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Bone resorption induced by A23187 is abolished by indomethacin: implications for second messenger utilised by parathyroid hormone

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Abstract

Parathyroid hormone acts on the osteoblast to induce osteoclastic bone resorption. Parathyroid hormone utilises cyclic AMP as a second messenger in osteoblasts, but may also cause an increase in cytoplasmatic free calcium ions ($[Ca^{2+}]_i$) in the same cell. To investigate the role of osteoblastic $[Ca^{2+}]_i$ in the induction of bone resorption, we have compared the effects of parathyroid hormone and the Ca^{2+}-ionophore, A23187, as well as the adenylate cyclase stimulating agent, forskolin, and the phorbol ester, phorbole 12,13 dibutyrate (PDB), on bone resorption in neonatal mouse calvarial bones. Parathyroid hormone (0.1 and 1 nM) dose dependently stimulated the release of prelabelled $^{45}Ca^{2+}$ in 72 h culture. Parathyroid hormone-induced bone resorption was not affected by the addition of 1 μM indomethacin to the incubation media, and was therefore, not mediated by local prostaglandin formation. A23187 stimulated the release of $^{45}Ca^{2+}$ at 1–10 nM. Above 100 nM, A23187 inhibited bone resorption. The A23187 (3 and 10 nM)-induced bone resorption was abolished by the cyclooxygenase inhibitor, indomethacin (1 μM), indicating that the stimulatory effect was mediated via prostaglandin formation. The adenylate cyclase stimulating agent, forskolin, dose dependently stimulated bone resorption at and above 1 μM. There was no additive or synergistic effect of forskolin and A23187 on $^{45}Ca^{2+}$ release. Forskolin-induced bone resorption was, as with parathyroid hormone but in contrast to ionophore-induced bone resorption, not abolished by indomethacin (1 μM). The protein kinase C activator, PDB, at 10 and 1000 nM stimulated the release of prelabelled $^{45}Ca^{2+}$. The stimulatory effect of the protein kinase C stimulating phorbol ester, PDB, on bone resorption was abolished by the addition of indomethacin. In summary, bone resorption induced by a Ca^{2+}-ionophore is abolished by indomethacin. This indicates that bone resorbing agents known to increase $[Ca^{2+}]_i$ subsequently enhance local prostaglandin formation. Bone resorption induced by the protein kinase C activator, PDB, was also abolished by indomethacin, whereas, forskolin and parathyroid hormone-induced bone resorption was unaffected. These data indicate that cyclic AMP, but not $[Ca^{2+}]_i$, is involved as a second messenger in parathyroid-induced bone resorption. © 1998 Elsevier Science B.V.

Keywords: Bone resorption; Ca^{2+}, intracellular; Prostaglandin formation

1. Introduction

Osteoclastic bone resorption is believed to be regulated via the osteoblasts (Rodan and Martin, 1981). A number of hormones and cytokines such as parathyroid hormone (Wong, 1986), $1,25(OH)_2D_3$ (Chambers et al., 1985), interleukin 1 (Thomson et al., 1986), and tumour necrosis factors (Thomson et al., 1987) have the capacity to act via the osteoblasts to induce osteoclastic bone resorption. The second messenger events that regulate the conversion of a bone matrix-producing osteoblast into a cell that participates in the resorption of bone are poorly understood. Parathyroid hormone was one of the first hormones shown to utilise the cAMP second messenger system (Chase and Aurbach, 1967), and cAMP is believed to be a mediator for many of the actions of parathyroid hormone in bone (Chase and Aurbach, 1970). However, several well known agents with bone resorbing capacity, e.g., parathyroid hormone (Löwik et al., 1985; Reid et al., 1987; Abou-Samra et al., 1992), prostaglandin E_2 (Yamaguchi et al., 1988), and $1,25(OH)_2D_3$ (Lieberherr, 1987; Civitelli et al., 1990), have been reported to cause an increase in cytoplasmatic free Ca^{2+} ($[Ca^{2+}]_i$) in osteoblasts. The bone resorbing agents, bradykinin and thrombin, also enhance $[Ca^{2+}]_i$ in

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osteoblasts (Lerner et al., 1987a; Gustafson and Lerner, 1983; Ljunggren et al., 1991a,b). All these data suggest that $[Ca^{2+}]_i$ plays an important role in the regulation of the activity of the osteoblasts. Earlier studies have demonstrated a stimulatory effect of ionophores on bone resorption in cultured rat long bones (Dziak and Stern, 1975, 1976; Lorenzo and Raisz, 1981). These studies led to the conclusion that $[Ca^{2+}]_i$ may be involved as a second messenger in parathyroid hormone induced bone resorption. However, recent studies again implicate cyclic AMP as the main second messenger for parathyroid hormone in osteoblasts (Ljunggren and Ljunghall, 1993; Ljunggren et al., 1993) and that activation of protein kinase A is linked to parathyroid hormone- and prostaglandin E_2-induced bone resorption (Yamaguchi et al., 1988; Kaji et al., 1992, 1996; Sugimoto et al., 1993). To further investigate the role of $[Ca^{2+}]_i$ as a second messenger that regulates bone resorption, we have studied the effect of the Ca^{2+}-ionophore, A23187, on bone resorption in neonatal mouse calvarial bones, in vitro. We have compared the resorbing effects of A23187 and the adenylate cyclase stimulating agent, forskolin, with the effect of parathyroid hormone, with regard to the involvement of prostaglandin formation.

