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Inhibition of vascular smooth muscle cell calcification by ATP-analogues

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ABSTRACT

Arterial medial calcification (AMC) has been associated with phenotypic changes in vascular smooth muscle cells (VSMCs) that reportedly makes them more osteoblast-like. Previous work has shown that ATP/UTP can inhibit AMC directly via P2 receptors and indirectly by NPP1-mediated hydrolysis to produce the mineralisation inhibitor, pyrophosphate (PP_i). This study investigated the role of P2X receptors in the inhibitory effects of extracellular nucleotides on VSMC calcification. We found that Bz-ATP, α,β-meATP and β,γ-meATP, inhibited calcification by up to 100%. Culture in a high phosphate medium (2mM) was associated with increased VSMC death and apoptosis; treatment with Bz-ATP, α,β-meATP and β,γ-meATP reduced apoptosis to levels seen in non-calcifying cells. Calcification was also associated with alterations in the protein levels of VSMC (e.g. SM22α, SMA) and osteoblast-associated (e.g. Runx2, osteopontin) markers; Bz-ATP, α,β-meATP and β,γ-meATP attenuated these changes in protein expression. Long-term culture with Bz-ATP, α,β-meATP and β,γ-meATP resulted in lower extracellular ATP levels and an increased rate of ATP breakdown. P2X receptor antagonists failed to prevent the inhibitory effects of these analogues suggesting that they act via P2X receptor-independent mechanisms. In agreement, the breakdown products of α,β-meATP and β,γ-meATP (α,β-meADP and methylene diphosphonate, respectively) also dose dependently inhibited VSMC calcification. Furthermore, the actions of Bz-ATP, α,β-meATP and β,γ-meATP were unchanged in VSMCs isolated from NPP1 knockout mice, suggesting that the functional effects of these compounds do not involve NPP1-mediated generation of PP_i. Together, these results indicate that the inhibitory effects of ATP analogues on VSMC calcification and apoptosis *in vitro* may be mediated, at least in part, by mechanisms that are independent of purinergic signalling and PP_i.

INTRODUCTION

Vascular calcification is a frequent consequence of chronic kidney disease, diabetes, atherosclerosis and ageing. It is the pathological deposition of calcium phosphate mineral, typically as hydroxyapatite, in the intimal and/or medial layers of the arteries and heart valves. Arterial medial calcification (AMC) develops within the tunica media of blood vessels and is characterised by increased vessel stiffness and decreased blood flow [1]. Originally, it was thought that AMC was a passive process due to high serum levels of calcium and phosphate; however, it is now widely stated in the literature that AMC shares some outward similarities to physiological bone formation. In particular, development of AMC is characterised by a loss of calcification inhibitors (e.g. pyrophosphate (PP$_i$), fetuin A, matrix gla protein), a gain of calcification inducers (e.g. alkaline phosphatase (TNAP)), and increased apoptosis [1,2]. Vascular smooth muscle cells (VSMCs) are the major cell type driving AMC and when maintained in a calcifying environment (high calcium and/or phosphate) these cells undergo phenotypic changes to take on some limited osteoblast-like characteristics [3-7].

ATP and related nucleotides (UTP, ADP, UDP) are important extracellular signalling molecules that act via purinergic P2 receptors to regulate cell proliferation, differentiation, survival and function in many tissues [8]. The P2 receptor family comprises seven P2X ion channels (P2X1-7) and eight P2Y G-protein coupled receptors (P2Y$_{1,2,4,6,11-14}$) [9,10]. The study of P2X receptor function has been aided by synthetic ATP analogues which act as receptor agonists with a degree of selectivity for the different subtypes. These include Bz-ATP (P2X7 and, weakly, P2X1), α,β-meATP (most potent at P2X1 and P2X3, also P2X4) and β,γ-meATP (P2X1, weakly P2X2 and P2X3) [11]. The recent development of numerous selective P2X receptor antagonists has also aided in the study of these receptors.

Whilst the role of purinergic signalling in the cardiovascular system has been well documented (see review [12]), the regulation of vascular calcification by extracellular nucleotides has been less well investigated [13]. To date most studies have reported protective effects with ATP and UTP being able to reduce AMC, valve calcification and intimal calcification [6,14-16]. However, the mechanisms underpinning these functional effects appear to vary between the different forms of calcification. For example, P2Y$_2$ receptor mediated signalling has been shown to promote the survival of aortic valve interstitial cells as well as protect against aortic valve calcification [14] and arterial intimal calcification [15]. In contrast, whilst ATP and UTP can inhibit VSMC calcification by preventing apoptosis, these effects do not appear to be mediated via the P2Y$_2$ receptor [6].

VSMCs, like many other cell types, release ATP constitutively [17,18]. Once released, extracellular nucleotides are rapidly hydrolysed by ecto-nucleotidases. One important product of these breakdown reactions is PP$_i$, a ubiquitous and potent physiochemical inhibitor of calcification [19]. PP$_i$ is generated from nucleotide triphosphates by ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs). VSMCs express high levels of NPP1 and the hydrolysis of locally released ATP by this enzyme is a major source of extracellular PP$_i$ [17,20]. Once generated, this PP$_i$ can then act locally to prevent VSMC calcification. In agreement, we recently reported that the inhibitory effects of ATP/UTP on AMC are attenuated in VSMCs derived from NPP1 knockout mice [6]. This suggests that the actions of these extracellular nucleotides are mediated by both P2 receptor-dependent and independent mechanisms (i.e. hydrolysis to produce PP$_i$).

Although earlier work from our laboratory showed that the P2Y$_2$ receptor did not mediate the effects of ATP and UTP on VSMC calcification [6], it was not defined whether any of the other P2 receptor subtypes were involved. The aim of this study was to determine which P2 receptor(s) mediates these functional effects. Thus, the more selective ATP analogues, Bz-ATP, α,β-meATP and β,γ-meATP, were

used to investigate the role of the P2X receptors in the inhibition of VSMC calcification by extracellular nucleotides.

METHODS

Reagents

All tissue culture reagents were purchased from Life Technologies (Paisley, UK); unless mentioned, all chemicals were purchased from Sigma Aldrich (Poole, UK). All P2X receptor antagonists were obtained from Tocris Bioscience (Bristol, UK) and all P2X receptor antibodies from Alomone Labs (Jerusalem, Israel). All other antibodies were purchased from Abcam (Cambridge, UK).

Animals

Primary VSMCs were isolated from C57BL/6J, NPP1 knockout (*Enpp1*^{-/-}) or NPP1 wildtype (*Enpp1*^{+/+}) mice. *Enpp1*^{-/-} and *Enpp1*^{+/+} VSMCs were isolated from animals on a 129Sv/TerJ background. The generation and characterisation of *Enpp1*^{-/-} animals has previously been described [21]. Animals were bred from heterozygote (*Enpp1*^{+/-}) breeding pairs due to the inability of homozygotes to breed. All mice were housed under standard conditions with free access to food and water. All procedures complied with the UK animals (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Research Ethics Committee.

