





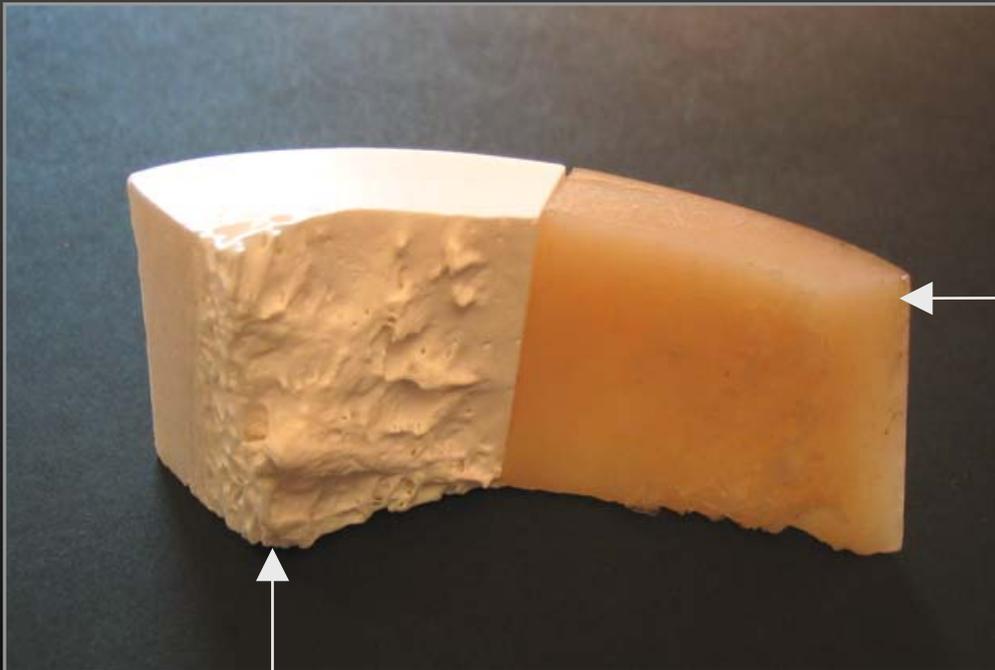
UCL

Calcium, phosphate & magnesium regulation

Tim Arnett

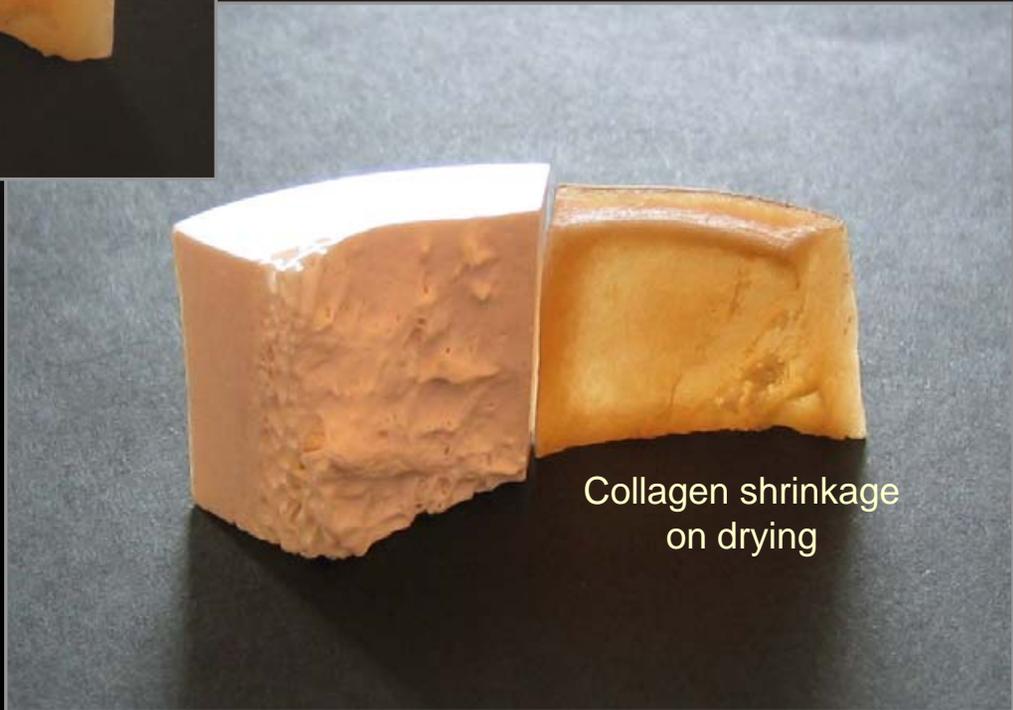
Department of Cell and Developmental Biology
University College London

Bone composition



Treated with hydrochloric acid
to dissolve mineral
leaves organic component
[mainly type 1 collagen] intact

Treated with bleach (hypochlorite) to
digest collagen
leaves mineral component
[hydroxyapatite – $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$]
intact



Collagen shrinkage
on drying

Ca²⁺ regulation

- Almost all of the Ca²⁺ in the body (>99%) exists as mineral deposits in the skeleton and teeth
- In man, plasma Ca²⁺ is tightly maintained at ~2.5mM
- Local concentrations of Ca²⁺ in extracellular fluid may vary more
- About 50% of plasma Ca²⁺ is ionised and diffusible

Why so precise ?

- Ca²⁺ is used as a vital second messenger within cells
- Ca²⁺ is necessary for normal blood coagulation, muscle contraction and nerve function; hypocalcaemia results in excitation of nerve and muscle cells leading to spasms, tetany and asphyxia
- Solubility of many Ca²⁺ salts (phosphate, carbonate, sulphate, oxalate) is low...
...increases in Ca²⁺ could lead to inappropriate precipitation (tissue mineralisation)

Major Ca²⁺ regulating hormones

- Parathyroid hormone (PTH)
- 1,25-dihydroxyvitamin D₃
- Calcitonin

Parathyroid hormone

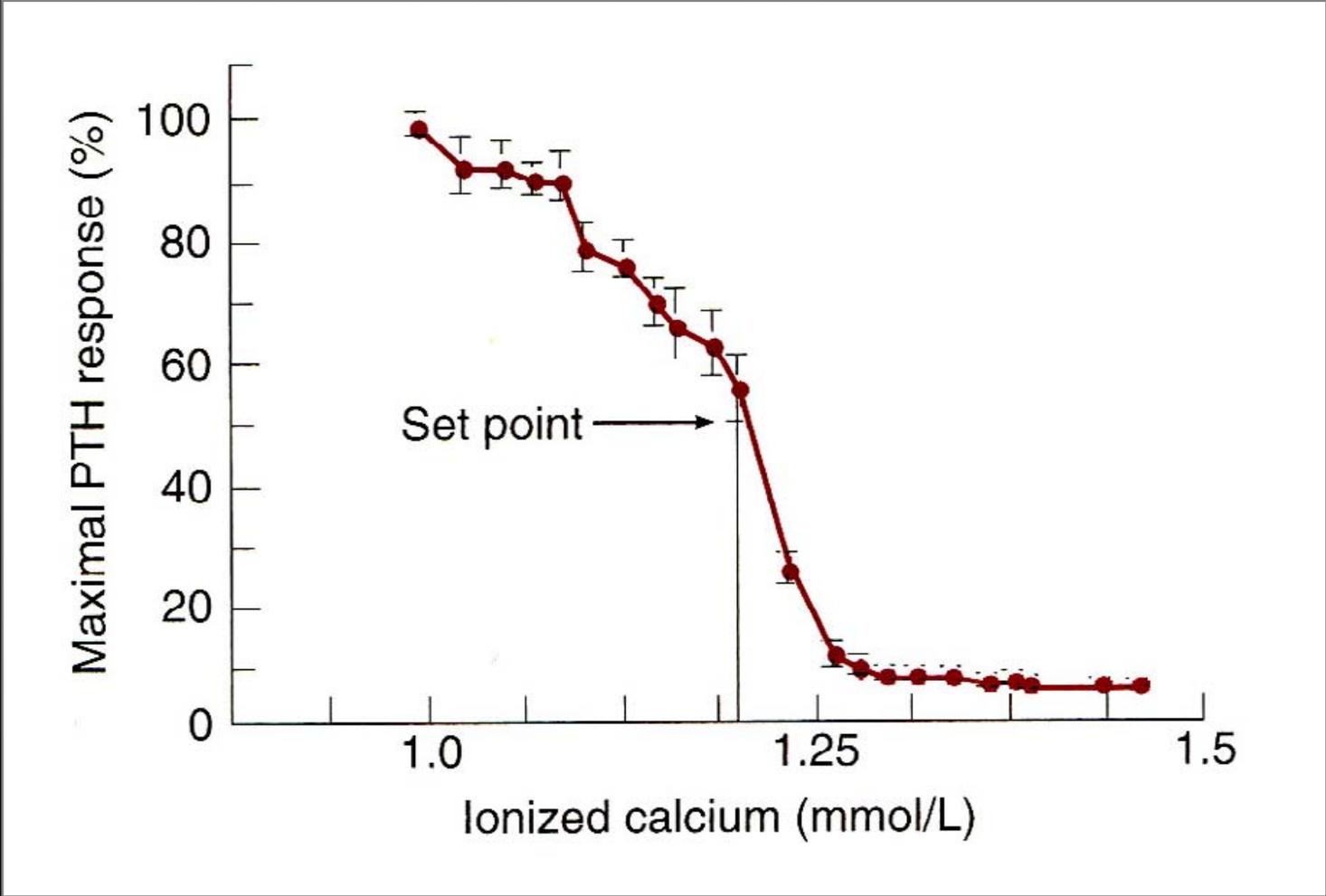
- Raises plasma Ca^{2+} - *key minute-to-minute regulator*
- 84 amino acid polypeptide secreted by chief cells of parathyroid glands (amphibia upwards) into bloodstream in response to small falls in local Ca^{2+} concentration
- Pulsatile secretion
- Normal plasma concentration $\cong 10\text{-}50$ pg/ml; plasma half life $\cong 10$ min
- (*synthetic 1-34 peptide has full biological activity*)
- Actions mediated through PTH / PTHrP receptor - coupled to adenylate cyclase through G_s & to phospholipase C via G_q signalling proteins

