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A REAPPRAISAL OF THE EFFECT OF EXTRACELLULAR CALCIUM ON OSTEOCLASTIC BONE RESORPTION

Tony J. Hall[1]

Ciba-Geigy Ltd., Research Department, Pharmaceuticals Division,
4002-Basel, Switzerland

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During bone resorption osteoclasts are exposed to high levels of extracellular calcium solubilized from bone mineral and it has been suggested that this may act as a physiological negative feedback to control the resorptive process. We have confirmed that calcium (1.5 - 20 mM) dose-dependently inhibits osteoclastic bone resorption when added at the start (t = 0 hr) of the 24 hr bone slice assay. When 20 mM calcium was added at t = 0, 1, 3 or 6 hr after osteoclast attachment to bone slices, resorption was inhibited by 100%, 100%, -10% and 6% respectively. In contrast, human calcitonin (1 ng/ml) inhibited bone resorption by 100%, 100%, 91% and 52% when added at t = 0, 1, 3 and 6 hr respectively. Osteoclasts were not seen on bone slices after 24 hr incubation when 20 mM calcium was added at t = 0 or 1 hr, but when calcium was added at t = 3 or 6 hr osteoclast numbers were similar to controls, indicating that 20 mM calcium is not toxic to osteoclasts. Human calcitonin did not significantly affect osteoclast numbers regardless of time of addition to the bone slice assay. The absence of osteoclasts on bone slices exposed to 20 mM calcium at early time points indicates that high levels of extracellular calcium prevent osteoclast adhesion to bone slices, and later addition of high Ca_e to the assay does not inhibit ongoing osteoclastic bone resorption.

Although the molecular processes involved in osteoclastic bone resorption have been extensively studied in recent years [1,2], what regulates the resorptive process is not clear. Calcitonin is well established as a circulating peptide inhibitor of osteoclastic bone resorption that is released from C-cells scattered in the thyroid gland in response to elevated serum calcium levels [3]. Osteoclasts express high numbers of calcitonin receptors [4] and calcitonin induces rapid cytoquiescence and inhibition of bone resorption by increasing intracellular levels of cyclic AMP and calcium in osteoclasts [4-6]. During bone resorption high levels of calcium (17 - 40

[1]Address all correspondence to: Dr. T.J. Hall, Ciba-Geigy Ltd., Pharmaceuticals Division, R-1056.P.05, CH-4002 Basel, Switzerland.

Abbreviations: Ca_e/Ca_i, extracellular/intracellular calcium; CTR, calcitonin receptor; DMA, dimethylamiloride; hCT, human calcitonin.

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mM) solubilized from hydroxyapatite have been measured in resorbing hemivacuoles [7], and several studies have shown that high concentrations of extracellular calcium (Ca_e) can inhibit osteoclastic bone resorption [8-10]. These observations have led to the hypothesis (excellently overviewed in ref. [11]) that these high local levels of Ca_e generated by a resorbing osteoclast may act on the cell to limit bone resorption [8,9]. Furthermore, it has been proposed that osteoclasts have a so-called "calcium receptor" that senses the high levels of hemivacuolar Ca_e and acts to inhibit further resorption by increasing intracellular levels of calcium (Ca_i) [8-10,12].

However, the resorbing osteoclast is a highly polarized cell [11,13] and whilst it is clear that the apical membrane of a bone resorbing osteoclast is likely to be exposed to high levels of Ca_e, calcium added at the start of the bone slice assay will interact with the basolateral membrane of non-polarized osteoclasts that have recently (within 20 min.) attached to bone. We have previously shown that osteoclasts are activated for bone resorption by attachment to bone slices and that this activation process can be prevented by addition dimethylamiloride (DMA), an Na^+/H^+-antiporter inhibitor, within 3 hr of osteoclast attachment to bone slices [14]; However, when DMA is added after 3 hr, it has no effect on osteoclastic bone resorption. We have now used this kinetic system to examine the effects of calcium on osteoclast activity in the bone slice assay, with human calcitonin (hCT) being used as a positive control for inhibition of resorption in the kinetic experiments. The results show that in the bone slice assay high levels of Ca_e do not inhibit ongoing osteoclastic bone resorption, but do inhibit osteoclast adherence to bone slices when added during the early stages of the assay.