Vascular smooth muscle cell (VSMC) calcification assay

Primary VSMCs were isolated from aortas of 6-8 week old mice. After removal of the adventitia, the aorta was opened to expose the endothelial layer under a dissection microscope. Tissues from 6-8 animals were pooled and incubated with trypsin (0.25% *w/v*) for 10 minutes to remove any remaining adventitia and endothelium. Tissues were incubated overnight in alpha Minimum Essential Medium, supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin (complete mixture abbreviated to αMEM) before being digested with 425U/ml collagenase type II (Worthington Biomedical Corporation, Lakewood, USA) for 5 hours. Isolated VSMCs were expanded in T25 tissue culture flasks in a humidified atmosphere of 5% CO_2-95% air at 37°C until confluent. Following seeding into 24-well plates at a density of 2.5 x 10^4 cells/well, VSMCs were cultured in calcifying medium (αMEM + 2mM sodium phosphate) for up to 14 days, with half medium changes every 3 days. Cells were treated with 1-100µM Bz-ATP, α,β-meATP or β,γ-meATP and, where appropriate, P2 receptor antagonists (0.1-10μM), for the duration of the culture; fresh agonist/antagonist was added at each medium change. The P2 receptor antagonists used were: PPADS (non-selective), NF110 (P2X3), 5-BDBD/PSB-12062 (P2X4) and A740003/AZ10606120 (P2X7). The antagonists were used at concentrations previously shown to abolish the functional effects of Bz-ATP and α,β-meATP on bone mineralisation in long term cultures [22].

Determination of VSMC calcification

Calcifying VSMCs were washed twice with phosphate buffered saline (PBS) and incubated with 0.6M HCl at room temperature for 24 hours. Calcium content was measured colorimetrically by stable interaction with *o*-cresolphthalein using a commercially available kit (Calcium Colorimetric Assay, Sigma-Aldrich, Poole, UK) and corrected for total protein concentration using the Bradford assay (Sigma-Aldrich, Poole, UK). Calcium deposition was visualised by alizarin red staining of VSMC cell layers as previously described [23].

Total RNA extraction and DNase treatment

Calcifying VSMCs were cultured for 14 days before total RNA was extracted using TRIZOL® reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (35U/ml) for 30 min at 37°C. The reaction was terminated by heat inactivation at

65°C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260nM. RNA was stored at −80°C until amplification by qRT-PCR.

Quantitative real time polymerase chain reaction (qRT-PCR)

VSMC RNA (50ng) was transcribed and amplified using the qPCRBIO SyGreen one-step qRT-PCR kit (PCR Biosystems, London, UK), which allows cDNA synthesis and PCR amplification to be carried out sequentially. qRT-PCR was performed according to manufacturer's instructions with initial cDNA synthesis (45°C for 10 min) and reverse transcriptase inactivation (95°C for 2 min) followed by 40 cycles of denaturation (95°C for 5 sec) and detection (60°C for 30 sec). All reactions were carried out in triplicate using RNAs derived from 4 different cultures. Data are presented as mRNA expression relative to P2Y$_2$ receptor expression. Primers sequences (forward/reverse): ***β-actin***, S: *gcc ttc ctt cct ggg tat gg* / AS: *tcc gat tca act cat act gc*; ***P2X1***, S: *tgt acg ggg aga aga acc tg /* AS: *tcc caa aca cct tga aga gg;* ***P2X2***, S: *cgt ctt cat cgt gca gaa aa* / AS: *cac ttt gtg ttc cga cat gg*; ***P2X3***, S: tac caa gtc ggt ggt tgt ga */* AS: *cca ccc cac aaa gta gga ga*; ***P2X4***, S: *gca ccc tcc acc atc tct aa* / AS: *aaa cct ctt gcc aga agc aa*; ***P2X5***, S: *ggg ctt tct tct gtg acc tg /* AS: *gtt ggc ctc aac ctc aac at*; ***P2X6***, S: *agc cat ggc ata aaa act gg /* AS: *gtg aag ttc ttg gcc tga gc*; ***P2X7***, S: *ggc act gga gga aaa ttt ga /* AS: *tga gca agt caa tgc aca ca*; ***P2Y$_1$***, S: *agg aaa gct tcc agg agg ag /* AS: *cgt gtc tcc att ctg ctt ga*; ***P2Y$_2$***, S: *gtc agc agt gac gac tca aga c /* AS: *tca gag gat atc agc ccc ttt a*; ***P2Y$_4$***, *agg aag cag cag aac acc at* / AS: *caa gga gtc tgc act ggt ca*; ***P2Y$_{12}$***, S: *cct gtg cgt cag aga cta caa g /* AS: *gga ttt act gcg gat ctg aaa g.*

Western blotting

Protein was extracted from control VSMCs and VSMCs treated with 25µM α,β-meATP, 10µM β,γ-meATP or 100µM Bz-ATP for 7 and 14 days. 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$_3$VO$_4$ and 2.5mg/ml deoxicolic acid). Cell homogenates were sonicated for 5 min before use. Protein concentrations from lysates were determined using the Bradford assay. Prior to loading total protein, samples were denatured by incubating at 95°C for 5 min in the presence of 5x reducing sample buffer (60 mM 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) using a wet tank blotter (Bio-Rad, Watford, UK) at 150 V for 1 hour. Membranes were then blocked with 5% non-fat milk and incubated with β-actin (1:1000), P2X receptor (1:200), SM22α (1:500), Acta2 (1:200), Runx2 (1:500) or osteopontin (OPN, 1:200) antibodies overnight at 4°C. Blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. A peroxidase detection system (Immobilon™ Western, Merck-Millipore, Watford UK) and ChemiDoc™ XRS+ system (Bio-Rad, Watford, UK) was used for visualisation of the immunoreactivity.

Cell number and viability assay

VSMCs were cultured for 14 days in medium supplemented with Bz-ATP, α,β-meATP or β,γ-meATP (1-100µM); fresh nucleotide was added at each medium change. Cell number and viability were determined using the CytoTox 96® colorimetric cytotoxicity assay (Promega UK, Southampton UK), as described previously [22]. Cell supernatants were collected to determine medium LDH levels (cell viability). To establish total cellular LDH levels, VSMCs were lysed with 1% Triton X-100 in water (lysis buffer, 15µl/ml of medium) for 1 hour. The LDH content of the supernatants and cell lysates were measured colorimetrically (490nm) according to the manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at 10^2 to 10^6/well. VSMC viability

(shown as percentage of dead cells) was calculated by expressing medium LDH as a percentage of the total cellular LDH.

Quantification of apoptosis by flow cytometry
VSMCs were cultured in control or calcification medium (± 1-100µM Bz-ATP, α,β-meATP, or β,γ-meATP) for 7 days. Apoptosis was assessed via flow cytometry using an annexin V antibody conjugated to fluorescein (Life Technologies, Paisley, UK), as per manufacturer's instructions. Briefly, VSMCs were detached using 0.25% trypsin and the resultant pellet washed in ice-cold PBS. This suspension was centrifuged and resuspended in 1X annexin-binding buffer (Life Technologies, Paisley, UK). A sample of this suspension was incubated with the annexin V antibody for 15 minutes, before analysis using a BD FACSCanto II Flow Cytometer (Becton, Dickinson and Company, Oxford, UK). Data were processed to calculate percentage apoptosis using Flowing Software (version 2.5.1) (Turku University, Finland).

Determination of alkaline phosphatase (TNAP) activity
VSMCs were cultured with 1-100µM Bz-ATP, α,β-meATP or β,γ-meATP for 14 days; fresh nucleotide was added at each medium change. TNAP activity was measured in cell lysates using a colorimetric assay (Anaspec, CA, USA), as previously described [22]. TNAP activity was normalised to cell protein using the Bradford assay.

Measurement of ATP release and breakdown
VSMCs were cultured with Bz-ATP (100µM), α,β-meATP (25µM) or β,γ-meATP (10µM) for 14 days. On the day of assay cells were switched to serum-free DMEM (without Bz-ATP, α,β-meATP or β,γ-meATP) and left for 60 minutes to allow any ATP released during the medium exchange to be degraded. After this time, samples were taken to measure basal ATP levels and cell viability. ATP (1µM) was then added to each well and samples taken after a further 2, 5, 10, 30 and 60 minutes to assess rate of ATP breakdown. ATP levels were measured luminetrically using the *luciferin-luciferase* assay, as described previously [24].