Parathyroid hormone

Increases plasma Ca^{2+} by:

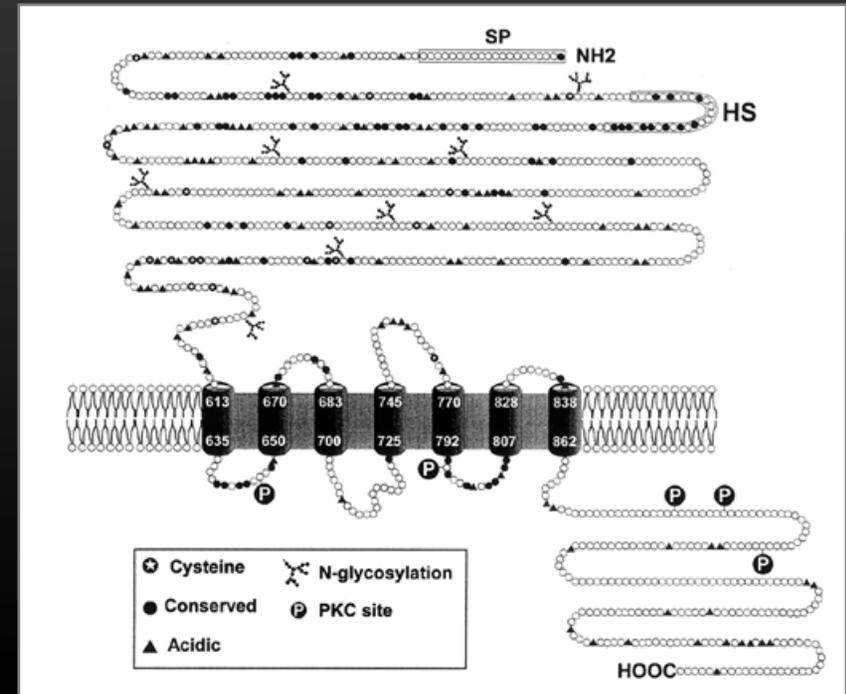
- \uparrow osteoclast formation (\uparrow RANKL) and resorptive activity
(direct & indirect actions)
- \uparrow Ca^{2+} reabsorption in distal renal tubules
- \uparrow $1,25(OH)_2D_3$ production by stimulating the activity of the critical 1α - hydroxylase enzyme in kidney
- *PTH is also anabolic for bone when administered intermittently...
... thought to involve suppression of sclerostin production by osteocytes
... not fully understood – PTH does not directly promote bone formation by cultured osteoblasts*

PTH production is very sensitive to plasma $[Ca^{2+}]$

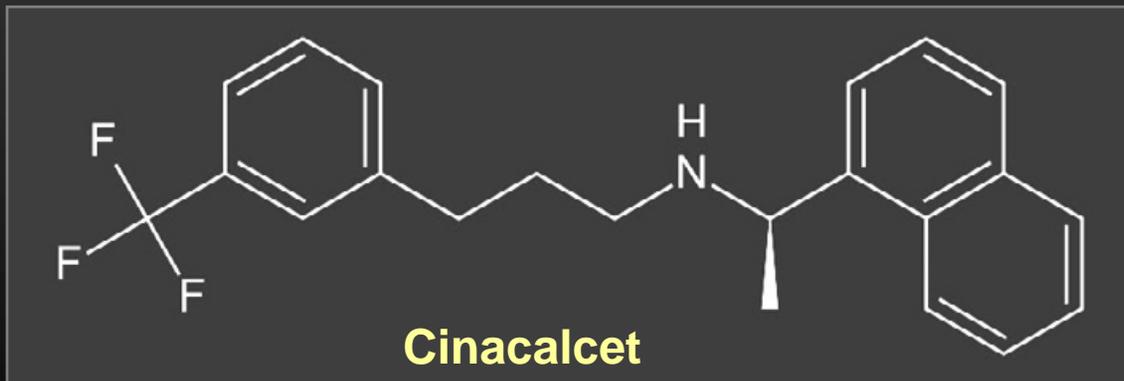


Ca²⁺ sensing receptor (CaSR)

- CaSR is a G-protein coupled receptor (GPCR)
- Molecular mechanism underlying Ca²⁺ sensing by parathyroid chief cells and renal tubules
- CaSR acts as the body's thermostat for Ca²⁺ or 'calciostat'
- Detects perturbations in the ionized Ca²⁺ of only a few percent, leading to alterations in parathyroid function & PTH secretion that are designed to normalise plasma Ca²⁺



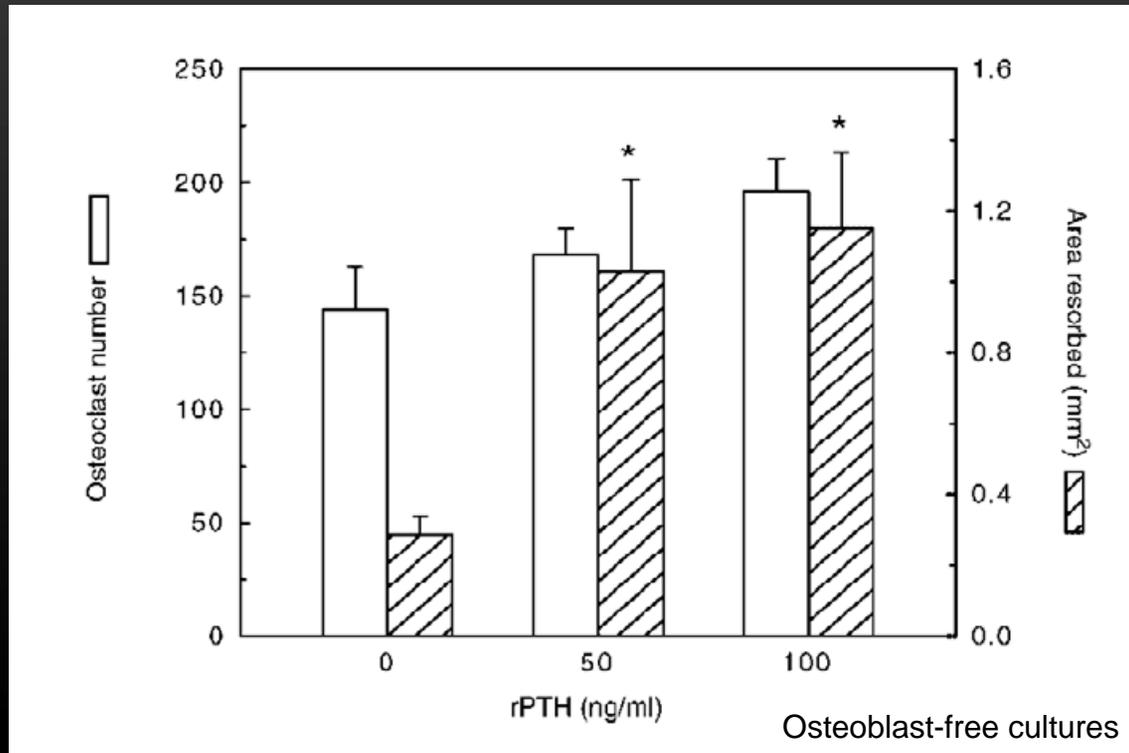
Calcimimetics



- Synthetic agonist which activates Ca^{2+} sensing receptor
- Treatment of hyperparathyroidism / hypercalcaemia

