10^{-5} M MK 801. At the end of the culture, each culture well received 10 μl of MTT at 5 mg/ml for 4 h. 100 μl of lysis buffer (10% SDS in 0.01 N HCl) were then added to each well and the plates were kept overnight at 37°C to allow complete dissolution of the reaction product. Absorbance of the solubilized MTT formazan products was read at 570 nm on a Dynatech MR7000 ELISA plate reader (Dynatech, Guernesey, UK). Results were expressed as the percentage of optical density, assuming absorbance of control untreated cells as 100%.

Osteoclast apoptosis. Rabbit osteoclasts, isolated as previously described, were plated onto glass coverslips in the presence or absence of 10^{-4} M MK 801 during 24 h. Osteoclast apoptosis was detected using Hoechst 33258 (Sigma) staining. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min, then stained with 1% (wt/vol) methylene blue and Hoechst 33258 (10 μg/ml). Cells were examined under a fluorescence microscope (Polyvar, Reichert, Germany) and the number of multinucleated osteoclasts showing chromatin condensation and/or nuclear fragmentation was assessed. Another method used for detection of osteoclast apoptosis was the Klenow *in situ* apoptosis detection kit, based on the TUNEL assay principle (Genzyme Diagnostics, Cambridge, MA). Biotinylated nucleotides were incorporated into the 3′-OH ends of the DNA fragments by the Klenow fragment of DNA polymerase I in apoptotic cells and were visualized using a streptavidin-horseradish peroxidase staining. Cells were counterstained with 1% methyl green solution and observed under a light microscope. In both assays, more than 100 osteoclasts were scored on each coverslip to measure the incidence of apoptosis. Osteoclasts treated with 10^{-4} M alendronate or 10^{-2} M calcium to induce their apoptosis were used as positive controls.

Actin staining in osteoclasts and confocal microscopy. After the adhesion period of 90 min, isolated rabbit osteoclasts plated onto glass coverslips or ivory slices were cultured without or with MK 801 at concentrations of 10^{-4} M or 5×10^{-5} M, in αMEM containing 10% FCS, for different times (5, 10, 30 min, 1, 4, 8 and 24 h). Cells were then fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 100% cold methanol for 5 min. After rinsing in PBS, cells were incubated with Tris Buffer Saline (TBS) containing 3% BSA to block nonspecific antigen sites. Cells were then incubated for 1 h at room temperature with a polyclonal antibody directed against actin (dilution 1/100) or with purified rabbit immunoglobulins as control, diluted in TBS containing 0.05% BSA. The cells were rinsed three times in TBS and incubated with the anti-rabbit second antibody conjugated with cyanine (dilution 1/500) for 1 h at room temperature. Slices and coverslips were mounted in Mowiol (Hoechst, Frankfurt, Germany). Osteoclasts were visualized using a laser scanning confocal microscope (Laser Scan Microscope, Zeiss, Germany). For each condition, the total number of osteoclasts by slice or coverslip was scored (over 100) and the percentage of osteoclasts showing an actin ring was determined.

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

RESULTS

NMDAR-Specific Antagonists Inhibit in Vitro Bone Resorption

Several specific antagonists of NMDAR that bind to different sites of the receptor (ligand, glycine, channel and polyamine sites) have been tested in an *in vitro* assay of bone resorption by rabbit osteoclasts. D-AP5 which binds to the ligand site, L-689,560 which acts on the glycine coactivation site of the receptor, and MK 801 and DEP that are specific channel blockers, all dose-dependently reduced the number of resorption lacunae on cortical bone slices compared to control (Fig. 1). In contrast, Arcaine sulphate, an antagonist of the modulatory polyamine site, had no effect on bone resorption. DEP and MK 801 were the most powerful inhibitors in this model. These results demonstrate