Measurement of Pyrophosphate (PP$_i$) levels
VSMCs were cultured until widespread calcification was evident before measurement of PP$_i$ levels. Culture medium was removed, cell layers washed and cells incubated in 10mM HEPES buffer containing 0.9% NaCl and 1% BSA, pH 7.4 for 1 hour. Bz-ATP (100µM), α,β-meATP (25µM), β,γ-meATP (10µM) or ATP (10-100µM) were added to the HEPES buffer and samples collected at 15 minutes after treatment. PP$_i$ levels were measured using a fluorescent assay kit (Pyrophosphate Assay Kit, Abcam, Cambridge, UK); assays were performed according to the manufacturer's instructions.

Statistical analysis
Data were analysed using GraphPad Prism 7 software (San Diego, CA). Statistical comparisons were made using a T-test or a one-way analysis of variance (ANOVA) with a post-hoc Bonferroni correction for multiple comparisons. Results shown are representative of experiments performed at least three times using cells obtained from different animal isolations. Each individual experiment comprised 4-12 technical replicates.

RESULTS

Expression of functional P2 receptors by calcifying VSMCs

Calcifying VSMCs (day 14 of culture) express mRNA for all P2X receptors and some P2Y receptors (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_{12}$) **(Fig. 1A)**. Western blot analysis of cell lysates after 7 and 14 days of culture revealed that calcifying VSMCs displayed protein expression of P2X3, P2X4, P2X5 and P2X7 receptors **(Fig. 1B)**. No protein expression of P2X1, P2X2 and P2X6 receptors was detected. For all P2X receptors, positive controls using osteoblast protein were carried out to show the antibodies were functional.

Inhibition of VSMC calcification by Bz-ATP, α,β-meATP and β,γ-meATP

Bz-ATP (100µM) and α,β-meATP (≥25µM) inhibited VSMC calcification by up to 95% **(Fig. 2A & 2B)**. β,γ-meATP was the most potent of the ATP-analogues tested as it completely prevented all calcification at concentrations of ≥10µM **(Fig. 2C)**. Comparatively, ATP (≥10µM) decreased VSMC calcification by up to 80% [6]. Representative phase contrast images of alizarin red stained cell layers in **Fig. 2D** show normal phosphate-induced VSMC calcification and the inhibitory actions of Bz-ATP (100µM), α,β-meATP (25µM) and β,γ-meATP (10µM)**.**

Bz-ATP, α,β-meATP and β,γ-meATP increase VSMC viability and decrease apoptosis

In calcifying VSMCs, treatment with Bz-ATP, α,β-meATP and β,γ-meATP (≥1µM) decreased the percentage of dead cells present by up to 72% (**Fig. 3A-3C**). However, there was no overall effect on total cell number **(Fig. 3D-3F)**. Apoptosis was increased up to 2.1-fold in calcifying VSMCs compared to control VSMCs **(Fig. 3G-3I)**. Treatment with 100µM Bz-ATP and β,γ-meATP (≥10µM) decreased the level of apoptosis seen in calcifying cells to a level similar to that in control VSMCs **(Fig. 3G & 3I)**. Culture with α,β-meATP (50µM) reduced the amount of apoptosis in calcifying cells by 25% **(Fig. 3H)**.

The effect of Bz-ATP, α,β-meATP and β,γ-meATP on calcification-induced changes in VSMC protein expression

The effect of Bz-ATP (100µM), α,β-meATP (25µM) and β,γ-meATP (10µM) on the protein expression of VSMC and osteoblast markers was investigated using western blotting **(Fig. 4)**. VSMC calcification was associated with lower protein levels of SM22-α and Acta2 at day 14 compared to day 7; treatment with Bz-ATP, α,β-meATP or β,γ-meATP appeared to prevent this reduction. Little or no OPN or Runx2 expression was detected in VSMCs at day 7 of culture; however, limited protein expression was evident by day 14. Protein levels of Runx2 and OPN appeared to be slightly reduced in VSMCs cultured with Bz-ATP, α,β-meATP and β,γ-meATP.

The inhibitory effects of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC calcification are not mediated by P2X receptors

A combination of selective and non-selective P2X receptor antagonists were then tested to identify which receptor subtype(s) mediates the effects of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC calcification. PPADs (a non-selective P2 receptor antagonist) did not prevent the inhibitory effects of Bz-ATP, α,β-meATP or β,γ-meATP **(Fig 5A-5C)**. Of the ATP-analogues tested, only α,β-meATP and β,γ-meATP activate the P2X3 receptor; however, receptor inhibition with NF110 had no effect on the actions of either compound (**Fig. 5D & 5E**). Selective antagonists for the P2X4 (5-BDBBD, PSB-12062) and P2X7 (A740003, AZ10606120) receptors also failed to prevent the inhibitory effects of α,β-meATP and Bz-ATP, respectively **(Fig. 5F-5I)**. Due to the extended nature of VSMC cultures, selective P2X receptor antagonists were not used at concentrations above 10µM because they resulted in obvious cell death.

Increased TNAP activity in VSMCs treated with Bz-ATP, α,β-meATP and β,γ-meATP
Treatment with Bz-ATP (100µM), α,β-meATP (≥25µM) and β,γ-meATP (≥10µM) increased TNAP activity up to 5.5-fold, 2.2-fold and 5.9-fold, respectively **(Fig. 6A-6C)**. The concentrations at which TNAP activity was increased corresponded to the levels at which calcification was significantly inhibited (**Fig. 2**).

The effect of Bz-ATP, α,β-meATP and β,γ-meATP on ATP release and breakdown
High concentrations of α,β-meATP and β,γ-meATP have been shown to inhibit ecto-nucleotidases and therefore slow ATP breakdown [17,25]. Basal extracellular ATP levels were 70% and 45% lower in VSMCs cultured with α,β-meATP and β,γ-meATP, respectively **(Fig. 6D)**. ATP levels were unchanged in Bz-ATP-treated VSMCs **(Fig. 6D)**. The breakdown of exogenously added ATP was faster in VSMCs cultured with Bz-ATP **(Fig. 6E)**, α,β-meATP **(Fig. 6F)** and β,γ-meATP **(Fig. 6G)**. The cells treated with α,β-meATP showed the largest difference in the rate of ATP breakdown. To avoid any potential confounding effects of the ATP analogues on the *luciferin-luciferase* reaction, all ATP measurements were performed using culture medium that did not contain Bz-ATP, α,β-meATP or β,γ-meATP.

The inhibitory effects of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC calcification are not mediated by breakdown to produce PP_i
Enpp1^{-/-} VSMCs were used to determine if any indirect actions involving NPP1 contributed to the effects of ATP-analogues. Deletion of NPP1 did not prevent or reduce the inhibitory actions of Bz-ATP, α,β-meATP or β,γ-meATP **(Fig. 7A-7C)**. Comparatively, the effects of ATP/UTP on VSMC calcification were attenuated in *Enpp1*^{-/-} cells, suggesting that some of the inhibitory actions of these nucleotides are due to NPP1-mediated breakdown to produce PP_i [6]. Addition of exogenous ATP (≥10µM) to VSMC cultures increased extracellular PP_i levels by up to 4µM within 15 minutes of treatment (**Fig. 7D**).Bz-ATP (100µM), α,β-meATP (25µM) and β,γ-meATP (10µM) only increased extracellular PP_i levels by a small amount (≤0.7µM) (**Fig. 7D**).