MATERIALS AND METHODS

Calcium chloride (Merck) was dissolved in deionised water at 2 M, sterile filtered and stored at 4°C. Human calcitonin (Ciba) was dissolved in PBS + 0.1% w/v bovine serum albumin (Sigma) at 1 mg/ml, sterile filtered and stored as aliquots at -20°C.

Bone resorption assay

The isolation and culture of osteoclasts for use in the bone resorption assay has been described in detail previously [14,15]. Briefly, femora and tibiae from neonatal rats were cleaned and the osteoclasts released by curetting the bones, followed by pipetting in HEPES-buffered medium 199 (Gibco). The cell suspension was allowed to sediment and attach onto bovine cortical bone slices (3 x 3 x 0.1 mm) for 15 min at 37°C, when the non-adherent cells were washed off and the bone slices cultured in 96 well plates in Eagle's MEM supplemented with 2 mM glutamine (Gibco) and 0.1% bovine serum albumin (Sigma). The compounds diluted in supplemented Eagle's MEM were added to the bone slices at the beginning of culture (t = 0) unless otherwise stated. After a total incubation period of 24 hr, the bone slices were formalin-fixed and stained with toluidine blue for assessment by reflected light

microscopy [16] using a Leitz Laborlux microscope fitted with a Kappa CF15/2 video camera (Gleichen, Germany). Bone resorption was quantified by measuring the total number and surface area of excavations in each bone slice. Osteoclasts on bone slices were identified and counted by virtue of their size, morphology and characteristic brown staining colour.

RESULTS

Inhibition of osteoclastic bone resorption by high Ca_e

Addition of Ca_e at the start of the bone slice assay, resulted in a concentration-dependent inhibition of resorption as assessed by inhibition of number of pits and the area resorbed per bone slice (Fig. 1). The area per resorption pit was the least sensitive parameter showing little effect of 5 mM and 10 mM Ca_e, but complete inhibition of resorption was seen with 20 mM Ca_e in each case. The IC_{50} from the area resorbed per bone slice parameter was 8 mM Ca_e.

Effect of time of addition of calcium or hCT on osteoclast activity and numbers/survival on bone

As shown in Figure 2(a), addition of calcium or hCT at t = 0 or 1 hr after osteoclast adherence to the bone slices resulted in complete inhibition of resorption during the 24 hr assay. However, addition of calcium at t = 3 and 6 hr had no significant effect on bone resorption during the subsequent culture period up to 24 hr. In contrast, hCT completely inhibited all resorption subsequent to its addition to the assay, the apparent loss of activity being explained by resorption having occurred prior to the

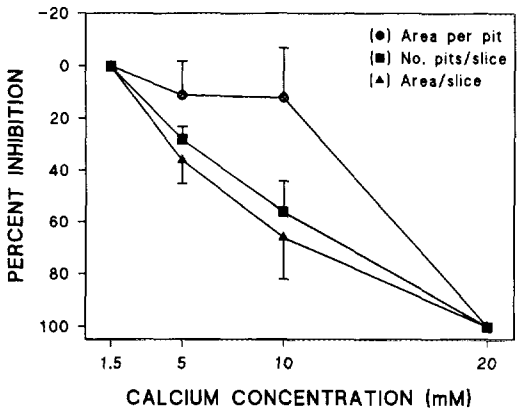

Figure 1. Effect of calcium on osteoclastic bone resorption.
Calcium chloride was added at the start of the 24 hr bone slice assay to produce the final concentrations of Ca_e shown. The normal concentration of calcium in the medium is 1.5 mM. The results shown are the mean ± SEM of 3 separate experiments (15 bone slices per point), the control values were: area resorbed/bone slice = 8.46 ± 3.19 x $10^{-3}\mu m^2$; number of pits/bone slice = 6.7 ± 2.1; area/pit = 1.25 ± 0.25 x 10^{-3} μm^2.