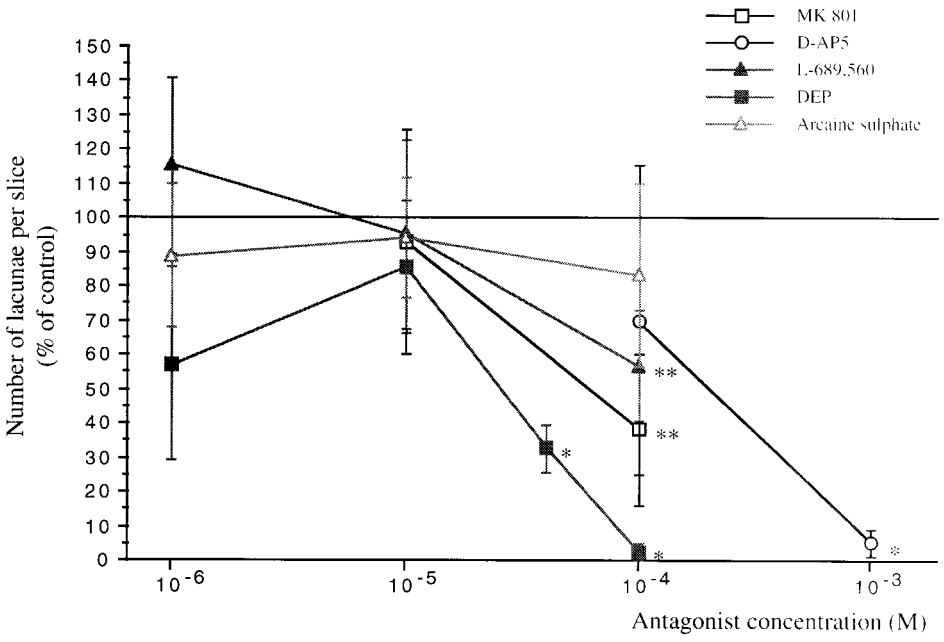

FIG. 1. Effect of different specific NMDAR antagonists on *in vitro* bone resorption. Five antagonists of NMDAR (MK 801; D-AP5; DEP; L-689-560; Arcaine sulphate) were added at different concentrations (10^{-3} M to 10^{-6} M) to rabbit osteoclasts cultured during 24 h onto cortical bone slices, and the number of resorption lacunae per slice was scored. Results expressed as percentage of control are means ± SD of three independent experiments, each performed in quadruplicate. $*P < 0.05$; $**P < 0.01$ vs untreated control.

that it is possible to inhibit *in vitro* bone resorption by blocking different sites of NMDAR.

The Antagonist of NMDAR, MK 801, Binds to Osteoclasts

Previous results have demonstrated that osteoclasts, osteoblasts and their precursors express NMDAR (4, 5). To determine if NMDAR antagonists inhibit bone resorption by acting directly on osteoclasts or indirectly through an action on other cells present in the culture, we examined the binding of [^{3}H]-MK 801 to the cell population isolated from rabbit long bones. Osteoclasts demonstrated a high level of [^{3}H]-MK 801 binding as assessed by autoradiography (Fig. 2A). Quantification of labeled multinucleated osteoclasts and monucleated cells has shown that while most of osteoclasts (>80%) were positive for [^{3}H]-MK 801 binding, only 20% of mononuclear cells present in the culture were labeled. The binding of [^{3}H]-MK 801 to the cells was specific since no labeling was observed in the presence of a 100 times excess of unlabeled MK 801 (Fig. 2B). These data indicate that osteoclasts are the main target of MK 801.

MK 801 Has No Effect on Osteoclast Attachment to Bone nor Cell Survival

To investigate the mechanisms involved in the inhibition of bone resorption induced by MK 801, we have studied the effect of this compound on osteoclast adhesion to bone, cell survival and osteoclast apoptosis. We found no significant difference in the total number of TRAP-positive multinucleated osteoclasts attached to ivory slices after 24 h of culture with or without 10^{-4} M MK 801 (Fig. 3A), indicating that this compound does not inhibit osteoclast attachment to bone. Survival of the total cell population containing the osteoclasts was evaluated by measuring the MTT reduction ability of these cells upon incubation for 24 h with MK 801 at 10^{-4} M or 5×10^{-5} M. Cell survival was not affected by MK 801 treatment for 24 h (Fig. 3B).

We next examined the effect of MK 801 on osteoclast apoptosis using Hoechst 33 258 (Figs. 4A, 4B, 4C) and a TUNEL assay (Figs. 4D, 4E, 4F). Quantification of apoptotic osteoclasts has been performed for each coverslip and the mean percentage of apoptic osteoclasts for each condition is illustrated in Fig. 4G. In control conditions, no osteoclast apoptosis was observed (Figs. 4A, 4D, 4G). Treatment of osteoclasts with MK 801 (10^{-4} M during 24 h) did not induce osteoclast apoptosis (Figs. 4B, 4E, 4G). In contrast, osteoclasts with characteristic changes of nuclear morphology and chromatin condensation were observed when treated with 10^{-4} M alendronate (Figs. 4C, 4F, 4G) or 10^{-2} M calcium (Fig. 4G).

MK 801 Inhibits Actin Ring Formation in Osteoclasts

We have examined the effect of MK 801 on actin ring formation in osteoclasts cultured on glass coverslips or