2. Materials and methods

2.1. Materials

Recombinant human parathyroid hormone 1–34, forskolin, phorbole 12,13 dibutyrate, 4α-phorbol 12,13 didecanoate and A23187 were purchased from Sigma Chemical, St. Louis, MO, USA; $^{45}CaCl_2$ from New England Nuclear Chemicals, Dreieich, Germany; Gibco CMRL 1066 medium from Life Technologies, Paisley, Scotland, UK; indomethacin was kindly provided by Merck, Sharp and Dohme, Haarlem, The Netherlands.

2.2. Bone resorption assay

Calvarial bones from new-born mice, 5–6 days old, were dissected out and divided into small pieces. The bone fragments were preincubated for 24 h in CMRL 1066 medium containing 1 μM indomethacin in order to increase the sensitivity of the assay (Lerner, 1987). Thereafter, the samples were rinsed three times in phosphate saline buffer and incubated in fresh CMRL 1066 medium, without indomethacin, for 3 h. Subsequently, each sample was incubated for 72 or 96 h with test substances in 400 μl CMRL 1066. The neonatal mice had been injected with 1.5 μCi $^{45}Ca^{2+}$ 4 days prior to dissection. Bone resorption was estimated by calculating the amount of $^{45}Ca^{2+}$ released into the culture medium from the bone fragments during the incubation. After culture, the amount of $^{45}Ca^{2+}$ was analysed by liquid scintillation in the culture medium and in remaining bones dissolved in HCl. Mobilisation of radioactivity was expressed as a percent of initial activity.

2.3. Statistics

Statistical evaluation of the data was performed using the analysis of variance (ANOVA) or Student's *t*-test for unpaired data.

3. Results

3.1. Effect of indomethacin on parathyroid hormone-induced bone resorption

Parathyroid hormone (0.1 and 1 nM) dose dependently stimulated the release of prelabelled $^{45}Ca^{2+}$ in 72 h culture. Parathyroid hormone-induced bone resorption was not affected by the addition of 1 μM indomethacin to the incubation media (Fig. 1).

3.2. Effect of indomethacin on A23187-induced bone resorption

The Ca^{2+}-ionophore, A23187, had a biphasic effect on the release of prelabelled $^{45}Ca^{2+}$, in vitro. At 1–100 nM, A23187 stimulated the release of $^{45}Ca^{2+}$ (Fig. 2A). Above 100 nM, however, A23187 inhibited bone resorption (Fig. 2A). The inhibition seen at higher concentrations might be

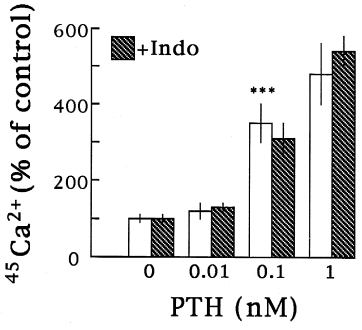

Fig. 1. Parathyroid hormone-induced bone resorption is not affected by indomethacin. Prelabelled neonatal mouse calvarial bones were dissected into small fragments. The samples were preincubated for 24 h in CMRL 1066 culture medium with indomethacin (1 μM), rinsed and subsequently incubated for 72 h in fresh CMRL 1066 with test substances. After the culture period, samples from the incubation media were withdrawn and the remaining bones were dissolved in HCl. The amount of radioactivity released from the bones during culture was estimated by liquid scintillation and expressed as percentage release of $^{45}Ca^{2+}$. Values represent pooled data from three separate experiments with six bones/group in each experiment, and are presented as means ± S.E.M. of % stimulation with control values set to 100%. ***Significantly different from control, $P < 0.001$, as determined by ANOVA.

