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Regulatory actions of 3’,5’-cyclic adenosine monophosphate on osteoclast function: possible roles of Epac-mediated signalling

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Abstract

Alterations in cellular levels of the second messenger 3'-5'-cyclic adenosine monophosphate $[cAMP]_i$ regulate a wide range of physiologically important cellular signalling processes in numerous cell types. Osteoclasts are terminally differentiated, multinucleated cells specialised for bone resorption. Their systemic regulator, calcitonin, triggers morphometrically and pharmacologically distinct, retraction ('R') and quiescence ('Q'), effects respectively on cell-spread area and protrusion-retraction motility paralleling its inhibition of bone resorption. Q effects were reproduced by cholera toxin-mediated G_s-protein activation known to increase $[cAMP]_i$, unaccompanied by the $[Ca^{2+}]_i$ changes contrastingly associated with R effects. We explore a hypothesis implicating cAMP-signalling involving guanine nucleotide-exchange activation of the small GTPase Ras-proximate-1 (Rap1) by exchange proteins directly activated by cAMP (Epac). Rap1 activates integrin clustering, cell adhesion to bone matrix, associated cytoskeletal modifications and signalling processes, and transmembrane transduction functions. Epac activation enhanced, whereas Epac inhibition or shRNA-mediated knockdown compromised the appearance of markers for osteoclast differentiation and motility following receptor activator of nuclear factor kappa-B ligand (RANKL) stimulation. Deficiencies in talin and Rap1 compromised in vivo bone resorption producing osteopetrotic phenotypes in genetically modified murine models. Translational implications for an Epac-Rap1 signalling hypothesis in relationship to N-bisphosphonate actions on prenylation and membrane localisation of small GTPases are discussed.

3'-5'-cyclic adenosine monophosphate in the regulation of specialised cell types

Alterations in cellular levels of the key second messenger 3'-5'-cyclic adenosine monophosphate $[cAMP]_i$ take place with alterations in the balance between its generation and hydrolysis. The messenger is generated by the activity of a range of adenylyl cyclase isoforms following hormone or neurotransmitter-mediated activation of their coupled heterotrimeric G-protein, $G_{s\alpha}$. Its subsequent hydrolysis to 5'AMP is brought about by one or more of a range of cAMP phosphodiesterases (PDEs)[1]. The resulting intracellular cAMP concentration, $[cAMP]_{i,}$ influences a wide range of physiologically important cellular signalling processes. The exact processes affected and the nature of their regulation varies with the particular specialisation of the cells concerned. Osteoclasts exemplify a terminally differentiated, highly specialised cell type which degrade mineralized matrix during normal and pathological bone turnover. Following the proliferation and migration of hemopoietic osteoclast progenitors to bone and their differentiation and fusion to form these multinucleated cells, osteoclast function involves their migration to and between resorption sites, prior to highly specialised and closely regulated resorption episodes terminated by cell retraction and regulated both by local extracellular Ca^{2+} and systemic calcitonin action[2].

Components of osteoclast motility and retraction properties following calcitonin action: the 'Q' and 'R' effects

Long-range regulation of osteoclast activity involves calcitonin action, for which $[cAMP]_i$ complements intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ as a cellular second messenger[3,4]. These two messenger systems exert distinct physiological effects consistent with their representing distinct activation pathways. Nevertheless both culminate in inhibition of bone resorptive activity. Calcitonin increased cAMP levels in both bone[5] and isolated osteoclasts[6]. In isolated rat osteoclasts, the lowest effective calcitonin concentrations replicated the effects of agents known directly to elevate $[Ca^{2+}]_i$ including elevated extracellular $[Ca^{2+}]$, and perchlorate and ionomycin challenge[7]. They induce retraction ('R' effect) of cell spread area and inhibit bone resorptive activity over 2-3 h following stimulus application whilst sparing cellular motility in the form of cell retraction and protrusion activity. These effects accompanied transient elevations in $[Ca^{2+}]_i$[8]. These effects were not replicated by calcitonin analogues such as amylin or calcitonin gene-related peptide (CGRP), yet the latter are similarly known to reduce bone resorption[9].

However, higher calcitonin concentrations additionally induced a quiescence ('Q' effect) in osteoclast motility, reflected in a cessation of cell retraction and protrusion activity that was distinct from the R effect[10]. It also reduced the margin ruffling implicated in resorptive hemivacuole formation and bone resorptive activity and increased secretion of the osteoclast-specific enzyme tartrate-resistant acid phosphatase (TRAP). The Q effect could be selectively replicated in an absence of the R effect by the calcitonin analogues amylin, β-CGRP[9], or the peptide CGRP fragment CGRP-(Val^8Phe^{37}), findings also accompanied by reduced bone resorption[9,11]. However, in contrast to agents associated with the R effect, none of these latter manoeuvres elevated osteoclast $[Ca^{2+}]_i$[9]. The Q effect was associated with reductions in supernatant TRAP and reduced osteoclast-mediated bone resorption in vitro.