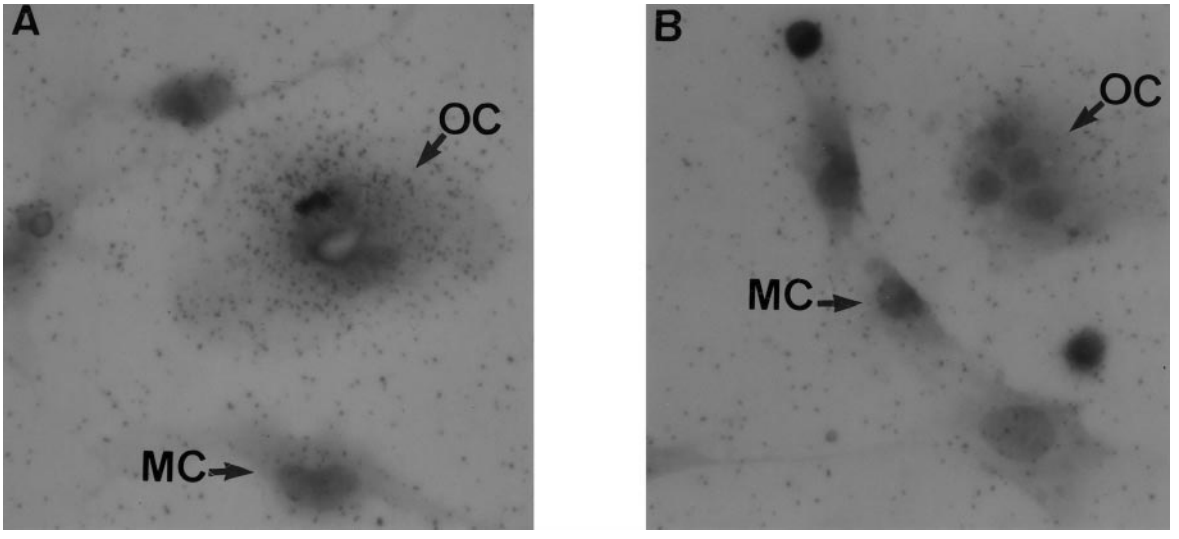

FIG. 2. Autoradiography of [^{3}H]-MK 801 binding to osteoclasts. Isolated osteoclasts were incubated 1 h with 10^{-7} M [^{3}H]-MK 801, without (A) or with (B) a 100 times excess of unlabeled MK 801. Two independent experiments were performed, each in duplicate. After fixation, cells were processed for autoradiography and counterstained with hematoxylin for microscopic observation (original magnification ×400). Numerous silver grains were observed in multinucleated osteoclasts but not in mononucleated cells (A). In the presence of an excess of unlabeled MK 801, no significant labeling was observed in osteoclasts (B).

ivory slices, as visualized with confocal microscopy (Fig. 5A). In control conditions, the percentage of osteoclasts with actin rings increased with time and reached 50% 1 h after osteoclast adhesion. MK 801 at 10^{-4} M induced a rapid inhibition of actin ring formation, as the percentage of osteoclasts showing actin rings decreased by 50% after 10 min of treatment with MK 801 (Figs. 5B, 5C). This decrease was maintained with time and was similar after 24 h of culture with MK 801 (Fig. 5C). Identical results were obtained when osteoclasts were plated onto ivory slices (Fig. 5D). These results demonstrate that MK 801 induces modifications of the actin organization in osteoclasts that may lead to osteoclast inactivation and inhibition of bone resorption.

DISCUSSION

We have previously reported that NMDAR are expressed by bone cells and are involved in *in vitro* bone resorption (4). As shown in this study, several specific antagonists of NMDAR, acting on different sites of this receptor (ligand, glycine and channel sites), inhibit bone resorption *in vitro*. NMDAR activation requires glutamate and glycine (22–24), that are both present in the medium used for osteoclast culture. Fetal calf serum added to cultures also contains glutamate and glycine, indicating that the concentrations of these agonists in the culture medium are largely sufficient to induce NMDAR activation in bone cells. Our results demonstrate that a number of modulators, competitive and noncompetitive antagonists, affect the activation of the receptor in these cells as demonstrated for neuronal cells (25, 26). The antagonist of the polyamine site had however no effect on bone resorption, and this might reflect the presence in the culture medium of large amounts of polyamines, brought by fetal calf serum or secreted by cells. The blockade of NMDAR activation by specific antagonists of the different active sites of the receptor represents a new target for regulating osteoclast activity and bone resorption. Numerous antagonists with therapeutic potential have been developed to block NMDAR activation in neuronal cells (23–26), that might be used to regulate this receptor in bone cells.

The cell population isolated from rabbit long bones is heterogenous and contains osteoblasts, stromal cells and hematopoietic cells, in addition to osteoclasts. We have previously demonstrated that NMDAR are expressed by both osteoclasts and osteoblasts, and possibly their precursors (4). Because many hormones and cytokines regulate bone resorption indirectly through osteoblasts that express receptors for these factors (27), we have determined if the NMDAR antagonist MK 801 was acting directly on osteoclasts. Autoradiography experiments have shown that [^{3}H]-MK 801 binds significantly to 80% of osteoclasts and only to 20% of mononuclear cells present in the culture, indicating that osteoclasts are the principal target of NMDAR antagonists among cells present in this population. These results are in line with the strong expression of NMDAR in osteoclasts (4), and confirm our previous electrophysiological study which shows that NMDAR are functional in mammalian osteoclasts and that their activation is blocked by specific antagonists such as MK 801 (7). A 1000 times lower concentration