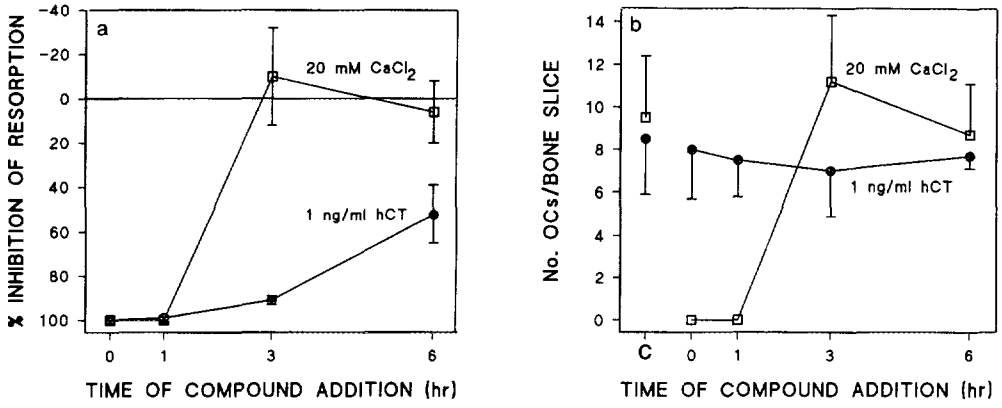

Figure 2. Effect of time of addition of 20 mM calcium on (a) bone resorption and (b) osteoclast numbers in the bone slice assay.
Calcium chloride was added to give a final concentration of 20 mM at various times after the start of the 24 hr bone slice assay. **(a)** The results shown are the mean ± SEM from 3 - 4 experiments (15 - 20 bone slices per point), and the control area resorbed per bone slice was 8.38 ± 1.58 x 10^{-3} μm^2. **(b)** Osteoclast numbers on bone slices after 24 hr incubation are shown as mean ± SEM for 15 - 20 bone slices per point, compared to controls (C).

addition of hCT (~10% at t = 3 hr and ~42% at t = 6 [14], inhibition by hCT was 91% and 52% respectively). These results showed that high Ca_e was inhibiting early events associated with osteoclast adherence to bone and activation for resorption. However, it was noted that with early addition of 20 mM Ca_e to the assay, no cells were present on the bone slices. Therefore, osteoclasts numbers were counted on the bone slices of the calcium and calcitonin kinetic experiments. The results in Figure 2(b) show that addition of hCT at any time did not significantly affect osteoclast numbers on bone, whereas osteoclasts were absent on bone slices when 20 mM calcium was added at t = 0 or 1 hr, but addition at t = 3 or 6 hr did not significantly affect osteoclast numbers. These results indicate that 20 mM calcium added at early time points inhibits osteoclast adherence to the bone slices and consequently no bone resorption is seen, whereas addition at later time points does not affect osteoclast numbers on the bone slices and does not inhibit their ability to resorb bone.

DISCUSSION

Several groups have reported that high concentrations of Ca_e can inhibit osteoclastic bone resorption in vitro [8-10]. It was also demonstrated that high Ca_e increased intracellular calcium (Ca_i) levels in osteoclasts [8,9], a phenomenon also observed with calcitonin [6]. A "calcium-receptor" was postulated to mediate this increase in osteoclast Ca_i [6,8,12] and recently Stroop et al. have shown that the calcitonin receptor (CTR) itself can act as "calcium-receptor" [17]. In addition, Silver et al. have reported that the calcium concentration in resorbing hemivacuoles is as high as 40

mM [7]. Taken together, these data resulted in the proposal of an attractive hypothesis that calcium accumulation in the hemivacuole acts to negatively regulate osteoclastic bone resorption [8-11], for example by causing osteoclast detachment and movement prior to another resorptive cycle [11].