These Q and R effects could be separated firstly by the application of terms quantifying a parameter, ρ, describing cell spread area changes resulting from cell retraction ('R') and a motility parameter μ summing retraction (Δr)-protrusion (Δp) activity during the Q effect in isolated osteoclasts (Fig. 1A)[10]. These demonstrated that calcitonin produced time-dependent reductions in both ρ and μ terms (Fig. 1B). In contrast, extracellular Ca^{2+} challenge selectively altered the ρ term with relatively little change in μ (Fig. 1C). Conversely, amylin challenge selectively altered the μ parameter whilst leaving ρ relatively unchanged (Fig. 1D). Secondly, use of CGRP-(Val^8Phe^{37}) as a calcitonin antagonist, abolished the calcitonin-induced Q but not the R effect of calcitonin. Thus, the Q and R effects of calcitonin action may reflect activation of pharmacologically distinct receptor targets and signalling pathways[12].

G-protein involvement in the Q and R effects: involvement of cAMP signalling

Different G-protein agonists recapitulated the Q or R effects either in isolation or in combination[4]. The universal G-protein agonist tetrafluoro-aluminate (AlF^{4-}) produced both Q and R effects together[4]. This was accompanied by a concentration-dependent inhibition of bone resorption, yet a paradoxically increased in TRAP enzyme secretion[13]. Pertussis toxin has been previously implicated in $G_{i/o}$ protein inhibition. In osteoclasts, it selectively induced an R effect, in the absence of an Q effect, suggestive of a novel Ca^{2+} dependent G-protein mediated mechanism. Thus, as also reported in connection with platelet aggregation and activation[14], as well as a number of other cell types[15], pertussis toxin may exert effects that increase osteoclastic $[Ca^{2+}]_i$ independent of its previously established ADP-ribosylation action on $G_{i/o}$. These findings were accompanied by reduced bone resorption though a relatively limited reduction in enzyme release. In contrast, the Q effect was replicated selectively, in the absence of an R effect, with challenge by the G_s protein stimulator cholera toxin[13,16]. This was similarly accompanied by a reduction in bone resorption but an enhanced TRAP secretion. The latter effects likely were mediated by an increase of $[cAMP]_i$ previously attributed to cholera toxin induced G_s protein activation. Thus, they were mimicked by the adenylate cyclase activator forskolin[16] and dibutyryl cyclic AMP[17,18], and potentiated by the phosphodiesterase inhibitor theophylline[17].

Protein kinase A vs. Epac mediated pathways involving cAMP

The $[cAMP]_i$-mediated actions *inhibiting* cellular function in the bone-resorbing osteoclast following calcitonin action complement their classically described *activating* effects in cardiomyocytes. The latter follow β_1 adrenergic stimulation which results in a cAMP-induced activation of protein kinase A (PKA) mediated phosphorylation activity of a wide range of regulatory molecules involved in cellular Ca^{2+}

signalling. Thus, PKA-mediated L-type Ca^{2+} channel phosphorylation enhances inward voltage-dependent L-type Ca^{2+} current and extracellular Ca^{2+} entry resulting from such channel opening during action potential excitation. The consequent sarcoplasmic reticular (SR) ryanodine receptor (RyR2)-Ca^{2+} channel mediated Ca^{2+}-induced Ca^{2+} release is further increased with RyR2 phosphorylation. These phosphorylation events thus markedly accentuate the elevations in systolic cytosolic [Ca^{2+}] resulting from excitation-contraction coupling. Conversely, PKA-mediated phosphorylation of the inhibitory peptide phospholamban increases the SR Ca^{2+}-ATPase mediated uptake of Ca^{2+} from cytoplasm to SR that reduces cytosolic [Ca^{2+}] to its background level during cardiomyocyte relaxation[19].

