

Rapid Report

Active NMDA glutamate receptors are expressed by mammalian osteoclasts

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1. The *N*-methyl-D-aspartate (NMDA) glutamate receptor, widely distributed in the mammalian nervous system, has recently been identified in bone. In this study, we have investigated whether NMDA receptors expressed by osteoclasts have an electrophysiological activity.
2. Using the patch clamp technique two agonists of the NMDA receptor, L-glutamate (Glu) and NMDA, were shown to activate whole-cell currents recorded in isolated rabbit osteoclasts.
3. The current–voltage (*I*–*V*) relationships of the currents induced by Glu (I_{Glu}) and NMDA (I_{NMDA}) were studied using Mg^{2+} -free solutions. The agonist-induced currents had a linear *I*–*V* relationship with a reversal potential near 0 mV, as expected for a voltage independent and non-selective cationic current.
4. I_{Glu} and I_{NMDA} were sensitive to specific blockers of NMDA subtype glutamate receptors, such as magnesium ions, (5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) and 1-(1,2-diphenylethyl) piperidine (DEP). The block of I_{Glu} and I_{NMDA} by these specific antagonists was voltage dependent, strong for negative potentials (inward current) and absent for positive potentials (outward current).
5. These results demonstrate that NMDA receptors are functional in rabbit osteoclasts, and that their electrophysiological and pharmacological properties in these cells are similar to those documented for neuronal cells. Active NMDA receptors expressed by osteoclasts may represent a new target for regulating bone resorption.

Different factors and mechanisms such as the endocrine system, mechanical stimuli, local growth factors and cytokines have been shown to participate in the control of bone remodelling. More recently, several studies have demonstrated that neurotransmitters, including neuropeptides and excitatory amino acids such as glutamate (Bjurholm *et al.* 1989; Lerner 1996; Chenu *et al.* 1998; Patton *et al.* 1998), may also actively contribute to the local regulation of bone metabolism. Bone is innervated (Calvo & Forteza-Vila, 1969), and nerve fibres containing neuropeptides or glutamate (Hohmann *et al.* 1986; Hill & Elde 1991; Serre *et al.* 1998) have been found in this tissue, in the vicinity of bone cells which express receptors for these neurotransmitters (Hohmann & Tashjian 1984; Bjurholm *et al.* 1992; Konttinen *et al.* 1996; Chenu *et al.* 1998; Patton *et al.* 1998). Although neuromediators might be involved in a local paracrine signalling between bone cells, these findings strengthen the hypothesis of a neuronal control of bone metabolism, previously suggested by a number of clinical

observations and *in vitro* studies (Konttinen *et al.* 1996; Lerner 1996).

L-Glutamate (Glu) is a widely used neuromediator in the central and peripheral nervous system that acts through two types of membrane receptors; ionotropic receptors (NMDA, α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate) which have a ionic channel structure, and metabotropic receptors which are members of the seven transmembrane family receptors coupled to protein G. Different subtypes of glutamate receptors (GluR) have been identified in bone, expressed by both bone-forming osteoblasts and bone-resorbing osteoclasts (Chenu *et al.* 1998; Patton *et al.* 1998). In particular, we have previously demonstrated that osteoclasts express the NMDA subtype of GluR and that specific antagonists of these receptors block *in vitro* bone resorption (Chenu *et al.* 1998), suggesting that activation of NMDA receptors is required for the resorptive function of these cells.

In this report, we first demonstrate, using electrophysiological studies, that NMDA receptors expressed by normal mammalian osteoclasts are functional and regulated by specific modulators, and that they may be involved in osteoclast signalling activated by Glu during bone remodelling.

Initial results from this work have been reported in abstract form (Espinosa *et al.* 1998).