PTH directly stimulates resorptive function of human osteoclasts

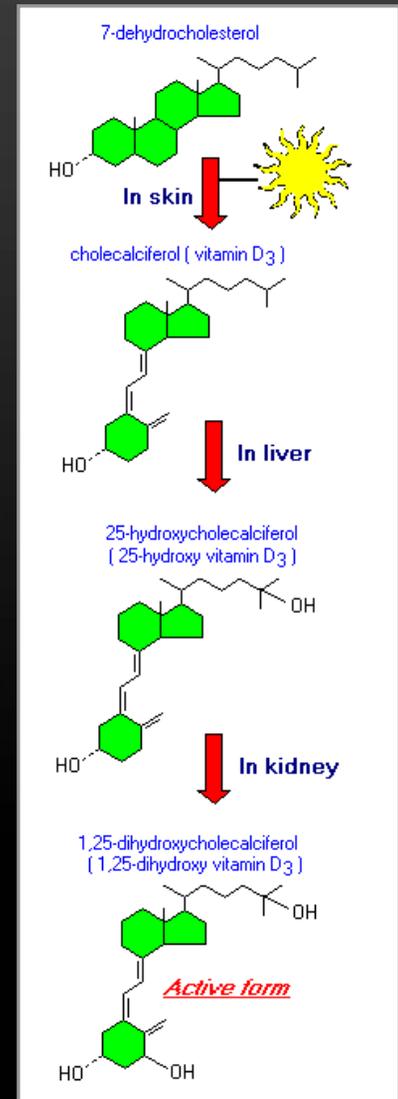
PTH1R highly expressed on mature osteoclasts



- Parathyroid hormone – related protein
- Action very similar to PTH at receptor level (high homology in 1-34 region)
- Paracrine agent expressed by many normal cell types (not controlled by Ca^{2+} & CaSR)
- Also expressed by tumour cells – contributes to hypercalcaemia of malignancy
- Important developmental actions: required for cartilage differentiation, tooth eruption, normal development of mammary glands... etc

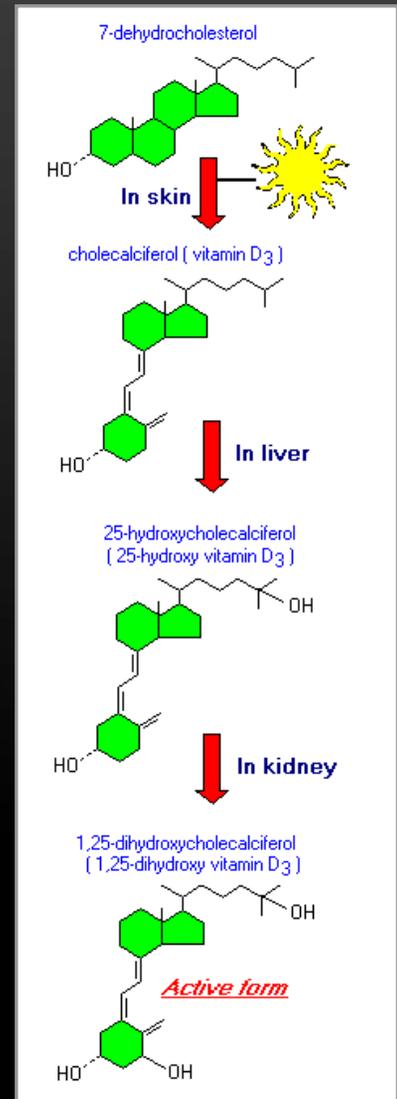
Vitamin D

- Vitamin D (cholecalciferol), a ‘seco-steroid’ has no biological activity
- 25-hydroxy vitamin D is main circulating metabolite (nanomolar range) - very low biological activity
- 1,25-dihydroxy vitamin D is active metabolite – circulating concentration $\approx 10\text{-}50\text{ pM}$
- 1,25-dihydroxy vitamin D is classed as a steroid hormone – acts via nuclear receptor (VDR)
- Vitamin D metabolites are fat soluble – slower action than peptide hormones – not involved in minute-to-minute regulation of plasma Ca^{2+}



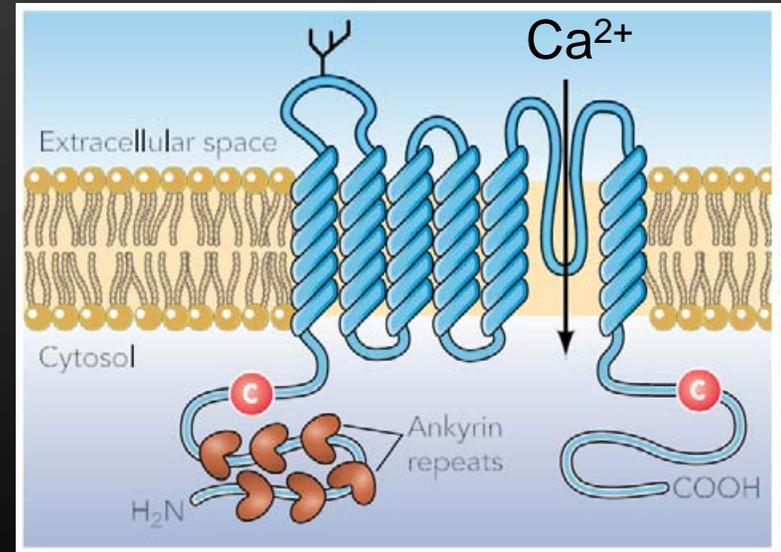
1,25(OH)₂D - actions

- ↑ Gut Ca²⁺ uptake
- ↑ Plasma Ca²⁺
- ↑ OC recruitment, activity (↑ RANKL)
- ↓ OB proliferation; ↑ OB (and skin cell) differentiation
- Required for normal matrix mineralisation. Acts mainly via promoting Ca²⁺ uptake - and thus ensuring adequate local Ca²⁺ supply; not much evidence for a direct effect on mineral deposition by normal osteoblasts
- deficiency → osteomalacia, rickets...



Ca²⁺ uptake / transport

- Ca²⁺ uptake from the intestine now thought to occur primarily via the epithelial **TRPV6** Ca²⁺ transport channel (which is strongly upregulated by 1,25(OH)₂D)
- Ca²⁺ reabsorption in the kidney occurs primarily via the epithelial **TRPV5** Ca²⁺ transport channel (upregulated by 1,25(OH)₂D and PTH)
- TRPV5 also expressed in osteoclast ruffled border



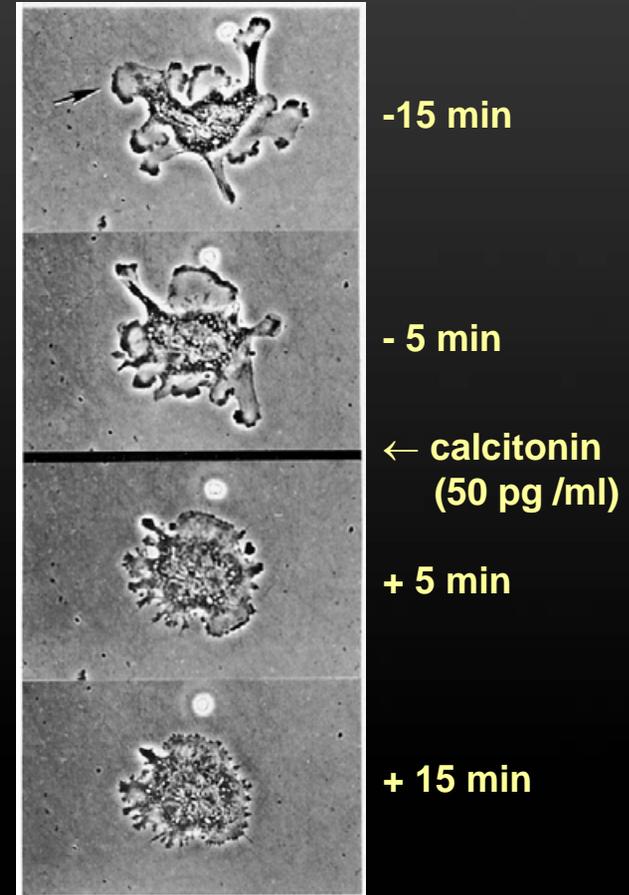
TRPV5/6 calciotropic channel

(ref: Hoenderop & Bindels (2008) Physiology 23: 32-40)

- 1,25(OH)₂D also increases expression of intracellular Ca²⁺ binding proteins, esp. calbindin D 9K (*old mechanism!*)

Calcitonin

- 32 amino acid peptide hormone secreted by parafollicular 'C' cells in thyroid / ultimobranchial gland (elasmobranchs upwards)
- ↓ plasma Ca^{2+} in young / hypercalcaemic animals
- ↓↓ osteoclast resorptive function & recruitment
- Inhibits Ca^{2+} and PO_4^{3-} reabsorption by the kidney tubules
- 'emergency' hormone; not much effect in normal adults