The results presented here confirm that Ca_e dose-dependently prevents osteoclastic bone resorption in the bone slice assay with an IC_{50} of 8 mM (Fig. 1), a value similar to that reported by Zaidi et al. [8,10]. Interestingly, the effective Ca_e for half-maximal (EC_{50}) stimulation of chicken osteoclast Ca_i was reported to be 6 mM [9] and the EC_{50} for the human CTR was estimated to be 8 - 10 mM [17].

However, it was noted in the present experiments that with high concentrations of Ca_e added at t = 0, few (10 mM) or no osteoclasts (20 mM) were seen on the bone slices, suggesting a possible effect of high Ca_e on osteoclast adhesion and/or survival on bone. Furthermore there are some other obvious concerns with the hypothesis discussed above. Firstly, bone resorbing osteoclasts are polarized cells [11,13] and high Ca_e in the hemivacuole, which is limited by the sealing zone, would be expected to interact selectively with the apical/ruffled border membrane, whereas calcium added early to the bone slice assay will clearly interact with the basolateral membrane of recently bone-attached osteoclasts ([11] and see below). Secondly, calcium inhibits resorption in the bone slice assay with an IC_{50} of around 5 to 8 mM and more or less completely at 20 mM ([8,10] and this paper), but Ca_e as high as 40 mM has been measured in resorbing hemivacuoles by microelectrode studies [7]. Furthermore, Silver et al. showed that the high levels of Ca_e were increased under osteoclasts reprobed 1 - 2 hr later [7], an observation that is clearly inconsistent with this hypothesis [11].

Therefore, kinetic experiments were performed to see if later addition of high Ca_e would also inhibit ongoing bone resorption. We have previously shown that there is a cellular activation period within the first 3 hr of the bone slice assay when osteoclasts are activated for resorption. Thus, inhibitors of the Na^+/H^+-antiporter will prevent resorption when added at t = 0 or 1 hr after osteoclast attachment, but not when added at t = 3 or 6 hr [14]. Consequently, if high Ca_e added at t = 3 or 6 hr inhibited subsequent bone resorption (approximately 90% and 55% of the total resorption measured at 24 hours [14]), this would support the notion of high Ca_e as a negative regulator of resorption. However, the kinetic study (Fig. 2) showed that while 20 mM added at t = 0 or 1 hr completely inhibited resorption and was associated with an absence of osteoclasts on the bone slices, addition of 20 mM Ca_e at t = 3 or 6 hr had no significant effect on bone resorption or on osteoclast numbers on the bone slices. In contrast, when hCT was added to the assay at different times, only a decrease in total resorption concomitant with resorption occurring before the addition of hCT to the assay was observed, and osteoclast numbers on the bone slices were the same as controls. The obvious conclusions from these experiments are (i) that early addition of 20 mM Ca_e prevents osteoclast adherence to bone and (ii) that later

addition of 20 mM Ca_e to the assay does not inhibit ongoing osteoclastic bone resorption.

While these results appear to contradict the hypothesis proposing a negative regulatory role of calcium on resorption, the status of the osteoclasts on bone slices at these different time points needs to be considered. During the first 3 hr of the assay it is likely that the osteoclasts adhere and spread on the bone slices, form sealing zones and polarize prior to bone resorption commencing. It is not surprising that addition of high Ca_e during this time interferes with these processes since Miyauchi et al. [9] have shown that expression of the adhesion structures of osteoclasts, podosomes [see 11,18], is inhibited by high Ca_e. Therefore, one may speculate that once the osteoclasts have formed a sealing zone (within 3 hr), high Ca_e cannot cause their detachment from bone because access to the apical/ruffled border membrane is prevented and therefore bone resorption is not inhibited. This would be a particularly attractive view if expression of the "calcium receptor" was restricted to the apical membrane of polarized, resorbing osteoclasts. However, if the CTR is indeed the "calcium receptor" on osteoclasts then this is clearly not the case, since hCT was active on the cells at all times and the CTR must therefore be expressed on the basolateral membrane, but not necessarily the apical/ruffled border membrane.