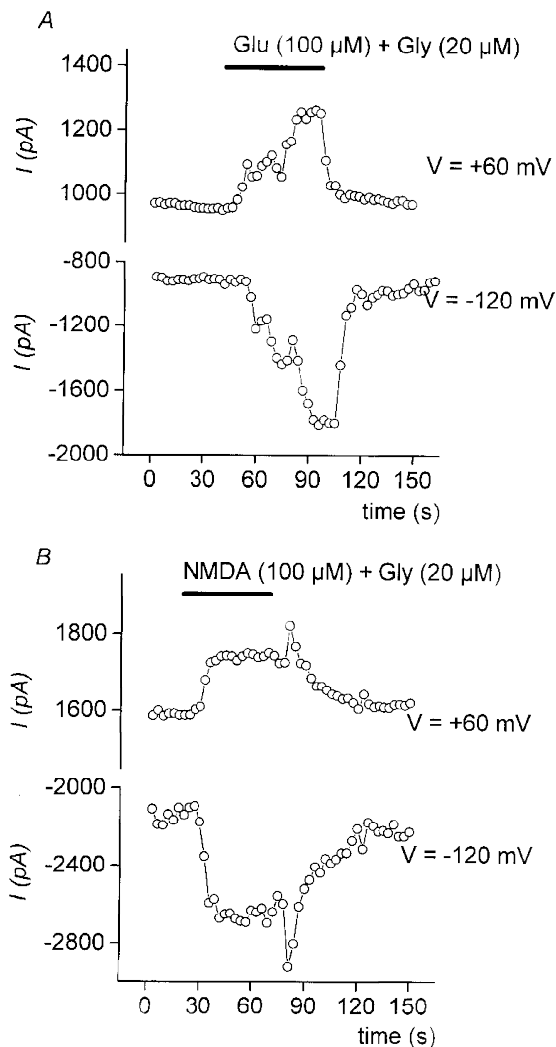


Figure 1. Extracellular L-glutamate (Glu) and NMDA activate a transient current in rabbit osteoclasts

Each point plotted in this figure represents the whole-cell current (expressed in pA) measured every 3 s at -120 mV or $+60$ mV during a voltage ramp protocol (-130 to $+60$ mV in 150 ms, from holding potential of -30 mV). A, Glu ($100 \mu\text{M}$ + Gly $20 \mu\text{M}$) and B, NMDA ($100 \mu\text{M}$ + Gly $20 \mu\text{M}$) were applied during the time indicated by the bar. They both induced an inward current at -120 mV and an outward current at $+60$ mV. The current was reaching a peak in ~ 60 s and was reversible after complete washout of the agonists.

METHODS

Materials

Medium 199 (M199) and α minimal essential medium (α MEM), fetal calf serum (FCS), penicillin, streptomycin and HEPES buffer were purchased from Life Technologies (Grand Island, NY, USA). Chemicals were obtained from ICN Biomedicals Inc. (Aurora, OH, USA). Agonists and antagonists of glutamate receptors (GluR) were purchased from Tocris Cookson (Bristol, UK).

Osteoclast isolation and culture

Osteoclasts were isolated from long bones of 1-day-old New Zealand rabbits that were killed by decapitation. The bones were removed, cleaned of soft tissues and bone marrow, then split and scraped into M199 containing 10% heat-inactivated fetal calf serum (FCS), 20 mM HEPES and 50 U ml^{-1} penicillin–streptomycin. Cells were centrifuged at low speed, resuspended in the same medium and seeded onto glass coverslips for 1 h at 37°C , 5% CO_2 . After this adhesion period, non-adherent cells were removed and coverslips were placed for 24 h in α MEM containing 2% heat-inactivated FCS, at pH 7.2.

Cells were always studied within 30 h of isolation. Electrophysiological recordings were performed on spread osteoclasts, identified as cells having multiple nuclei (> 3).

All experiments were carried out according to the guidelines defined by the animal welfare committee of the National Institute of Health and Medical Research (INSERM).