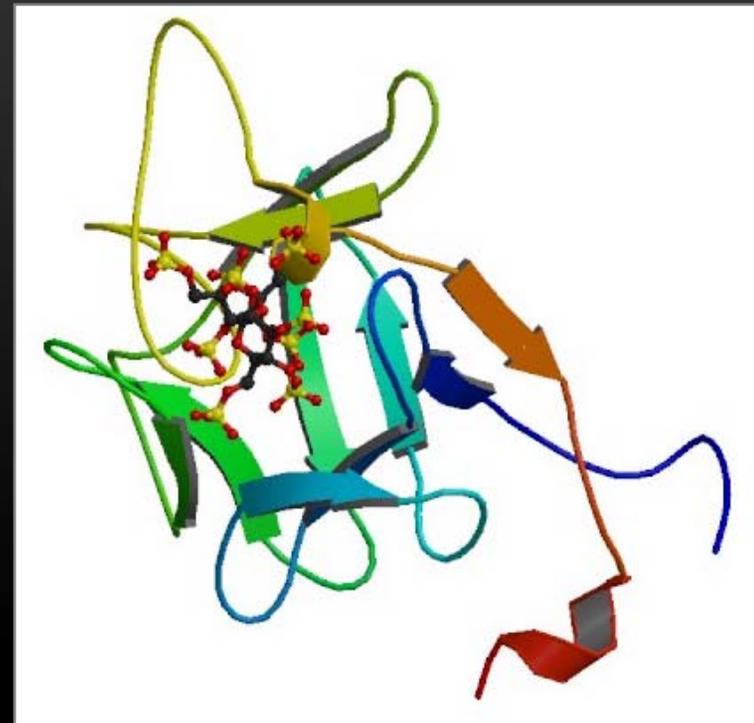
Time-lapse sequence showing rapid inhibition of rat osteoclast motility in response to salmon calcitonin (Arnett & Dempster, *Endocrinology*, 1987)

PO₄³⁻ regulation

- Plasma PO₄³⁻ is less tightly regulated than Ca²⁺ - normal range is ~0.8-1.5mM in adult humans
- **PTH** decreases PO₄³⁻ reabsorption from proximal tubules of kidney – but this is compensated by enhanced 1,25-(OH)₂ vitamin D-mediated PO₄³⁻ uptake from intestine
- **FGF23** is the key circulating PO₄³⁻-lowering hormone. Reduces PO₄³⁻ reabsorption in proximal renal tubules and also decreases PO₄³⁻ absorption from the intestine
- Calcitonin inhibits PO₄³⁻ reabsorption in renal tubules
- PO₄³⁻ stimulates PTH secretion from the parathyroids... mechanism not understood
- Is there a PO₄³⁻ receptor (analagous to Ca²⁺-sensing receptor) ?
... no real evidence at present

FGF-23

- Phosphaturic hormone; 251 amino acid protein, produced by bone (osteocytes)
- Identified in 2000 via gain-of function mutations associated with autosomal dominant hypophosphatemic rickets
- Prior to discovery, it was hypothesised that a protein existed which performed the function of FGF23. This protein was known as phosphatonin
- Acts through several FGF receptor subtypes in concert with **Klotho**, a co-receptor for FGF23,



- Klotho identified in 1997: affected / deficient mice show premature ageing and altered mineral homeostasis resulting in osteopenia
- Klotho is a type I transmembrane protein with β -glucuronidase activity in extracellular domain; expressed in kidney and parathyroids, co-localises with TRPV5
- Klotho is required for normal PO_4^{3-} elimination
- Lowering blood phosphate levels by restricting dietary PO_4^{3-} intake or by blocking vitamin D function in Klotho / FGF23-deficient mice rescues not only hyperphosphatemia but also many ageing-like phenotypes

ie, PO_4^{3-} retention is toxic – and responsible for accelerated ageing...

Review: Kuro-O M 2008 Trends Endocrinol Metab 19: 239-245

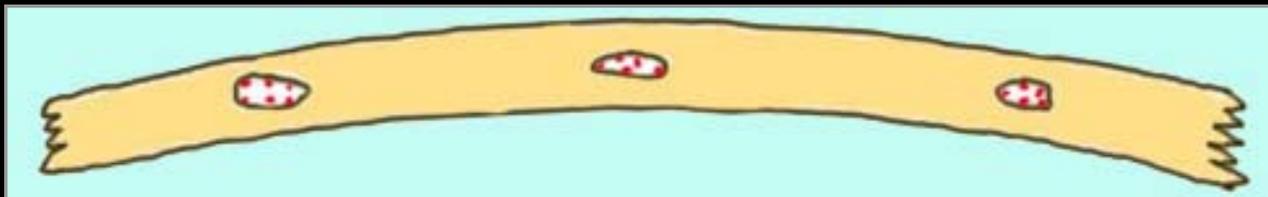
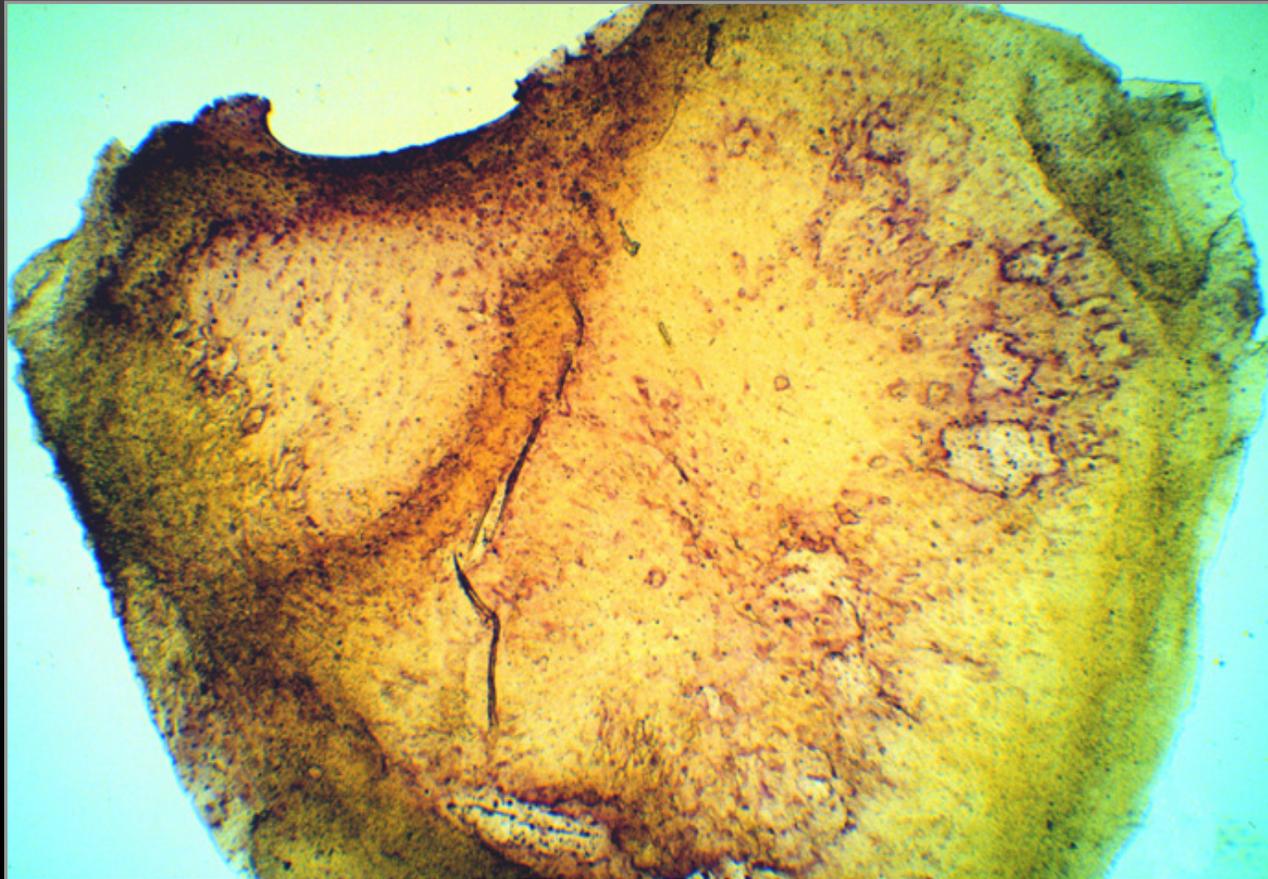
Regulation of bone cell function by Ca^{2+} & PO_4^{3-}

- Ca^{2+} has a minor inhibitory action on osteoclasts at concentrations >10 mM (ie, 4 x higher than physiological)
- PO_4^{3-} exerts a strong, reversible inhibitory action on osteoclast function at concentrations >2 mM (ie, close to physiological) (*Yates et al, JBMR 1991*)
- Ca^{2+} & PO_4^{3-} both promote mineral deposition by differentiated osteoblasts