However, cAMP also exerts PKA-independent effects. In sino-atrial node cells, cAMP binding to hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels mediating pacemaker (I_f) ionic currents increases heart rate[20]. Furthermore, there has been much recent interest about the role of exchange proteins directly activated by cAMP (Epac)[21–24]. In cardiomyocytes, Epac signaling offers an alternative or co-existent cAMP-dependent regulatory mechanism that operates downstream of β adrenergic receptor activation but upstream of the RyR2-mediated Ca^{2+}-induced SR Ca^{2+} release underlying excitation-contraction coupling[25,26]. Thus, the cAMP analog 8-(4-chlorophenylthio)-2'-O-methyladenosine 3', 5'-cyclic monophosphate (8-CPT) when applied at concentrations preferentially activating Epac rather than PKA[27] elicited spontaneous, or increased amplitudes of electrically evoked, cytosolic Ca^{2+} transients, as well as spontaneous propagated cytosolic Ca^{2+} waves, in rat or mouse cardiac myocytes[23]. Such phenomena potentially increase electrogenic Na^+/Ca^{2+} exchange current activity accounting for the pro-arrhythmic triggering electrophysiological activity that was observed accompanying such alterations in Ca^{2+} homeostasis[23,28–32]. These effects also followed isoproterenol-mediated adrenergic agonist activation in the presence of the PKA inhibitor H-89[33]. They thus appear to reflect a cAMP-mediated, PKA-independent regulatory mechanism, specifically acting on excitation-contraction coupling through modulation of RyR2 activation[23]. Thus they were abolished with genetic ablation of Epac2, β_1 adrenoreceptor, Ca^{2+}/calmodulin-dependent protein kinase II-δ (CaMKIIδ) and RyR2-S2814 phosphorylation[34]. Furthermore, murine *Epac1*$^{-/-}$, *Epac2*$^{-/-}$ and *CaMKIIδ*$^{-/-}$ hearts showed normal indices for other cardiac functions. These included in vivo cardiac structure, ratios of heart to body weight, cardiac contractility or pressure overload–induced hypertrophy, and their responses to adrenergic dobutamine challenge, and cellular SR Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger protein expression levels, and Ca^{2+} content[34].

Since their discovery, Epac proteins have been implicated in a wide range of critical physiological actions of cAMP[35]. The present article speculates on a possible role for this system in osteoclast function in the context of available evidence. Epac exists in three isoforms. Of these, Epac1, Epac2 and Repac all include a Ras-association domain (RA)[36] (Fig. 2). Epac1 and Epac2 are membrane bound proteins reflecting their possession of a dishevelled-Egl-10-pleckstrin (DEP) domain. Epac1 contains a regulatory, cyclic mononucleotide-binding (CNB) domain with a natively disordered, N-terminal extension[37] permitting activation by direct cAMP binding[38,39]. Epac2 contains an additional lower affinity amino-terminal cAMP-binding (CNB) site[37,40]. This is N-terminal to the DEP domain and has a 20-fold lower cAMP affinity than the conserved CNB. These regulatory domains are joined via a Ras exchange motif (REM) and an intervening RA domain to the cell division cycle phosphatase-25 (CdC25) homology domain (CDC25HD) responsible for guanine nucleotide-exchange activity. The isoform related to Epac, Repac, only includes a catalytic region, consistent with it being constitutively active or having a separate regulatory site[37,41]. Of the isoforms directly regulated by cAMP, cellular expression of Epac1 appears ubiquitous, whereas Epac2 occurs preferentially in brain, pituitary, and adrenal gland. In contrast to Epac1 and to the apparent $[Ca^{2+}]_i$-independence of the osteoclastic Q effect, Epac2 has been associated with regulation of processes dependent upon Ca^{2+} signaling[25,42]. Thus, besides producing growth arrest in neuroendocrine cells, Epac2 located at cardiomyocyte transverse tubules may regulate potentially arrhythmogenic SR Ca^{2+} leak and/or Ca^{2+} induced Ca^{2+} release through SR RyR2-Ca^{2+} release channels[23,43]. Epac2 has also been implicated in endoplasmic reticular Ca^{2+} dependent secretary events in pancreatic cells[44–48]. It has been suggested that in these situations it acts through activation of a novel phosphoinositide (PI) phospholipase C isoform (PLCe), protein kinase C (PKC), and CaMKII[35]. Epacs can also function as guanine nucleotide-exchange factors (GEFs) facilitating exchange of GDP for GTP binding in small guanine nucleotide-binding (G) proteins. In general, GEFs activate signalling by catalyzing the exchange from G-protein-bound GDP to GTP. In contrast, GTPase-activating proteins (GAPs) terminate such signalling by inducing GTP hydrolysis. GEFs promote the GTP-bound, active, form. GAPs promote the GDP-bound, inactive, form (Fig. 3). Both GEFs and GAPs contain multiple domains mediating regulation by both extracellular signals and localized events[49].

