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P2 receptor expression, signaling and function in osteoclasts

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1. ABSTRACT

Skeletal development and bone remodeling depend on the coordinated activity of osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively. Mature osteoclasts result from the fusion of precursor cells, and they are large, multinucleated, highly specialized cells. Cellular release of ATP and UTP occurs in response to a variety of stimuli including mechanical stimulation, which occurs in the bone environment. ATP and UTP or their metabolites can then act on P2 receptors in the plasma membrane to induce various responses in bone cells. The influence of these receptors on osteoclast physiology and bone physiology in general is beginning to be understood, but much work is still required. This review focuses on P2 receptors in osteoclasts, their expression, signaling and function in the regulation of osteoclast formation, resorptive activity and survival.

2. INTRODUCTION

2.1. Osteoclast biology

Osteoclasts originate from the fusion of mononucleated precursor cells which derive from bone marrow cells of the monocyte/macrophage lineage (1). Even though many regulatory signals are involved in this complex process of osteoclast formation (osteoclastogenesis), macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) ligand (RANKL) have been identified as key factors (2). Exposure of monocytes to a combination of M-CSF and RANKL is sufficient to induce osteoclastogenesis *in vitro* (3). Many other signaling molecules acting downstream of these key factors are necessary for osteoclastogenesis. For example, mice lacking functional NF-κB display osteopetrosis, as the result of complete inhibition of osteoclast formation (4, 5). After differentiation, mature osteoclasts continue to be regulated by multiple extracellular signals, which influence

functions such as migration, bone resorption and, ultimately, programmed cell death.

Osteoclasts are highly motile cells whose motion depends on the extension and retraction of pseudopodia/lamellipodia (6-8). Reversible integrin-mediated adhesion of pseudopodia to the extracellular matrix allow the generation of contractile force (9). The main integrin expressed by osteoclasts is $\alpha_v\beta_3$ (10-12). Osteoclasts isolated from mice lacking the integrin β_3 subunit display significant impairment of pseudopodia spreading and deficient resorptive activity, giving rise to an osteopetrotic phenotype (13, 14). Chemotactic factors influence osteoclast migration (7, 15, 16). A resorption trail is often observed behind a resorbing osteoclast as it migrates along the bone surface *in vitro*, indicating that migration and bone resorption are not mutually exclusive processes (17).

At sites of resorption, osteoclasts attach to the surface of bone, forming a circumferential zone – the sealing zone – where plasma membrane proteins (e.g., $\alpha_v\beta_3$ integrins) bind to the extracellular matrix (18). This region of the cell is characterized by the presence of a ring of actin filaments, which delimits a specialized membrane referred to as the ruffled membrane due to its multiple invaginations (18, 19). The osteoclast isolates the region of bone surface that lies below the ruffled membrane – the so-called resorption lacuna – where resorption takes place. This process involves dissolution of the bone mineral as well as enzymatic degradation of the organic matrix. Dissolution of the mineral is enabled by acidification of the resorption lacuna, which is mediated by vacuolar-type ATP-driven proton pumps that are highly localized at the ruffled membrane. Degradation of the organic components is carried out by secreted proteases, such as cathepsin K (19).

The overall rate of bone resorption depends on the number of osteoclasts present in the bone and the intensity of their resorptive activity. An increase in osteoclast survival (prolonged life span) results in more resorptive osteoclasts present in bone and higher overall rate of resorption (20). Apoptosis is tightly regulated by signals received by the cell, which eventually activate an internal death program. In this regard, it is known that osteoclasts undergo apoptosis when exposed to a variety of stimuli (21-23). Indeed, subtle changes in the rate of osteoclast apoptotic death are proposed to have significant impact on overall bone resorption and remodeling (20, 21, 23).

2.2. P2 receptors

P2 receptors are activated by purines and pyrimidines (ATP, ADP, UTP, UDP, UDP-glucose) and are classified into two families, the metabotropic P2Y receptors and the ionotropic P2X receptors. These receptors are present in a variety of cell types (24), including osteoclasts (25-27). P2Y are G protein-coupled receptors, with seven transmembrane domains. P2X receptor channels are formed by three subunits, assembled either as homo- or heterotrimers. A number of excellent reviews have summarized P2 receptor biology and physiological functions (24, 28-31).

3. EXPRESSION, SIGNALING AND FUNCTION OF P2X RECEPTORS IN OSTEOCLASTS

Seven members of the P2X receptor family are currently known: $P2X_{1-7}$. Gating of a non-selective cation channel occurs upon binding of extracellular ATP. The crystal structure of the *Danio rerio* (zebrafish) $P2X_4$ homotrimeric receptor has recently been solved (Figure 1) (32). This outstanding discovery will certainly boost our understanding of P2X receptors. The structure reveals a membrane pore formed by the three transmembrane helices (TM2; one from each subunit of the trimer). The three suggested intersubunit ATP binding sites are ~45 Å from the ion channel domain. Conserved residues located at the interface between the transmembrane domain and the extracellular domain are proposed to propagate conformational changes from the ATP binding sites to the gate.

Among the mechanisms involved in P2X receptor signaling are changes in membrane potential, protein-protein interactions and Ca^{2+} influx through the channel (28). Although there is evidence for the expression of $P2X_{2,4,7}$ in osteoclasts (Table 1), electrophysiological studies only support the functional expression of $P2X_4$ and $P2X_7$ (33, 34).

3.1. $P2X_2$ receptors

Expression of $P2X_2$ receptors in rat osteoclasts has been shown by immunocytochemistry and *in situ* hybridization (35). However, there is no electrophysiological evidence for functional expression of this receptor at the plasma membrane of osteoclasts. Thus, it is possible that $P2X_2$ receptors are located in intracellular compartments. In this regard, it has been reported recently that another subtype of P2X receptor ($P2X_4$) resides mainly within intracellular compartments in rodent microglia and macrophages, where it may play important roles in organelles such as phagosomes. Trafficking of $P2X_4$ receptors between intracellular compartments and the plasma membrane was observed and it was enhanced by microglial activation with lipopolysaccharide (36). Hence, it is possible that – as happens with $P2X_4$ receptors in microglia and macrophages – $P2X_2$ receptors may be present intracellularly in osteoclasts.

3.2. $P2X_4$ receptors

Rat osteoclasts show expression of $P2X_4$ receptors by immunocytochemistry and *in situ* hybridization (35). Transcripts coding for $P2X_4$ were also detected by RT-PCR in rabbit osteoclasts purified by micromanipulation (33).

Naemsch *et al.* monitored currents activated by ATP and other purinergic agonists in rabbit osteoclasts using the whole-cell patch-clamp technique (33). When the cells were held at a negative potential (-30 mV), a biphasic pattern of ATP-activated currents was observed. A transient inward current was activated first, followed by a transient

Table 1. Expression of P2X receptors in mammalian osteoclasts

Subtype	Model	Species	Approach used	Signaling	Proposed function	Refs.
$P2X_2$	OCs[1] isolated from long bones	Rat	Immunolabeling *In situ* hybridization	Not determined	Not determined	(35)
$P2X_4$	OCs isolated from long bones	Rabbit Rat	Electrophysiology Immunolabeling *In situ* hybridization RT-PCR	Depolarization by non-selective cation current	Not determined	(33) (35) (43)
$P2X_7$	OCs isolated from long bones OCs derived from bone marrow cells OCs derived from peripheral blood cells	Human Mouse Rabbit Rat	Ca^{2+} fluorometry Electrophysiology Fluorescent dye uptake Immunolabeling Quantification of apoptosis RT-PCR Use of $P2X_7$-deficient mice	Ca^{2+} influx Depolarization by non-selective cation current Syk-dependent microtubule deacetylation PKC activation NF-κB translocation Pore formation and membrane permeabilization	Cytolysis Disruption of F-actin structures, cytoskeletal reorganization Decreased resorption Enhanced resorption Enhanced apoptosis Fusion of osteoclast precursors Intercellular signaling	(34) (35) (47) (48) (50) (54) (58) (59) (60) (62) (65) (77)