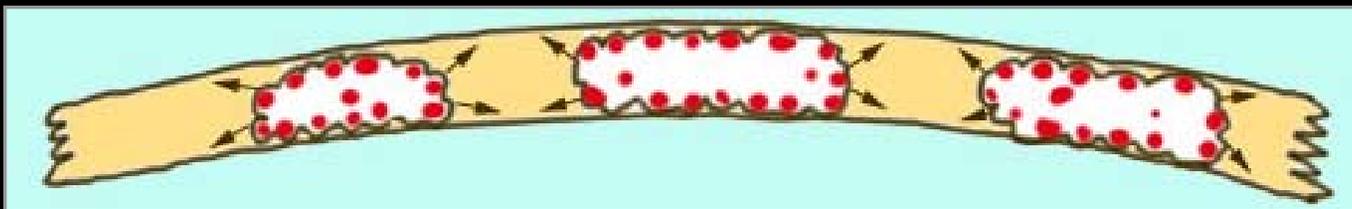
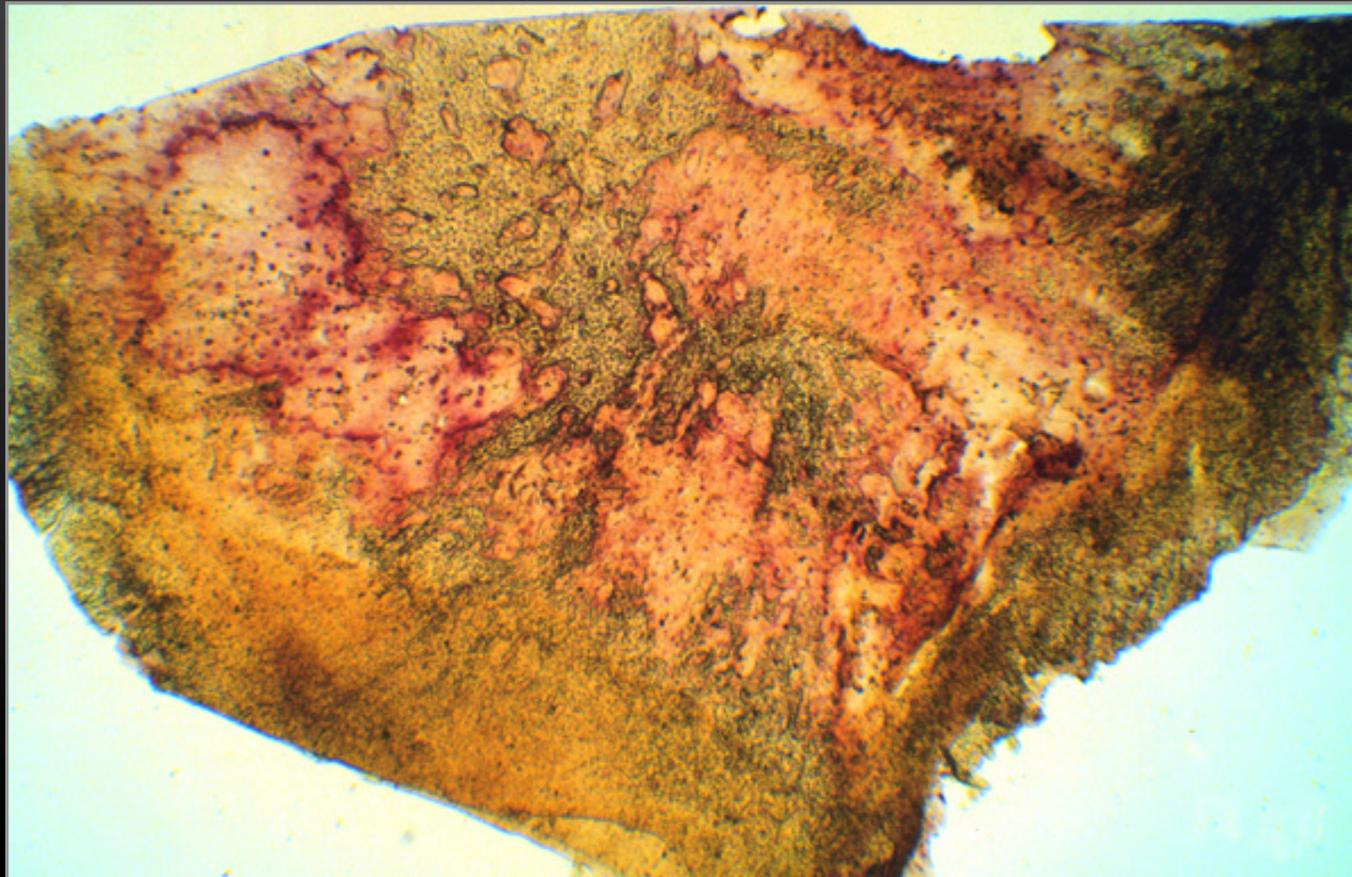
Mineralisation

- Ca^{2+} and PO_4^{3-} regulation must be understood in relation to mineralisation
- Total body Ca content in adults is about 1000 g, of which 99% exists as hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] in the mineral phase of bone
- Mineralisation is a physicochemical process – but ‘managed’ by osteoblasts and osteocytes
- Release of mineral from bone largely under cellular control (osteoclasts)...

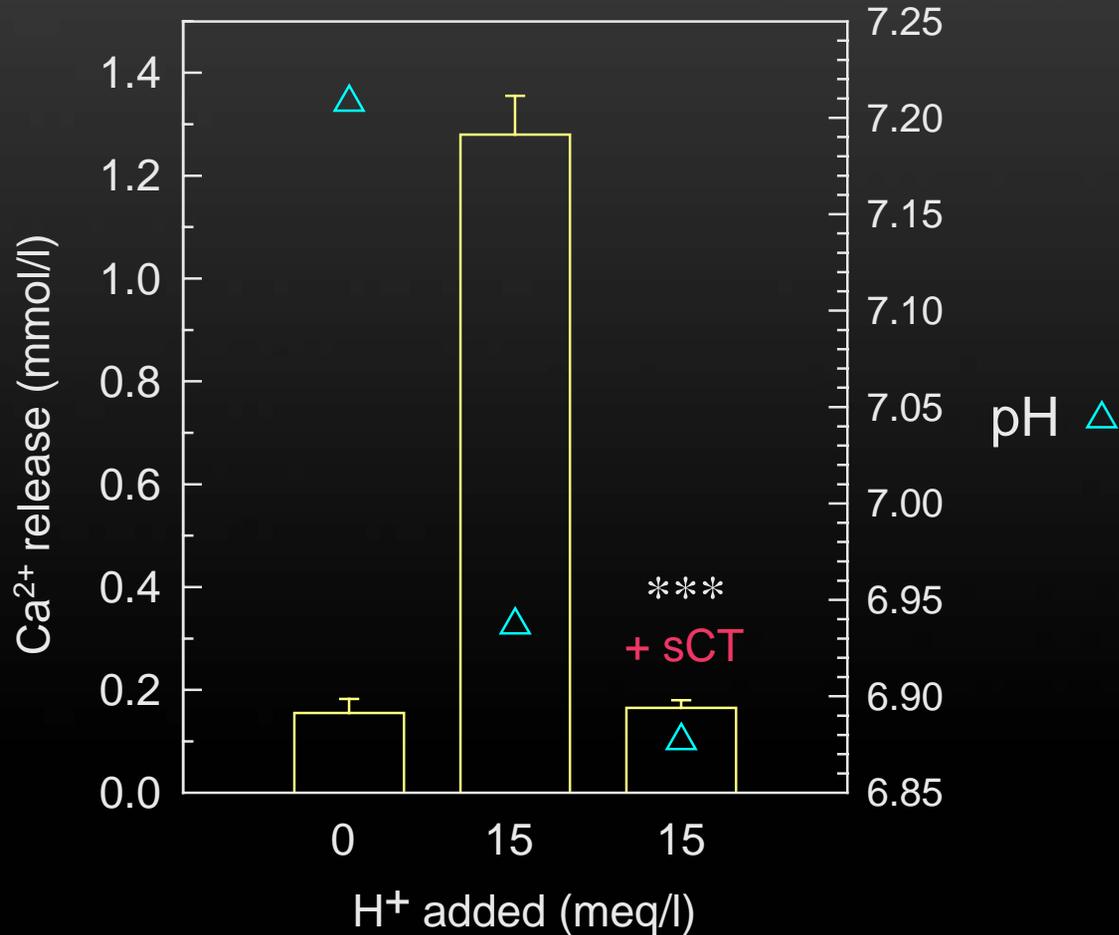
TRAP stained calvarial bone 3 day culture - *control*



Resorption of calvarial bone stimulated by acidosis (pH 7.01)