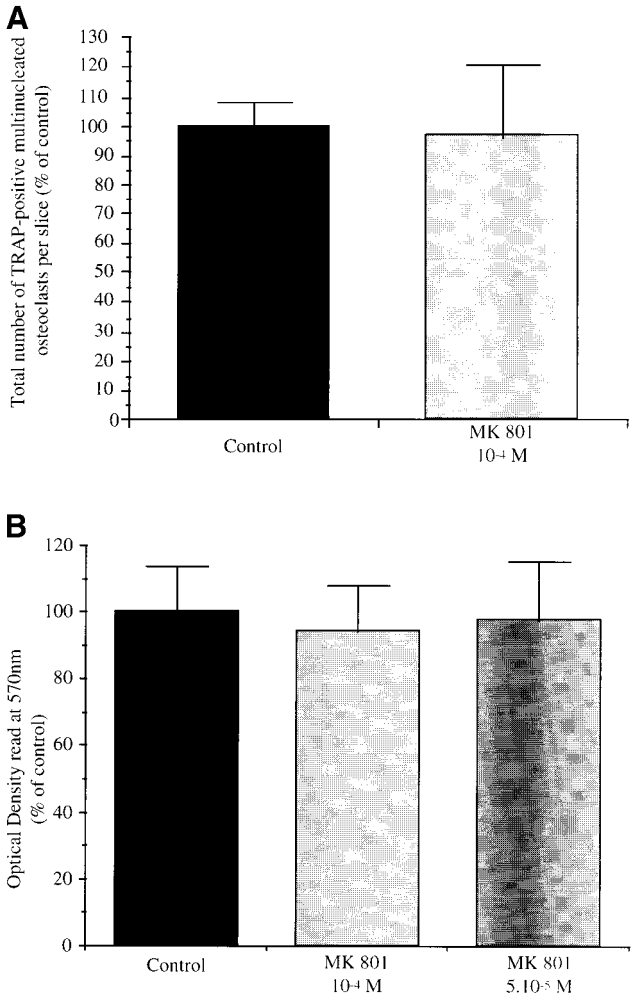

FIG. 3. Effect of MK 801 on osteoclast adhesion to bone and cell survival. (A) Isolated osteoclasts were plated on ivory slices in absence or in presence of 10^{-4} M MK 801. After 24 h of culture, cells were fixed and stained for TRAP, and the number of TRAP-positive multinucleated osteoclasts was scored on each slice. Results expressed as percentage of control are means ± SD of two independent experiments, each performed in quadruplicate. (B) Isolated osteoclasts were cultured for 24 h without or with MK 801 at 10^{-4} M or 5×10^{-5} M. Cell viability was determined using the colorimetric MTT assay. Absorbance of solubilized MTT products was measured at 570 nm and results expressed as percentage of untreated control are means ± SD of 10 wells in two independent experiments.

of [^{3}H]-MK 801 was however sufficient to label osteoclasts in the autoradiography experiment compared to the efficient concentration of this antagonist in bone resorption and electrophysiology experiments. This is probably due to the high sensitivity of the autoradiography technique. A significant labelling of osteoclasts may be obtained with a low number of bound molecules of MK 801 per cell, while inhibition of bone resorption by MK 801 may require a simultaneous activation of almost all NMDAR expressed by osteoclasts. This may also explain the lower percentage of osteoclasts (30%) that respond to MK 801 in our electrophysiological study (7), since a low number of active NMDAR per osteoclast may not allow a measurable response in electrophysiology.

Bone resorption depends on osteoclast attachment to the mineralized matrix, and osteoclast cytoskeletal reorganization and polarization that lead to actin ring formation (8–13). This ring structure corresponds to the sealing zone formation, a specialized adhesion structure responsible for the tight attachment of osteoclasts to bone. Actin ring formation is one indicator of osteoclast functional activity (14, 28, 29). Inhibitors of bone resorption may specifically act on these different stages of osteoclast activation and/or induce osteoclast apoptosis (14–20, 30). To determine the mechanism of action of MK 801 on osteoclasts, we have tested its effect on each step of osteoclast activation and on cell survival. MK 801 had no effect on the number of attached osteoclasts to bone slices, indicating that it does not affect the initial phase of osteoclast adhesion to bone. Survival of the total cell population present in the culture was not affected by MK 801, which did not either induce osteoclast apoptosis, while alendronate and high extracellular calcium concentrations promoted osteoclast apoptosis in our culture model as already shown (31, 32). These data demonstrate that high concentrations of MK 801 have no toxic effect on osteoclasts. In contrast, MK 801 rapidly decreased the percentage of osteoclasts with actin rings, cultured on both glass coverslips and bone slices. Actin ring formation is dependent on matrix substrates, matrix proteins and integrins (33). In our culture conditions, actin ring formation in osteoclasts was identical on both substrates and increased similarly with time of culture. Our results suggest that MK 801 blocks actin ring formation in osteoclasts but may also disrupt actin rings, as observed for osteoclasts cultured 10 min on glass coverslips with MK 801. Various inhibitory agents of osteoclast function have already been shown to disrupt actin ring (14). The decrease of the percentage of osteoclasts with actin rings, observed in the presence of MK 801, is indicative of a role of NMDAR in actin organization and sealing zone formation required for bone resorption.