Breakdown products of α,β-meATP and β,γ-meATP also inhibit VSMC calcification
The breakdown product of α,β-meATP, α,β-meADP, dose-dependently (≥10µM) inhibited VSMC calcification by up to 95% **(Fig. 8A).** Methylene diphosphonate (≥10µM), which is formed by the hydrolysis of β,γ-meATP, reduced VSMC calcification by up to 80% **(Fig. 8B)**.

DISCUSSION

This study found that the synthetic ATP-analogues and P2X receptor agonists, Bz-ATP, α,β-meATP and β,γ-meATP, all inhibited VSMC calcification *in vitro*. These actions appear to be mediated, at least in part, by preventing apoptosis and reducing calcification-induced phenotypic changes in the VSMCs. Despite pharmacological studies using both non-specific and selective P2X receptor antagonists, this investigation could not attribute these functional effects to activation of a particular P2X receptor subtype(s). Furthermore, the effects of these ATP analogues did not involve NPP1-mediated generation of PP_i and the breakdown products of α,β-meATP and β,γ-meATP (α,β-meADP and methylene diphosphonate, respectively) also inhibited VSMC calcification. Taken together, these findings suggest that these compounds may be acting via mechanisms that are independent of both P2X receptor activation and NPP1-mediated hydrolysis to prevent VSMC calcification.

Accumulating evidence shows that extracellular nucleotides are protective against the different forms of vascular calcification [6,14-16]. However, the P2 receptor subtypes which mediate these effects appear to vary; for example, the inhibition of valve and intimal calcification is thought to involve $P2Y_2$ receptor activation [14,15]. In contrast, our previous work showed that the effects of ATP and UTP on VSMC calcification were not mediated via the $P2Y_2$ receptor [6]. Since ATP is the universal P2 receptor agonist, this study investigated whether P2X receptor signalling contributes to the inhibitory effects of ATP on calcification. Like endogenous extracellular nucleotides, we found that Bz-ATP, α,β-meATP and β,γ-meATP also reduce VSMC calcification *in vitro*, with β,γ-meATP being the most potent.

VSMCs expressed mRNA for each P2X receptor but protein was only detected for the P2X3, P2X4, P2X5 and P2X7 receptors. Bz-ATP, α,β-meATP and β,γ-meATP each display a degree of selectivity for the different P2X receptors and therefore our initial findings suggested involvement of the P2X3, P2X4 and/or P2X7 receptor subtypes. Subsequent pharmacological analysis showed that selective antagonists for the these receptors did not attenuate any of the actions of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC calcification. A non-selective P2 receptor antagonist (PPADS) was also without effect. This failure to block the actions of Bz-ATP, α,β-meATP and β,γ-meATP pharmacologically suggests that these compounds are acting via P2X receptor-independent mechanisms to elicit their functional effects on calcification, apoptosis and gene expression. However, further work using knockout or knockdown approaches would be necessary to fully exclude involvement of P2X receptors.

Earlier work has shown that a calcifying environment can trigger VSMC apoptosis [2]. This increased apoptosis can then contribute to further development of AMC since the apoptotic bodies can act as a nucleation site for the formation of hydroxyapatite crystals [2]. Similar to endogenous extracellular nucleotides [6], Bz-ATP, α,β-meATP and β,γ-meATP act in a protective manner by decreasing VSMC apoptosis to levels similar to that seen in non-calcifying cells. Fewer apoptotic bodies would result in less sites where hydroxyapatite formation can be initiated, thus contributing to the reduced calcification observed in treated VSMCs.

AMC is also associated with phenotypic changes that result in reduced expression of VSMC marker genes and increased expression of osteoblast-associated genes [1]. In agreement, we found that the development of calcification was associated with decreased Sm22α and Acta2 protein expression and small increases in Runx2 and OPN protein expression. Treatment with Bz-ATP, α,β-meATP and β,γ-meATP attenuated these calcification-induced changes in gene expression. This suggests that these compounds can modulate the phenotypic alterations that occur in VSMCs when in a calcifying environment. It is unclear from the present study whether these actions are a direct consequence of

Bz-ATP, α,β-meATP and β,γ-meATP-induced signalling or a secondary consequence of the reduced apoptosis/calcification. However, given that no P2X receptor subtype was attributed to the functional effects of these compounds, the latter seems more plausible.

The metabolism of extracellular nucleotides is complex and involves numerous different enzymes with overlapping specificities [26]. Previous work has shown that β,γ-meATP and α,β-meATP (≥30μM although 300μM is more commonly used) can inhibit ecto-nucleotidase (NTPdases and NPPs) activity which slows nucleotide metabolism and leads to ATP accumulation [17,25,27]. Since ATP inhibits VSMC calcification [6,16], we investigated whether α,β-meATP, β,γ-meATP and Bz-ATP were acting indirectly via increased ATP levels. We found that extracellular ATP levels were in fact lower in VSMCs cultured long term with α,β-meATP and β,γ-meATP. Furthermore, the breakdown of exogenously added ATP was faster in VSMCs that had been cultured long term with these compounds. This suggests that α,β-meATP and β,γ-meATP are not inhibiting VSMC ecto-nucleotidase activity and may even be increasing enzyme activity or expression. The concentrations of α,β-meATP and β,γ-meATP studied here were, at 25μM and 10μM, respectively, lower than the levels at which enzyme inhibition has been reported [17,25]. Nonetheless, at these concentrations, these compounds reduced VSMC calcification by >85%. If decreased ecto-nucleotidase activity and the resultant ATP accumulation were contributing to the functional effects of these compounds it would be expected to be evident using these concentrations.

NPP1 deletion leads to decreased extracellular PP$_i$ levels and the development of vascular calcification [28]. We have shown that the inhibitory effects of ATP and UTP on VSMC calcification are partially mediated indirectly via the actions of NPP1 to generate PP$_i$ [6]. Studies here using *Enpp1$^{-/-}$* VSMCs showed that deletion of NPP1 had no effect on the inhibitory actions of α,β-meATP, β,γ-meATP or Bz-ATP. Furthermore, treatment with Bz-ATP, α,β-meATP or β,γ-meATP only lead to marginal increases in extracellular PP$_i$ levels. Taken together, these data suggest that these analogues are not hydrolysed by NPP1 to produce significant amounts of PP$_i$.

TNAP is a crucial enzyme in bone mineralisation and increased expression and activity has been associated with promoting the development of AMC [3,29]. The inhibition of bone mineralisation by ATP, UTP and ATP-analogues is in part mediated by decreased TNAP expression and activity [22,30]. Interestingly, ATP and UTP, at levels which inhibit VSMC calcification, promote TNAP activity in calcifying VSMCs [6]. This study observed a similar stimulatory effect in VSMCs cultured with Bz-ATP, α,β-meATP and β,γ-meATP. The reason for this counter-intuitive increase in activity remains to be determined but, given that these compounds can modulate the activity of other ecto-nucleotidases [17,25,27], their effects could reflect direct interactions with the TNAP enzyme. However, the basal TNAP activity in calcifying mouse VSMCs *in vitro* is low (being approximately 100-fold lower than mineralising osteoblasts) and calcification cannot be induced using low, physiologically relevant levels of the TNAP substrate, β-glycerophosphate (≤2mM) [6]. Therefore, the relative importance of this enzyme in ATP metabolism and driving calcification in this experimental model of AMC remains unclear.