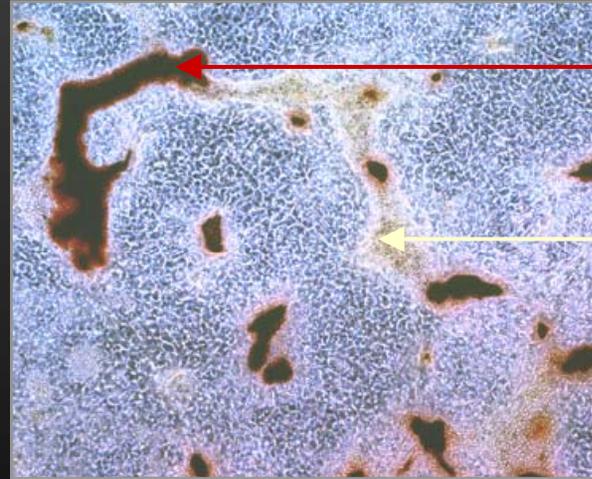
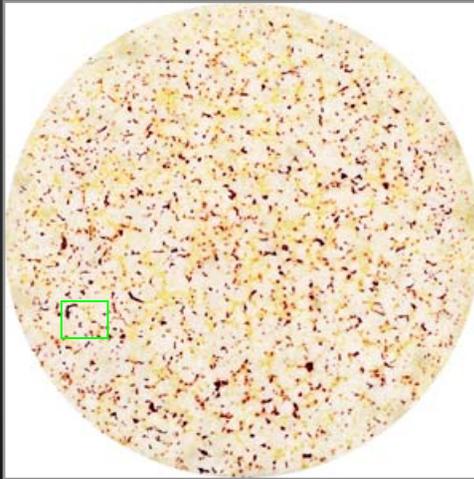
Ca²⁺ release stimulated by acidosis is blocked by salmon calcitonin



- Major function of $1,25(\text{OH})_2\text{D}$ and PTH for the bone mineralisation process is to maintain $[\text{Ca}^{2+}] \times [\text{PO}_4^{3-}]$ solubility product in circulation / ECF in a supersaturated state (<4.5), resulting in passive mineralisation of the collagen matrix (osteoid) laid down by osteoblasts
- *So why don't all tissues mineralise ?*
- 'Vascular calcification - a passive process in need of **inhibitors**' (Schinke & Karsenty, 2000)
- **Matrix Gla protein**: MGP $^{-/-}$ mice exhibit lethal arterial calcification and inappropriate calcification of cartilage (in mice); MGP expressed by vascular smooth muscle cells but not bone cells
- **ASARM** peptides (Clemens Löwik)
- Pyrophosphate
- ATP & other nucleotide triphosphates...

ATP and UTP selectively inhibit bone mineralisation

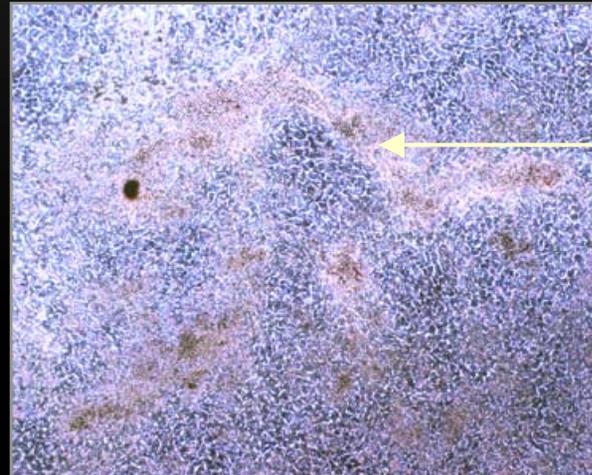
control



mineral stained with Alizarin red

un-mineralised collagenous matrix

10 μ M ATP / UTP

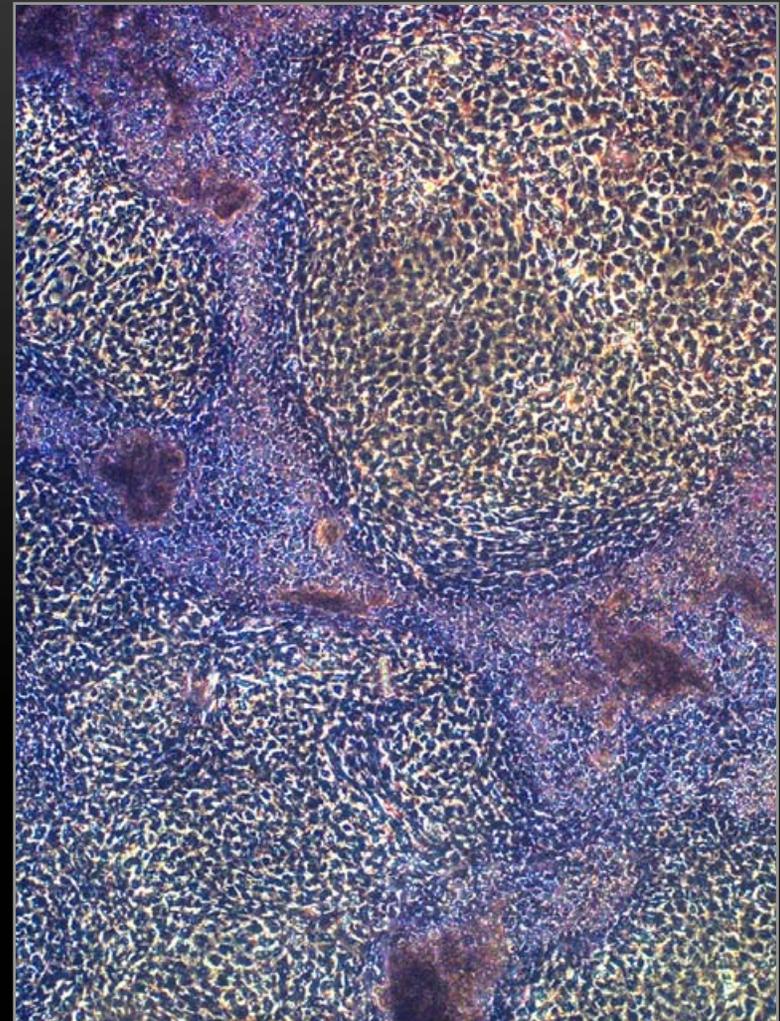
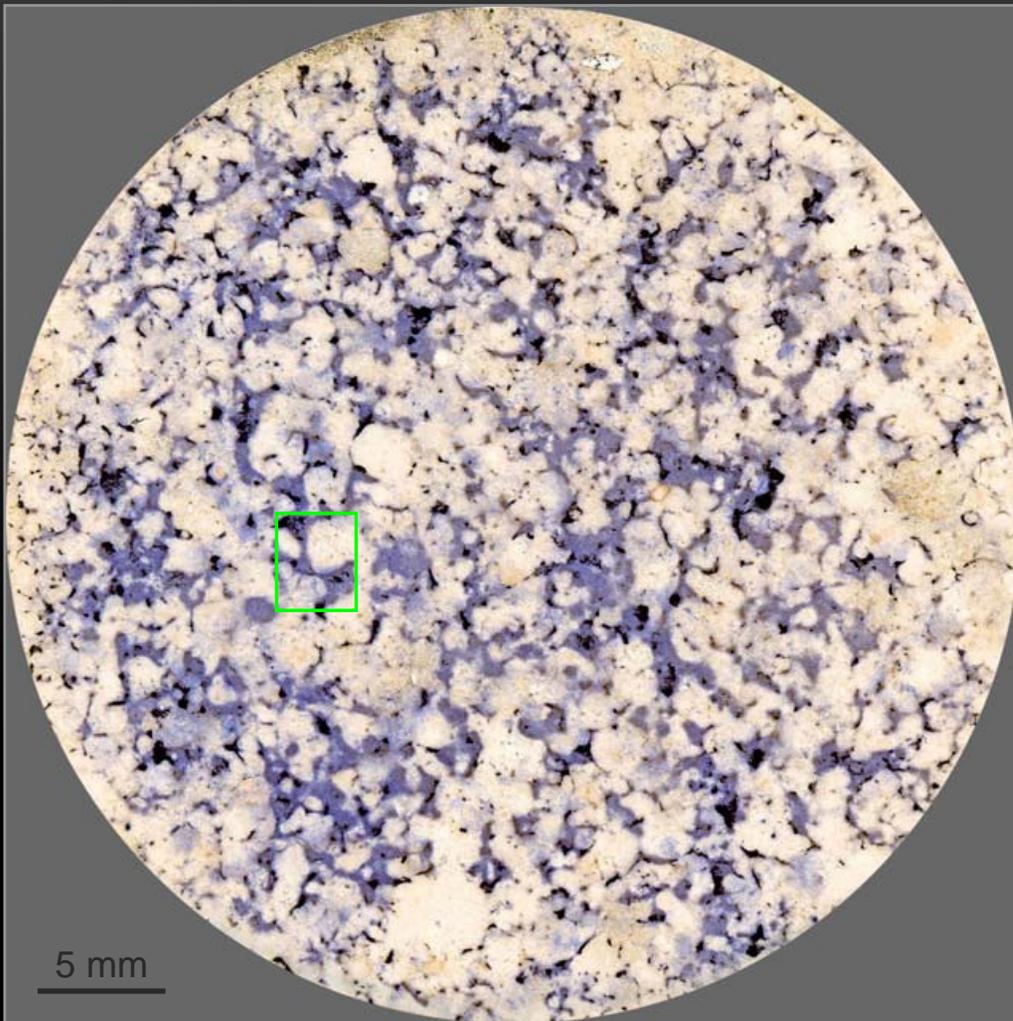


