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LACK OF EFFECT OF ADENOSINE ON THE FUNCTION OF RODENT OSTEOBLASTS AND OSTEOCLASTS *IN VITRO*

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ABSTRACT

Extracellular ATP, signalling through P2 receptors, exerts well-documented effects on bone cells, inhibiting mineral deposition by osteoblasts and stimulating the formation and resorptive activity of osteoclasts. The aims of this study were to determine the potential osteotropic effects of adenosine, the hydrolysis product of ATP, on primary bone cells *in vitro*. We determined the effect of exogenous adenosine on 1) the growth, alkaline phosphatase (TNAP) activity and bone-forming ability of osteoblasts derived from the calvariae of neonatal rats and mice and the marrow of juvenile rats; 2) the formation and resorptive activity of osteoclasts from juvenile mouse marrow. RT-PCR analysis showed marked differences in the expression of P1 receptors in osteoblasts from different sources. Whilst the A_1 and A_{2B} adenosine receptors were expressed by all primary osteoblasts, A_{2A} receptor expression was limited to rat bone marrow and mouse calvarial osteoblasts and the A_3 receptor to rat bone marrow osteoblasts. We found that adenosine had no detectable effects on cell growth, TNAP activity or bone formation by rodent osteoblasts *in vitro*. The analogue 2-chloroadenosine, which is hydrolysed more slowly than adenosine, had no effects on rat or mouse calvarial osteoblasts but increased TNAP activity and bone formation by rat bone marrow osteoblasts by 30-50% at a concentration of 1µM. Osteoclasts were found to express the A_{2A}, A_{2B} and A_3 receptors; however, neither adenosine (≤100µM), nor 2-chloroadenosine (≤10µM) had any effect on the formation or resorptive activity of mouse osteoclasts *in vitro.* These results suggest that adenosine, unlike ATP, is not a major signalling molecule in bone.

INTRODUCTION

The effects of extracellular purines and pyrimidines on cell surface receptors have been extensively studied for over 40 years [1]. ATP and related compounds exert their physiological affects via seven P2X ligand-gated ion channel receptors and eight P2Y G-protein coupled receptors that are expressed in most tissues [2,3].

The roles played by P2X and P2Y receptors in regulating the function of bone cells have received considerable attention in recent years [4]. Osteoblasts, the bone-forming cells, express multiple P2 receptors [5,6], in a differentiation-dependent manner [7,8] and respond to extracellular nucleotides with a prompt increase in intracellular calcium [7-10]. Importantly, exogenous ATP, UTP and other nucleotide analogues also potently inhibit mineralisation of bone formed by osteoblasts in culture [8,11,12]. Moreover, endogenous ATP released by osteoblasts also appears to act as a significant local inhibitor of mineralisation [13]. The action of UTP, together with data from pharmacological studies using other selective P2 receptor agonists and antagonists indicated that $P2Y_2$, P2X1 and P2X7 receptors could be involved in mediating the inhibition of mineralisation by ATP [8,12,13].

Osteoclasts, the bone-resorbing cells also express most P2 receptors [14] and respond to extracellular nucleotides with elevation of intracellular calcium [15]. Moreover, extracellular ATP, ADP and UDP have been shown to increase the formation and resorptive activity of primary rodent osteoclasts *in vitro* [14,16-18]. Pharmacological evidence now suggests that the pro-resorptive action of ATP and related molecules are mediated by the $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ receptors [14,17-19].

Adenosine is a hydrolysis product of ATP and is formed extracellularly by the actions of ecto-nucleotidases. There are four major families of these ecto-enzymes: (1) ecto-nucleoside triphosphate diphosphohydrolases (NTPdases), which hydrolyse ATP to ADP and finally AMP; (2) ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs) that hydrolyse ATP to AMP, with the release of pyrophosphate; (3) alkaline phosphatases, which sequentially remove single phosphate groups and can hydrolyse ATP through to adenosine; and (4) ecto-5'nucleotidase, that hydrolyses AMP to adenosine. Extracellular adenosine concentrations are usually <300nM [20] but can rise to approximately 1μM under conditions of physiological stress [21].

The physiological actions of adenosine have been studied for over 90 years. Adenosine acts via the G-protein coupled P1-receptors, found on the surface of many cell types. The P1 receptor family can be subdivided into the A_1, A_{2A}, A_{2B} and A_3 receptors [22]. The A_{2A} and

A_{2B} adenosine receptors are predominantly stimulatory and are coupled to G_s to stimulate cAMP signalling; the A_1 and A_3 receptors are mainly Gi coupled and act to inhibit cAMP signalling [22].

Both osteoblasts and osteoclasts have been reported to express all four P1 receptor subtypes [23-27]. However, the actions of extracellular adenosine on bone cells appear to be somewhat less clear-cut than those of ATP. Synthetic adenosine analogues caused a receptor-mediated rise in cAMP levels in calvarial osteoblast-like cells [28] but adenosine had no effect on intracellular calcium levels in these cells [7]. Two independent groups failed to find an effect of adenosine on the formation of mineralised bone nodules by rat calvarial osteoblasts [11,29]. However, a more recent study indicated that adenosine, acting via the A_{2B} receptor, may increase the osteogenic differentiation of rat long bone mesenchymal stem cells [24]. In addition, a synthetic A_{2B} receptor agonist has been shown to increase bone formation, and bone marrow osteoblasts from A_{2B} receptor knockout mice display reduced levels of bone formation [30]. Recently, it has also been reported that stimulation of the A_{2A} receptors can enhance bone regeneration [31].

The study of Lerner *et al* (1987) found that adenosine analogues had no effect on the resorption of cultured mouse calvarial bones. Adenosine was later shown to be without effect on the formation or resorptive activity of primary rodent osteoclasts *in vitro* [16,17]. Adenosine was also reported to have no effect on intracellular calcium levels in rabbit osteoclasts *in vitro* [32]. However, more recent work has indicated that adenosine, acting through the A_{2A} receptor, may stimulate the formation of osteoclasts from human peripheral blood cells [25]. In contrast, Mediero *et al* (2012) [33] found that A_{2A} receptor agonists inhibited mouse osteoclast formation *in vitro*. Blockade or deletion of the A_1 receptor has additionally been reported to reduce the formation of mouse osteoclasts in culture [23]; however, the same group also found that stimulation of the A_1 receptor had no effect on mouse osteoclasts [34].

The aim of the present study was to determine the direct actions of adenosine on normal osteoblasts and osteoclasts, using well-characterised assays that measure the accepted physiological functions (i.e. bone formation and bone resorption) of these cells.

METHODS

Reagents

All tissue culture and molecular biology reagents were purchased from Life Technologies (Paisley, UK) unless stated otherwise. Chemical reagents were purchased from Sigma Aldrich (Poole, UK). 2-chloroadenosine, GR79236, BAY606583, CGS15943 and pentostatin were purchased from Tocris (Bristol, UK). P1 receptor antibodies were obtained from Alomone (Jerusalem, Israel), the β-actin antibody from Abcam (Cambridge, UK) and HRP-conjugated secondary antibodies from Jackson Immunoresearch Laboratories (Philadelphia, USA).

Primary bone cell culture

This study used osteoblasts from several sources namely rat / mouse calvaria and rat bone marrow. Osteoclasts were obtained from mouse bone marrow. These methods represent the most widely used and well validated methods for obtaining primary bone cells for *in vitro* research.

Rat / mouse calvarial osteoblasts: primary cells were derived from the calvarial bones of 2-4 day old rats (Sprague-Dawley) and mice (C57BL/6 or 129/SvTerJ). Osteoblasts were obtained using methods similar to those previously described [35-37]. Briefly, calvariae were digested using 0.25% trypsin for 10 minutes, 0.2% collagenase in Hank's buffered salt solution (HBSS) for 30 minutes, and finally 0.2% collagenase in HBSS for 60 minutes, all at 37ºC. The first two digests were discarded and cells from the final digest were resuspended in Dulbecco's modified essential medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin (mixture abbreviated to 'DMEM'). Due to increased nutritional requirements [36], mouse cells were resuspended in α-modified essential medium supplemented with 10% foetal calf serum, 70μg/ml gentamicin, 50U/ml penicillin, 50 μg/ml streptomycin, 0.125 μg/ml amphotericin (mixture abbreviated to 'α-MEM').