Native NMDAR consist of one or more NR1 subunits, NR2 subunits, and possibly the more recently identified NR3 subunit, which all contain an extracellular domain, 4 transmembrane domains and an intracellular domain (3, 34, 35). In neuronal cells, several studies have demonstrated biochemical and functional interactions between NMDAR subunits and cytoskeleton proteins (36–38), and similar associations may exist in osteoclasts. Direct interactions between NR1 and NR2 subunits and cytoskeletal proteins such as actin or α-actinin have been documented, as well as indirect ones through specific intracellular proteins such as the family of PDZ-containing proteins (39–42). Recently, several studies have described the interactions of PDZ-

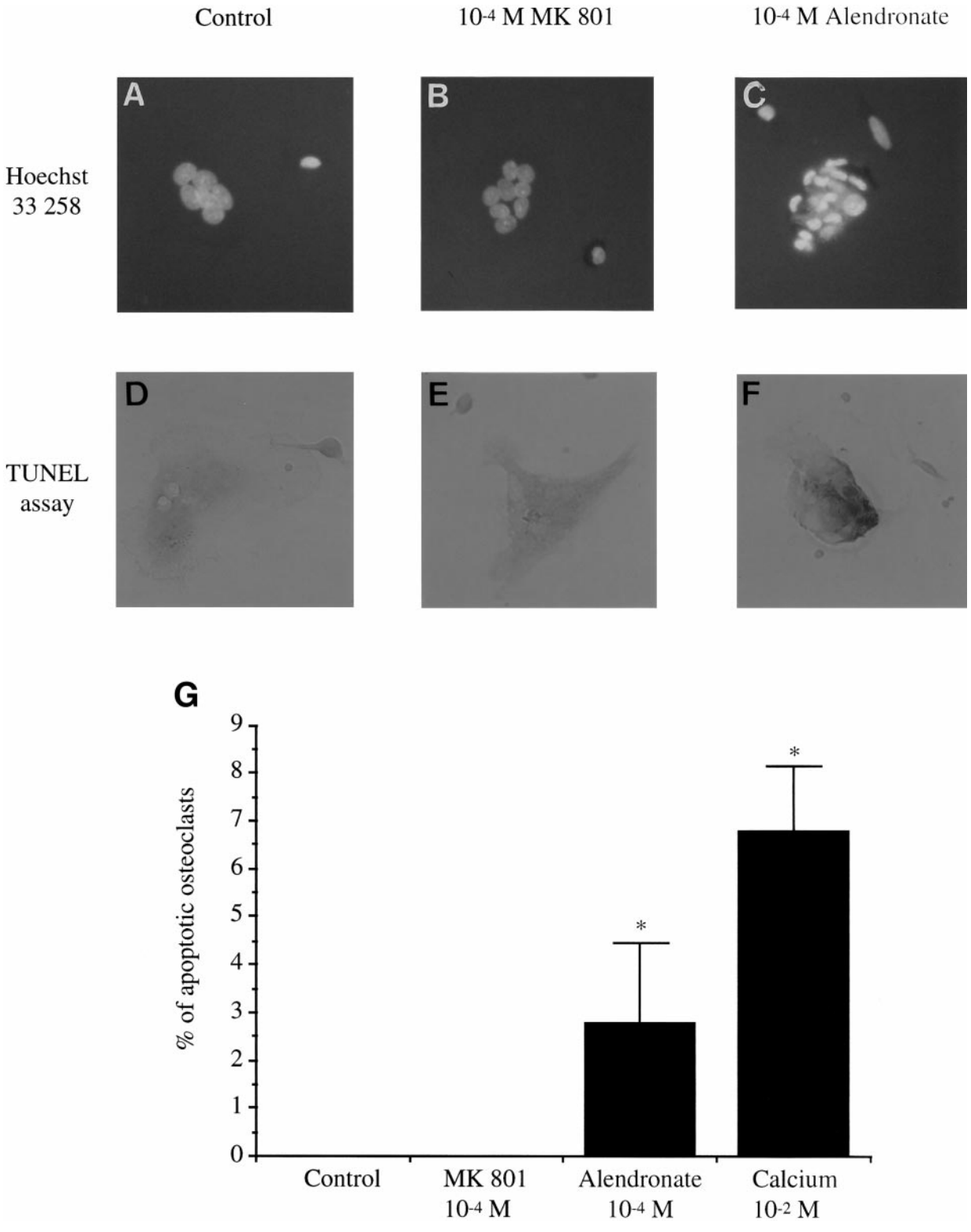

FIG. 4. Effect of MK 801 on osteoclast apoptosis. Osteoclasts were cultured on glass coverslips for 24 h in control conditions (A, D) or in the presence of 10^{-4} M MK 801 (B, E), 10^{-4} M alendronate (C, F) or 10^{-2} M calcium. Osteoclast apoptosis was visualized after Hoechst staining (A, B, C) or by TUNEL assay (D, E, F) (original magnification ×400). (G) The number of apoptotic osteoclasts assessed by Hoechst staining was scored on each coverslip; results are means ± SD of the percentage of apoptotic osteoclasts, scored in two independent experiments performed in quadruplicate. *$P < 0.05$ vs untreated control.