un-mineralised collagenous matrix only

Organic matrix deposition unaffected

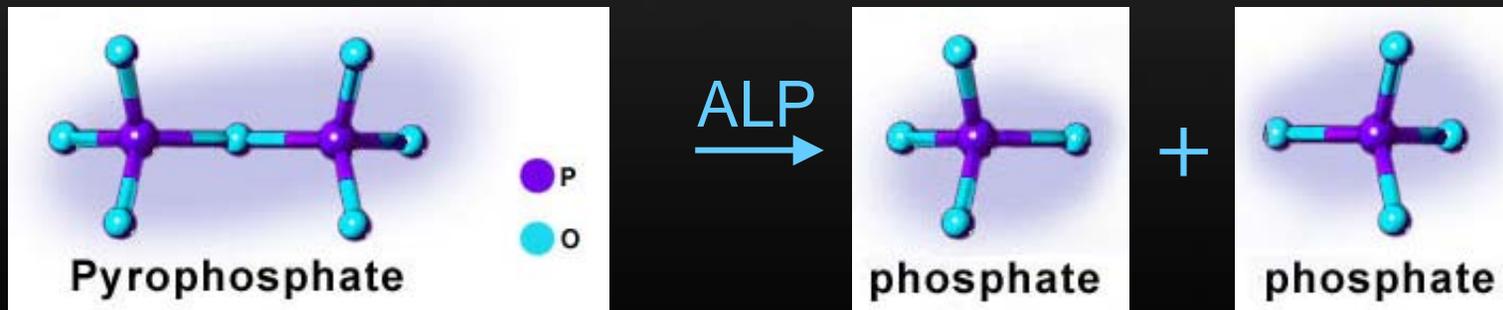
Alkaline phosphatase

Differentiated osteoblasts express high levels of alkaline phosphatase (ALP)
(which is selectively inhibited by ATP / UTP... evidence for P2Y₂ receptor involvement)



Pyrophosphate and alkaline phosphatase

Major function of ALP in bone is to hydrolyse pyrophosphate (PPi), a key inhibitor of mineralisation



→ enables mineralisation

Pyrophosphate: a potent inhibitor of mineralisation



- Inorganic pyrophosphate (PPi) in the low micromolar range inhibits mineralisation (Fleisch 1962 & 1966)

no. 5844 September 1, 1962 NATURE 911

feeding palm, the magnitude of the potential change thereby produced in *I* and *E* being 60 per cent of that in *G* and *J*. By applying the test pairs at various places during the night or estimated after hyperpolarization, it was shown that the depression of the low hyperpolarization or depolarization approximately paralleled the amplitude of the after-hyperpolarization.

The prolonged after-hyperpolarizations following impulses in mammalian C fibres and frog myelinated nerves show also a temporal summation. The ionic process of these after-hyperpolarizations has been related to the active transport of sodium and potassium ions that would hyperpolarize the membrane by depletion of the extracellular potassium¹⁴ or by driving an electrostatic sodium current¹⁵. In this respect, the after-hyperpolarization in motoneurons, which is generated by the increased potassium conductance, appears to be a unique phenomenon. The special significance of the motoneuron after-hyperpolarization has already been discussed^{16,17}.

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Mechanism of Calcification: Inhibitory Role of Pyrophosphate

The mechanism of calcification has been considerably clarified recently¹. Collagen fibrils were shown to possess in situ the property of nucleating hydroxyapatite precipitation and of triggering mineralization^{2,3}. But why does only part of the collagen present in the organism calcify? Recently, we suggested that the activating sites of collagen and crystal growth could be blocked in the organism by a plasma inhibitor⁴. Indeed we showed plasma to contain one or several substances highly inhibitory to apatite precipitation. The technique used was to determine *in vitro*, at physiological conditions, the minimum ion product (MIP) necessary for hydroxyapatite precipitation. After addition of plasma, the rise of this minimum product revealed the presence of inhibitor. As the inhibitor was shown to be destroyed by alkaline phosphatase, and the pyrophosphates to have a strong inhibitory activity at concentrations as low as 10⁻⁶ M, we suggested that pyrophosphate to be a pyrophosphate. However, to our knowledge, no such compound has so far been demonstrated in plasma of any mammal.

As the concentration of plasma of this hypothetical pyrophosphate was found to be very low (10⁻⁶ M), we first searched for it in urine, where it was implied to exist, according to our test, in a much higher concentration. After isolation, its nature was determined as inorganic pyrophosphate^{5,6}. The mean daily excretion of pyrophosphate in healthy male was

found to be 2-10 mg expressed as phosphorus (x = 2.920), in adult young women it is below 1 mg, but it increases with age. By its highly inhibitory action, the urine pyrophosphate accounts for the supermineralization of urine in calcium and phosphorus. Our finding that its mean daily excretion falls to 1.06 mg (x = 0.17) in uraemic men, suggests that its action is present both *in vivo* and *in vitro*.

The presence of pyrophosphate in urine suggests that the plasma inhibitor is of a similar composition. Indeed, in experiments recently completed, we could isolate from plasma a phosphorus compound highly inhibitory of hydroxyapatite precipitation which, after purification, migrates on paper chromatography in the same way as inorganic pyrophosphate, with three different solvents (Solvent I: isopropanol, 70; water, 30; 20 per cent ammonium hydroxide, 10; trichloroacetic acid, 4 g; Solvent II: methanol, 70; 2 N ammonium hydroxide, 30; Solvent III: n-propanol, 30; ethanol, 30; water, 30; 25 per cent ammonium hydroxide, 1). Its concentration, although very low (10⁻⁶ M), easily inhibits apatite precipitation. The action at this level cannot be explained by a lower ionic calcium concentration due to the formation of a complex, but it is probably a 'poisoning' of crystal growth.

The following mechanisms of calcification in therefore suggested: Hydroxyapatite precipitation can occur at a physiological concentration of calcium and phosphorus, due to the nucleating function of collagen. This property, together with crystal growth, is inhibited by plasma pyrophosphate, which prevents the collagen that is not to be mineralized. For collagen to calcify, pyrophosphate must be destroyed *in vivo* by the enzyme pyrophosphatase, which was shown to be present in mineralizing tissue^{7,8}.

We thank Prof. H. Flämig, Interceptor, World Health Organization, for translating this manuscript. This work was supported by the grant A-4307 of the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

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EFFECT OF PYROPHOSPHATE ON HYDROXYAPATITE AND ITS IMPLICATIONS IN CALCIUM HOMEOSTASIS

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INVESTIGATIONS OF THE physical and chemical properties of calcium phosphate crystals *in vitro* and of factors modifying these properties have contributed much to our understanding of calcium homeostasis *in vivo*. Such work has not, however, enabled a precise description of the relationship between the calcium ions of blood and those of bone. It is uncertain how whole living bone maintains higher concentrations of calcium and phosphate in solution than does extracted bone mineral.

We have been particularly interested in the possible role of various inhibitors of crystallization in the main-

the crystals converted only 0.43 per cent of their phosphorus into pyrophosphate. The length of the crystals as seen under the electron microscope was about 1000 Å. This indicated that the crystals were predominantly a non-calcium deficient hydroxyapatite and that they did not contain significant amounts of octocalcium phosphate or tri-calcium phosphate hydrate.