Osteoblasts were cultured for 4 days in 75 cm^2 flasks in a 5% CO_2 atmosphere at 37ºC until confluent. Upon confluence, rat cells were then plated into 6-well trays in DMEM further supplemented with 2mM β-glycerophosphate, 50μg/ml ascorbate and 10nM dexamethasone (mixture abbreviated to 'supplemented DMEM') [35-37]. Mouse cells were plated into 6 well trays in α-MEM further supplemented with 2mM β-glycerophosphate and 50 μg/ml ascorbate (mixture abbreviated to 'supplemented α-MEM'). Osteoblasts were treated with 1nM–100μM adenosine, 2-chloroadenosine, GR79235 (selective A_1 receptor agonist), BAY606583 (a selective A_{2B} receptor agonist), CGS15943 (a non-selective P1 receptor antagonist), pentostatin (an adenosine deaminase inhibitor), ATP or PBS (vehicle) for the duration of the culture. Half-medium changes were performed every third day of culture. Experiments were terminated by fixing the cells in 2.5% glutaraldehyde for 5 minutes. Cell culture plates were imaged at 800 dpi using a flat-bed scanner (Epson Perfection 4990 Photo) and the total area of bone nodules formed was quantified by image analysis, as described previously [35-37].

Rat bone marrow osteoblasts: Primary rat osteoblasts of bone marrow / stromal cell origin were obtained from the long bones of 6-week old Sprague-Dawley rats. The epiphyses were cut across and the marrow was flushed out of the bones using PBS. The collected cells were suspended in α-MEM and pre-cultured in a 75 cm^2 flask in 5% CO_2 at 37ºC. After 24 hours all the α-MEM was replaced in order to eliminate non-adherent cells; adherent stromal cells were cultured for a further 2 days until confluent. Upon confluence, cells were plated into 6-well trays and cultured as described above.

Mouse osteoclasts: Osteoclasts were formed from precursors flushed from the bone marrow of 8-week old mice using previously described methods [38]. Cells were pre-incubated in a 75 cm^2 flask containing modified essential medium supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin and 100nM prostaglandin E_2 (abbreviated as 'MEM'), supplemented with 2.5ng/ml macrophage colony stimulating factor (M-CSF; R&D Abingdon, UK) in 5% CO2 at 37ºC. After 24 hours the non-adherent mononuclear cells remaining in the culture medium were collected. The cells were re-suspended in MEM supplemented with 10ng/ml M-CSF and 3ng/ml receptor activator of NF-κB (RANKL) (R&D Abingdon, UK) and seeded onto 5 mm-diameter ivory discs in a 96 well tray (10^6 cells/disc). After a further 24 hours the ivory discs were transferred into 6-well trays and cultured for 6 days at pH 7.30. Discs were cultured for the final 2 days in medium acidified to pH 6.90 to activate osteoclastic resorption [38] before fixation in 2.5% glutaraldehyde and staining to demonstrate tartrate-resistant acid phosphatase (TRAP). Osteoclasts were identified as TRAP-positive cells with ≥2 nuclei. The numbers of osteoclasts and area resorbed per disc were evaluated 'blind' using transmitted and reflected light microscopy, as described previously [38].

Alkaline phosphatase activity

Osteoblast tissue non-specific alkaline phosphatase (TNAP) activity was measured in cell lysates taken defined stages of osteoblast differentiation (proliferating, differentiating, mature, mature bone-forming) using a colorimetric kit (Anaspec, CA, USA), as previously described [35]. TNAP activity was normalised to cell protein using the Bradford reagent (Sigma-Aldrich, Poole, UK).

Cell number and viability assays

Osteoblast cell number was measured at regular intervals throughout the culture period using a commercially available kit (CytoTox 96, Promega UK, Southampton, UK), as previously described [13]. This assay measures the activity of lactate dehydrogenase (LDH), a cytosolic enzyme which is released on cell lysis.

RNA extraction and RT-PCR

Osteoblasts were cultured in 6-well trays for up to 28 days and total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions. Osteoclasts were cultured on 1cm diameter dentine discs for up to 10 days before RNA extraction. RNA was treated with RNase-free DNase I (Promega, Madison, USA) for 30 min at 37°C to remove contaminating genomic DNA. The reaction was terminated by heat inactivation at 65°C for 10 minutes. Total RNA was quantified spectrophotometrically by measuring absorbance at 260nm. cDNA was synthesised from approximately 1μg of RNA using Superscript III reverse transcriptase, oligo dT, RNasin and a deoxyribo-nucleotide mix.

The cDNA produced from osteoblast and osteoclast RNA was amplified by PCR using 1U GoTaq DNA polymerase, 1.5mM $MgCl_2$, 0.8μM nucleotide mix (Promega, Madison, USA) and 0.5μM primers (MWG Biotech, Ebersberg, Germany). The primer sequences used for rat and mouse RT-PCR are shown in **Table 1**.

Western blot

Protein was extracted from mature rat calvarial osteoblasts and osteoclasts. Cell layers were lysed in ice-cold radio immunoprecipitation (RIPA) lysis buffer (50mM Tris HCl pH 7.4, 150mM NaCl, 5mM EDTA, 0.1% SDS 1mM phenyl methyl sulfonyl fluoride (PMSF), 1mg/ml aprotinin, 1mM Na_3VO_4 and 2.5mg/ml deoxicolic acid). Cell homogenates were sonicated for 5 min and stored at -80°C for at least half an hour before use. Protein concentrations from lysates were determined using the Bradford assay (Sigma Aldrich, Gillingham, Dorset, UK). Prior to loading total protein samples were denatured by incubating at 95°C for 5 min in the presence of 5x reducing sample buffer (60mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue). Protein samples (20μg/lane) were loaded into SDS-PAGE (10%) gels and transferred onto a polyvinylidenifluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) by the use of a wet tank blotter (Bio-

Rad, Hercules, CA, USA) at 150 V for 1 hour. Membranes were then blocked with 5% non-fat milk and incubated with one of the P1 receptor antibodies (1:200) or β-actin (1:1000) overnight at room temperature. After washing, blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature (1:10,000). A peroxidase detection system (Immobilon™ Western, Millipore UK, Watford, UK) was used for the visualisation of the immunoreactivity.

Statistics

Statistical comparisons were made using one-way analysis of variance (ANOVA) and adjusted for multiple comparisons using the Bonferroni method. Calculations were performed using In Stat 3 (GraphPad, San Diego, CA). All data are presented as means ± SEM for 6 - 12 biological replicates. Results are representative of experiments performed at least three times, using cells from different animals.

RESULTS

Rodent osteoblasts and osteoclasts express P1 receptor mRNA *in vitro*

Total RNA was extracted from mature, bone-forming osteoblasts derived from rat calvaria (day 14), rat bone marrow (day 17) and mouse calvaria (day 28). RT-PCR showed mRNA expression of the A_1 and A_{2B} receptors in rat calvarial osteoblasts and all P1 receptors in rat bone marrow osteoblasts (**Fig. 1A).** Mouse calvarial osteoblasts expressed mRNA for A_1, A_{2A} and A_{2B} receptors but not the A_3 receptor **(Fig. 1A).**

RNA was extracted from mature, resorbing osteoclasts (day 10 of culture) for investigation of P1 receptor expression. Osteoclasts were found to express mRNA for the A_{2A}, A_{2B} and A_3 receptors **(Fig.1A)**

Total protein was extracted from mature rat calvarial osteoblasts and mouse osteoclasts. Western blot analysis revealed expression of A_{2A} and A_{2B}, receptor protein in osteoblasts; A_1 and A_3 receptor protein was not detected (**Fig. 1B**). Osteoclasts were found to express protein for all four adenosine receptors (**Fig.1B**).