Electrophysiology

The conventional whole-cell patch-clamp configuration has been used. Macroscopic currents were recorded with RK-400 amplifier (Bio-Logic, Grenoble, France), and data were registered and analysed using pCLAMP6 software (Axon Instruments). Current signals were filtered at 3 kHz and digitized at 10 kHz using an analog/digital converter (DigiData 1200, Axon instruments). Borosilicate glass pipettes had a resistance of 2–3 $\text{M}\Omega$ and seal resistances were always superior to 1 $\text{G}\Omega$. Current–voltage (I – V) relationships were obtained using a voltage ramp protocol defined by a 15 ms pulse from -30 mV holding potential (V_h) to -130 mV, followed by a ramp from -130 mV to $+60$ mV over 150 ms. This protocol was compared with a conventional step protocol, composed of four pulses of 85 ms from $V_h = -30$ mV to -100 , -50 , 0 and $+50$ mV, with a short return to V_h during 15 ms between each pulse. Each protocol was applied every 3 s. Experiments were performed at room temperature (21 – 25°C).

Cells were continuously superfused at 3 ml min^{-1} using a bath solution flowing by gravity from a set of five capillaries. Agonists and antagonists of GluR were applied using this system. The time to switch between different solutions was ≤ 3 s. Na^+ external control solution (Tyrode solution) contained (mM): NaCl 145, CsCl 5, CaCl_2 2, glucose 5, HEPES 10, pH at 7.4 adjusted with NaOH. The pipette-filling solution contained (mM): CsCl 15, NaCl 5, aspartic acid 100, EDTA 5, HEPES 20, pH at 7.2 adjusted with CsOH. Glutamate and NMDA were added to Tyrode solution at $100 \mu\text{M}$ with $20 \mu\text{M}$ glycine.

RESULTS

Glutamate and NMDA-induced currents in osteoclasts

Osteoclasts were voltage clamped at a holding potential (V_h) of -30 mV that allowed experiments over a long time period (> 30 min) to be performed. However, because the

Glu (I_{Glu})- or NMDA (I_{NMDA})-elicited current at -30 mV was small, the induced current modifications were followed using a voltage ramp protocol applied every 3 s in Cs^+ solution. The mean holding current for all tested cells, measured at -30 mV, was -272 ± 54 pA. In order to minimize possible stretch activated currents, cells were continuously superfused with control solution and we have checked that application of the vehicle was not affecting the current. Glutamate ($100 \mu\text{M}$) and glycine ($20 \mu\text{M}$) activated a current, measured at different membrane potentials (-130 to $+60$ mV), which was inward at negative potentials and outward at positive potentials (Fig. 1). Three to ten seconds after agonist addition, the current increased and reached a peak in 30 to 60 s representing 15–20% of the control

current (Glu: $15 \pm 6\%$, $n = 12$; NMDA: $18 \pm 17\%$, $n = 10$). No significant differences were observed between currents elicited by Glu and NMDA, the specific agonist of the NMDA subtype GluR. Responses to GluR agonists were observed in 12/39 cells (30.7%) for Glu and 10/34 (29.4%) for NMDA. Glu- and NMDA-induced currents had variable activation time courses and different reversal phases which could be attributed to the various times needed to reach homogeneous concentrations of agonists due to their high concentrations ($100 \mu\text{M}$) and/or to different times required for their complete wash out.

No dynamic currents were observed. The time courses of the whole-cell currents had not shown dynamic components in control conditions and after Glu application (Fig. 2*A* and *B*).