[1] Abbreviations: OCs – osteoclasts

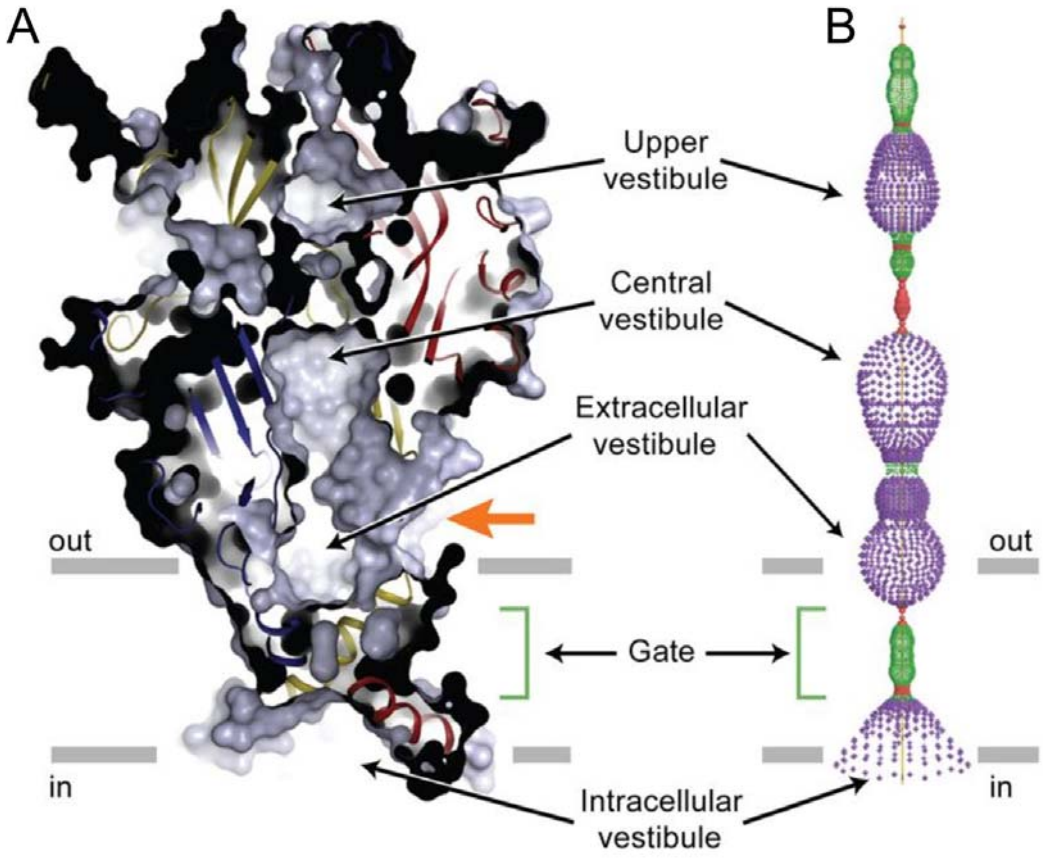

Figure 1. Crystal structure of *Danio rerio* $P2X_4$ receptor. (A) Sagittal section, showing a closed conformation of the pore. The gate is located about halfway across the membrane bilayer. Four vestibules (upper, central, extracellular and intracellular) are located on the molecular three-fold axis, with the extracellular vestibule connected to the bulk solution through a fenestration (orange arrow). (B) Pore-lining surface. Each color represents a different radius range measured from the receptor centre (red: <1.15 Å, green: 1.15–2.3 Å, and purple: >2.3 Å). Reproduced with permission from (32).

outward current (Figure 2A). The outward current was dissociated from the initial current (i.e., only outward current was activated) by stimulation with the P2Y agonist ADPβS (Figure 2B). Additional experiments showed that this latter component is a Ca^{2+}-dependent K^+ current activated by P2Y receptor-mediated release of Ca^{2+} from intracellular stores (33, 37).

The initial transient inward current reversed near 0 mV when the pipette and bath solutions contained

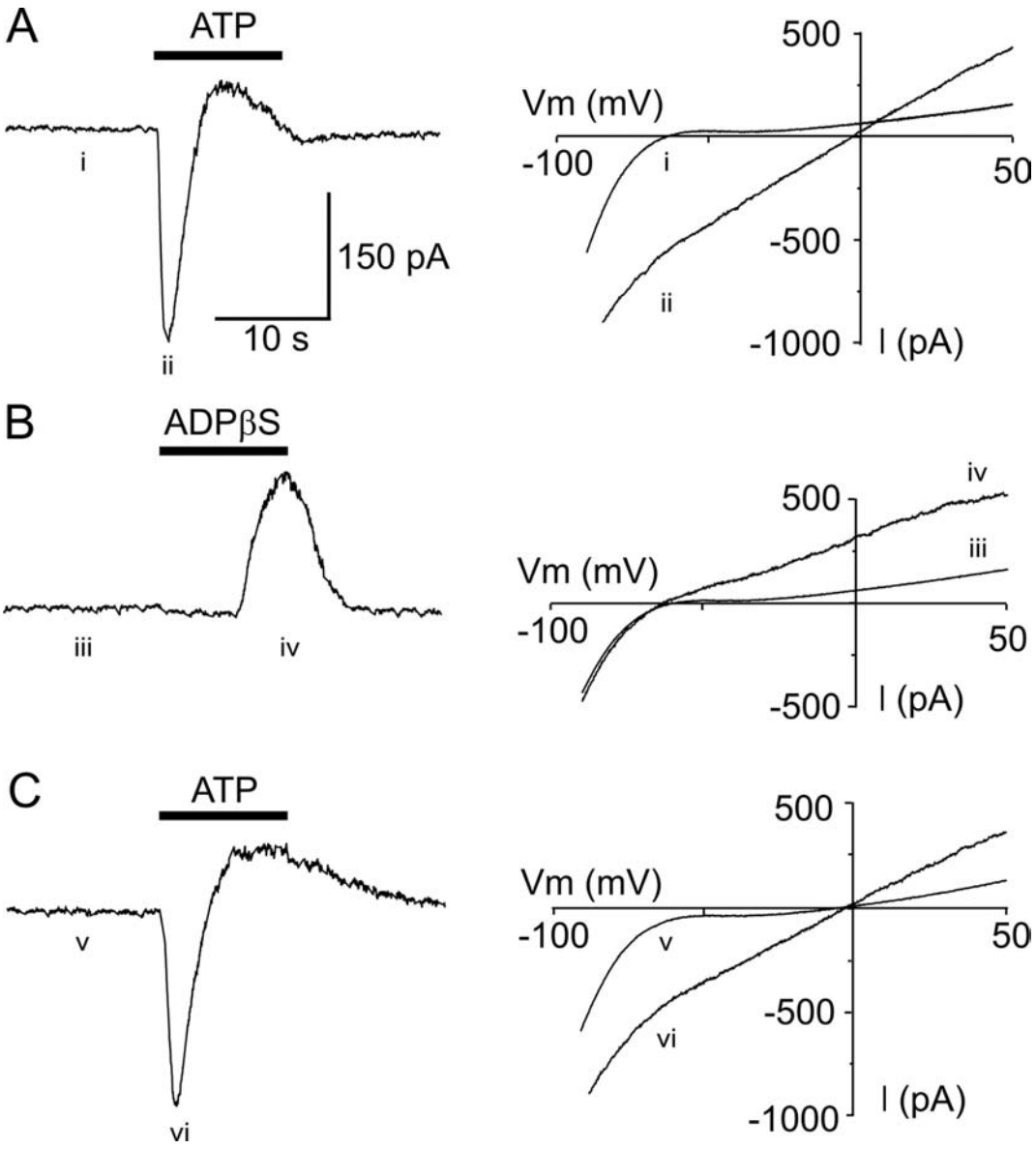

Figure 2. Evidence for functional P2X and P2Y receptors in rabbit osteoclasts. Whole-cell currents (left) were recorded at a holding potential of -30 mV. At the times indicated by roman numerals i-vi, voltage ramps were applied to obtain current-voltage curves (right). (A) The basal, resting current before purinergic stimulation showed marked inward rectification (i, right). ATP (100 μM) activated both inward and outward currents. The initial inward current reversed close to 0 mV (ii, right), consistent with non-selective P2X cation current. The outward current reversed at more negative potential (not shown). (B) After a 5-minute recovery, the same osteoclast was stimulated with ADPβS (100 μM), inducing only the outward current (iv, left). Note that the current activates after a latency period, consistent with an indirect mechanism of activation (i.e., through elevation of intracellular Ca^{2+}). In addition, the negative reversal potential of this current (-67 mV; iv, right) indicates that it is mediated by K^+-selective channels. Therefore, it is proposed that ADPβS activates P2Y receptors, which in turn induce an increase in $[Ca^{2+}]_i$ and activation of Ca^{2+}-dependent K^+ channels. (C) After a 5-minute recovery period, biphasic current was again activated by ATP (100 μM), indicating that the differences between the responses to ATP and ADPβS cannot be explained by deterioration of the osteoclast over time. The pipette and bath solutions contained standard, physiological concentrations of K^+ and Na^+. Reproduced with permission from (33).

physiological concentrations of K^+ and Na^+ (Figure 2A and C), indicating that the current was non-selective for Na^+ and K^+. Furthermore, when extracellular Na^+ concentration was lowered from 135 mM to 5 mM, there was a dramatic shift (~ 30 mV) in the reversal potential towards more negative values (33). This is expected for channels with high Na^+ conductance. Reversal potential of the current did not change when chloride in the pipette solution was

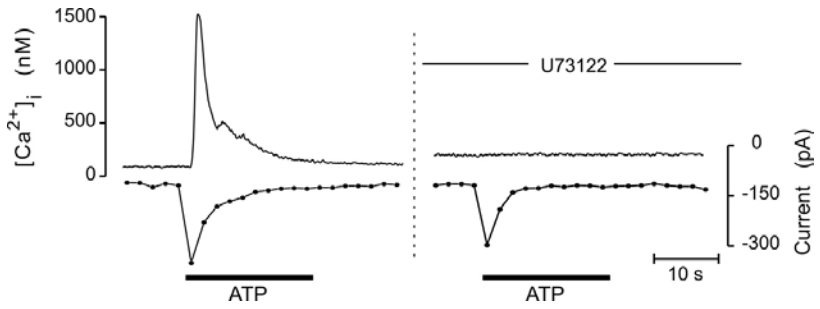

Figure 3. No rise of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) accompanies activation of $P2X_4$ cation current in rat osteoclast. $[Ca^{2+}]_i$ and membrane currents were studied with combined patch-clamp and fluorescence recording. ATP (20 μM) was applied at the times indicated by the horizontal bars below the traces. Control response is illustrated (left), showing that ATP induced a large elevation of $[Ca^{2+}]_i$ as well as inward current, measured at -60 mV. To eliminate the contribution of P2Y receptor-induced Ca^{2+} release from intracellular stores, the same cell was treated with the phospholipase C inhibitor U73122 (10 μM, bath application) for 5 min before stimulation. After such treatment, no rise of $[Ca^{2+}]_i$ was elicited by ATP, even though there was little change in the $P2X_4$ current (right). This indicates that Ca^{2+} entry through $P2X_4$ channels was negligible. Reproduced with permission from (43).

replaced by aspartate, indicating that no significant anion conductance was activated by ATP. Moreover, replacement of K^+ by Cs^+ in the pipette solution did not affect the reversal potential of the transient inward current, indicating that no Cs^+-sensitive K^+ channels were involved. Hence, these experiments established the expression of ATP-activated, non-selective cation channels in osteoclasts.