The effects of P1 receptor agonists on bone formation

Rodent calvarial osteoblasts and rat bone marrow osteoblasts were cultured for up to 28 days with adenosine, 2-chloroadenosine and ATP. Rat calvarial osteoblasts were also additionally treated with the more selective agonists, GR79236 (A_1) and BAY606583 (A_{2B}). Representative light microscopy images of adenosine and 2-chloroadenosine-treated cell layers are shown in **Fig. 2.** In cultures of calvarial and bone marrow osteoblasts, adenosine had no effect on the level of bone formation (**Fig. 3A-3C**). 2-chloroadenosine was without effect in calvarial osteoblasts (**Fig. 3D & 3E**) but caused a small stimulatory effect at 1μM in bone marrow osteoblasts (**Fig. 3F**). Concentrations of ≥10μM 2-chloroadenosine appeared to have toxic effects, resulting in a complete abolition of bone formation. ATP (≥10μM) inhibited mineralisation by ≤90% and ≤85% in calvarial and bone marrow osteoblasts, respectively (**Fig. 3G -3I**). Treatment with GR79236 and BAY606583 also had no effect on bone formation by osteoblasts (**Fig. 4A & 4B).**

Endogenous adenosine does not affect bone formation

Rat osteoblasts were cultured with CGS15943, a non-selective P1 receptor antagonist, and pentostatin, an adenosine deaminase inhibitor, to determine whether endogenous adenosine influences bone formation. Both CGS15943 and pentostatin (≤1μM) had no effect on the level of bone formation **(Fig. 4C & 4D).**

Increased TNAP activity in bone marrow osteoblasts treated with 2-chloroadenosine

The effect of adenosine and 2-chloroadenosine on TNAP activity was measured in calvarial and long bone osteoblasts at different stages of differentiation (proliferating, differentiating, mature, mature-bone forming). Adenosine had no effect on TNAP activity (**Fig. 5A, 5B, 5C**). 2-chloroadenosine (1μM) was without effect in calvarial osteoblasts (**Fig. 5D, 5E**) but increased TNAP activity by ≤48% in rat bone marrow osteoblasts (**Fig. 5F**). This effect was evident in differentiating (day 11), mature (day 14) and mature, bone forming (day 17) osteoblasts.

Osteoblast numbers are unaffected by adenosine or 2-chloroadenosine

Calvarial and long bone marrow osteoblasts were cultured for up to 28 days with adenosine or 2-chloroadenosine; cell numbers were estimated at the different stages of osteoblast differentiation using a lactate dehydrogenase assay. Adenosine had no effect on calvarial or long bone osteoblast numbers at any time point at concentrations up to 100μM (**Fig. 6A-C)**. 2-chloroadenosine did not influence cell number in cultures of rat calvarial osteoblasts at concentrations up to 10μM (**Fig. 6D).** In mouse calvarial and rat long bone osteoblasts, ≤1μM 2-chloroadenosine had no effect on cell number but 10μM 2-chloroadenosine was toxic, resulting in widespread cell death **(Fig. 6E & 6F)**.

Lack of effect of adenosine or 2-chloroadenosine on osteoclast formation and resorptive activity

The effect of adenosine, 2-chloroadenosine and ATP was examined in cultures of mouse osteoclasts cultured on ivory discs. Representative light microscopy images of treated osteoclasts are shown in **Fig. 7A.** At all the concentrations tested, adenosine (**Fig. 7B, 7E**) and 2-chloroadenosine (**Fig. 7C, 7F**) had no effect on osteoclast number or the amount of resorption per osteoclast. In contrast, ATP increased osteoclast formation up ≤75% and bone resorption by up to 2-fold **(Fig. 7D, 7G)**.

DISCUSSION

The role of adenosine in the regulation of bone cell function has been a significant area of study (see review [39]) yet published data presents conflicting results. The aim of this investigation was to clarify the functional effects of adenosine on osteoblasts and osteoclasts. We found that adenosine and the selective P1 receptor agonists, GR79236 (A_1) and BAY606583 (A_{2B}), had no effect on osteoblast number and / or bone formation. However, 2-chloroadenosine (a synthetic, universal P1 receptor agonist) modestly increased TNAP activity and bone formation by rat bone marrow osteoblasts, but did not affect rat and mouse calvarial osteoblasts. Osteoclast formation and activity was also unaffected by adenosine or 2-chloroadenosine. In contrast, the established osteogenic inhibitory effects [12] and osteoclastic stimulatory effects [17] of ATP were readily observed in all cells.

The work presented here showed no effects of adenosine, 2-chloroadenosine, GR79236 or BAY606583 on calvarial-derived osteoblasts; this is in broad agreement with previous studies which showed exogenous adenosine had no effect on cultured rat osteoblasts [11,29]. However, our results are at variance with several studies which found that adenosine or adenosine analogues, acting via the A_{2A} or A_{2B} receptors, stimulate the differentiation and function of human and rodent bone marrow osteoblasts and promote bone regeneration [24,30,31,40]. Our data also do not concur with the reported inhibitory effects of adenosine analogues, acting via A_1 or A_{2A} receptors, on the differentiation of rodent osteoblast-like cells [41] or human osteoblasts [40]. We did observe small stimulatory effects of 2-chloroadenosine on rat bone marrow osteoblasts. This synthetic agonist is more potent than adenosine and is hydrolysed more slowly [42]. Differences in agonist pharmacology may therefore explain why we observed small effects with this analogue but adenosine was inactive in bone marrow osteoblasts.

It is possible that significant differences in osteoblast culture methodologies also contributed to the divergent results between studies. Bone formation *in vitro* can be influenced by a number of variables including use of glucocorticoids (which are strongly osteogenic for rat but not mouse-derived cells *in vitro* [36,43] in the culture medium, culture duration, cell seeding densities, tissue culture medium, β-glycerophosphate concentration and the age of the animals from which the cells were isolated.

The breakdown of ATP released by cells represents a key source of extracellular adenosine. Osteoblasts release ATP constitutively [44,45] and can generate low micromolar concentrations of adenosine *in vitro* [21,27]. Therefore, the possibility remains that endogenous adenosine exerts effects on osteoblasts that are not enhanced further by the addition of exogenous adenosine. To investigate this possibility further, rat osteoblasts were cultured with a non-selective P1 receptor antagonist, CGS15943, to block all adenosine-mediated signalling. Cells were also cultured with pentostatin which inhibits adenosine deaminase and prevents adenosine breakdown to inosine. Both CGS15943 and pentostatin had no effect on the level of bone formation *in vitro*. Taken together, this suggests that signalling mediated by endogenous adenosine does not exert a significant effect on calvarial osteoblast function.

Earlier work has shown that osteoblasts express all the adenosine receptors [24,27]. The present study examined the expression profile of P1 receptors in mature, bone forming osteoblasts from different sources. In agreement with the previous studies, rat bone marrow expressed low levels of mRNA for all four receptors. However, calvarial osteoblasts displayed more restricted expression at both the protein and mRNA level. Osteoblasts expressed mRNA for the A_1 receptor yet no protein was detected, suggesting this receptor is not translated. In contrast, we found that osteoblasts expressed A_{2A} receptor protein but not mRNA. This discrepancy suggests that expression of A_{2A} receptor mRNA is below the threshold that can be detected by conventional PCR. The limited adenosine receptor expression on calvarial osteoblasts may also contribute to the lack of functional effects seen in these cells.

Previous work reported that P1 receptor expression by human and rat bone marrow mesenchymal stem cells and osteoblasts is strongly dependent on differentiation [40,24], as is P2 receptor expression by rat calvarial osteoblasts [7]. Although not investigated here, it is possible that P1 receptor expression in osteoblasts is also affected by differentiation.