Our finding that Bz-ATP, α,β-meATP and β,γ-meATP do not appear to act directly via P2X receptors or indirectly via altered ecto-nucleotidase activity raises the question of their exact mechanism of action. Whilst historically considered to be relatively non-hydrolysable, it is now known that these ATP analogues can be metabolised to some extent [17,31]. Bz-ATP can be sequentially broken down to produce Bz-adenosine, whilst α,β-meATP can produce α,β-meADP [31]. β,γ-meATP can be hydrolysed to produce methylene diphosphonate, a bisphosphonate-like compound. Bisphosphonates adhere strongly to mineralised matrix and have been shown to inhibit both vascular calcification [16,32-34] and

bone mineralisation [35,36]. This study found that both methylene diphosphonate and α,β-meADP blocked VSMC calcification at a similar potency to the parent compounds. Bz-adenosine could not be commercially obtained and so it was not possible to determine whether this molecule mediated the functional effects of Bz-ATP. Taken together these findings suggest that some of the actions of β,γ-meATP and α,β-meATP are mediated indirectly via these breakdown products. However, since previous work has also shown that these ATP-analogues have some weak agonist activity at P2Y receptors [37], involvement of one of these receptors (except the P2Y$_2$ receptor) cannot be discounted.

This study provides clear evidence that ATP-analogues can reduce VSMC calcification and apoptosis and prevent changes in gene expression *in vitro*. However, it also indicates that the P2X receptors are not involved in these actions. Instead our findings suggest that some of the functional effects observed are mediated indirectly via compounds generated during hydrolysis.

FIGURE LEGENDS

Figure 1. *Expression of P2 receptors by calcifying VSMCs*

(A) Calcifying VSMCs express mRNA for all P2X receptors and some of the P2Y receptors. Results are shown as mRNA expression relative to $P2Y_2$ receptor expression (dotted line). Data are presented as box and whisker plots (min-to-max values, n = 5 RNA sets, * = $p<0.05$, ** = $p<0.01$). **(B)** Western blot analysis shows that calcifying VSMCs expression protein for the P2X3, P2X4, P2X5 and P2X7 receptors; protein for the P2X1, P2X2 and P2X6 receptors was not detected. The positive control (+ve) was protein isolated from mature osteoblasts.

Figure 2. *Inhibition of VSMC calcification by Bz-ATP, α,β-meATP and β,γ-meATP*

Treatment with **(A)** Bz-ATP (100µM) and **(B)** α,β-meATP (≥25µM) reduced VSMC calcification by up to 95%. **(C)** β,γ-meATP (≥10µM) completely prevented VSMC calcification. Data are presented as box and whisker plots (min-to-max values, n = 6 replicate wells, *** = $p<0.001$) and are representative of an experiment performed at least three times. **(D)** Representative phase contrast images of alizarin red stained cell layers show the inhibitory effects of Bz-ATP, α,β-meATP and β,γ-meATP (regions of calcification highlighted by the arrows)**.** Scale bar = 100µm.

Figure 3. *The effect of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC viability and apoptosis*

Treatment with **(A)** Bz-ATP, **(B)** α,β-meATP and **(C)** β,γ-meATP (≥1µM) reduced the percentage of dead cells present by up to 72%. There was no effect on total cell number in **(D)** BzATP, **(E)** α,β-meATP and **(F)** β,γ-meATP treated VSMCs. **(G)** Bz-ATP reduced the level of apoptosis in calcifying VSMCs to a level similar to control cells. **(H)** α,β-meATP (50µM) decreased VSMC apoptosis by 25%. **(I)** β,γ-meATP (≥10µM) prevented the VSMC apoptosis induced by a calcifying environment. Data are presented as box and whisker plots (min-to-max values, n = 4-6 replicate wells, * = $p<0.05$, *** = $p<0.001$) and are representative of an experiment performed at least three times.

Figure 4. The effect of *Bz-ATP, α,β-meATP and β,γ-meATP on calcification-induced changes in protein expression*

Western blot analysis showed calcification was associated with reduced protein levels of SM22-α, and Acta2 whilst Runx2 and OPN protein levels were increased. Bz-ATP, α,β-meATP and β,γ-meATP appeared to attenuate the calcification-associated alterations in gene expression in VSMCs after 14 days of culture. Blots are representative of 3 experiments with similar results.

Figure 5. *P2X receptor antagonists do not prevent the actions of Bz-ATP, α,β-meATP and β,γ-meATP on VSMC calcification*

(A-C) The non-selective P2 receptor antagonist, PPADS, did not prevent the inhibitory effects of Bz-ATP, α,β-meATP and β,γ-meATP. **(D-E)** NF110, a selective P2X3 antagonist, **(F-G)** P2X4 receptor antagonists and **(H-I)** P2X7 receptor antagonists were also without effect. Data are presented as box and whisker plots (min-to-max values, n = 6 replicate wells, *** = $p<0.001$) and are representative of an experiment performed at least three times.

Figure 6. The effect of *Bz-ATP, α,β-meATP and β,γ-meATP on TNAP activity and ATP release and breakdown.*

(A) Bz-ATP, **(B)** α,β-meATP and **(C)** β,γ-meATP (≥10µM) increased VSMC TNAP activity by up to 5.9-fold. **(D)** Basal extracellular ATP levels were 70% and 45% lower in VSMCs cultured for 14 days with α,β-meATP (25µM) and β,γ-meATP (10µM), respectively. Bz-ATP did not affect basal ATP levels. The

breakdown of exogenously added ATP was faster in VSMCs-treated with **(E)** Bz-ATP, **(F)** α,β-meATP and **(G)** β,γ-meATP. Data are presented as box and whisker plots (min-to-max values, n = 6 replicate wells) or means ± SEM (n = 6-12 replicate wells, * = p<0.05, ** = p<0.01, *** = p<0.001) and are representative of an experiment performed at least three times.

Figure 7: *The effects of Bz-ATP, α,β-meATP and β,γ-meATP are not mediated indirectly via NPP1*
The effects of **(A)** Bz-ATP, **(B)** α,β-meATP and **(C)** β,γ-meATP on VSMC calcification were not prevented or reduced by deletion of NPP1. **(D)** Treatment with ATP increased extracellular PP_i levels by up to 4μM; Bz-ATP, α,β-meATP and β,γ-meATP increased PP_i levels by ≤0.7μM. Data are presented as box and whisker plots (min-to-max values, n = 6 replicate wells, *** = p<0.001) and are representative of an experiment performed at least three times.

Figure 8. *Breakdown products of α,β-meATP and β,γ-meATP also inhibit VSMC calcification*
(A) α,β-meADP (≥10μM) and **(B)** methylene diphosphonate (≥10μM) inhibit VSMC calcification by up to 95% and 80%, respectively. Data are presented as box and whisker plots (min-to-max values, n = 6 replicate wells, *** = p<0.001) and are representative of an experiment performed at least three times.

REFERENCES

1. Zhu D, Mackenzie NC, Farquharson C, Macrae VE (2012) Mechanisms and clinical consequences of vascular calcification. Front Endocrinol(Lausanne) 3: 95.

2. Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, et al. (2000) Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. Circ Res 87: 1055-1062.

3. Narisawa S, Harmey D, Yadav MC, O'Neill WC, Hoylaerts MF, et al. (2007) Novel inhibitors of alkaline phosphatase suppress vascular smooth muscle cell calcification. J Bone Miner Res 22: 1700-1710.

4. Zhu D, Mackenzie NC, Millan JL, Farquharson C, Macrae VE (2011) The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. PLoS ONE 6: e19595.