P2X subtypes display diverse current kinetics in response to ATP. Activation of the current occurs rapidly after ATP exposure in most subtypes. However, when exposure is prolonged, a rapid decline to basal levels is observed in some subtypes ($P2X_{1,3}$ – fast desensitization), whereas it is persistent in other subtypes ($P2X_{5,7}$ – slow desensitization). The inward current described in rabbit osteoclasts had a time course of desensitization that was well described by a single exponential, with an intermediate time constant of ~ 4 seconds (33). This value falls close to those reported for the $P2X_4$ receptor expressed in HEK-293 cells (38, 39).

The osteoclast current was elicited by 100 μM ATP, ATPγS or ADP, but not by 100 μM ADPβS, UTP or α,β-methylene-ATP (33). This agonist profile rules out the involvement of some P2X receptors – such as $P2X_1$ or $P2X_3$ – but does not discriminate between $P2X_2$ and $P2X_4$ (40). In addition, the current was potentiated by Zn^{2+}. However, both $P2X_2$ and $P2X_4$ receptors are potentiated by Zn^{2+} (40, 41).

Experiments making use of the P2X receptor antagonists Cibacron blue (CB) and suramin gave further evidence for the involvement of $P2X_4$ receptors (33). Both CB and suramin (100 μM each) failed to inhibit the current. CB is a weak antagonist of $P2X_4$ receptors (IC_{50} >300 μM), but a potent antagonist of $P2X_2$ receptors (IC_{50} 0.6-0.8 μM) in HEK-293 cells (42). Thus, the lack of inhibition by CB points to $P2X_4$ receptors rather than $P2X_2$ receptors. In the same way, it is well known that suramin is a poor antagonist at the recombinant $P2X_4$ receptors (IC_{50} >300 μM), whereas it is a more potent antagonist of recombinant $P2X_2$ receptors (IC_{50} 8-10 μM) (40). Taken together, these results established that the transient inward ATP-activated current described in rabbit osteoclasts by Naemsch *et al.* is mediated by $P2X_4$ receptors.

3.2.1. Surprising lack of Ca^{2+} permeability of $P2X_4$ receptors in osteoclasts

Weidema *et al.* investigated whether $P2X_4$ receptors induce Ca^{2+} entry when activated in rat and rabbit osteoclasts (43). Their conclusion, supported by three convincing lines of evidence, was that Ca^{2+} entry through $P2X_4$ receptors in osteoclasts is null or negligible.

First, it was observed that elevation in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) elicited by 20 μM ATP was abolished by 100 μM CB. This concentration of ATP is sufficient to activate osteoclast $P2X_4$ receptors (33) as well as P2Y receptors (44). Since 100 μM CB is unable to block the $P2X_4$-mediated current (33), then Ca^{2+} entry through $P2X_4$ channels should still be expected in the presence of CB (provided that the $P2X_4$ channels are permeable to Ca^{2+}). Since no Ca^{2+} response was observed in the presence of CB, the authors concluded that there was no Ca^{2+} entry through $P2X_4$ receptor channels.

Second, the Ca^{2+} responses to agonists which activate $P2X_4$ receptors as well as P2Y receptors (e.g., 2-methylthioadenosine 5'-triphosphate, 2-MeSATP) were virtually identical in the presence and absence of extracellular Ca^{2+} (37, 43). This result indicates negligible Ca^{2+} entry through $P2X_4$ receptors.

Third, when osteoclasts were stimulated with 20 μM ATP, both $P2X_4$ and P2Y receptors were activated. To eliminate the contribution of P2Y receptors to the Ca^{2+} response elicited by ATP, osteoclasts were incubated with the phospholipase C inhibitor U73122. By means of simultaneous measurement of membrane currents and $[Ca^{2+}]_i$, it was observed that osteoclasts incubated with U73122 and stimulated with 20 μM ATP did not show Ca^{2+} responses. However, they still displayed the typical $P2X_4$-mediated inward current (Figure 3). This was a direct demonstration that, even when $P2X_4$ receptor channels are functional,

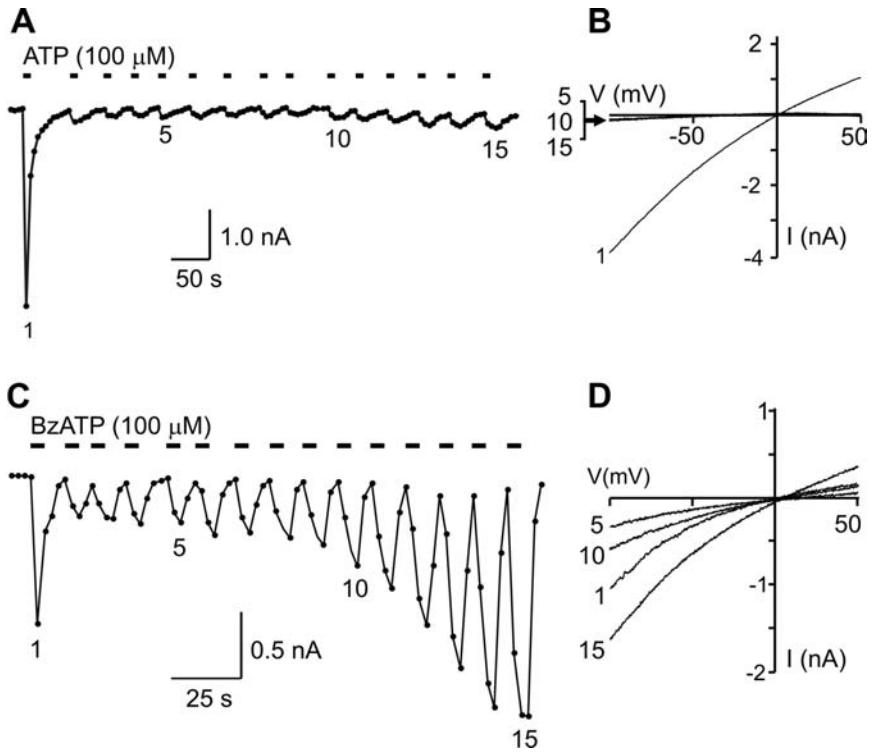

Figure 4. Repeated application of nucleotides to rabbit osteoclasts induces an initial $P2X_4$ current that desensitizes, followed by an activity-dependent $P2X_7$ current. Representative traces from osteoclasts stimulated either with 100 μM ATP (upper row) or 100 μM BzATP (lower row). Whole-cell currents (left) were recorded at a holding potential of -30 mV (pipette solution contained Cs^+ to block K^+ currents). Bars above the current traces represent agonist applications. Voltage ramps were applied to obtain current-voltage curves (right) at the times indicated by the numbers. Nucleotide-induced currents were obtained by subtraction of the basal, resting current. (A) Initial stimulation of an osteoclast with 100 μM ATP elicited a large inward $P2X_4$ current, whereas successive applications of ATP resulted in little response, due to $P2X_4$ desensitization. (B) The corresponding current-voltage curves for this cell indicate that the initial ATP-induced current had a reversal potential near 0 mV, as expected for the non-selective cation pore of $P2X_4$. (C) Another osteoclast was stimulated with the more potent $P2X_7$ receptor agonist BzATP (100 μM). After the initial response, successive stimulations led to the progressive development of an inward current of increasing amplitude. The results are consistent with initial activation of $P2X_4$ receptors, followed by their desensitization and progressive recruitment (priming) of $P2X_7$ receptors with successive stimulations. (D) The current-voltage curves for the cell in panel C indicate that both the initial current that desensitizes (the $P2X_4$ current) and the progressively developing current (the $P2X_7$ current), had reversal potentials near 0 mV. Reproduced with permission from (34).

there is no detectable entry of Ca^{2+}. The reason why $P2X_4$ receptors in rat and rabbit osteoclasts have negligible Ca^{2+} permeability is not clear. The $P2X_4$ receptor cloned from rat brain has high Ca^{2+} permeability when expressed in a heterologous system (45). An interesting hypothesis to account for this observation is the existence of cell-specific endogenous regulators of $P2X_4$ ion permeability.