Several studies have shown that A_1 receptors and A_{2A} receptors can form homomers [46,47] or A_1-A_{2A} heteromers [48]. A_1-P2Y_1 and A_1-P2Y_2 adenosine-receptor-ATP-receptor G-protein heteromers have also been reported [49,50]. This receptor dimerisation may lead to alterations in downstream signalling and cellular responses to P1 receptor agonists, potentially contributing to the different effects observed between cell types.

Available data regarding the effects of adenosine and P1 receptors on osteoclasts are also conflicting. Consistent with earlier studies we show that osteoclasts express all four adenosine receptors, albeit at a low level [23,25]. However, we demonstrate that adenosine has no effect on the formation or resorptive activity of mouse osteoclasts grown on dentine. These results are in agreement with some previous investigations [16,17] but differ from others. For example, A_{2A} receptor agonists have been shown to both inhibit [33] and stimulate [25] the formation and activity of osteoclasts. Kara and colleagues [51] found that A_1 receptor antagonists decreased osteoclast formation and resorption. However, we found that mouse osteoclasts only express low levels of A_1 receptor protein. Pellegatti *et al* [25] also reported that the A_1 receptor was only weakly expressed by osteoclasts formed from human peripheral blood. Taken together, this suggests that the A_1 receptor is unlikely to play a role in regulating osteoclast function. It should be noted that the culture conditions used by the above studies varied considerably. Osteoclast formation and activity is strongly influenced by factors including RANKL/M-CSF concentration, substrate (dentine, bone or plastic), pH, source of cells and culture duration [38]. Thus, different experimental conditions combined with variations in P1 receptor expression may account for the disparity between studies.

The lack of effect of adenosine on osteoblasts and osteoclasts in this study suggests that the P1 receptors are not critical in regulating bone cell function directly. However, A_1 and A_{2B} receptor knockout mice are reported to display increases in the trabecular and/or cortical bone [23,30], whilst A_{2A} receptor knockout mice have decreased cortical and trabecular bone [33]. At present, the A_3 receptor knockout has not been investigated for specific changes in the bone; however, no overt changes in phenotype have been noted [52]. Adenosine receptors display widespread expression and are involved in many biological processes including coronary vasodilation [53], VEGF production, angiogenesis [54,55] and nerve transmission [56]. Thus it is possible that the changes in bone mass seen in the knockout mouse models are occurring indirectly via actions on other tissues.

This study used established and well validated assays for measuring accepted bone cell function. We clearly show that supraphysiological concentrations of adenosine did not affect rodent osteoblasts or mouse osteoclasts. In rat bone marrow osteoblasts, 2-chloroadenosine exerted small effects on TNAP activity and bone formation, but only when added to cell cultures in extremely high concentrations. 2-chloroadenosine was without effect on the other cell types used. We also provide evidence to suggest endogenous adenosine does not influence bone formation. Taken together these data suggests that adenosine has very little direct effect on osteoblast and osteoclast function.

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Table 1

Primer sequences used for RT-PCR analysis of rodent P1 receptor mRNA expression. GAPDH = glyceraldehyde-3-phosphate dehydrogenase

Rat primer 5' – 3'	**Sense**	**Anti-sense**
A_1 receptor	CTCCATTCTGGCTCTGCTCG	CTCCATTCTGGCTCTGCTCG
A_{2A} receptor	CCATGCTGGGCTGGAACA	GAAGGGGCAGTAACACGAACG
A_{2B} receptor	TGGCGCTGGAGCTGGTTA	GCAAAGGGGATGGCGAAG
A_3 receptor	AGAGCTAGGTCCACTGGC	GCACATGACAACCAGGGGGATGA
β-actin	GTTCGCCATGGATGACGAT	TCTGGGTCATCTTTTCACGG
Mouse primer 5' – 3'	**Sense**	**Anti-sense**
A_1 receptor	CTACCTTCTGCTTCATCGTA	ACAAGACAGTGGTGACTCAG
A_{2A} receptor	CTATTGCCATCGACAGATAC	GAACAACTGCAGTCAGAAAG
A_{2B} receptor	CCACCAACTACTTTCTGGTA	AACAGTAAAGACAGTGCCAC
A_3 receptor	TCATTGTCTCCCTAGCACT	GACARCRRCRACARCARCCG
GAPDH	CTCACTCAAGATTGTCAGCA	GTCATCATACTTGGCAGGTT

FIGURE LEGENDS

Figure 1. ***Expression of P1 receptors by rodent bone cells***

(A) Rat calvarial osteoblasts express A_1 and A_{2B} receptor mRNA whilst rat bone marrow osteoblasts showed expression of all four adenosine receptors. Mouse calvarial osteoblasts express the A_1, A_{2A} and A_{2B} receptors. Mouse osteoclasts expressed mRNA for the A_{2A}, A_{2B} and A_3 receptors. Positive control: rat/mouse brain. **(B)** Western blot analysis shows that rat calvarial osteoblasts express low levels of A_{2A} and A_{2B} receptor protein. Mouse osteoclasts express protein for all four of the adenosine receptors. Images are representative of experiments performed using mRNA and protein from three separate cell populations.

Figure 2. ***Effects of adenosine and 2-chloroadenosine on mineralised bone nodule formation by rodent osteoblasts***

Representative images (n=5) showing alizarin red-stained mineralised bone nodules, viewed by phase contrast microscopy (left) and low power reflected light scans (right). Adenosine (≤ 100µM) had no effect on bone formation by rat calvarial, mouse calvarial or rat bone marrow osteoblasts (cultured on plastic for 14, 28 and 17 days, respectively). 2-chloroadenosine (1µM) caused a modest increase in bone formation by rat bone marrow osteoblasts only. Scale bars: left, 1mm; right, 1cm.

Figure 3. ***Effects of adenosine, 2-chloroadenosine and ATP on mineralised bone nodule formation by cultured rodent osteoblasts***

Adenosine had no effect on mineralised nodule formation in cultures of **(A)** rat calvarial, **(B)** mouse calvarial or **(C)** rat bone marrow osteoblasts. 2-chloroadenosine was also without effect in **(D)** rat calvarial or **(E)** mouse calvarial osteoblasts but caused **(F)** a ~50% increase in nodule formation by rat bone marrow osteoblasts (1µM only). The complete abolition of bone formation at concentrations of **(E, F)** 10µM and **(D)** 100µM 2-chloroadenosine suggests toxicity at these levels. ATP inhibited bone formation by **(G)** rat calvarial, **(H)** mouse calvarial and **(I)** rat bone marrow osteoblasts by up to 90%. Data are means ± SEM for 6 replicate determinations, n=5; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 4. ***The effects of selective adenosine receptor agonists and endogenous adenosine on bone formation by osteoblasts***

At concentrations up to 10µM the selective **(A)** A_1 agonist, GR79236, and **(B)** A_{2B} agonist, BAY606583 did not affect mineralised bone nodule formation by rat calvarial osteoblasts. **(C)** The non-selective adenosine receptor antagonist, CG15943, and **(D)** the adenosine deaminase inhibitor, pentostatin, also had no effect on the level of bone formation. Data are means ± SEM for 6 replicate determinations (n=3).

Figure 5. ***Effects of adenosine and 2-chloroadenosine on alkaline phosphatase (TNAP) activity of rodent osteoblasts***

Culture with adenosine had no effect on TNAP activity in **(A)** rat calvarial, **(B)** mouse calvarial and **(C)** rat bone marrow osteoblasts at any stage of culture. 2-chloroadenosine had no effect on TNAP activity in **(D)** rat and **(E)** mouse calvarial osteoblasts but **(F)** increased TNAP activity by up to 48% in rat bone marrow osteoblasts. Data are means ± SEM for 6 replicate determinations (n=3-5): * = $p<0.05$, ** = $p<0.01$.