5. Shroff RC, Shanahan CM (2007) The vascular biology of calcification. Semin Dial 20: 103-109.

6. Patel JJ, Zhu D, Opdebeeck B, D'Haese P, Millan JL, et al. (2018) Inhibition of arterial medial calcification and bone mineralization by extracellular nucleotides: The same functional effect mediated by different cellular mechanisms. J Cell Physiol 233: 3230-3243.

7. Kapustin AN, Davies JD, Reynolds JL, McNair R, Jones GT, et al. (2011) Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. Circ Res 109: e1-12.

8. Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87: 659-797.

9. Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 16: 433-440.

10. Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64: 445-475.

11. North RA, Surprenant A (2000) Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40: 563-580.

12. Burnstock G, Ralevic V (2014) Purinergic signaling and blood vessels in health and disease. Pharmacol Rev 66: 102-192.

13. Fish RS, Klootwijk E, Tam FW, Kleta R, Wheeler DC, et al. (2013) ATP and arterial calcification. Eur J Clin Invest 43: 405-412.

14. Cote N, El HD, Pepin A, Guauque-Olarte S, Ducharme V, et al. (2012) ATP acts as a survival signal and prevents the mineralization of aortic valve. J Mol Cell Cardiol 52: 1191-1202.

15. Qian S, Regan JN, Shelton MT, Hoggatt A, Mohammad KS, et al. (2017) The P2Y$_2$ nucleotide receptor is an inhibitor of vascular calcification. Atherosclerosis 257: 38-46.

16. Villa-Bellosta R, Sorribas V (2013) Prevention of vascular calcification by polyphosphates and nucleotides- role of ATP. Circ J 77: 2145-2151.

17. Prosdocimo DA, Douglas DC, Romani AM, O'Neill WC, Dubyak GR (2009) Autocrine ATP release coupled to extracellular pyrophosphate accumulation in vascular smooth muscle cells. Am J Physiol Cell Physiol 296: C828-C839.

18. Lohman AW, Billaud M, Isakson BE (2012) Mechanisms of ATP release and signalling in the blood vessel wall. Cardiovasc Res 95: 269-280.

19. Fleisch H, Bisaz S (1962) Mechanism of calcification: inhibitory role of pyrophosphate. Nature 195: 911.

20. Orriss IR, Arnett TR, Russell RG (2016) Pyrophosphate: a key inhibitor of mineralisation. Curr Opin Pharmacol 28: 57-68.

21. Sali A, Favaloro JM, Terkeltaub R, Goding JW (1999) Germline deletion of the nucleoside triphosphate pyrophosphohydrolase (NTPPPH) plasma cell membrane glycoprotein (PC-1) produces abnormal calcification of the periarticular tissues. In: Vanduffe L, Lemmens R, editors Ecto-ATPases and related ectonucleotides, Mastricht, the Netherlands: Shaker Publishing BV: 267-282.

22. Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G, et al. (2012) The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of P2X receptors. Bone 51: 389-400.

23. Taylor SE, Shah M, Orriss IR (2014) Generation of rodent and human osteoblasts. BoneKey Rep 3: 585.

24. Orriss IR, Knight GE, Utting JC, Taylor SE, Burnstock G, et al. (2009) Hypoxia stimulates vesicular ATP release from rat osteoblasts. J Cell Physiol 220: 155-162.

25. Joseph SM, Pifer MA, Przybylski RJ, Dubyak GR (2004) Methylene ATP analogs as modulators of extracellular ATP metabolism and accumulation. Br J Pharmacol 142: 1002-1014.

26. Zimmermann H, Zebisch M, Strater N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8: 437-502.

27. Villa-Bellosta R, Wang X, Millan JL, Dubyak GR, O'Neill WC (2011) Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. Am J Physiol Heart Circ Physiol 301: H61-68.

28. Mackenzie NC, Huesa C, Rutsch F, Macrae VE (2012) New insights into NPP1 function: lessons from clinical and animal studies. Bone 51: 961-968.

29. Sheen CR, Kuss P, Narisawa S, Yadav MC, Nigro J, et al. (2015) Pathophysiological role of vascular smooth muscle alkaline phosphatase in medial artery calcification. J Bone Miner Res 30: 824-836.

30. Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, et al. (2007) Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both $P2Y_2$ receptors and pyrophosphate. Endocrinology 148: 4208-4216.

31. Kukley M, Stausberg P, Adelmann G, Chessell IP, Dietrich D (2004) Ecto-nucleotidases and nucleoside transporters mediate activation of adenosine receptors on hippocampal mossy fibers by P2X7 receptor agonist 2'-3'-O-(4-benzoylbenzoyl)-ATP. J Neurosci 24: 7128-7139.

32. Sugitani H, Wachi H, Murata H, Sato F, Mecham RP, et al. (2003) Characterization of an in vitro model of calcification in retinal pigmented epithelial cells. J Atheroscler Thromb 10: 48-56.

33. Bauer C, le Saux O, Pomozi V, Aherrahrou R, Kriesen R, et al. (2018) Etidronate prevents dystrophic cardiac calcification by inhibiting macrophage aggregation. Sci Rep 8: 5812.

34. Miyai K, Ariyasu D, Numakura C, Yoneda K, Nakazato H, et al. (2015) Hypophosphatemic rickets developed after treatment with etidronate disodium in a patient with generalized arterial calcification in infancy. Bone Rep 3: 57-60.

35. Orriss IR, Key ML, Colston KW, Arnett TR (2009) Inhibition of osteoblast function in vitro by aminobisphosphonates. J Cell Biochem 106: 109-118.

36. Idris AI, Rojas J, Greig IR, van't Hof RJ, Ralston SH (2008) Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation in vitro. Calcif Tissue Int 82: 191-201.