3.3. $P2X_7$ receptors

In comparison with other P2X subtypes, $P2X_7$ receptors exhibit distinctive features, such as the requirement for relatively high concentrations of ATP for activation and 10-30 times more potent activation by 2',3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP) than by ATP itself. Formation of large pores that allow permeation of hydrophilic molecules – up to 900 Da – is another feature of $P2X_7$ receptor activation in many cell types, although this has also been reported for $P2X_2$ and $P2X_4$ receptors (28).

Expression of $P2X_7$ receptors in osteoclasts is well documented. In human osteoclasts, mRNA coding for $P2X_7$ receptor has been detected by RT-PCR (46-48) and expression of the receptor at the protein level has been shown by immunocytochemistry (47-50). As well, in rabbit (34) and rat (35) osteoclasts, $P2X_7$ expression has been shown by immunocytochemistry.

3.3.1. Electrophysiology

In rabbit osteoclasts, Naemsch *et al.* characterized $P2X_7$-mediated currents using the whole-cell, patch-clamp technique (34). The patch pipette solution contained CsCl to avoid contamination by Ca^{2+}-dependent K^+ current. Application of 100 μM ATP induced a current that activated and desensitized quite rapidly (Figure 4A and B), which was probably mediated by $P2X_4$ receptors. In contrast, when 100 μM BzATP or a higher concentration of ATP (1 mM) was applied, another current with distinctive

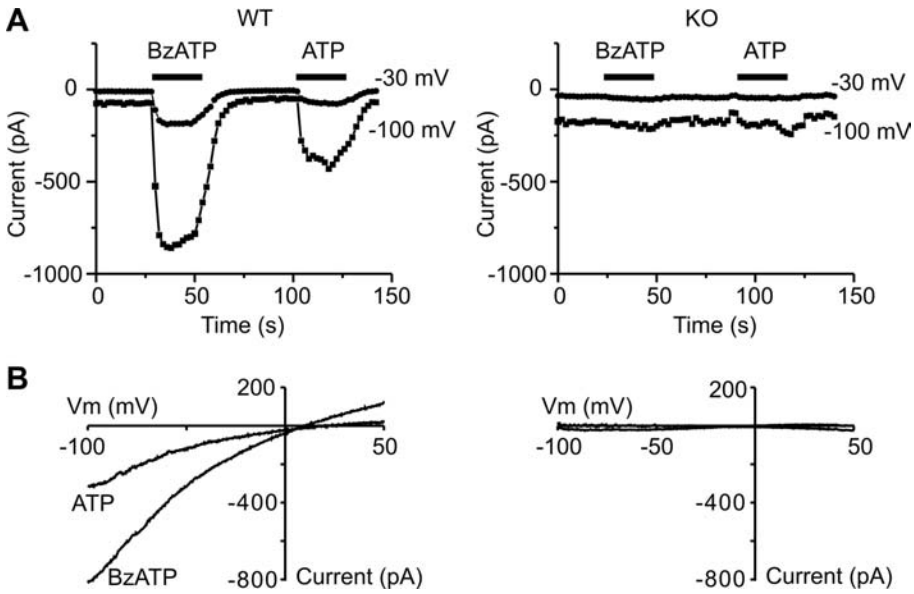

Figure 5. Nucleotide-induced currents compared in osteoclasts isolated from wild-type and *P2rx7*-deficient mice. Whole-cell currents were recorded with the pipette containing Cs^+ to block K^+ currents. The amplitude of the current at -30 and -100 mV (upper row) and the current-voltage curves (lower row) were obtained from voltage ramps applied every two seconds. (A) BzATP (300 μM) or ATP (1 mM) caused development of slowly deactivating inward current in wild-type osteoclasts (WT, left). In contrast, neither BzATP nor ATP elicited inward current in osteoclasts from *P2rx7* knockout mice (KO, right). (B) The current-voltage curve of the nucleotide-activated current – determined by subtraction of the basal current – is plotted for an osteoclast from a wild-type mouse (left) and from a knockout mouse (right). The BzATP- and ATP-activated currents of osteoclasts from wild-type mice showed little desensitization and reversed direction near 0 mV, consistent with $P2X_7$ current. Reproduced with permission from (54).

characteristics was observed. In these experiments, ATP or BzATP still evoked the initial transient $P2X_4$ current, but it was followed by current that progressively increased in amplitude with successive stimulations (Figure 4C), resembling the activity-dependent behavior of $P2X_7$ receptors (51, 52).

This activity-dependent current was inwardly rectifying and had a reversal potential close to 0 mV (Figure 4D), as expected for the non-selective cation pore of $P2X_7$. When ATP or BzATP stimulation was maintained for several seconds, the current did not desensitize but was sustained (34) – a typical feature of $P2X_7$-mediated current (28). In contrast to the behavior of the $P2X_4$-mediated current, the activity-dependent current was inhibited by Zn^{2+} (as well as Ca^{2+} and Mg^{2+}) (34), resembling $P2X_7$ receptors (53). Oxidized ATP or pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (100 μM) inhibited the activity-dependent current (34), consistent with $P2X_7$ receptors but not $P2X_4$ receptors (40).

In another study, osteoclasts were isolated from control mice and mice in which the gene encoding $P2X_7$ (*P2rx7*) had been disrupted (54). Electrophysiological responses to BzATP or high concentrations of ATP were then compared. A current similar to that described in rabbit osteoclasts was found in the control (wild-type) murine osteoclasts, but it was completely absent in osteoclasts isolated from *P2rx7*-deficient (*P2rx7*$^{-/-}$, knockout) animals (Figure 5).

3.3.2. Permeabilization of the plasma membrane

ATP-induced permeabilization of the plasma membrane to molecules up to 900 Da is a hallmark of $P2X_7$ expression. However, there are some exceptions where $P2X_7$ receptors are functionally expressed, but pore formation is not observed (55-57). The molecular identity of the permeation pathway responsible for this phenomenon remains controversial. Pore formation is usually evidenced by cellular uptake of normally impermeant fluorescent dyes (such as ethidium or YO-PRO®) or by means of electrophysiology. In the latter case, an increase in permeation to large cations (such as N-methyl D-glucamine$^+$, $NMDG^+$) is manifested by time-dependent shifts in the reversal potential of the current.

ATP-induced fluorescent dye uptake has been reported in human (47, 48) and mouse (58, 59) osteoclasts. However, in rabbit osteoclasts, the reversal potential of the BzATP-activated current does not change over time in the presence of $NMDG^+$ (34).

3.3.3. Signaling

3.3.3.1. Ca^{2+} influx

Ca^{2+} influx through $P2X_7$ receptors may contribute importantly to signaling in osteoclasts. Simultaneous patch-clamp and Ca^{2+} fluorescence recordings showed that successive stimulation of rabbit osteoclasts with BzATP resulted in progressively increasing elevations of $[Ca^{2+}]_i$. These Ca^{2+} signals were strongly correlated with the activity-dependent current

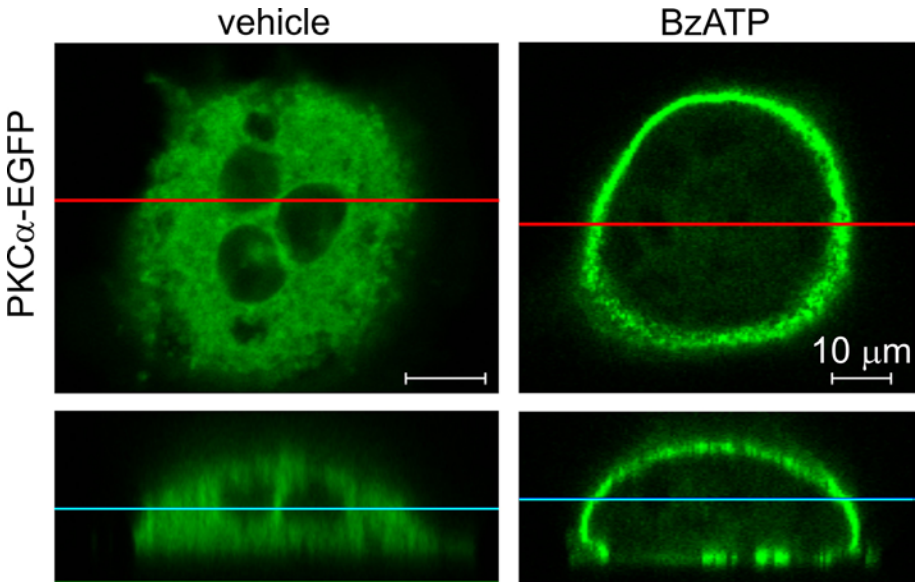

Figure 6. $P2X_7$-induced translocation of PKCα to the plasma membrane of osteoclasts. Osteoclasts were generated from the bone marrow of wild-type mice. Enhanced green fluorescent protein (EGFP)-tagged PKCα was expressed by adenoviral transduction. Nucleotide-induced translocation of PKCα-EGFP was monitored by confocal microscopy. PKCα-EGFP showed typical cytoplasmic localization in a vehicle-treated osteoclast (left). Within 2 minutes, BzATP (150 μM) induced translocation of PKCα-EGFP to the plasma membrane of another osteoclast (right). The lower panels are reconstructed *z*-stack images of the osteoclasts shown above. PKCα-EGFP translocated selectively to the upper (basolateral) membrane. Red horizontal lines indicate location of the *z*-stacks. Blue horizontal lines indicate location of the *x-y* confocal images shown above. Reproduced with permission from (60).

mediated by the $P2X_7$ receptor (34). Indeed, removal of extracellular Ca^{2+} abolished these increases in $[Ca^{2+}]_i$. The $P2X_7$ receptor antagonists oxidized ATP and PPADS inhibited both the activity-dependent Ca^{2+} responses and currents elicited by BzATP (34). Since $P2X_7$ and P2Y-mediated Ca^{2+} signals are expected to have different time courses and amplitudes, divergent cellular responses may arise from activation of $P2X_7$ or P2Y receptors in osteoclasts.