Figure 6. ***Osteoblast number is unaffected by adenosine and 2-chloroadenosine***

Treatment with adenosine had no effect on cell number in cultures of **(A)** rat calvarial, **(B)** mouse calvarial and **(C)** rat bone marrow osteoblasts at any stage. 2-chloroadenosine (≤1μM) was also without effect in **D)** rat calvarial, **(E)** mouse calvarial and **(F)** bone marrow osteoblasts. [0] indicates that there were no viable cells present suggesting toxicity at concentrations of ≥10μM 2-chloroadenosine. Data are means ± SEM for 6 replicate determinations (n=3-5).

Figure 7. ***Lack of effect of adenosine and 2-chloroadenosine on the formation and resorptive activity of mouse osteoclasts***

Osteoclasts were generated in 10 day cultures of mouse marrow cells on ivory discs, in the presence or absence of adenosine**,** 2-chloroadenosine or ATP. Cells were acidified to pH 6.90 on day 8 of culture to activate resorption. **(A)** Representative transmitted light images of cultures, showing tartrate-resistant acid phosphatase-positive multinucleated osteoclasts (large red cells) and resorption pits (tan areas); scale bar = 50 μm. **(B, E)** Adenosine and (**C, F)** 2-chloroadenosine were without effect on osteoclast formation and resorptive activity. **(D, G)** ATP increased osteoclast formation by ≤75% and resorption by 2-fold. Data are means ± SEM for 8 replicate determinations (n=3); * = $p<0.05$; ** = $p<0.01$; ** = $p<0.001$.

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Figure 1

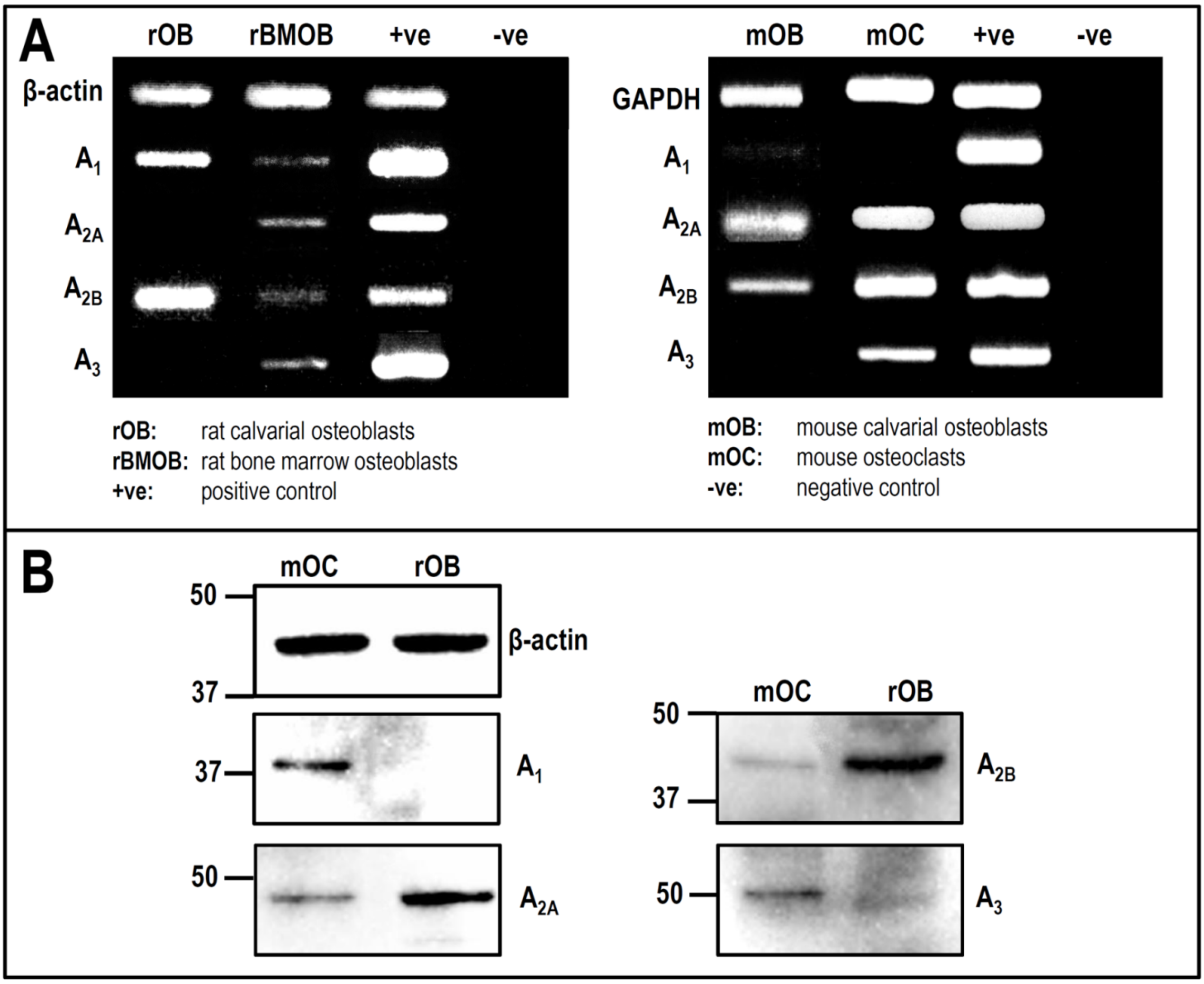
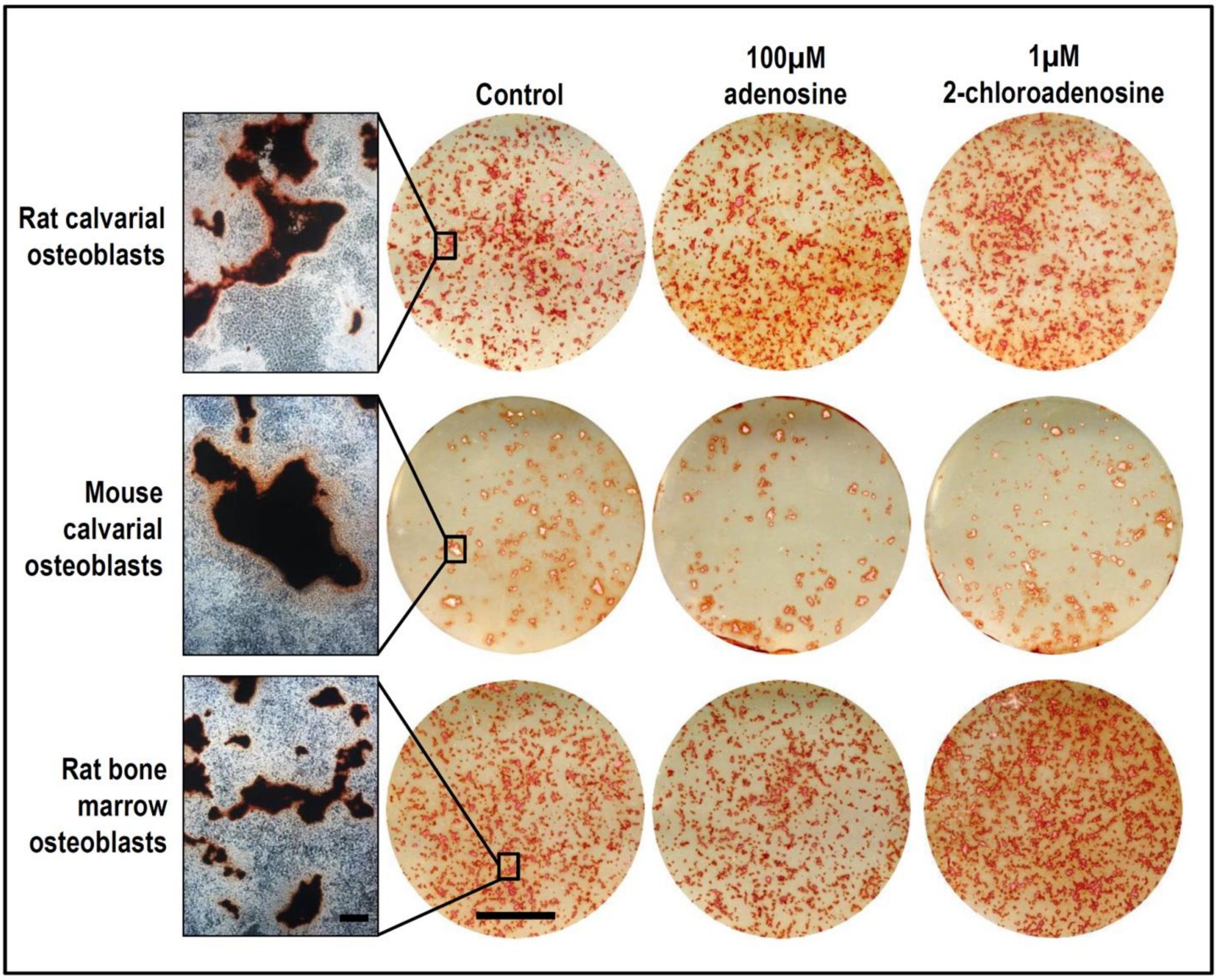
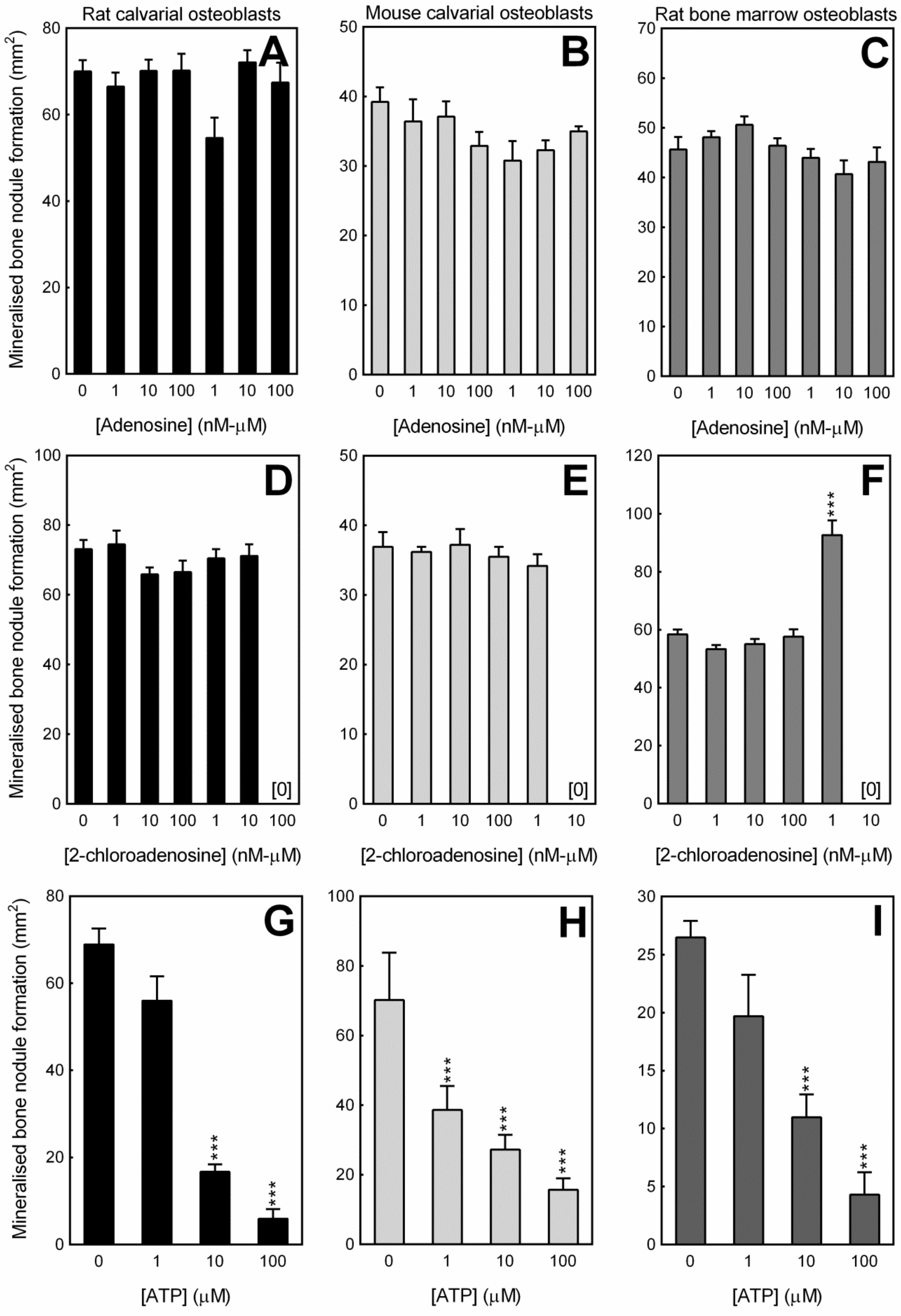