Rap1 as a target for Epac-mediated regulation

Amongst the small guanine nucleotide-binding (G) proteins that cycle between GDP- and GTP-bound forms, Ras proteins, resident at the cytoplasmic surface of cell membranes, are of particular importance. They act as cellular switches in signal transduction[50]. The Ras-proximate or Ras-related proteins (Rap) constitute one group within the Ras protein superfamily[39]. These contain a C-terminal tetrapeptide sequence motif Cys-Ali-Ali-X, where Ali is an aliphatic amino acid and X is any amino acid. This constitutes the site of membrane attachment via isoprenylation for ras, nuclear lamins and γ-subunits of the heterotrimeric G-proteins. Rap1 is thus localized to the cytosolic side of internal membranes such as endosomes and secretory granules compatible with roles in recruiting components for vesicle formation and/or transport.

In contrast to Ras, in which mutations are associated with cell proliferation and human neoplasms, genetic studies suggest that of Rap proteins, Rap1 is primarily involved in cell adhesion and cell junction formation[51]. Rapl proteins, exist as two similar isoforms in many animal tissues, Rap1a and Rap1b, differing by only a few amino acids[42,51]. Rap2a (previously Rap2) proteins are found mostly in platelets and brain tissue. They have been less extensively studied than Rap1, but have been implicated in Epac2-dependent growth arrest in neuroendocrine cells, and Ca^{2+}-dependent secretary events in pancreatic cells[44,45]. Rap2b has been reported in *Escherichia coli* systems.

Ras and Rap are regulated by different GEFs and GAPs[52]. Epac is likely one of a range of these GEFs regulating the Rap1 member of this family (Fig. 3). Thus, in addition to transmembrane receptors such as receptor tyrosine kinases, heterotrimeric G-protein-coupled receptors, cytokine receptors and cell-adhesion molecules[53], common second messengers such as cAMP, Ca^{2+} and diacylglycerol appear involved in transducing extracellular signals to Rap1[54]. Rap1 can also be activated by PKA-mediated phosphorylation but the functional significance of this is unclear but may even concern Rap1 inactivation[55], activation of other messengers such as B-raf [56], and involve an indirect, Ca^{2+}-dependent pathway[57].

Rap1 has been implicated in a range of processes dependent on integrin-dependent activation, exemplified in its requirement for phagocytosis by mouse macrophages and by *Dictyostelium discoideum*. It is also implicated in other processes that might involve integrin-mediated cell adhesion, morphogenesis, haematopoiesis, leukocyte migration and tumour invasion. It has been suggested that Rap1 activates integrins by stimulating cell surface receptors through currently uncertain mechanisms[58–61]. Rap1 has also been implicated in the recruitment to and activation of Phg2 at the leading edge of chemotaxin *D. discoideum* cells required for myosin II phosphorylation and disassembly in pseudopodial formation [62].

Epac modification alters osteoclast development and function

The selective Epac activator, 8-CPT, enhanced, whilst the Epac inhibitor brefeldin A reduced the osteoclast differentiation that followed stimulation by receptor activator of nuclear factor κ-B ligand (RANKL)[63] in primary murine and human bone marrow culture studies. Such differentiation accompanied cellular morphological alterations from centrally-sited nuclei surrounded by a ring of F-actin and absence of podosomes to large multinucleate osteoclasts with a peripheral podosome belt, showing TRAP production.

Similar findings were made in RAW264.7 cells in which these findings were further confirmed by the additional, recently available, Epac activation inhibitors 4-methylphenyl-2,4,6-trimethylphenylsulfone (ESI-05) and 3-[5-(*tert*-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile (ESI-09). Silencing either Epac1 or Epac2 by shRNA prevented this differentiation in the RAW264.7 cells. These findings were reflected in mRNA levels of the differentiation markers cathepsin K, nuclear factor of activated T cells c1 (NFATc1) and osteopontin, and reduced numbers of formed resorption pits in dentine slides. The findings paralleled reductions in RANKL-induced Rap1 activation, and in reduced activation of proteins that were involved in actin cytoskeletal changes. The latter included reductions in the Ras homolog

RhoA, involved in stress fibre formation, cell cycle regulation and cell development, and Ras-related C3 botulinum toxin substrate 1 (Rac1), involved in lamellipod formation and cell-cell adhesion, with Epac1 or Epac2 suppression. They included reductions in cdc42, involved in filopodial assembly, with Epac1, but not Epac2, suppression and reductions in focal adhesion kinase (FAK), involved in cellular adhesion, with Epac2, but not Epac1, suppression[64,65]. Direct activation of Rac1 and FAK is necessary for actin cytoskeleton rearrangements during osteoclast differentiation. Finally, Epac1 or Epac2 silencing reduced the RANKL-induced cytoplasm to nuclear translocation of p50/p105 nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) associated with normal osteoclast differentiation[66].