3.3.3.2. Signaling through PKC

Protein kinase C (PKC) comprises a family of serine/threonine protein kinases which play important roles in intracellular signaling. The conventional isoforms, such as PKCα and PKCβ1, depend on increases in $[Ca^{2+}]_i$ for activation. Upon activation, PKC is recruited to lipid membranes, where it phosphorylates proteins involved in signaling. Armstrong *et al.* investigated whether $P2X_7$ receptors induce activation of PKC in osteoclasts (60). Mouse bone marrow cells and macrophage RAW 264.7 cells were differentiated into multinucleated osteoclasts and osteoclast-like cells, respectively. Confocal imaging was used to localize enhanced green fluorescent protein (EGFP)-tagged PKC. Stimulation of these cells with BzATP (150 μM) induced transient translocation of PKCα and PKCβI to the plasma membrane (Figure 6), but did not induce translocation of the novel, Ca^{2+}-independent isoform PKCδ (60).

Low concentration of ATP or UTP (150 μM) failed to induce PKC translocation, ruling out the involvement of P2Y receptors or other members of the P2X family, apart from $P2X_7$. In addition, BzATP failed to induce PKC translocation in osteoclasts derived from the bone marrow of *P2rx7*$^{-/-}$ mice, confirming that $P2X_7$ receptors mediate this response. Time courses of BzATP-induced elevations in $[Ca^{2+}]_i$ and translocation of PKC were tightly correlated. Moreover, chelation of external Ca^{2+} with EGTA abolished PKC translocation.

In summary, the evidence strongly supports that $P2X_7$-mediated Ca^{2+} influx is responsible for activation of conventional PKCα and PKCβI isoforms in osteoclasts (60). However, the signaling functions of PKC downstream of the $P2X_7$ receptor in osteoclasts are not yet well understood.

3.3.3.3. Signaling through NF-κB

The transcription factor NF-κB resides in the cytoplasm in its inactive form. After activation, it translocates to the cell nucleus, where it regulates transcription of several genes involved in immune responses, inflammation, cell survival and cancer (61). This transcription factor plays an important role in osteoclast development (4, 5). Korcok *et al.* studied whether $P2X_7$ acts upstream of NF-κB activation in osteoclasts isolated from mice and rabbits (62). Stimulation with BzATP induced nuclear translocation of NF-κB in both rabbit and mouse cells. NF-κB translocation effect was transient, reaching a maximum about 30 minutes after exposure to the agonist and returning to basal levels an hour later. Low concentration

of ATP (10 μM) or adenosine (10 μM) failed to reproduce the effects of BzATP, consistent with the involvement of $P2X_7$ receptors. Furthermore, the BzATP-induced translocation was not observed in osteoclasts isolated from *P2rx7*$^{-/-}$ mice.

3.3.4. Physiological roles of $P2X_7$ receptors in osteoclasts

3.3.4.1. Role of $P2X_7$ in the formation of multinucleated osteoclasts

Fusion of precursor cells to form multinucleated osteoclasts is a complex phenomenon not completely understood. The discovery that a high expression level of $P2X_7$ receptors in macrophages promotes multinucleated giant cell formation (63) encouraged the idea that these receptors are involved in the formation of mature osteoclasts. Evidence in support of this possibility includes: 1) osteoclast formation can be prevented by incubation with an anti-$P2X_7$ antibody (48); 2) prolonged exposure to ATP results in extensive internalization of $P2X_7$ receptors and blocks the ability of RAW 264.7 cells to fuse into multinucleated osteoclast-like cells (59); and 3) some $P2X_7$ receptor antagonists block the formation of osteoclast-like cells from human blood monocytes *in vitro* (64).

However, observations using *P2rx7*$^{-/-}$ mice indicated that $P2X_7$ receptors do not play an essential role in the formation of these cells *in vivo* and *in vitro*. Two independent groups have reported that knockout animals have multinucleated osteoclasts (54, 65). In fact, the number of osteoclasts in trabecular bone is higher in knockout compared to wild-type animals (54). Since $P2X_7$ expression renders some cells more susceptible to apoptosis, this observation may be related to potentiation of osteoclast survival rather than enhanced osteoclastogenesis. In addition, no differences were observed between wild-type and knockout mice regarding the ability of osteoclast precursors to differentiate and fuse into multinucleated osteoclasts *in vitro* (54, 65).

3.3.4.2. Role of $P2X_7$ in osteoblast-osteoclast and osteoclast-osteoclast communication

Signaling between osteoblasts and osteoclasts is important to maintain the balance between bone formation and resorption (66). ATP released during mechanical stimulation could play an important role in intercellular communication. In this regard, it has been suggested that $P2X_7$ receptors in osteoclasts mediate mechanically induced intercellular signaling between osteoblasts and osteoclasts, and among osteoclasts (47). It was proposed that mechanical stimulation causes ATP release, which induces $[Ca^{2+}]_i$ elevation in adjoining cells. Lack of desensitization and sensitivity of the response to oxidized ATP led to the suggestion that this response involves $P2X_7$ receptors in osteoclasts. However, oxidized ATP is not a specific $P2X_7$ antagonist (28), making it difficult to rule out the possible involvement of other subtypes of Ca^{2+}-mobilizing P2 receptors. Regardless of which P2 receptors underlie the responses, these findings are important and more research is clearly needed in this area.

3.3.4.3. Role of $P2X_7$ in regulation of the osteoclast cytoskeleton

Remodeling of the actin cytoskeleton has been recently proposed as a novel function for osteoclast $P2X_7$ receptors. Stimulation of osteoclasts with a high concentration of ATP or with BzATP rapidly (within a few minutes) induced disruption of filamentous actin belts, whereas low concentrations of ATP had no significant effect (50, 67). This disruption was transient and actin reorganization was observed afterwards. Hazama and coworkers also reported that actin reorganization was accompanied by microtubule deacetylation, resulting in enhanced osteoclastic bone resorption (50).

3.3.4.4. Skeletal phenotype of *P2rx7* knockout mice

Ke *et al.* described the skeletal phenotype resulting from disruption of the gene encoding the $P2X_7$ receptor in mice (54). Femurs and tibias of these mice and matched littermate controls were analyzed in detail. In femurs, it was observed that knockout animals had significant reduction in periosteal circumference (bone diameter), but not in length (Figure 7A and B). This result indicates that $P2X_7$ has a role in regulating radial bone growth (periosteal bone formation) and expansion of the bone marrow cavity, but not longitudinal bone growth. In addition, femurs displayed significant reduction in cortical bone content and total bone content, as evaluated by peripheral quantitative computed tomography. In tibias, trabecular bone displayed an increased number of osteoclasts and a significant reduction in mass. This result suggests that loss trabecular bone mass was caused by increased osteoclast-mediated resorption. In addition, periosteal bone formation was significantly decreased (Figure 7C).

Although both male and female knockouts showed these features, they were more pronounced in males. This suggests a link between sex hormones and $P2X_7$ signaling. In this regard, an electrophysiological study indicates that the $P2X_7$ receptor is inhibited by 17β-estradiol (68). It has also been reported that estrogen attenuates $P2X_7$ receptor-mediated apoptosis of uterine cervical cells by blocking calcium influx (69).

Ke and coworkers also studied whether osteoclastogenesis was affected in $P2X_7$-deficient mice. As mentioned above, osteoclasts with normal appearance were isolated from their long bones. In addition, when bone marrow cells were treated with M-CSF and RANKL, no significant difference in osteoclast formation was observed in wild-type and *P2rx7*$^{-/-}$ cultures (54).

Interestingly, the skeletal phenotype of a second *P2rx7*$^{-/-}$ mouse model has been described by Gartland and coworkers (65). In contrast to the findings of Ke *et al.* (54), these mice showed no overt skeletal phenotype with the exception of thicker cortical bones than wild-type controls. This discrepancy may be due to the presence of a splice variant that escaped deletion in these *P2rx7*$^{-/-}$ mice, resulting in inadvertent tissue-specific expression of functional $P2X_7$ receptors (70, 71). However, it has yet to be reported whether escape from deletion occurs in osteoclasts from these knockout animals.