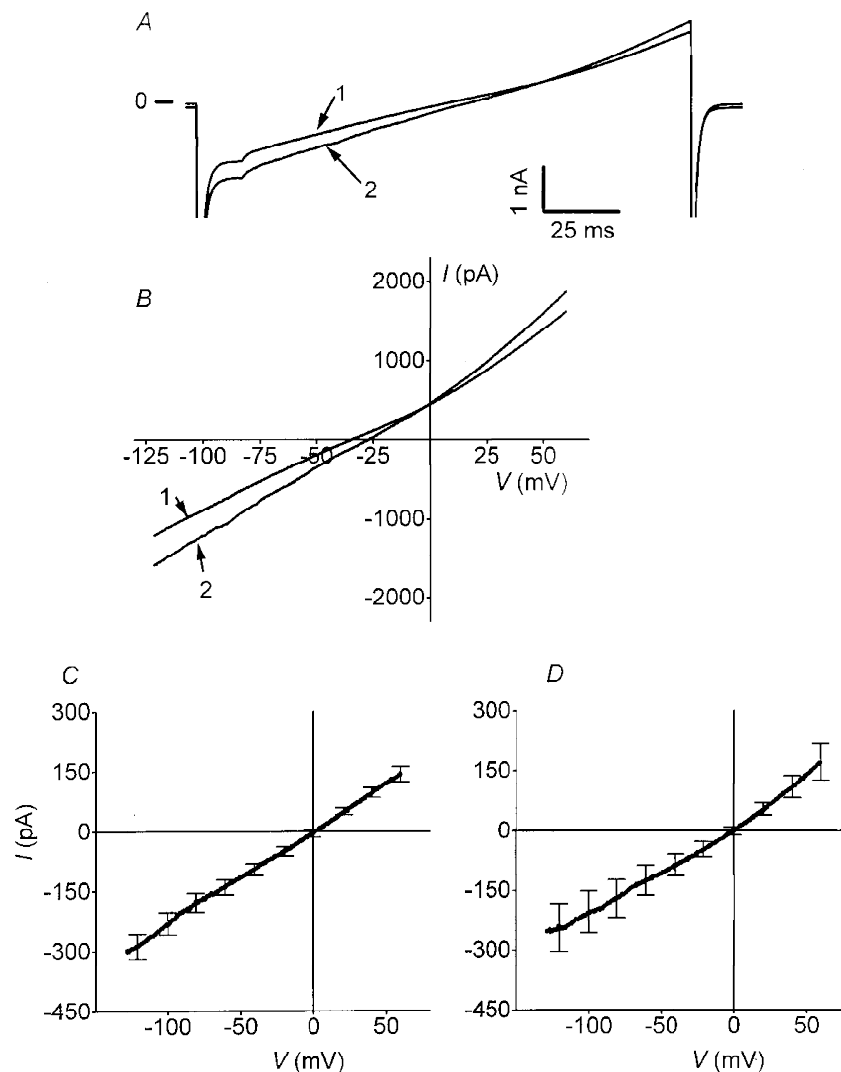


Figure 2. Glutamate and NMDA elicit a voltage-independent non-selective cation current

A, time courses of the whole-cell currents measured during the voltage ramp stimulation for control (1) and Glu application (2). *B*, I - V relationships of the whole-cell currents determined by a voltage ramp protocol, in control condition (1) and at the peak response to Glu (2). Mean currents (means \pm s.e.m.) induced by Glu (*C*) ($100 \mu\text{M}$ + Gly $20 \mu\text{M}$, $n = 12$) and NMDA (*D*) ($100 \mu\text{M}$ + Gly $20 \mu\text{M}$, $n = 10$) were calculated by subtracting the control current from the agonist-induced current at the peak of response. They were both linear with a reversal potential close to 0 mV (minimum and maximum values being -25 and $+21$ mV, respectively).