A
rOB
rBMOB
+ve
-ve
β-actin
A1
A2A
A2B
A3
mOB
mOC
+ve
-ve
GAPDH
A1
A2A
A2B
A3
rOB: rat calvarial osteoblasts
rBMOB: rat bone marrow osteoblasts
+ve: positive control
mOB: mouse calvarial osteoblasts
mOC: mouse osteoclasts
-ve: negative control
B
mOC
rOB
50
37
β-actin
37
A1
50
A2A
mOC
rOB
50
37
A2B
50
A3

Figure 2



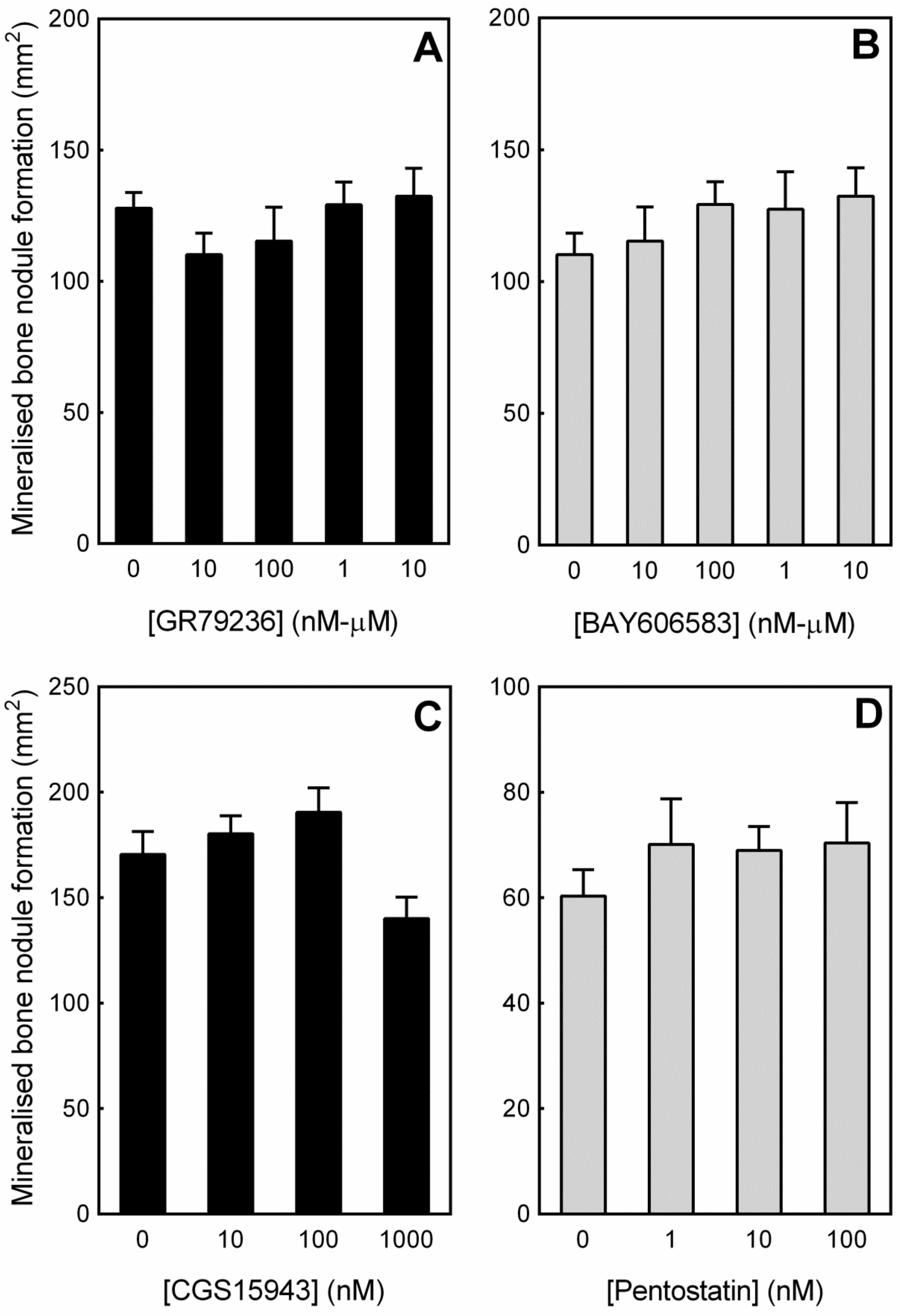
Control
100µM adenosine
1µM 2-chloroadenosine
Rat calvarial osteoblasts
Mouse calvarial osteoblasts
Rat bone marrow osteoblasts