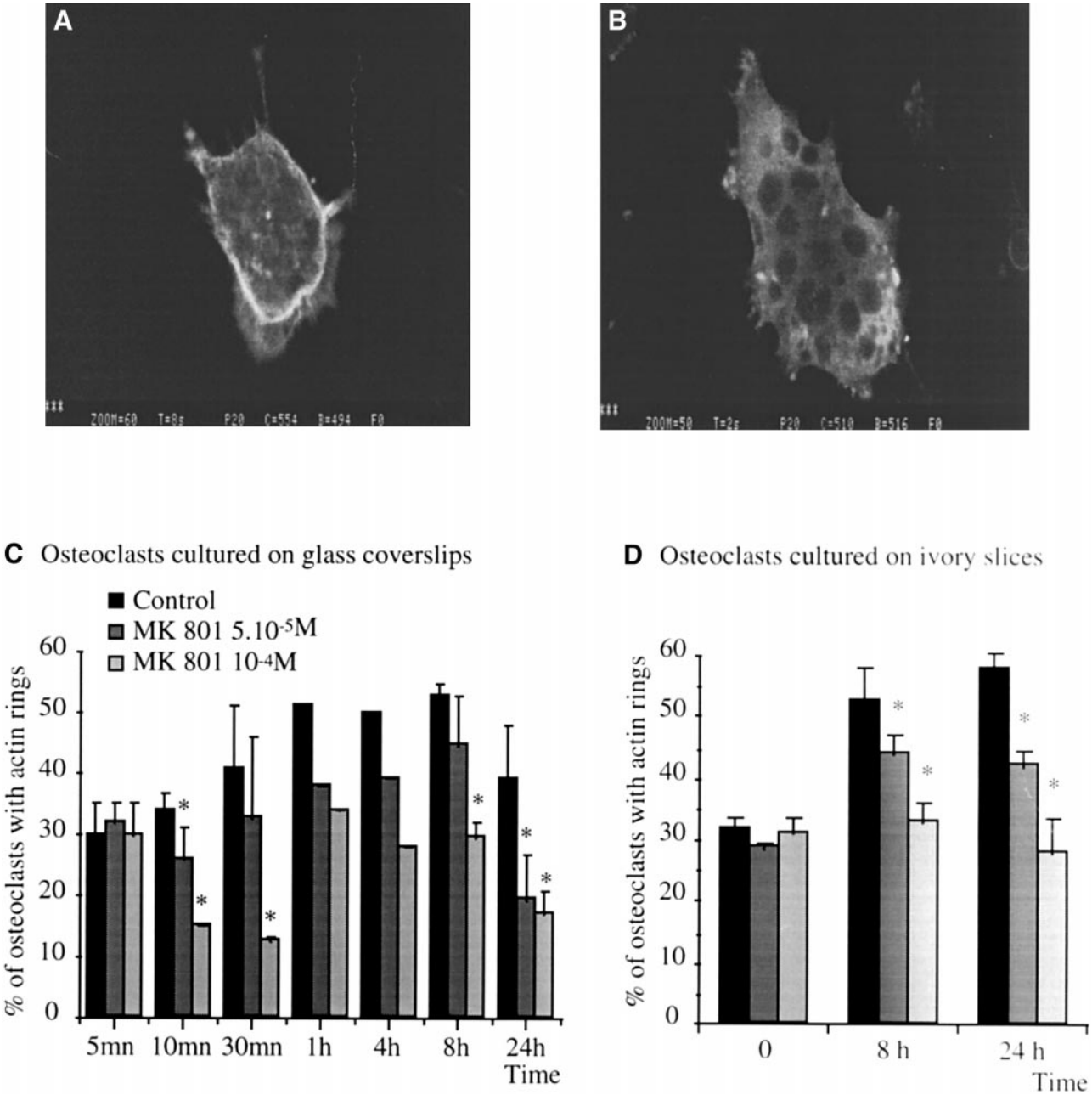

FIG. 5. MK 801 inhibits actin ring formation in osteoclasts. (A, B) After an attachment period of 90 min on glass coverslips, isolated osteoclasts were cultured without (A) or with 10^{-4} M MK 801 (B) for 10 min. Actin rings were detected in osteoclasts using a specific antibody directed against F-actin and observed using confocal microscopy. (C, D) A 24 h kinetic of actin ring formation was performed in osteoclasts cultured on glass coverslips (C) or ivory slices (D), in absence or presence of 10^{-4} M or 5×10^{-5} M MK 801. The percentage of osteoclasts with actin rings was scored. Results are means ± SD of two independent experiments, each performed in duplicate (data for 1 h and 4 h time points represent single observation only). *$P < 0.05$ vs untreated control.