In summary, the early addition of high Ca_e to the bone slice assay inhibits osteoclast attachment and not resorption per se. Later addition of high Ca_e to the assay does not inhibit ongoing osteoclastic bone resorption. However, the role of hemivacuolar Ca_e in negatively regulating osteoclastic bone resorption remains an attractive hypothesis that cannot easily be proved or disproved using the bone slice assay, since calcium cannot be added directly to the hemivacuole of resorbing osteoclasts.

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REFERENCES

1. Marks, S.C., and Popoff, S.N. (1988) Am. J. Anat. **183:** 1-44.
2. Chambers, T.J., and Hall, T.J. (1991) Vitamins and Hormones **46:** 41-86.
3. Austin, L.A. and Heath, H. (1981) N. Engl. J. Med. **304:** 269-278.
4. Nicholson, G.C., Moseley, J.M., Seyton, P.M., Mendelsohn, F.A.O. and Martin, T.J. (1986) J. Clin. Invest. **78:** 355-360.
5. Chambers, T.J., and Magnus, C.J. (1982) J. Pathol. **136:** 27-39.
6. Malgaroli, A., Meldolesi, J., Zambonin-Zallone, A. and Teti, A. (1989) J. Biol. Chem. **264:** 14342-14347.
7. Silver, I.A., Murrills, R.J. and Etherington, D.J. (1988) Exp. Cell Res. **175:** 266-276.

8. Zaidi, M., Datta, H.K., Patchell, A., Moonga, B. and MacIntyre, I. (1989) Biochem. Biophys. Res. Comm. **163:** 1461-1465.
9. Miyauchi, A., Hruska, K.A., Greenfield, E.M., Duncan, R., Alvarez, J., Barattolo, R., Colucci, S., Zambonin-Zallone, A., Teitelbaum, S.L. and Teti, A. (1990) J. Cell. Biol. **111:** 2543-2552.
10. Zaidi, M., Kerby, J., Huang, C.L.-H., Alam, A.S.M.T., Rathod, H., Chambers, T.J. and Moonga, B.S. (1991) J. Cell. Physiol. **149:** 422-427.
11. Teti, A., Marchisio, P.C. and Zambonin-Zallone, A. (1991) Am. J. Physiol. **261:** C1-C7.
12. Zaidi, M., Alam, A.S.M.T., Huang, C.L.-H., Pazianas, M., Bax, C.M.R., Bax, B.E., Moonga, B.S., Bevis, P.J.R. and Shankar, V.S. (1993) Cell Calcium **14:** 271-277.
13. Baron, R., Chakraborty, M., Chatterjee, D., Horne, W., Lomri, A. and Ravesloot, J.-H. (1993) Handbook Exp. Pharmacol. **107:** 111-147.
14. Hall, T.J., Schaueblin, M., and Chambers, T.J. (1992) Biochem. Biophys. Res. Comm. **188:** 1097-1103.
15. Chambers, T.J., McSheehy, P.M.J., Thompson, B.M., and Fuller, K. (1985) Endocrinol. **116:** 234-239.
16. Walsh, C.A., Beresford, J.N., Birch, M.A., Boothroyd, B. and Gallagher, J.A. (1991) J. Bone Min. Res. **6:** 661-671.
17. Stroop, S.D., Thompson, D.L., Kuestner, R.E. and Moore, E.E. (1993) J. Biol. Chem. **268:** 19927-19930.
18. Zambonin-Zallone, A., Teti, A., Grano, M., Rubinacci, A., Abbadini, M., Gaboli, M. and Marchisio, P.C. (1989) Exp. Cell Res. **182:** 645-652.