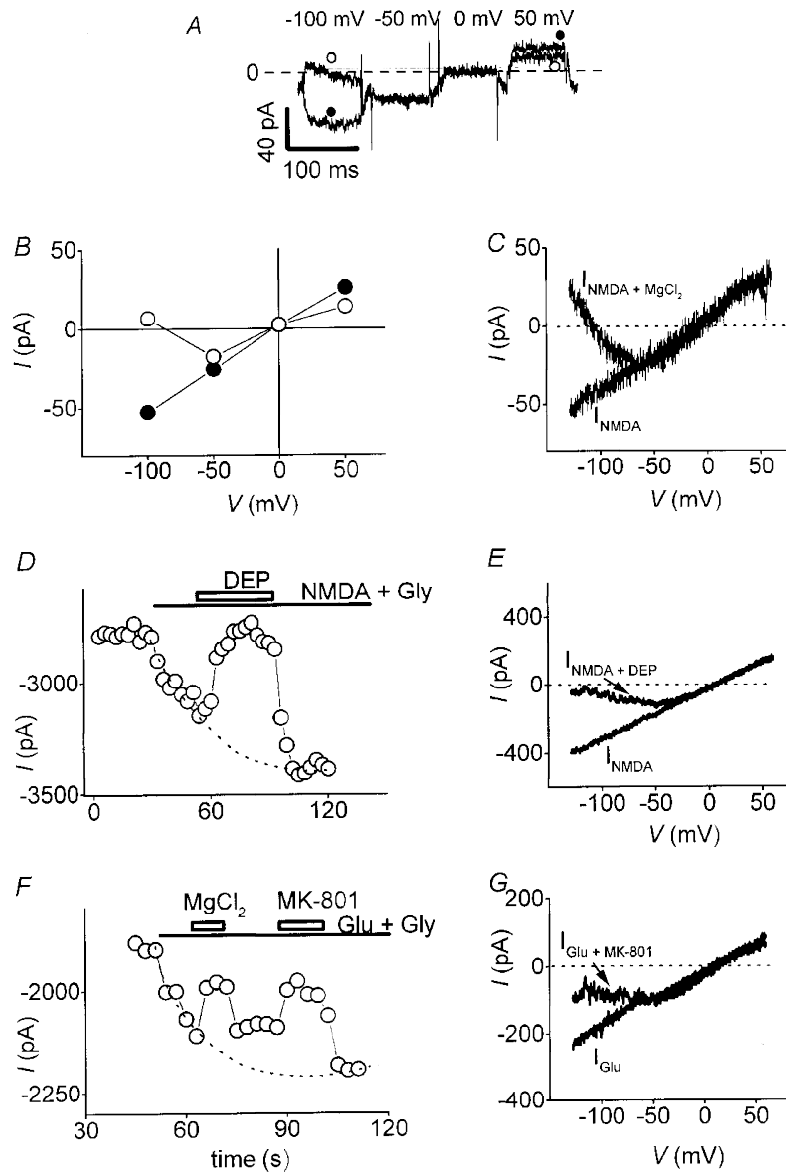


Figure 3. Mg²⁺, DEP and MK-801 induce a voltage-dependent block of NMDA receptor activation in osteoclasts

A, osteoclast was held at -30 mV and submitted to various potentials. Traces represent NMDA-induced current (I_{NMDA}) in the presence (○) or absence (●) of 2 mM extracellular Mg²⁺. I_{NMDA} was calculated by subtracting the control current from I_{NMDA} at the peak of response. B and C, I - V relationships of I_{NMDA} recorded in the presence (○) or absence (●) of 2 mM extracellular Mg²⁺ ($V_h = -30$ mV), using a voltage step (B) or a voltage ramp (C) protocol. Both I - V curves showed a strong voltage-dependent block of I_{NMDA} by external magnesium, illustrated by the negative slope of the current between -120 mV and -50 mV. D and F, NMDA (100 μM + Gly 20 μM) or Glu (100 μM + Gly 20 μM) were applied continuously (as indicated by the bar) and currents were measured at -120 mV ($V_h = -30$ mV). Channel blockers, 50 μM DEP (D) and 50 μM MK-801 (F), were added during the time designated by rectangles. These compounds blocked I_{NMDA} and I_{Glu} respectively and this inhibition was reversible after removal of the antagonists. Dotted lines in D and F were fitted by eye. E and G, I - V relationships of I_{NMDA} (E) obtained with a voltage ramp protocol, in absence (trace marked I_{NMDA}) or in presence of DEP (trace marked $I_{\text{NMDA}} + \text{DEP}$); I - V relationships of I_{Glu} (G) obtained with a voltage ramp protocol, in absence (trace marked I_{Glu}) or in presence of MK-801 (trace marked $I_{\text{Glu}} + \text{MK-801}$).

The agonist-induced currents (I_{Glu} and I_{NMDA}) were calculated by subtracting the control current elicited by a ramp before agonist application (trace 1) from the agonist-induced current at its peak response (trace 2). The mean currents and standard deviations, calculated for 20 mV intervals, are reported in Fig. 2C and D. Similar amplitudes and I - V relationships were obtained for Glu- and NMDA-induced currents. Using internal Cs^+ solution, I - V relationships of I_{Glu} and I_{NMDA} were linear with a reversal potential of 0 mV, indicative of a cationic non-selective current; in K^+ solution, the reversal potential was similarly close to 0 mV (not shown). As described for neuronal cells (Nowak *et al.* 1984), no rectification was observed for I_{Glu} and I_{NMDA} in conditions where intracellular magnesium was buffered by 5 mM EDTA added in the pipette solution (Johnson & Ascher, 1990) (Fig. 2C and D). In Cs^+ solution, chloride concentrations were adjusted to shift the chloride equilibrium potential to negative potentials (E_{Cl} ca -50 mV), hence suppressing a possible contribution of a chloride current.