Based on findings from the *P2rx7* knockout mouse described by Ke and coworkers (which have no

Table 2. Expression of P2Y receptors in mammalian osteoclasts

Subtype	Model	Species	Approach used	Signaling	Proposed function	Refs.
$P2Y_1$	OCs[1] isolated from long bones OCs derived from bone marrow cells	Human Rabbit Rat	Immunolabeling *In situ* hybridization RT-PCR	Ca^{2+} release from stores	Enhanced osteoclastogenesis Enhanced resorption	(47) (79) (84)
$P2Y_2$	OCs isolated from long bones Osteoclastoma cell lines	Human Rabbit Rat	*In situ* hybridization RT-PCR	Ca^{2+} release from stores	Not determined	(35) (79) (85) (86)
$P2Y_6$	OCs isolated from long bones	Rabbit Rat	Ca^{2+} fluorometry RT-PCR Use of selective agonist	Ca^{2+} release from stores NF-κB translocation	Increased survival	(79)

[1] Abbreviations: OCs – osteoclasts

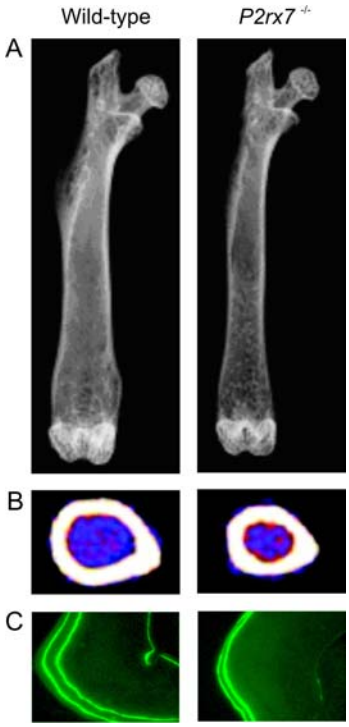

Figure 7. The $P2X_7$ receptor is involved in the regulation of periosteal bone formation. (A) Radiographs of femurs from adult male wild-type and *P2rx7*-deficient (*P2rx7*$^{-/-}$) mice. In comparison with wild-type controls, *P2rx7*$^{-/-}$ mice had femurs of smaller diameter, but similar length. (B) Differences in bone diameter were shown by peripheral quantitative computed tomography of a femoral shaft from adult male wild-type and *P2rx7*$^{-/-}$ mice. (C) Rates of periosteal bone formation, determined by double calcein labels. Calcein (green fluorescence) was administered twice to wild-type and *P2rx7*$^{-/-}$ mice within a 10-day period. The inter-labeling distance, which indicates the rate of periosteal mineral apposition and bone formation, was significantly smaller in *P2rx7*$^{-/-}$ mice. Reproduced with permission from (54).

fnctional $P2X_7$ receptors), $P2X_7$ receptors appear to be required for normal skeletal growth and anabolic responses to mechanical stimulation (72). Although $P2X_7$ receptors do not appear to be essential for osteoclastogenesis or bone resorption, the balance between bone formation and resorption may be affected in the knockout animals. Since $P2X_7$ plays a role in osteogenesis (73) and may contribute to osteoblast-osteoclast communication (47), the absence of this receptor could result in misbalance between the activities of these cell types.

3.3.4.5. $P2X_7$ polymorphisms in humans

Several genetic polymorphisms have been reported for the $P2X_7$ receptor. In particular, the Glu496Ala polymorphism results in a receptor which is deficient in ATP-induced pore formation (ethidium uptake) and promotion of apoptotic cell death (74). However, its function as an ion channel remains unaffected (75). The Glu496Ala polymorphism affects function but does not affect trafficking of the receptor to the plasma membrane (74). In contrast, another polymorphism, Ile568Asn, prevents normal trafficking and function of the receptor (76). Ohlendorff *et al.* have reported that 10-year fracture incidence in postmenopausal women is significantly associated with the Glu496Ala and Ile568Asn polymorphisms of the $P2X_7$ receptor (77). The authors also show that the Glu496Ala polymorphism results in decreased susceptibility of osteoclasts to ATP-induced apoptotic death. Impaired osteoclast apoptosis may enhance overall bone resorption, consistent with the increase in facture incidence observed by Ohlendorff and coworkers (77) and with the phenotype of the *P2rx7*$^{-/-}$ mouse described by Ke *et al.* (54). More details can be found in this special issue's review on P2 receptor polymorphisms and bone (78).

4. EXPRESSION, SIGNALING AND FUNCTION OF P2Y RECEPTORS IN OSTEOCLASTS

Currently, the P2Y receptor family is known to be composed of eight members: $P2Y_{1,2,4,6,11,12,13,14}$. They are G protein-coupled receptors and, in many cases, activate phospholipase C as a downstream effector for the formation of inositol 1,4,5-trisphosphate, which in turn causes release of Ca^{2+} from intracellular stores. Different P2Y receptors can be distinguished by their pharmacological properties. For example, $P2Y_1$ receptors are activated preferentially by ADP. On the other hand, $P2Y_2$ receptors are activated by ATP and UTP with approximately the same potency, and $P2Y_6$ receptors by UDP (44). When ATP or UTP are used as agonists, the results should be interpreted cautiously because the responses could be mediated by the nucleotides themselves or by their metabolites (e.g., ADP, UDP) generated by ectonucleotidase activity.

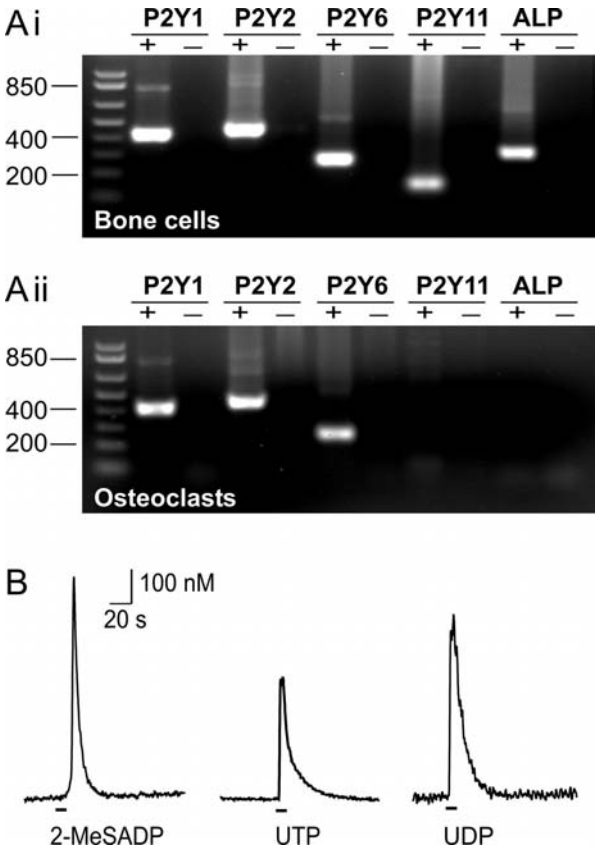

Figure 8. Rabbit osteoclasts express $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. (A) P2Y receptor transcripts were identified by RT-PCR. Cells were obtained from the long bones of neonatal rabbits and were processed to isolate mRNA (Bone cells; Ai). In other samples, osteoclasts were purified using pronase E, followed by removal of non-osteoclasts by micromanipulation prior to mRNA isolation (Osteoclasts; Aii). In both cases, samples were divided into those that did (+) and did not (–) undergo reverse transcription as a control. PCR was then carried out to detect mRNA encoding for P2Y receptors or alkaline phosphatase (ALP, an osteoblast marker). Whereas the bone cell samples displayed mRNA transcripts for $P2Y_{1,2,6,11}$ and ALP, samples from purified osteoclasts only contained transcripts for $P2Y_{1,2,6}$. Hence, it was proposed that $P2Y_{11}$ transcripts are not present in osteoclasts, but come from contaminating cells. (B) Purinergic agonist-induced Ca^{2+} signals in fura-2-loaded osteoclasts. At the time indicated by the bars below the traces, 2-MeSADP ($P2Y_1$ agonist, 10 μM), UTP ($P2Y_2$ agonist, 10 μM) or UDP ($P2Y_6$ agonist, 10 μM) was applied to single osteoclasts. The resulting transient elevations in $[Ca^{2+}]_i$ are consistent with the presence of functional $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. Reproduced with permission from (79).

The precise roles of P2Y receptors in osteoclasts remain to be fully established. Nevertheless, biochemical and functional evidence indicates their expression in osteoclasts (Table 2). Korcok *et al.* investigated P2Y receptor expression by RT-PCR (79). Transcripts for $P2Y_{1, 2, 6,11}$ as well as alkaline phosphatase (an osteoblast marker) were observed in samples obtained from cultured rabbit bone marrow cells – containing osteoclasts and other cell types. In contrast, when an additional step was carried out to purify osteoclasts, only $P2Y_{1,2,6}$ transcripts were observed (Figure 8A). It is worth noting that expression of $P2Y_4$ was not investigated in this work. However, a previous study using *in situ* hybridization indicated that rat osteoclasts do not express $P2Y_4$ (35). Therefore, these results indicate that $P2Y_{1,2,6}$ receptors are expressed by rabbit osteoclasts, whereas $P2Y_{11}$ and alkaline phosphatase transcripts are the result of contamination by other cell types.