containing proteins (PSD-95/SAP90, chapsyn/PSD-93, SAP102 and CIPP) with NMDAR in the nervous system (42–44). They allow the clustering and anchoring of these receptors in the postsynaptic membrane necessary for synaptic transmission (36, 37). PSD-95 is highly expressed by osteoclasts (our unpublished results), and it is also possible that NMDAR in these cells are directly associated with cytoskeletal proteins and PSD-95.

NMDAR activation has been shown to induce modifications of cytoskeleton organization that are involved in many neuronal cell functions (45). Our results suggest that as for neuronal cells, the NMDA channel activity in osteoclasts may be important for the cy-

toskeletal organization of the cell necessary for its function of bone resorption. NMDAR activation is regulated by protein-tyrosine phosphorylation, and several studies have shown that NMDAR expressed by neuronal cells are regulated by the protein-tyrosine kinase Src (46, 47). Another similarity with the nervous system is that this kinase is also present at high levels in osteoclasts and plays a critical role in the cytoskeleton organization of these cells (48, 49). In Src−/− mice, osteoclasts do not form actin rings and are inactive (48). It would be interesting to investigate whether Src also regulates the activity of NMDAR in osteoclasts, leading to the formation of actin organization in these cells.

Present data demonstrate that in the presence of an antagonist of NMDAR, osteoclasts do not form actin rings and do not resorb bone, suggesting that activation of these receptors may be required for the formation of the sealing zone necessary for bone resorption. The blockade of this function by specific antagonists of different sites of NMDAR, as we showed in this report, could represent a new possibility to develop therapeutics for pathologies associated with an increased bone resorption.