Pharmacology

To confirm the involvement of the NMDA subtype GluR, we have tested the sensitivity of Glu- and NMDA-elicited currents to three well known NMDA specific channel blockers: divalent cations Mg^{2+} which have been well characterized as NMDA channel blockers (Ascher & Nowak 1988); (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine (MK-801), the most largely used non-competitive NMDA receptor antagonist in neuronal studies (MacDonald *et al.* 1991); and 1-(1,2-diphenylethyl) piperidine (DEP), a high affinity antagonist at the ion channel on NMDA receptor which is a conformationally flexible homeomorph of MK-801 (Rogawski 1993). When applied to the external face, these antagonists interact with the opened channel pore and block it in a voltage-dependent manner, with a stronger blockade at hyperpolarized potentials (Nowak *et al.* 1984; Honey *et al.* 1985).

NMDA receptor antagonists were tested during a continuous application of Glu or NMDA, and were added during the activation phase of the current in order to differentiate their effects clearly from the current desensitization observed during long-time exposure to agonists. Figure 3 illustrates the effects of these compounds on agonists-induced currents. External magnesium (2 mM) induced a strong voltage-dependent block of the NMDA-induced current, demonstrated by voltage step (Fig. 3A and B) or voltage ramp (Fig. 3C) protocols. The current was highly blocked at strong negative potentials while unaffected or slightly reduced at potentials superior to -50 mV (Fig. 3A). I - V relationships of I_{NMDA} measured with both protocols showed a negative slope between -100 and -50 mV (Fig. 3B and C). The outward current at strong negative potentials and the mild block of I_{NMDA} at potentials superior to +50 mV observed in the presence of magnesium might be

the consequence of an inhibition of the outwardly rectifying chloride current by these ions, previously shown to be activated in osteoclasts (Sakai *et al.* 1999).

DEP (50 μM ; Fig. 3D) and MK-801 (50 μM ; Fig. 3F) also reversibly blocked currents elicited by NMDA and Glu. DEP was a stronger blocker of the current than MK-801. The effects of these NMDA channel blockers on I_{NMDA} and I_{Glu} obtained with a voltage ramp protocol are illustrated on Fig. 3E and G. The inward part of the current was blocked while its outward part was unaffected. As for magnesium, I - V relationships obtained with MK-801 and DEP had a negative slope for potentials inferior to -50 mV (J-shaped curve). These data clearly demonstrate a voltage-dependent block of I_{NMDA} by non-competitive antagonists, a major characteristic of the electrophysiological behaviour of NMDA Glu receptors.

DISCUSSION

Our results demonstrate for the first time the presence of active NMDA receptors in rabbit osteoclasts. We have shown that in Mg^{2+} -free medium, extracellular application of NMDA receptor agonists activated a cationic non-selective and non-voltage-dependent conductance in these cells. In the presence of extracellular magnesium, the NMDA-induced current showed a strong outward rectification. This voltage-dependent block of current by external magnesium ions is also a main characteristic of NMDA receptors, the accepted mechanism being that Mg^{2+} ions block the open channel when the driving force is negative (inward current). Other non-competitive specific NMDA channel antagonists have been described in neuronal cells, which block these receptors in a voltage-dependent way (MacDonald *et al.* 1991). Our pharmacological studies have demonstrated a strong voltage-dependent inhibition of Glu- and NMDA-induced currents by MK-801 and DEP, two well known antagonists of NMDA receptors, confirming the involvement of this subtype of GluRs in the electrophysiological responses of osteoclasts to Glu and NMDA. The similarity between I_{Glu} and I_{NMDA} , as well as the abilities of magnesium, MK-801 and DEP to block Glu-induced currents, are in favour of a prevalent activation of NMDA receptors in these cells. Previously AMPA receptors have also been shown to be expressed by bone cells (Chenu *et al.* 1998). Although we have not yet any electrophysiological evidence for activation of AMPA GluR in osteoclasts, we cannot totally exclude a possible contribution of non-NMDA GluRs to Glu-induced currents in these cells.