Integrins are potential effectors for Rap1 in regulating osteoclast motility and bone resorption

Integrins form a family of heterodimeric adhesion receptors involved in cell–matrix and cell–cell interactions central to a wide variety of cell functions, including leukocyte homing and activation, cell responses to mechanical stress, apoptosis, and tumour growth and metastases[67]. Osteclast motile and secretory activity similarly involve integrin-dependent, cell–matrix and cell–cell interactions[68]. Osteoclast activity involves cell migration, adhesion to bone surfaces, and formation of a tight clear sealing zone that encloses a resorption lacuna. After insertion of secretory vesicles, a ruffled border comprising a highly convoluted membrane facing the bone surface is formed.

Mammalian osteoclasts express the $\alpha_v\beta_3$ integrin/vitronectin receptor, at high levels, as well as the collagen/laminin receptor $\alpha_2\beta_1$ and the vitronectin/fibronectin receptor $\alpha_v\beta_1$[69]. The α and β integrin cytoplasmic domains do not themselves have enzymatic activities. However, integrins interact with a range of matrix, and cytoskeletal and signaling molecules (Fig. 4). First, integrins mediate osteoclast adhesion to the bone surface by interacting with extracellular matrix proteins within the bone matrix. Rat osteoclasts adhere in an $\alpha_v\beta_3$-dependent manner to matrix proteins containing RGD (Arg-Gly-Asp) sequences, including vitronectin, osteopontin, bone sialoprotein and a cryptic RGD-site in denatured collagen type I, and in a $\alpha_2\beta_1$- and RGD-dependent manner to collagen type I[70,71]. Correspondingly, antibodies raised to α_2, β_1 and $\alpha_v\beta_3$ inhibit bone resorption[72,73]. Second, integrin-ligand binding induces receptor clustering causing formation of focal adhesion contacts, recruitment of and linkage to cytoskeletal molecules including paxillin, vinculin, gelsolin and F-actin with an organisation of intracellular cytoskeletal complexes, additionally leading to changes in cell shape, spreading and motility[74,75]. Thirdly, integrins mediate transmembrane transduction of signals in both inside-out and outside-in directions relating organization of the actin network and the composition of the focal adhesions, through the cytoskeletal protein talin which links integrins to the actin cytoskeleton, and is an essential mediator of integrin activation[76]. Fourthly, a wide range of signaling pathways including those involving phosphatidylinositol 3-kinase, c-Src, protein tyrosine kinase 2 β (PYK2) and p130^{cas} are $\alpha_v\beta_3$ integrin-dependent[77,78].

Rap1 and its interaction with integrins are essential to osteoclast bone resorptive activity

Studies in genetically modified murine models suggest that both Talin and Rap1 are critical for osteoclastic bone resorptive activity in vivo[79]. Talin1-deficient osteoclast precursors showed normal osteoclast differentiation markers following exposure to macrophage colony-stimulating factor (M-CSF) and RANKL. However, they showed compromised attachment to and migration over bone substrate. The talin-deficient mice correspondingly showed osteopetrotic phenotypes. Mice with a talin deletion made late in the course of osteoclastogenesis showed a reduction in both ovariectomy-induced osteoporosis and periarticular osteolysis associated with inflammatory arthritis. Osteoclast-specific deletion of Rap1 (CtsK-Rap1), which promotes talin/β-integrin recognition, also yielded mice with an osteopetrotic phenotype. In both these examples, this was more severe, with up to fivefold increases in bone mass, than the phenotype associated with $\alpha_v\beta_3$-deficiency, likely due to the additional added failed activation of β_1 integrins.

Translational implications of Epac and Rap1 signalling: relationships to bisphosphonate action