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REFERENCES

1. Mayer, M. L., and Westbrook, G. L. (1987) *Prog. Neurobiol.* **28,** 197–276.
2. Ozawa, S., Kamiya, H., and Tsuzuki, K. (1998) *Prog. Neurobiol.* **54,** 581–618.
3. Hollmann, M. (1996) *in* The Ionotropic Glutamate Receptors (Monaghan, D. T., and Wenthold, R. J., Eds.), pp. 39–79, Humana Press Inc., Totowa, NJ.
4. Chenu, C., Serre, C. M., Raynal, C., Burt-Pichat, B., and Delmas, P. D. (1998) *Bone* **22,** 295–299.
5. Patton, A. J., Genever, P. G., Birch, M. A., Suva, L. J., and Skerry, T. M. (1998) *Bone* **22,** 645–649.
6. Mason, D. J., Suva, L. J., Genever, P. G., Steuckle, S., Hillam, R. A., and Skerry, T. M. (1997) *Bone* **20,** 199–205.
7. Espinosa, L., Itzstein, C., Cheynel, H., Delmas, P. D., and Chenu, C. (1999) *J. Physiol.* **518,** 47–53.
8. Roodman, G. D. (1996) *Endocr. Rev.* **17,** 308–332.
9. Kanehisa, J., Yamanaka, T., Doi, S., Turksen, K., Heersche, J. N. M., Aubin, J. E., and Takeuchi, H. (1990) *Bone* **11,** 287–293.
10. Teti, A., Marchisio, P. C., and Zambonin Zallone, A. (1991) *Am. J. Physiol.* **261,** C1–C7.
11. Lakkakorpi, P. T., and Väänänen, H. K. (1991) *J. Bone Miner. Res.* **6,** 817–826.
12. Aubin, J. E. (1992) *J. Bone Miner. Res.* **7,** 365–368.
13. Väänänen, H. K., and Horton, M. (1995) *J. Cell Sci.* **108,** 2729–2732.
14. Suda, T., Nakamura, I., Jimi, E., and Takahashi, N. (1997) *J. Bone Miner. Res.* **12,** 869–879.
15. Hughes, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R., and Boyce, B. F. (1995) *J. Bone Miner. Res.* **10,** 1478–1487.
16. Kameda, T., Miyazawa, K., Mori, Y., Yuasa, T., Shiokawa, M., Nakamaru, Y., Mano, H., Hakeda, Y., Kameda, A., and Kumegawa, M. (1996) *Biochem. Biophys. Res. Commun.* **220,** 515–519.
17. Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., and Boyce, B. F. (1996) *Nature Med.* **2,** 1132–1136.
18. Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y, Hiroi, E., Hiura, K., Kameda, A., Yang, N. N., Hakeda, Y., and Kumegawa, M. (1997) *J. Exp. Med.* **186,** 489–495.
19. Dempster, D. W., Moonga, B. S., Stein, L. S., Horbert, W. R., and Antakly, T. (1997) *J. Endocrinol.* **154,** 397–406.
20. Hiroi-Furuya, E., Kameda, T., Hiura, K., Mano, H., Miyazawa, K., Nakamaru, Y., Watanabe-Mano, M., Okuda, N., Shimada, J., Yamamoto, Y., Hakeda, Y., and Kumegawa, M. (1999) *Calcif. Tissue Int.* **64,** 219–223.
21. Weichert, H., Blechshmidt, I., Schröder, S., and Ambrosius, H. (1991) *Allerg. Immunol.* **37,** 139–144.
22. McBain, C. J., and Mayer, M. L. (1994) *Physiol. Rev.* **74,** 723–760.
23. Danysz, W., and Parsons, C. G. (1998) *Pharmacol. Rev.* **50,** 597–664.
24. Dannhardt, G., and Kohl, B. K. (1998) *Curr. Med. Chem.* **5,** 253–263.
25. MacDonald, J. F., Bartlett, M. C., Mody, I., Pahapill, P., Reynolds, J. N., Salter, M. W., Schneiderman, J. H., and Pennefather, P. S. (1991) *J. Physiol.* **432,** 483–508.
26. Rogawski, M. A. (1993) *Trends Pharmacol. Sci.* **14,** 325–331.
27. Martin, T. J., and Ng, K. W. (1994) *J. Cell Biol.* **56,** 357–366.
28. Suzuki, H., Nakamura, I., Takahashi, N., Ikuhara, T., Matsuzaki, K., Isogai, Y., Hori, M., and Suda, T. (1996) *Endocrinology* **137,** 4685–4690.
29. Razzouk, S., Lieberherr, M., and Cournot, G. (1999) *Eur. J. Cell Biol.* **78,** 249–255.
30. Helfrich, M. H., Nesbitt, S. A., Dorey, E. L., and Horton, M. A. (1992) *J. Bone Miner. Res.* **7,** 335–343.
31. Luckman, S. P., Hughes, D. E., Coxon, F. P., Russell, R. G. G., and Rogers, M. J. (1998) *J. Bone Miner. Res.* **13,** 581–589.
32. Lorget, F., Mentaverri, R., Ducellier, V., Meddah, B., Kamel, S., Maamer, M., and Brazier, M. (1999) *J. Bone Miner. Res.* **14,** F172.
33. Nakamura, I., Takahashi, N., Sasaki, T., Jimi, E., Kurokawa, T., and Suda, T. (1996) *J. Bone Miner. Res.* **11,** 1873–1879.
34. Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall, J. E., Dikkes, P., Conner, D. A., Rayudu, P. V., Cheung, W., Chen, H. S., Lipton, S. A., and Nakanishi, N. (1998) *Nature* **393,** 377–381.
35. Sun, L., Margolis, F. L., Shipley, M. T., and Lidow, M. S. (1998) *FEBS Lett.* **441,** 392–396.
36. Sheng, M., and Wyszynski, M. (1997) *BioEssays* **19,** 847–853.
37. Ehlers, M. D., Mammen, A. L., Lau, F. T., and Huganir, R. L. (1996) *Curr. Opin. Cell Biol.* **8,** 484–489.
38. Van Rossum, D., and Hanisch, U. K. (1999) *Trends Neurosci.* **22,** 290–295.

39. Fanning, A. S., and Anderson, J. M. (1998) *in* Current Topics in Microbiology and Immunology: Protein Modules in Signal Transduction (Pawson, A. J., Ed.), Vol. 228, pp. 209–233, Springer-Verlag, Berlin/Heidelberg.
40. Rosenmund, C., and Westbrook, G. L. (1993) *Neuron* **10,** 805–814.
41. Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M., and Sheng, M. (1997) *Nature* **385,** 439–442.
42. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) *Science* **269,** 1737–1740.
43. Kurschner, C., Mermelstein, P. G., Holden, W. T., and Surmeier, D. J. (1998) *Mol. Cell. Neurosci.* **11,** 161–172.
44. Nagano, T., Jourdi, H., and Nawa, H. (1998) *J. Biochem.* **124,** 869–875.
45. Shorte, S. L. (1997) *J. Neurophysiol.* **78,** 1135–1143.
46. Yu, X. M., Askalan, R., Keil, G. J. II, and Salter, M. W. (1997) *Science* **275,** 674–678.
47. Yu, X. M., and Salter, M. W. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 7697–7704.
48. Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992) *J. Clin. Invest.* **90,** 1622–1627.
49. Insogna, K. L., Sahni, M., Grey, A. B., Tanaka, S., Horne, W. C., Neff, L., Mitnick, M., Levy, J. B., and Baron, R. (1997) *J. Clin. Invest.* **100,** 2476–2485.