Activation kinetics of NMDA receptors studied in neuronal cells using fast agonist application systems have shown two components: a rapid transient peak (100 ms duration) and a stable steady state (Sather *et al.* 1992). As we used a slow switch system to apply the agonists, it has not been possible to compare Glu-induced peaks of response. It is also possible

that this slow application of agonists could have induced a pre-desensitization of receptors, reducing the maximum current amplitude (Sather *et al.* 1992). However, we have shown that the steady states of NMDA- and Glu-elicited currents were similar in osteoclasts compared with neuronal cells.

While the role of functional NMDA receptors in neuronal cells has been well described, the physiological significance of these receptors on osteoclasts is presently unknown. Although GluRs are the major neurotransmitter receptors in the vertebrate central nervous system, they have been recently discovered in non-neuronal tissues such as pancreas (Gonoi *et al.* 1994; Inagaki *et al.* 1995; Weaver *et al.* 1996), lung (Said *et al.* 1995) and in plants (Lam *et al.* 1998). Moreover, they could play a physiological role in these tissues since GluRs expressed by pancreatic cells are involved in the control of insulin and glucagon secretion by Glu (Bertrand *et al.* 1993). There is not yet any evidence for a mechanism involved in a local secretion of excitatory amino acids in bone. Glutamate may originate from bone cells and act as a paracrine signal between them, but it may also be released under specific stimuli from nerve fibres located in the vicinity of bone cells.

In neuronal cells, the activity of NMDA receptors depends on a pre-depolarization of the post-synaptic cell to abolish the voltage-dependent block by magnesium, induced by stimulation of non-NMDA glutamate receptors. One important characteristic of osteoclasts is that they can alternate between two stable membrane potentials of approximately -75 and -15 mV (Sims & Dixon, 1989). At -75 mV NMDA receptors are blocked by magnesium, while at -15 mV they allow a calcium influx into the cell. The switch from -75 mV to -15 mV could be triggered by a depolarizing current, due to activation of other types of receptors such as non-NMDA receptors if they are expressed, or P2X ATP receptors which have been recently described in osteoclasts (Weidema *et al.* 1997). The discovery of functional NMDA receptors in osteoclasts opens an interesting putative pathway for regulating bone resorption. Previous results by our group have shown that some antagonists of NMDA Glu receptors block *in vitro* bone resorption (Chenu *et al.* 1998). NMDA receptor activation induces changes in internal calcium concentration and modifications of membrane potential which may modulate the driving force for H^+ and facilitate its electrogenic pumping out of the cell, as it may also change the driving force for calcium ions. Calcitonin which inhibits bone resorption has been shown to modify both membrane potential and internal calcium concentration (Okabe *et al.* 1998).

Active NMDA receptors are heterodimers of NR1 and NR2A, B, C or D subunits and the properties of these channel receptors have been shown to be modulated by their

subunit composition (Williams 1997). NR1 is the major subunit that has been identified in bone cells but the composition of functional NMDA receptors on these cells is still unknown, although NR2D has been identified in bone marrow cells by RT-PCR (Chenu *et al.* 1998; Patton *et al.* 1998).

In this study, applications of NMDA receptor agonists have elicited a current in 30% of tested osteoclasts. This heterogeneity of responses is in line with a variability of expression of NR1 previously demonstrated in these cells using immunocytochemistry. The relationship between the expression of functional NMDA receptors by osteoclasts and their bone resorbing activity will be very important to determine in the future. The demonstration of active NMDA glutamate receptors in osteoclasts represents a novel possibility for regulating bone resorption in physiological and pathological conditions such as osteoporosis.

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