Figure 3



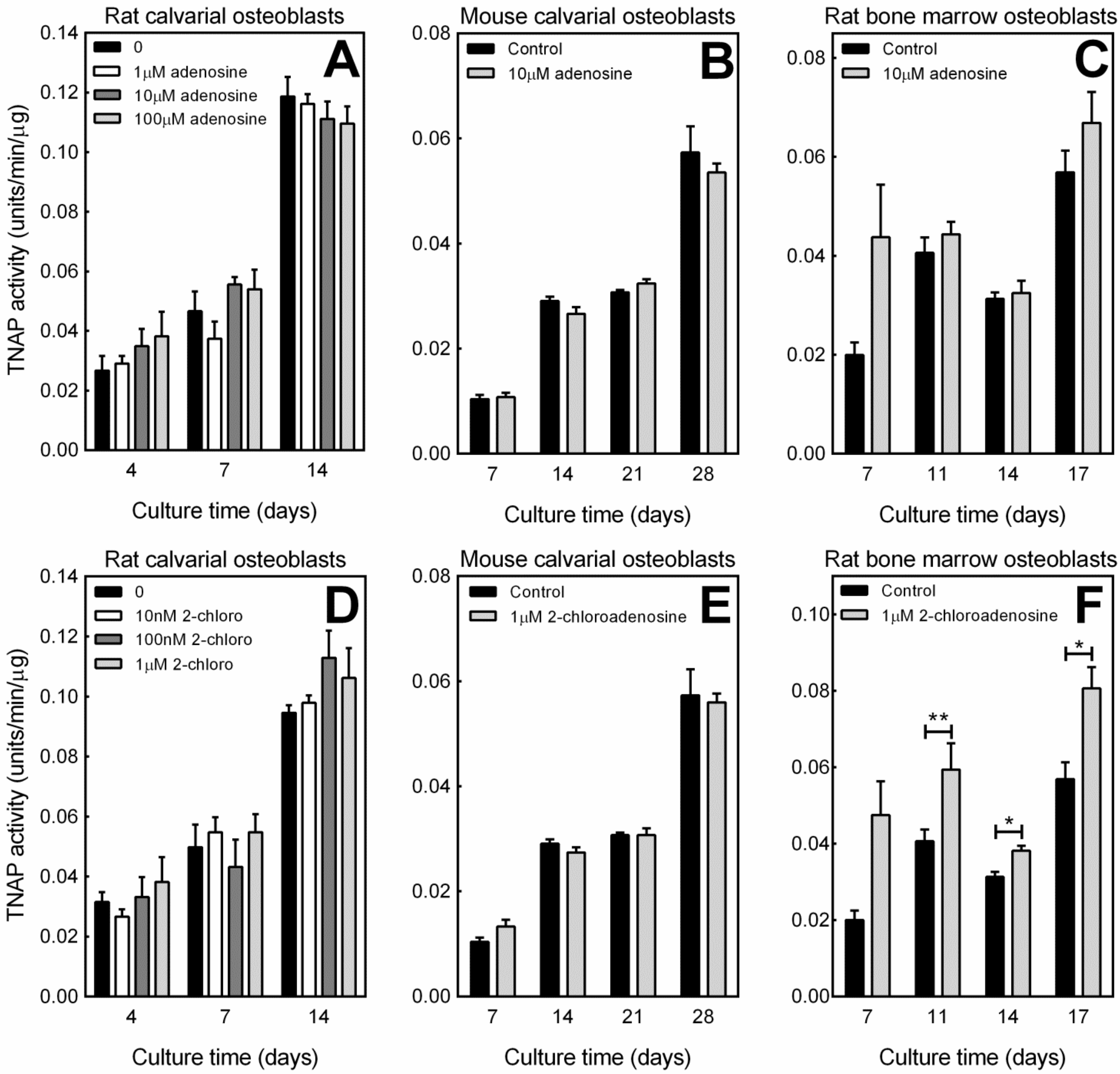
Rat calvarial osteoblasts
Mouse calvarial osteoblasts
Rat bone marrow osteoblasts
Mineralised bone nodule formation (mm^2)
A
B
C
0 1 10 100 1 10 100
[Adenosine] (nM-μM)
D
E
F
[0]
[2-chloroadenosine] (nM-μM)
G
H
I
***
0 1 10 100
[ATP] (μM)