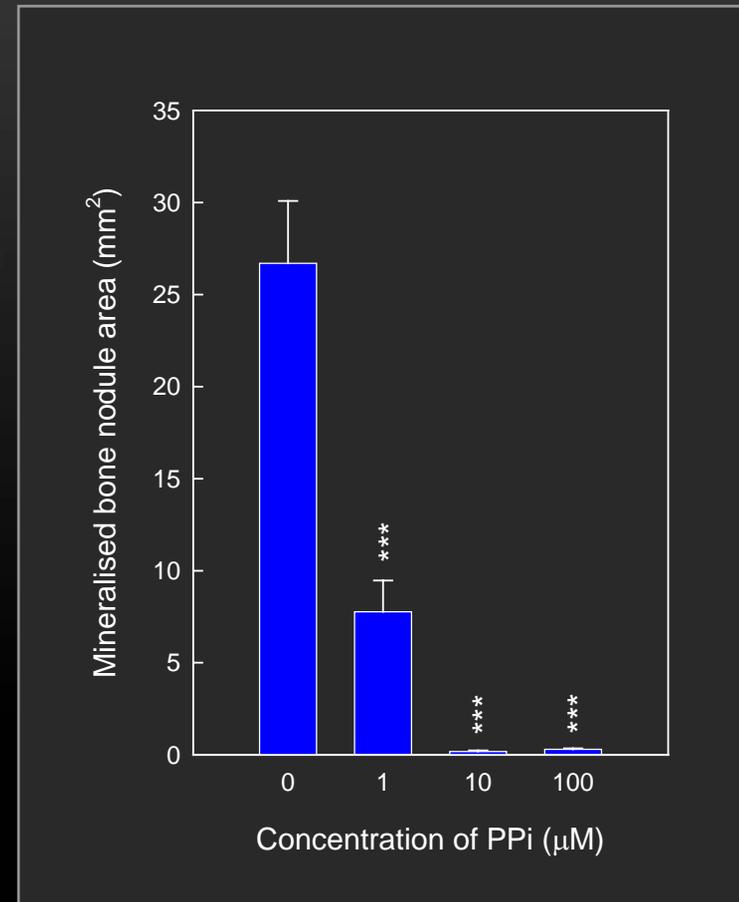
To investigate binding properties, hydroxyapatite crystals were equilibrated for 24 h at 4°C in a 0.155 molar potassium chloride solution, buffered at pH 7.4 with 0.01 molar barbital. Inorganic ³²P-labelled pyrophosphate was

Pyrophosphate: a potent inhibitor of mineralisation

- PPI is generated from many phosphate-containing molecules, including ATP
- Members of the ecto-phosphodiesterase / pyrophosphatase (ENPP) family hydrolyse:

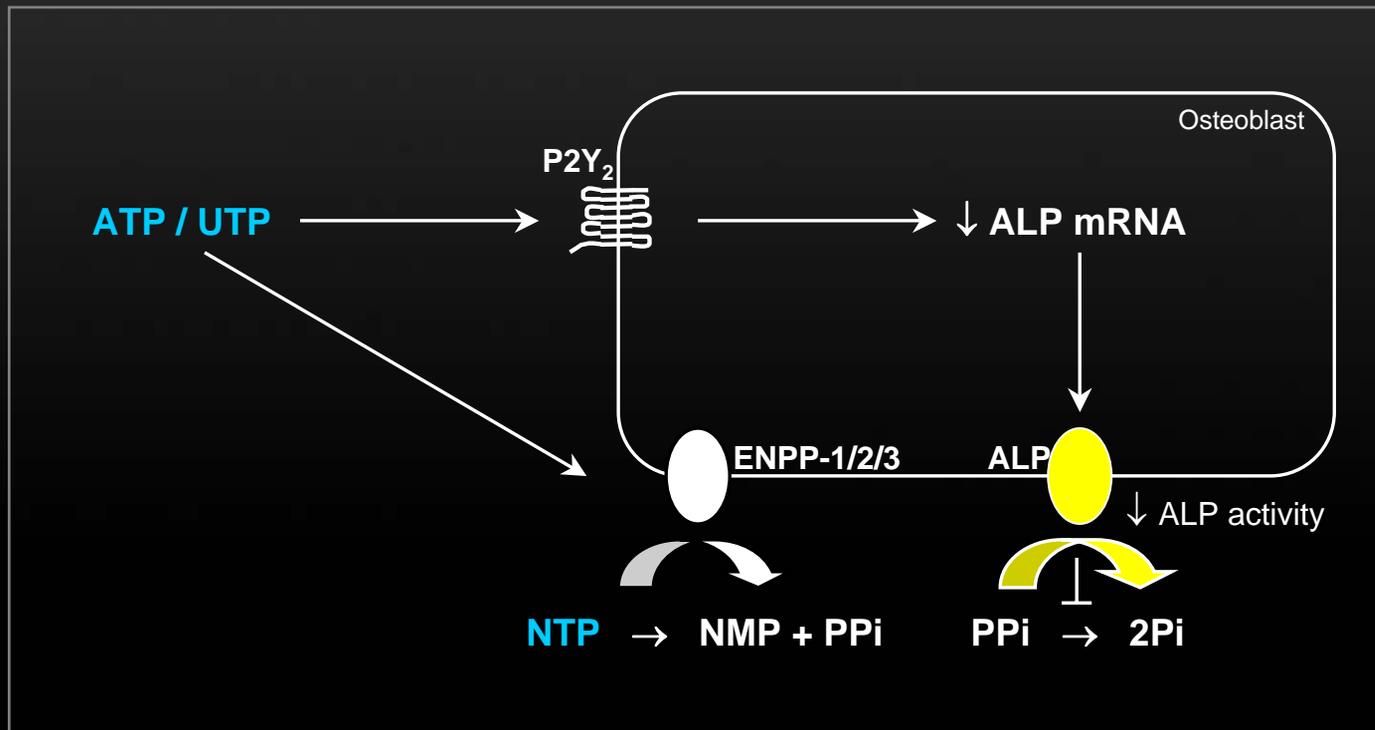


Could PPI generated by hydrolysis of ATP & UTP contribute to inhibition of bone mineralisation ?



Dual inhibitory action of ATP / UTP on bone mineralisation

1. Via P2Y₂ receptor
2. Via PPI generated by ENPPs

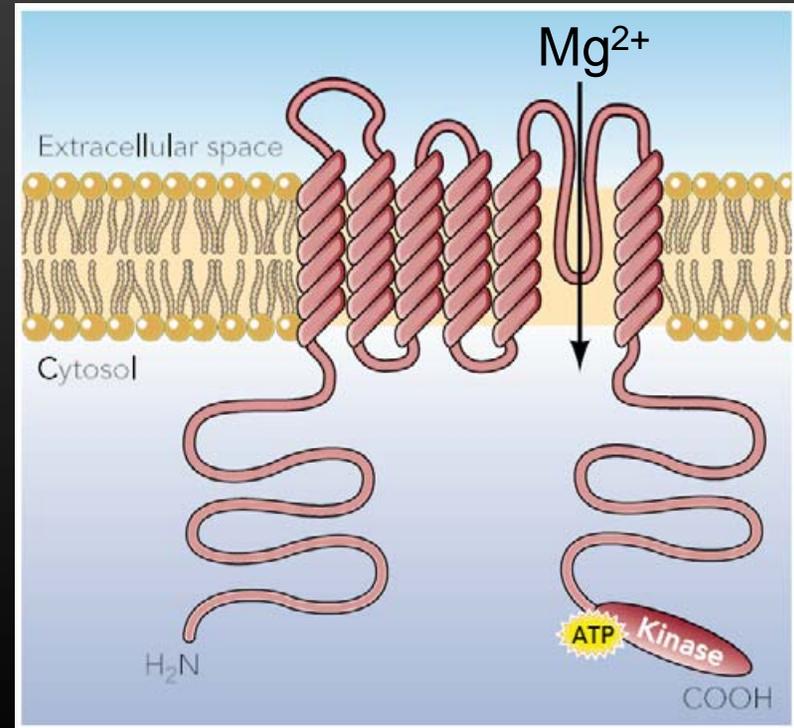


Mg²⁺ homeostasis

- Total adult body content of Mg²⁺ is ~25g
- Mg²⁺ salts have similar solubility profiles to those of Ca²⁺
- ~66% of Mg²⁺ located in the skeleton as mineral deposits (phosphate, carbonate)
- ~33% of Mg²⁺ in body is intracellular....
Mg²⁺ required for ≥300 biochemical reactions in the body
- ≤1% of Mg²⁺ in the body exists in soluble form in the extracellular fluid
- Plasma concentration of magnesium (Mg²⁺) also closely regulated (to about 0.85 mM); mechanisms involved still not well understood...

Mg²⁺ homeostasis

- Ca²⁺ -sensing receptor (in parathyroids) also senses Mg²⁺
- Mg²⁺ involved in regulation of cardiovascular function; deficiency can cause cardiac arrhythmia and impaired control of blood pressure (and also impaired PTH secretion)
- **TRPM6** channels (expressed in renal tubules) thought to be important for transport of Mg²⁺)
- The membrane protein GPCR6A is activated by Mg²⁺ as well as other divalent cations (eg, Ca²⁺, Sr²⁺)
- Mg²⁺ uptake from intestine not regulated by 1,25(OH)₂D



TRPM6 magnesiotropic channel

(see: Hoenderop & Bindels, Physiology 23: 32-40; 2008)

- ASBMR Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 6th edition (2006) – *downloadable from ASBMR website*
 - Ch 1-7: Morphogenesis, structure & cell biology of bone
 - Ch 8-12: Skeletal physiology
 - Ch 13-18: [Mineral homeostasis](#)
 - Ch 19-25: [Clinical evaluation of bone & mineral disorders](#)
 - Ch 26-41: [Disorders of serum minerals](#)
 - Ch 42-58: Osteoporosis
 - Ch 59-66: Metabolic bone diseases
 - Ch 67-70: Cancer & bone
 - Ch 71-74: Genetic & developmental disorders
 - Ch 75-76: Acquired disorders
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 - Ch 83-87: Dental biology
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