A possible translational outcome of testing a hypothesis implicating involvements of Epac regulation of Rap1 activity in osteoclast function may include targeted therapeutic intervention with reduced side effects, directed at bone diseases. Of these, osteoporosis is a widespread, often initially asymptomatic, insidious, disease of progressive bone loss and skeletal deterioration increasing fragility and liability to bone fracture. Its manifestations become commoner with age, particularly in postmenopausal women, affecting <1 in 2 women and <1 in 5 men over age 50 y. Bisphosphonates provide effective therapy: postmenopausal women receiving alendronate for 10 years showed fewer clinical vertebral fractures than those altered to placebo at 5 years in the Fracture Intervention Trial Long-term Extension (FLEX)[80]. Similarly, women who received 6 annual infusions of zoledronic acid showed fewer vertebral fractures than those altered to placebo after 3 years in the Health Outcomes and Reduced Incidence with Zoledronic acid Once Yearly–Pivotal Fracture Trial (HORIZON) extension[81]. Experimental studies report that relatively high (~10 μM) concentrations of nitrogen-containing bisphosphonates prevent post-translational prenylation of small GTPases not only in Rac, Rho, Cdc42, and Rab but also in Rap1 proteins, not only in J774 macrophages but also osteoclasts in vitro and in vivo[82,83]. This effect was attributed to inhibition of cholesterol biosynthetic pathways generating farnesyl pyrophosphate and geranyl diphosphate[84] substrates for post-translational protein prenylation. Prenylation of small GTPases enables their localization to the correct subcellular membranes and interaction with regulatory proteins[85]. Conversely, inhibition of such protein geranyl-geranylation by nitrogen containing bisphosphonates resulted in cytoskeletal disruption, reduced resorptive activity and osteoclast apoptosis[86,87]. Although the concentrations at which these effects occurred are unlikely to be reached in clinical practice, these findings broadly draw attention to Rap1 prenylation as a potential therapeutic target amenable to upstream approaches to therapy through the Epac-Rap signalling system. This could lead to alternative treatment strategies potentially avoiding some side effects of conventional bisphosphonate therapy[88,89].

Summary of abbreviations.

Δp, cell protrusion increment; Δr, cell retraction increment; 5'AMP, 5' adenosine monophosphate; 8-CPT, 8-(4-chlorophenylthio)-2'-O-methyladenosine 3', 5'-cyclic monophosphate; *A,* cell spread area; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cAMP, 3'-5'-cyclic adenosine monophosphate; CdC25, cell division cycle phosphatase-25; CDC25HD, CdC25 homology domain; CGRP, calcitonin gene-related peptide; CNB, cyclic mononucleotide-binding domain; DEP, dishevelled-Egl-10-pleckstrin domain; Epac, exchange protein directly activated by cAMP; ESI-05, 4-methylphenyl-2,4,6-trimethylphenylsulfone; ESI-09, 3-[5-(*tert*-butyl)isoxazol-3-yl]-2-[2-(3-chlorophenyl)hydrazono]-3-oxopropanenitrile; FAK, focal adhesion kinase; FLEX, Fracture Intervention Trial Long-term Extension; GAP, GTPase-activating protein; GDP, guanine diphosphate; GEF, guanine nucleotide-exchange factor; GTP, guanine triphosphate; G_x, guanine nucleotide-binding protein [x = s, stimulatory, i, inhibitory, o, other); HCN, hyperpolarisation-activated cyclic nucleotide-gated; HORIZON, Health Outcomes and Reduced Incidence with Zoledronic acid Once Yearly–Pivotal Fracture Trial; I_f, sino-atrial node pacemaker current; M-CSF, macrophage colony-stimulating factor; μ, cell motility parameter; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; PDE, phosphodiesterase; PI, phosphoinositide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PYK2, Protein tyrosine kinase 2β; Q, cell quiescence; R, cell retraction; Rac1, Ras-related C3 botulinum toxin substrate 1; RANK, nuclear factor κ-B; RANKL, nuclear factor κ-B ligand; Rap, Ras-proximate or Ras-related protein; Ras, rat sarcoma; RA, Ras association domain; REM, Ras exchange motif; Repac, related to Epac; ρ, cell retraction parameter; RGD, Arg-Gly-Asp; RhoA, Ras homolog A; RyR2, ryanodine receptor type 2; SR, sarcoplasmic reticulum; *t*, time; TRAP, tartrate-resistant acid phosphatase.

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Conflicts of interest.

None declared.

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Figure legends

Figure 1. Osteoclast motility properties.

(A) Measurement by overlaying cell margin outlines obtained at successive 2 min intervals (numbered 1-4) in time, t, in order to measure changes in normalised cell spread area $\rho = A(t)/A(t_0)$ and protrusion Δp and retraction Δr activity at the cell margin, used to derive an index for cell motility $\mu = (\Delta p + \Delta r)/A(t)$. The effect of (B) 300 pM human calcitonin (hCT) (C) 20 mM extracellular [Ca^{2+}] and (D) 250 nM amylin applied at $t = 0$, on motility and cell spread area. Note that calcitonin affects both but extracellular [Ca^{2+}] and amylin selectively influence the time course of one of the two parameters μ or ρ (reproduced by permission from [10]).

Figure 2. Basic organization of Epac isoforms.