37. Jacobson KA, Ivanov AA, de Castro S, Harden TK, Ko H (2009) Development of selective agonists and antagonists of P2Y receptors. Purinergic Signal 5: 75-89.
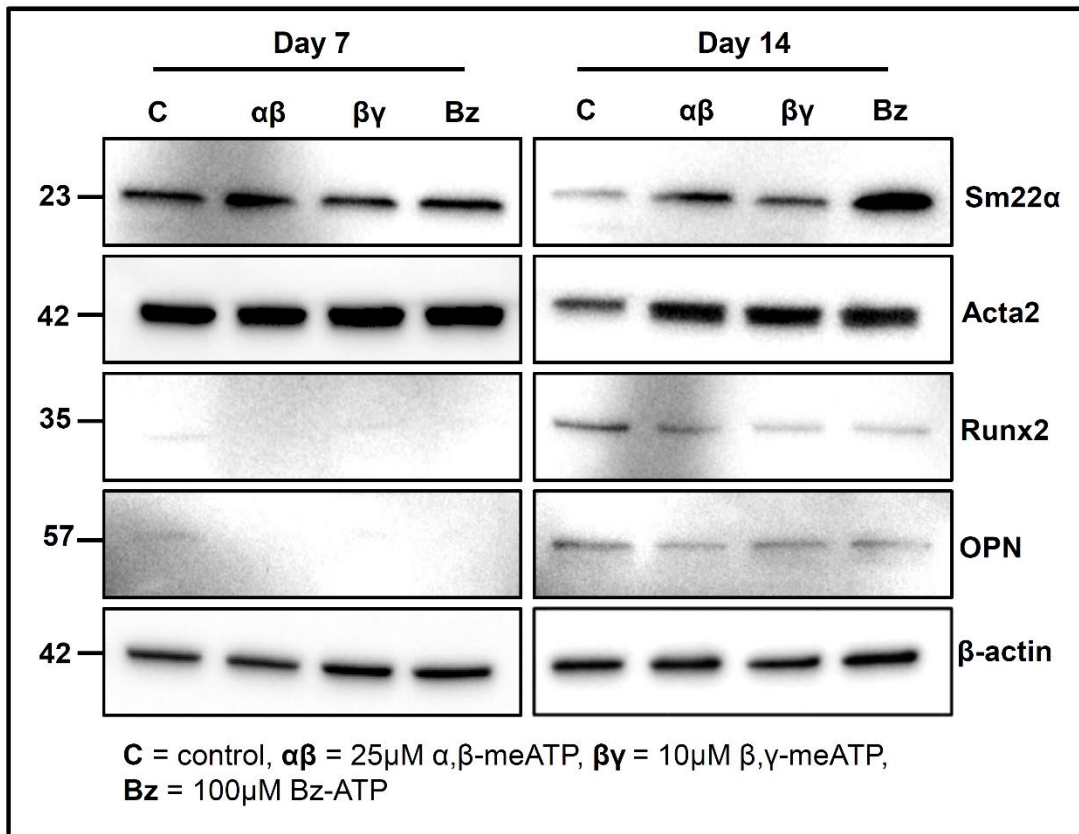
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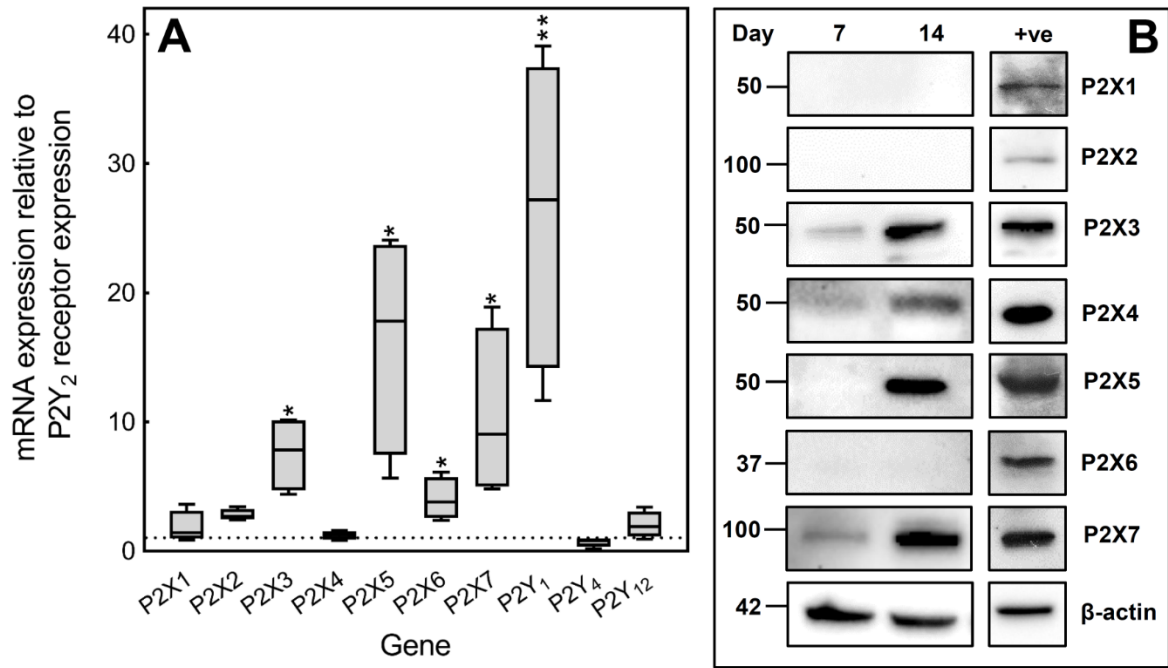
Figure 2