Figure 4



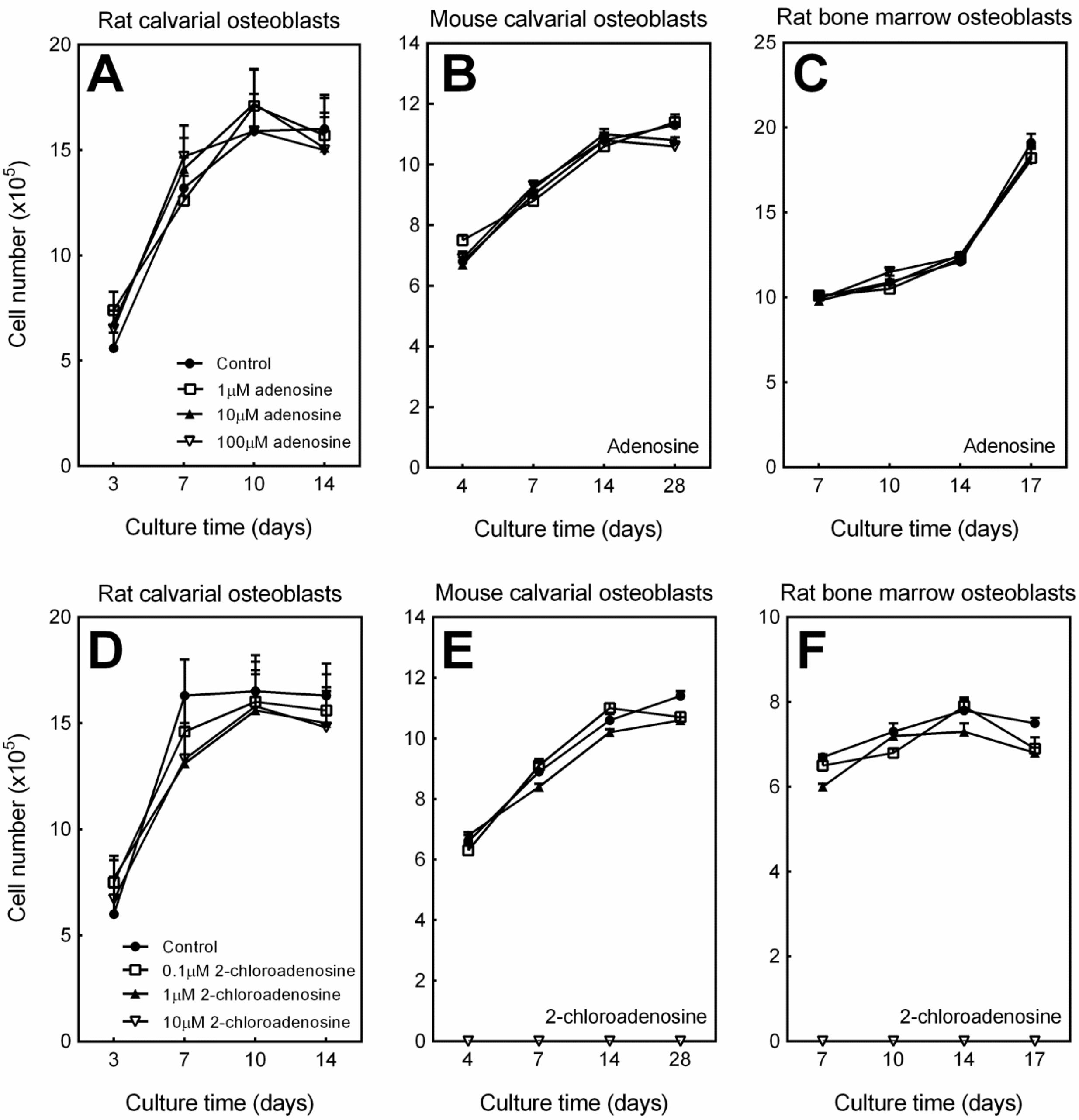
A
B
C
D
Mineralised bone nodule formation (mm2)
200
150
100
50
0
0
10
100
1
10
[GR79236] (nM-μM)
[BAY606583] (nM-μM)
250
1000
[CGS15943] (nM)
80
60
40
20
[Pentostatin] (nM)

Figure 5



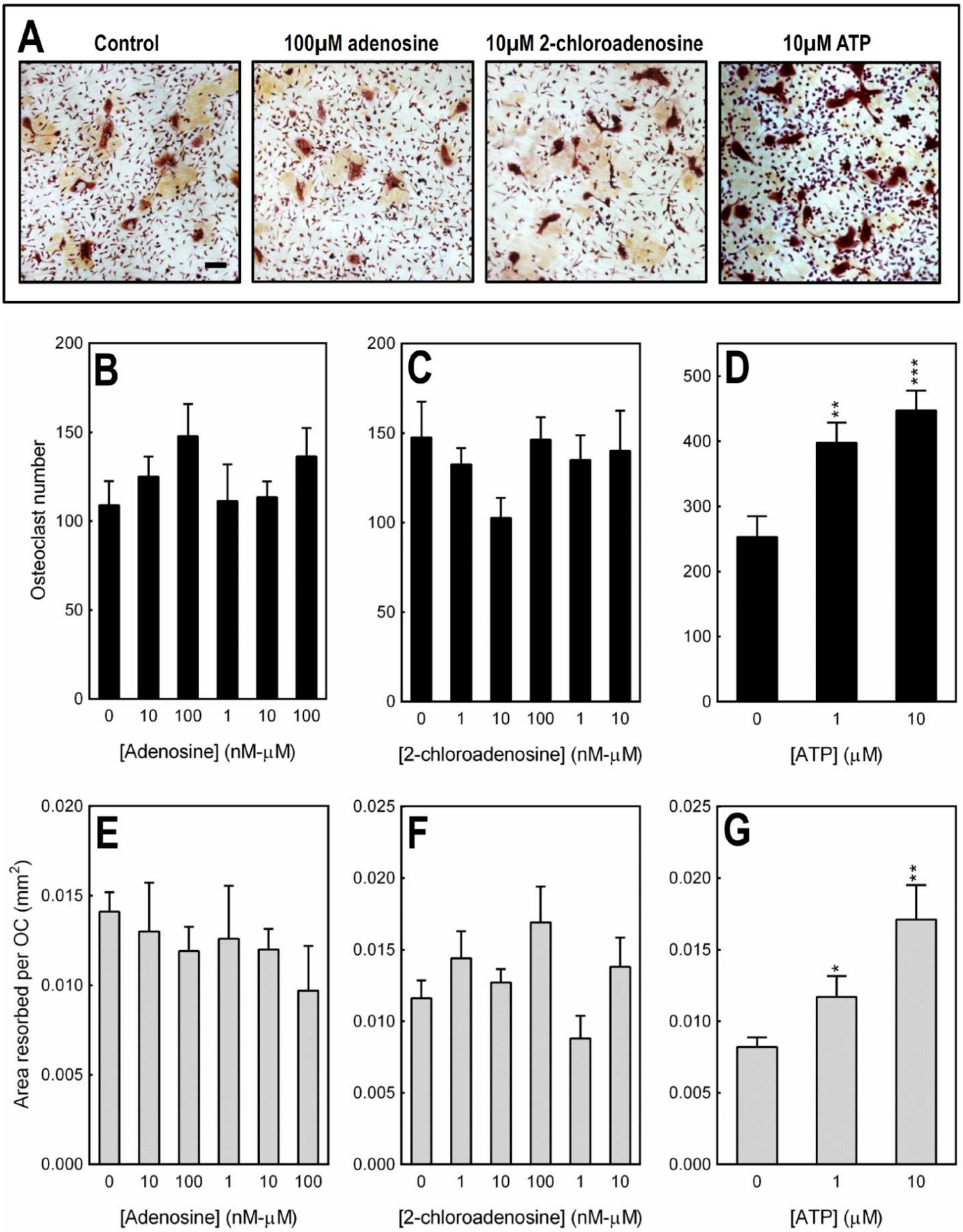
Rat calvarial osteoblasts
A
0
1μM adenosine
10μM adenosine
100μM adenosine
TNAP activity (units/min/μg)
Culture time (days)
Mouse calvarial osteoblasts
B
Control
10μM adenosine
Culture time (days)
Rat bone marrow osteoblasts
C
Control
10μM adenosine
Culture time (days)
Rat calvarial osteoblasts
D
0
10nM 2-chloro
100nM 2-chloro
1μM 2-chloro
TNAP activity (units/min/μg)
Culture time (days)
Mouse calvarial osteoblasts
E
Control
1μM 2-chloroadenosine
Culture time (days)
Rat bone marrow osteoblasts
F
Control
1μM 2-chloroadenosine
Culture time (days)

Figure 6



Rat calvarial osteoblasts
A
Control
1μM adenosine
10μM adenosine
100μM adenosine
Cell number (x10^5)
Culture time (days)
Mouse calvarial osteoblasts
B
Adenosine
Rat bone marrow osteoblasts
C
Adenosine
D
Control
0.1μM 2-chloroadenosine
1μM 2-chloroadenosine
10μM 2-chloroadenosine
E
2-chloroadenosine
F
2-chloroadenosine

Figure 7


A
Control
100μM adenosine
10μM 2-chloroadenosine
10μM ATP
B
C
D
E
F
G
Osteoclast number
Area resorbed per OC (mm2)
[Adenosine] (nM-μM)
[2-chloroadenosine] (nM-μM)
[ATP] (μM)
0 10 100 1 10 100
0 1 10 100 1 10
0 1 10
0 50 100 150 200
0 100 200 300 400 500
0.000 0.005 0.010 0.015 0.020
0.000 0.005 0.010 0.015 0.020 0.025