4.1. Physiological evidence for the presence of P2Y receptors in osteoclasts

4.1.1. Ca^{2+} signals

In many cases, activation of P2Y receptors induces Ca^{2+} release from intracellular stores. Early studies in rabbit osteoclasts revealed that ATP-induced elevations in $[Ca^{2+}]_i$ occurred even in the absence of extracellular Ca^{2+} (80, 81). Osteoclasts loaded with GDPβS (a blocker of G protein activation) failed to generate these Ca^{2+} responses (81), indicating that G protein-coupled receptors were involved.

When studied using fluorescent Ca^{2+} dyes, ADP, but not AMP or adenosine, elicited elevations in $[Ca^{2+}]_i$ in rabbit and rat osteoclasts (43). This result rules out the participation of adenosine receptors in Ca^{2+} signaling. The response to ADP was probably mediated by $P2Y_1$ receptors, because it was very similar to responses elicited

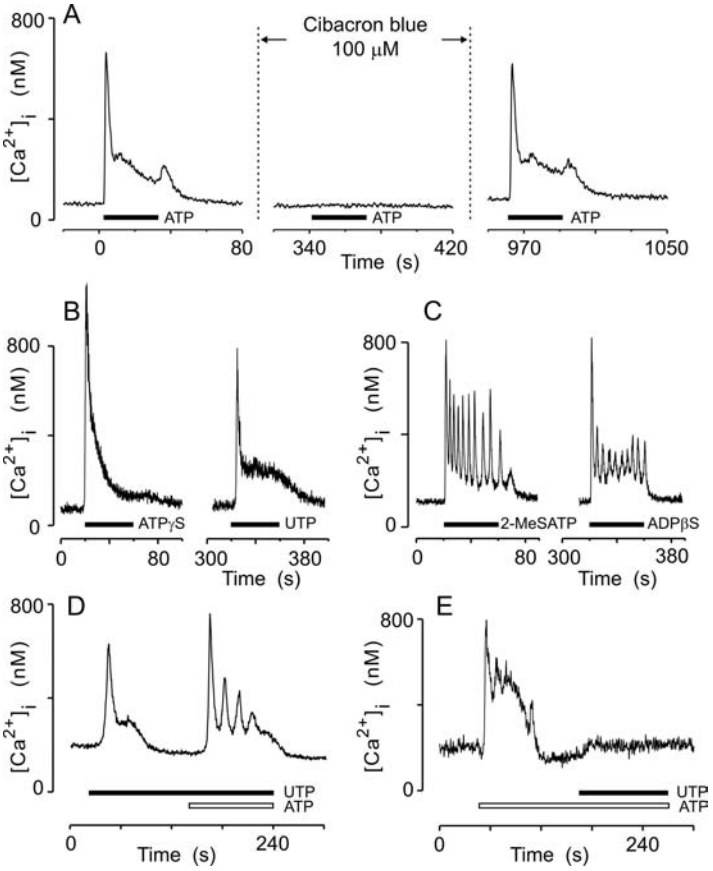

Figure 9. Multiple subtypes of P2Y receptors mediate Ca^{2+} signaling in mammalian osteoclasts. Nucleotides were applied to single fura-2-loaded rat and rabbit osteoclasts, as indicated by the bars below the traces. (A) Stimulation with ATP (20 μM) induced a rapid and transient increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase. The purinergic antagonist Cibacron blue (100 μM) abolished the response (middle panel). Following washout and recovery, the osteoclast again responded to ATP (right). (B) ATPγS (10 μM), a poorly hydrolysable analog of ATP, induced a Ca^{2+} response with transient and sustained components. After 3 min recovery, the osteoclast responded to UTP (10 μM) in a similar fashion. (C) Oscillatory Ca^{2+} signals were observed in some osteoclasts. 2-MeSATP (1 μM) elicited a rise of $[Ca^{2+}]_i$ followed by oscillations. Following 3 min recovery, a similar response was elicited by ADPβS (1 μM). (D) and (E) Evidence for multiple nucleotide receptors in individual cells. Prolonged application of UTP (100 μM) induced a transient elevation of $[Ca^{2+}]_i$ that desensitized after ~50 s. Subsequent stimulation with ATP (100 μM) in the continued presence of UTP elicited an oscillatory Ca^{2+} response (panel D). When the agonists were applied in the reverse order to another osteoclast, ATP induced the typical rise of $[Ca^{2+}]_i$ but a subsequent stimulation with UTP elicited little response (panel E). Since UTP stimulates $P2Y_2$ receptors that desensitize, the response to ATP in the continued presence of UTP (panel D) cannot be mediated by $P2Y_2$ receptors, but by other nucleotide receptors present in the osteoclast. Hence, the osteoclast of panel D must express at least two subtypes of purinoceptors. Reproduced with permission from (43).

by the potent $P2Y_1$ agonist ADPβS. Interestingly, application of the $P2Y_1$ selective agonists ADPβS or 2-MeSATP resulted in oscillations in $[Ca^{2+}]_i$ in some cells (Figure 9C). The oscillations persisted even in the absence of extracellular Ca^{2+} (37). Therefore, these oscillations depend on dynamic Ca^{2+} release from intracellular stores. $Ca^{2+}]_i$ elevations were also observed in response to UTP (10 μM) (43), an agonist of $P2Y_2$ receptors (Figure 8B and Figure 9). Furthermore, prolonged application of UTP elicited a transient response that declined to basal levels after a couple of minutes. The time course of the response is determined by $P2Y_2$ receptor desensitization due to prolonged exposure to the agonist (82). When the initial UTP response had declined to basal levels, application of ATP in the continued presence of UTP elicited a large transient elevation in $[Ca^{2+}]_i$ (Figure 9D). This result indicates that individual osteoclasts can express more than one subtype of P2Y receptor.

4.1.2. Activation of Ca^{2+}-dependent K^+ channels

P2Y receptor-induced Ca^{2+} release from intracellular stores activates Ca^{2+}-dependent K^+ channels in osteoclasts. Electrophysiological studies in rat and human osteoclasts indicate that this ATP-induced current: 1) is blocked by intracellular Cs^+; 2) has a reversal potential very close to the predicted K^+ equilibrium potential; 3) has a single-channel conductance about 50 pS; and 4) closely follows changes in $[Ca^{2+}]_i$ (37, 83). The time course of this

current depends on the purinergic agonist employed. When ATP was used, it was usually preceded by a $P2X_4$-mediated transient inward current (Figure 2A). When ADPβS was used, only the transient outward calcium-dependent K^+ current was observed (Figure 2B). This is because ADPβS does not activate $P2X_4$ receptors (33). In contrast, when 2-MeSATP was used, oscillations in the amplitude of this K^+ current could be observed in some cells. These oscillations closely followed the oscillations in $[Ca^{2+}]_i$ induced by 2-MeSATP (37). Activation of this current hyperpolarizes osteoclasts, whereas activation of P2X receptors has the opposite effect on membrane potential. Thus, it is possible that these channels contribute to the fine tuning of membrane potential in osteoclasts.

4.2. $P2Y_1$ receptors

$P2Y_1$ receptor mRNA has been detected by RT-PCR in human (47) and rabbit (79) osteoclasts. Expression of the $P2Y_1$ receptor has also been demonstrated by immunocytochemistry and *in situ* hybridization in rat osteoclasts (84). Agonists of $P2Y_1$ – such as ADP, ADPβS and 2-MeSADP – induce Ca^{2+} responses in osteoclasts (Figs. 8B and 9C).

ADP enhances osteoclastic resorption (84). Concentrations as low as 20 nM had a stimulatory effect, consistent with the high sensitivity of $P2Y_1$ receptors for ADP (44). Furthermore, MRS-2179 – a $P2Y_1$ receptor antagonist – blocked this stimulatory effect on resorption. Degradation products of ADP (AMP and adenosine) had no effect. ADP also stimulated the differentiation of precursor cells into osteoclasts (84); thus, ADP could stimulate resorption by enhancing osteoclastogenesis. Since osteoblasts also express $P2Y_1$, it is not clear whether the observed effects were due to a direct action of ADP on osteoclast $P2Y_1$ receptors or an indirect effect mediated by $P2Y_1$ receptors on osteoblasts (84). Activation of osteoblast $P2Y_1$ receptors could induce the expression of RANKL or other factors that stimulate osteoclasts.

4.3. $P2Y_2$ receptors

The $P2Y_2$ receptor was cloned from a human osteoclastoma cDNA library. Moreover, mRNA coding for $P2Y_2$ was detected by RT-PCR in osteoclast-like cells obtained from an osteoclastoma (85). Expression of $P2Y_2$ receptors in rat osteoclasts was also shown by *in situ* hybridization (35, 86).

Very little is known about the role of $P2Y_2$ receptors in osteoclasts. In fact, it has been reported by some authors that UTP – a potent $P2Y_2$ agonist – fails to elicit Ca^{2+} responses in osteoclasts (47, 81, 86), whereas others have reported Ca^{2+} responses (37, 79, 87) (see also Figs. 8 and 9). The reason for this discrepancy is not clear; it may be due to differences among species or different experimental conditions.