Figure 3

Figure 4

C = control, **αβ** = 25µM α,β-meATP, **βγ** = 10µM β,γ-meATP,
Bz = 100µM Bz-ATP
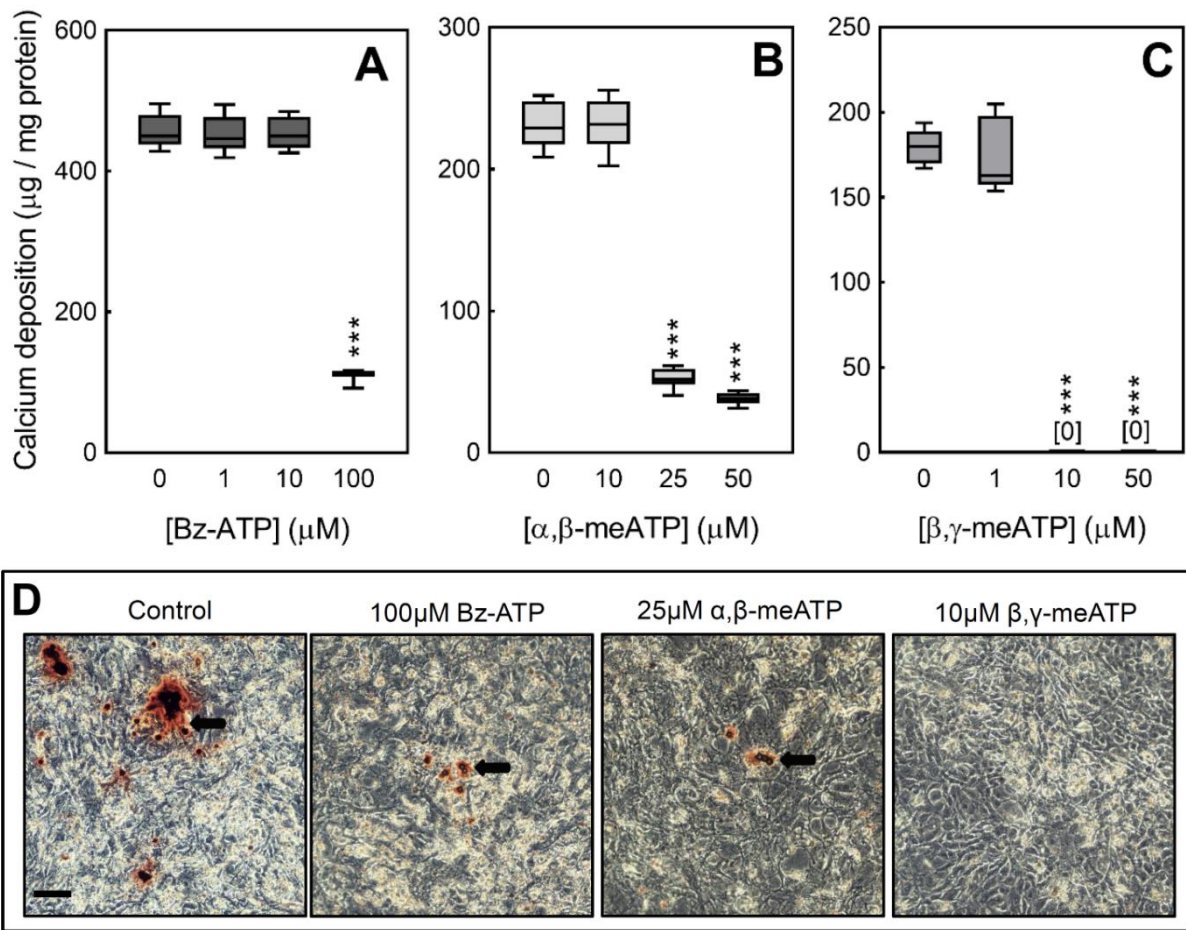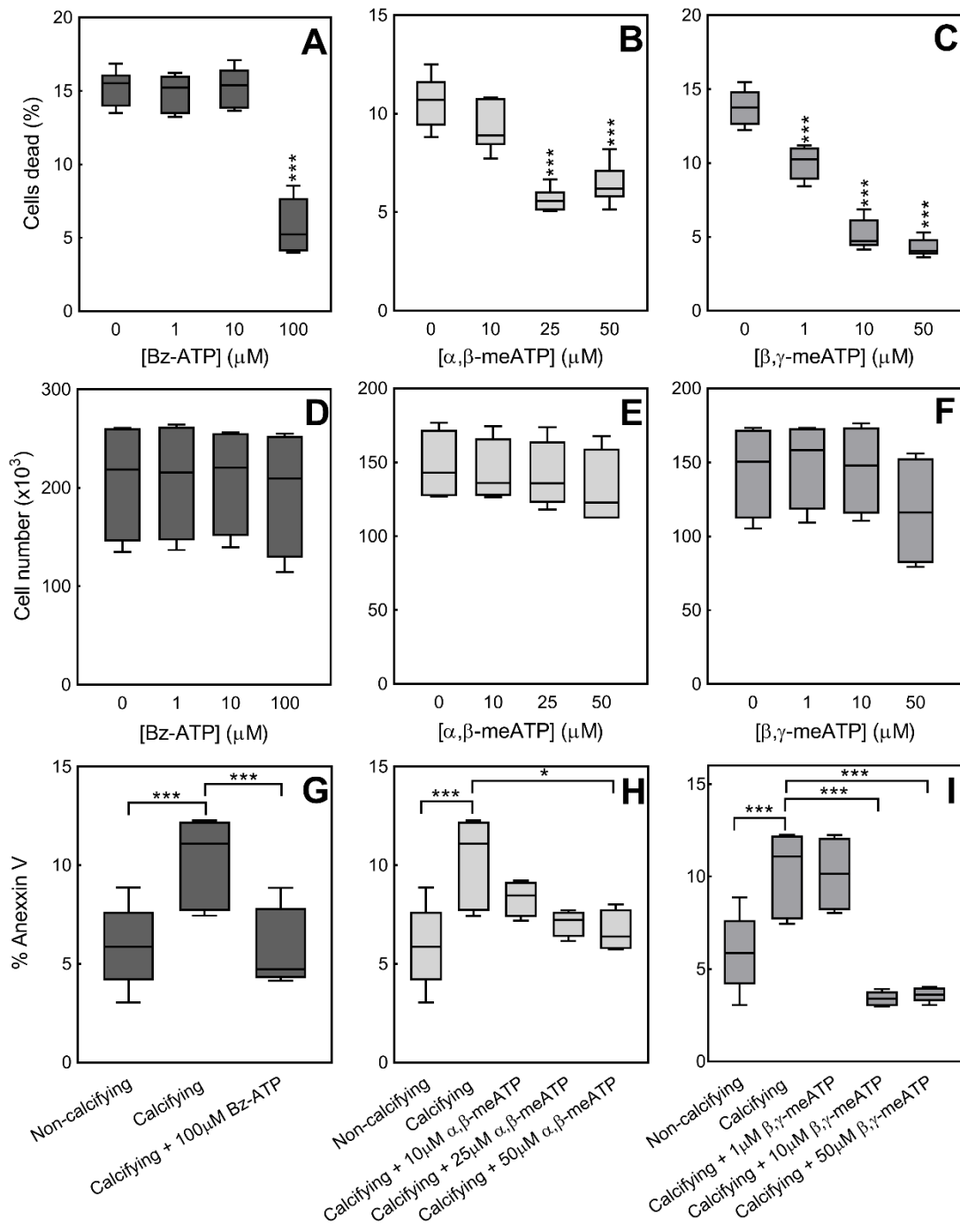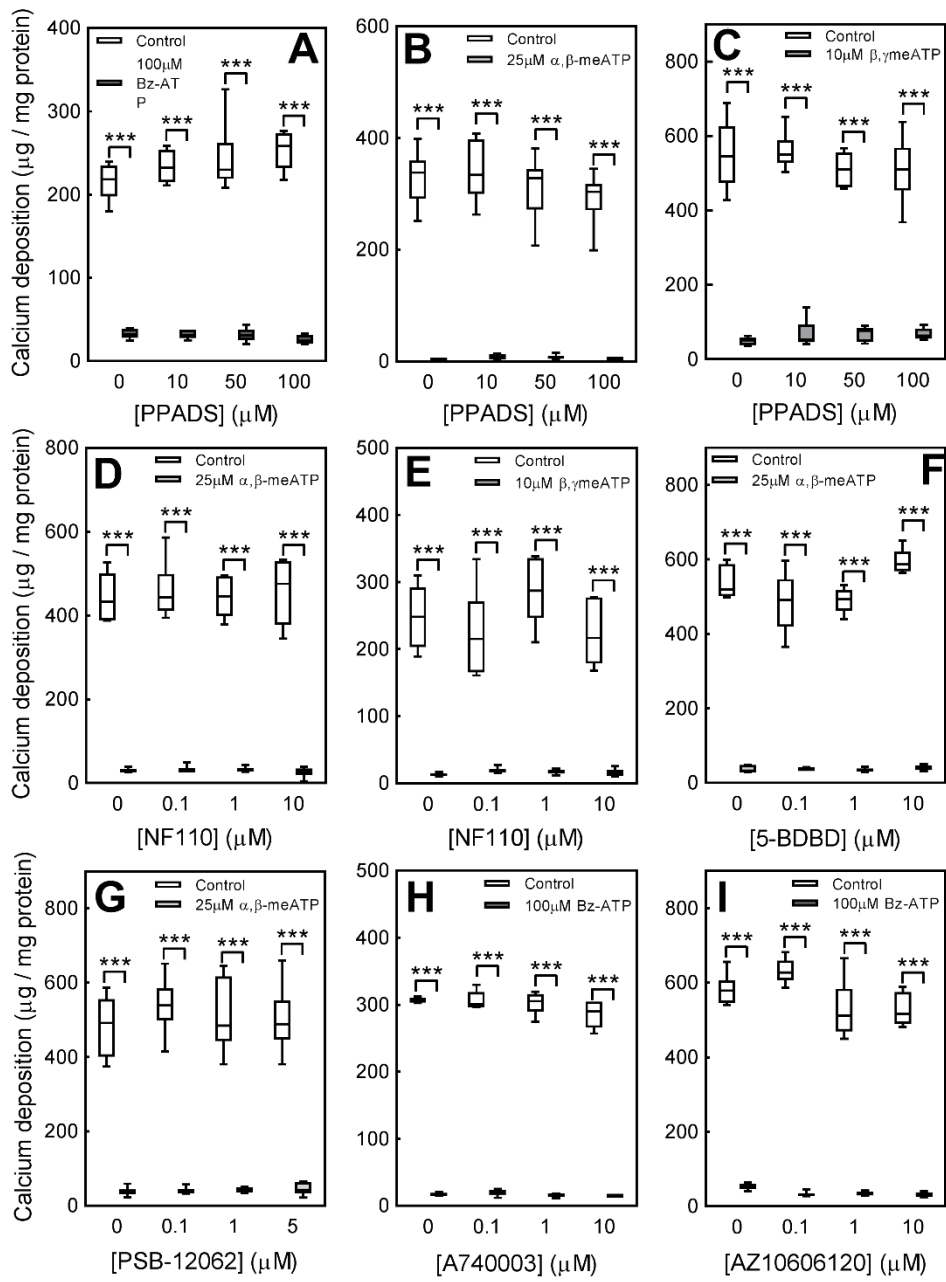
Figure 5

Figure 6

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Figure 8