In Epac1 the regulatory region contains membrane localizing dishevelled-Egl-10-Pleckstrin (DEP) and regulatory, cyclic mononucleotide-binding (CNB) domains. Epac2 further includes an additional CNB domain N-terminal to the DEP domain. The regulatory domains are joined via a Ras exchange motif (REM) and a ras association (RA) domain to the CdC25 homology domain (CDC25HD) responsible for GEF activity. The isoform related to Epac, Repac, only includes the catalytic region.

Figure 3. Rap1 activation by the Epac signalling system

Activation of Epac by the direct action of cAMP (A) enhancing the guanine nucleotide-exchange factor (GEF) function of Epac. (B) The increased exchange of GDP for GTP binding results in activation of the Ras-proximate-1 (Rap1).

Figure 4. Consequences of osteoclast integrin activation

Following (A) activation by Rap1, $\alpha_v\beta_3$ integrins (B) mediate osteoclast adhesion to the bone surface by interacting with extracellular bone matrix proteins containing RGD (Arg-Gly-Asp) sequences, including vitronectin, osteopontin and bone sialoprotein. Integrin-ligand binding induces receptor clustering causing formation of focal adhesion contacts, recruitment of cytoskeletal molecules with organisation of intracellular cytoskeletal complexes leading to changes in cell shape, spreading and motility. Integrins also mediate transmembrane transduction of signals in both inside-out and outside-in directions relating organization of the actin network and the composition of the focal adhesions, through the cytoskeletal protein talin which links integrins to the actin cytoskeleton. Finally, a wide range of phosphatidylinositol 3-kinase, c-Src, PYK2 and $p130^{cas}$ signaling pathways are $\alpha_v\beta_3$ integrin-dependent.

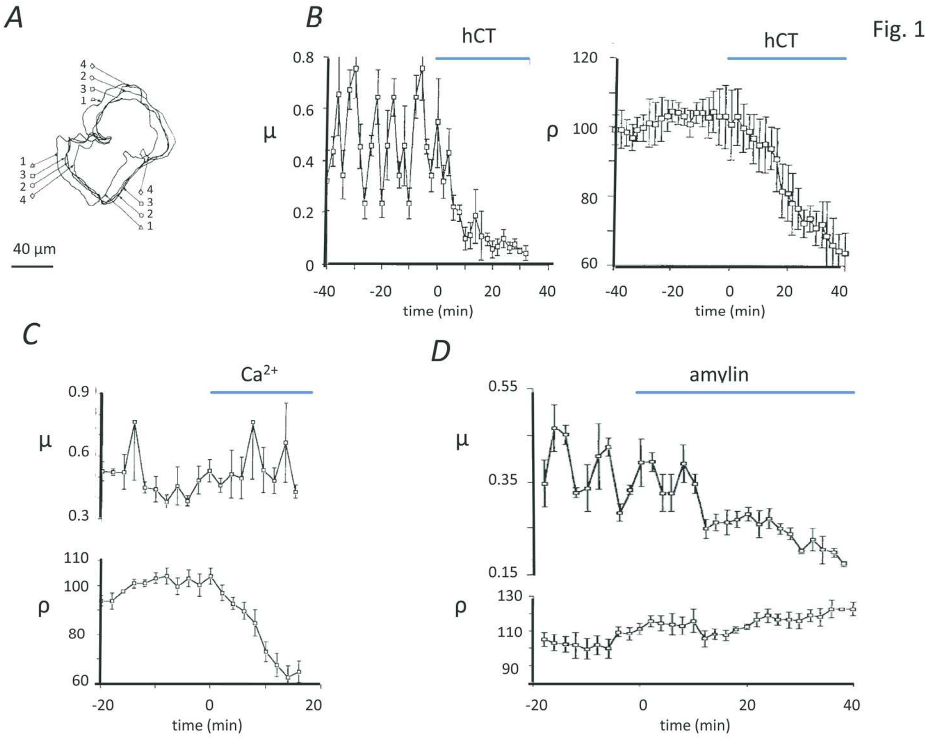

Figure 1

190x142mm (300 x 300 DPI)

Fig. 2

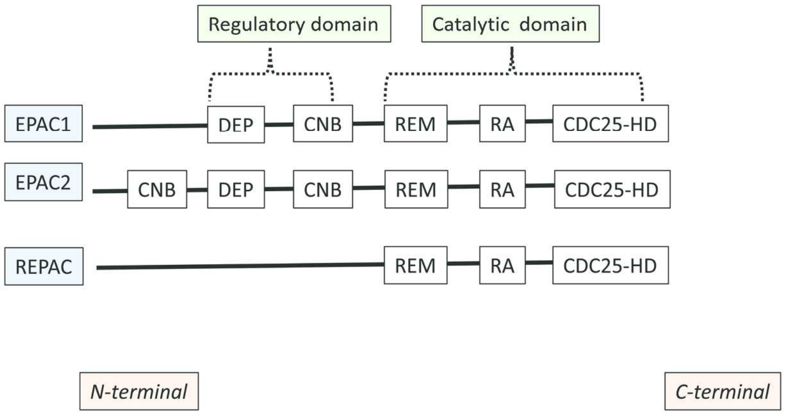

Figure 2

190x142mm (300 x 300 DPI)