4.4. $P2Y_6$ receptors

Korcok *et al.* studied the role of $P2Y_6$ receptors signaling through NF-κB in the regulation of osteoclast survival (79). A stable analog of diuridine 5'-triphosphate – INS48823 – was used in this study, because it is a selective agonist at $P2Y_6$ receptors (88). Stimulation of osteoclasts with the $P2Y_6$ receptor agonists UDP or INS48823 elicited a Ca^{2+} response. Even though agonists at other subtypes of P2Y receptors (ADP and UTP) also elicited Ca^{2+} responses, only UDP and INS48823 were able to induce translocation of NF-κB from the cytosol to the nuclei (Figure 10A). This result suggests that an increase in $[Ca^{2+}]_i$ alone is not sufficient for NF-κB activation; other conditions must be met. Furthermore, stimulation with UDP or INS48823 (but not ADP or UTP) significantly increased osteoclast survival (79). This increase in survival was mediated by NF-κB, since SN50 – a cell-permeable peptide inhibitor of NF-κB – abolished the effect. This peptide also abolished translocation of NF-κB to the nuclei induced by the $P2Y_6$ agonists. Therefore, the $P2Y_6$ receptor stimulates osteoclast survival by a mechanism dependent on NF-κB.

As mentioned above, an extension in osteoclast life span may have important physiological consequences (20, 21, 23). $P2X_7$ receptors seem to promote apoptosis in osteoclasts (77), whereas $P2Y_6$ receptors have the opposite action (79). The net effect of P2 receptor activation on osteoclast survival could be determined by integration of these signals.

5. SUMMARY AND PERSPECTIVE

A number of different P2 receptors are functionally expressed in osteoclasts. Currently, most evidence points to $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_6$; however, it is likely that a wider picture involving more P2 receptor subtypes will emerge in the future. Despite the important advances made in the field, a better understanding of the mechanisms involved and the roles that P2 receptors play in osteoclasts is still necessary.

P2 receptors are likely to influence osteoclast physiology at many, if not all, stages of their life cycle, from formation to death. Regarding formation, an early report indicates that low ATP concentrations (0.2-2 μM) stimulate formation, whereas higher concentrations (20-200 μM) reduce or block it (89). This result suggests that $P2X_7$ receptors, which are activated by high ATP concentrations, suppress osteoclast formation or survival. Other receptors, such as $P2Y_1$ might account for the stimulatory effect observed. Other reports suggest that $P2X_7$ receptors regulate osteoclast formation (48, 59, 64); however, it is clear that $P2X_7$ receptors are not essential for osteoclastogenesis since *P2rx7*$^{-/-}$ mice possess multinucleated osteoclasts (54, 65).

Resorption by osteoclasts is modulated by P2 receptors, but the detailed mechanisms are not completely understood. ATP stimulates resorption up to about six-fold with a maximum effect occurring at low concentrations (0.2-2 μM) (89). It is possible that at least part of this stimulatory effect is mediated by $P2Y_1$ receptors, since ADP has a potent stimulatory effect on resorption, which is blocked by the $P2Y_1$ antagonist MRS-2179 (84). The $P2X_7$ receptor appears to have the opposite effect on resorption. It is possible that induction of apoptosis accounts for the reduction in overall resorptive activity. Furthermore, signs

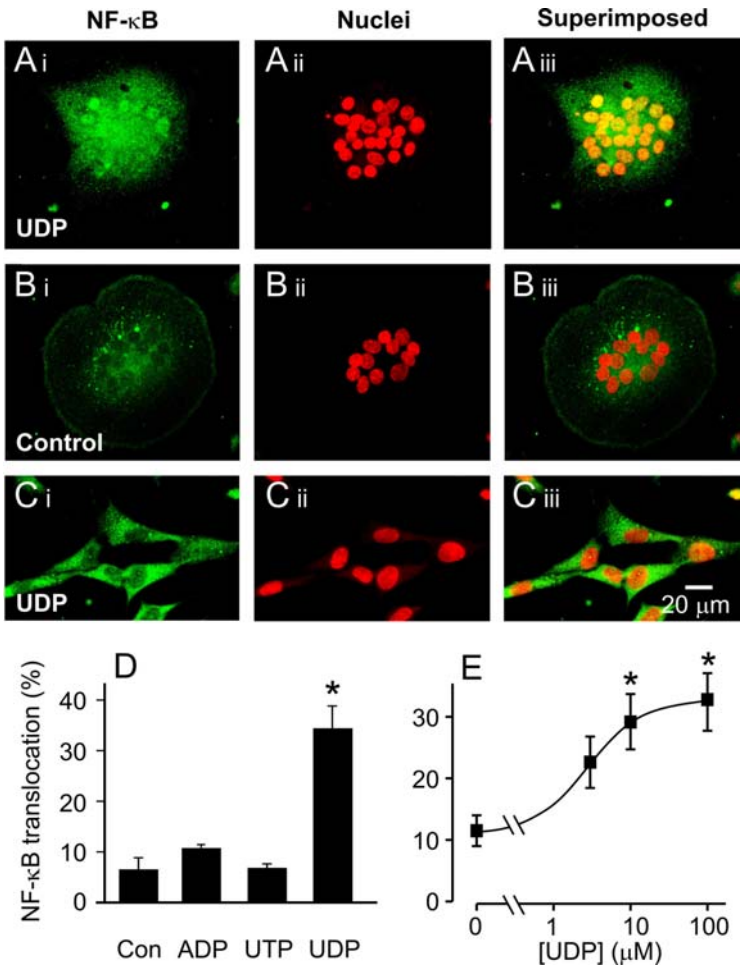

Figure 10. Activation of $P2Y_6$ receptors induces nuclear translocation of NF-κB in rabbit osteoclasts. Osteoclasts (A and B) and stromal cells (C) were incubated at 37°C with UDP (10 μM; a $P2Y_6$ agonist) or vehicle (labeled as Control, in B) for three hours. Following fixation, the p65 subunit of NF-κB was visualized by immunofluorescence (green, left panels) and cell nuclei were stained with TOTO-3 (red, middle panels). (A) Nuclear translocation of NF-κB is observed in osteoclasts incubated for 3 hours with UDP (yellow in superimposed image Aiii). (B) No translocation of NF-κB is observed in control osteoclasts treated with vehicle. (C) In contrast to osteoclasts, stromal cells stimulated with UDP show no translocation of NF-κB. (D) Osteoclasts were incubated with vehicle (Con), ADP, UTP or UDP (10 μM each) for three hours and processed as above (A-C). Only UDP induced a significant increase in the percentage of cells with nuclear localization of NF-κB, consistent with the involvement of $P2Y_6$ receptors. (E) UDP elevates the percentage of cells showing nuclear localization of NF-κB in a concentration-dependent manner (incubation time of 3 hours). Reproduced with permission from (79).

of increased resorptive activity are observed when $P2X_7$ receptor activity is reduced by genetic modification in mice (54), or loss-of-function polymorphisms in humans (77).

Osteoclast death is a tightly regulated phenomenon. In this regard, evidence to date indicates that P2 receptors play a role in regulating osteoclast survival. $P2X_7$ receptors promote osteoclast death by apoptosis (77) and $P2Y_6$ receptors suppress osteoclast apoptosis by a mechanism dependent on NF-κB (79).

Taking into account the influence that P2 receptors have on osteoclasts, there is considerable excitement about the potential of P2 receptors as targets for drugs that inhibit resorption (with relevance to diseases such as osteoporosis, periodontitis, rheumatoid arthritis and tumor-induced osteolysis). Previous reviews have considered these possibilities (25, 90-92). However, there are still no clear solutions and more research is needed.

6. ACKNOWLEDGMENT

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Abbreviations: ADPβS: adenosine 5'-*O*-(2-thiodiphosphate); ATPγS: adenosine 5'-*O*-(3-thiotriphosphate); BzATP: 2',3'-*O*-(4-benzoylbenzoyl)-ATP; $[Ca^{2+}]_i$: concentration of cytosolic free Ca^{2+}; CB: Cibacron blue; EGFP: enhanced green fluorescent protein; EGTA: ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GDPβS: guanosine-5'-*O*-(2-thiodiphosphate); IC_{50}: half maximal inhibitory concentration; M-CSF: macrophage colony stimulating factor; 2-MeSADP: 2-methylthioadenosine 5'-diphosphate; 2-MeSATP: 2-methylthioadenosine 5'-triphosphate; NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells; $NMDG^+$: N-methyl D-glucamine$^+$; PKC: protein kinase C; PPADS: pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid; RANKL: receptor activator of NF-κB (RANK) ligand; RT-PCR: reverse transcription polymerase chain reaction.

Key Words: Apoptosis, ATP, Bone, Calcium, Calcium-activated potassium channels, Electrophysiology, Ion channel, NF-κB, Nucleotide, Osteoclastogenesis, Osteoclasts, P2 receptors, *P2RX7*, P2X receptors, $P2X_7$, P2Y receptors, Patch clamp, Protein kinase C, Purinergic, Resorption, Signaling, Survival, Review

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