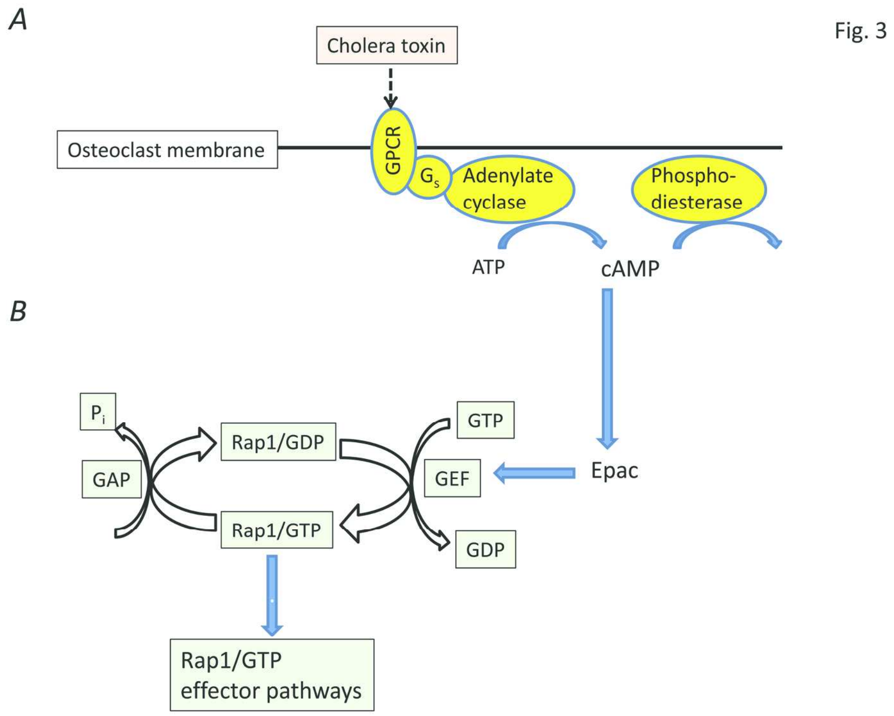

Figure 3

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Fig. 4

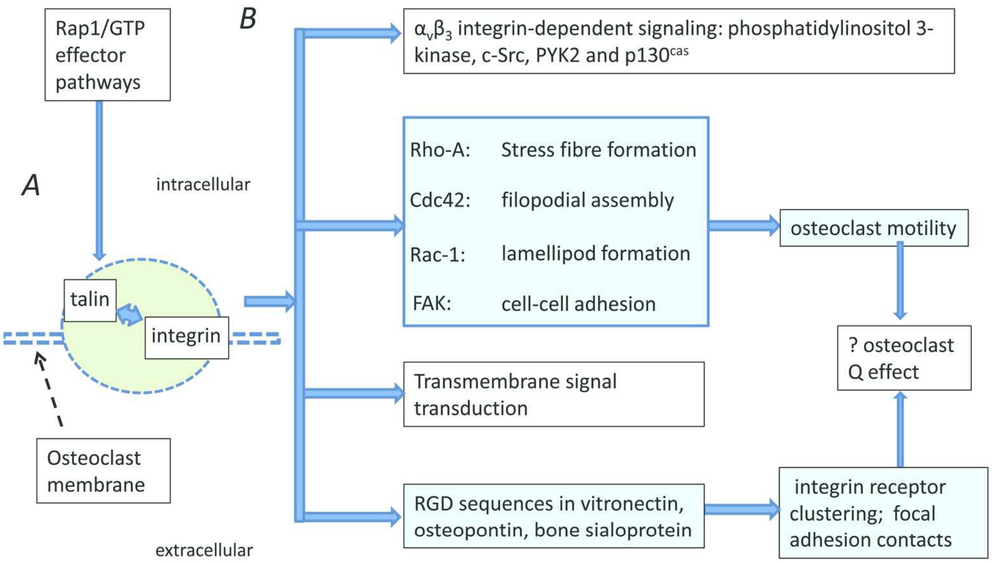

Figure 4

190x142mm (300 x 300 DPI)