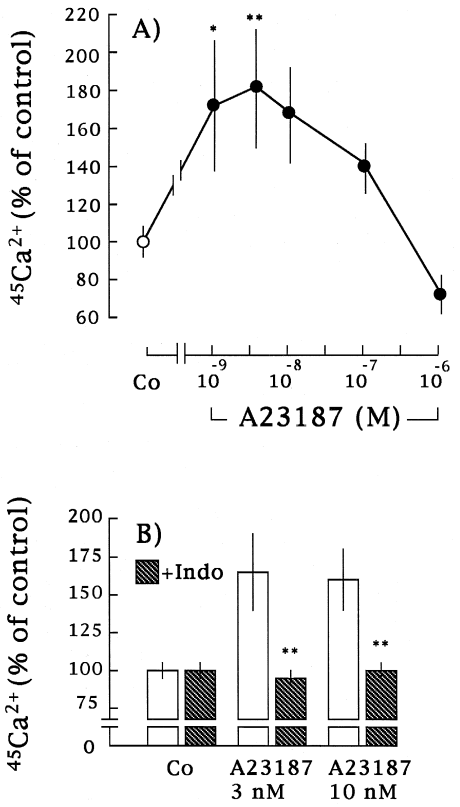

Fig. 2. Effect of the Ca^{2+}-ionophore A23187 on bone resorption in vitro. A $^{45}Ca^{2+}$ release was measured as described in legend to Fig. 1. (A) Dose-response of the effect of A23187 on bone resorption. Values represent pooled data from three separate experiments with six bones/group in each experiment, and are presented as means ± S.E.M. of % stimulation with control values set to 100%. *, **Significantly different from control, $P < 0.05$ and $P < 0.01$, respectively, as determined by ANOVA. (B) Effect of indomethacin on A23187 induced bone resorption. Neonatal mouse calvarial bones were incubated with A23187 ± indomethacin 1 μM. Values represent pooled data from three separate experiments with six bones/group in each experiment, and are presented as means ± S.E.M. of % stimulation with control values set to 100%. **Significantly different from treatment group with the same dose of A23187 but without indomethacin, $P < 0.01$ as determined by Student's *t*-test for unpaired data.

a cytotoxic effect, or possibly be due to depletion of ATP in the resorbing cells (Dziak and Stern, 1976). The A23187-induced bone resorption (3–10 nM) was abolished by the addition of 1 μM indomethacin to the incubation media (Fig. 2B).

3.3. Effect of indomethacin and A23187 on forskolin-induced bone resorption

The adenylate cyclase stimulating compound, forskolin, dose dependently stimulated the release of prelabelled $^{45}Ca^{2+}$ (0.1–10 μM, 96 h culture, Fig. 3A). In some experiments, the addition of the cyclooxygenase inhibitor, indomethacin (1 μM), had a slight inhibitory effect also on the release of $^{45}Ca^{2+}$ induced by forskolin (Fig. 3B). This might be explained by a cAMP-dependent induction of cyclooxygenase in osteoblastic cells (Oshima et al., 1991). The stimulatory effect of forskolin on bone resorption was not additive to or synergistic with bone resorption induced by A23187 (10 nM; Fig. 3C).

3.4. Effect of indomethacin on bone resorption induced by phorbole 12,13 dibutyrate and 4α-phorbol 12,13 didecanoate

The protein kinase C activator, PDB, at 10 and 1000 nM stimulated the release of prelabelled $^{45}Ca^{2+}$ (Fig. 4A). The stimulatory effect of PDB on bone resorption was abolished by the addition of indomethacin (1 μM). No effect on bone resorption was seen on stimulation of the

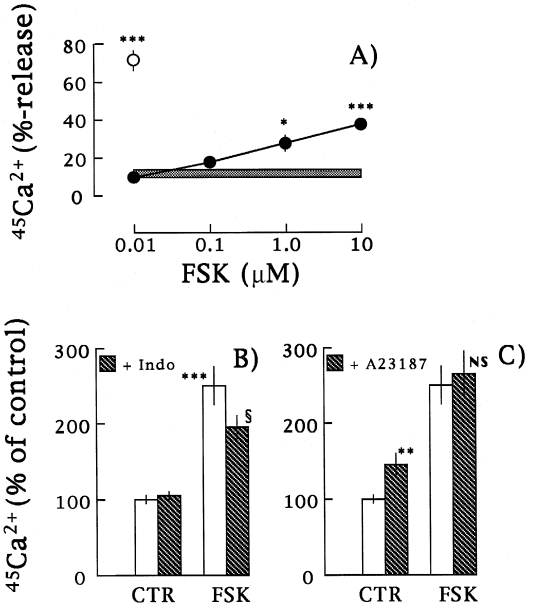

Fig. 3. Effect of forskolin on bone resorption. A $^{45}Ca^{2+}$ release was measured as described in legend to Fig. 1. (A) Dose-response of forskolin-induced bone resorption. Values represent means ± S.E.M. for six bone fragments. Shaded area represents mean ± S.E.M. for untreated controls. *, ***Significantly different from untreated control, $P < 0.05$ and $P < 0.001$, respectively, as determined by ANOVA, ●, forskolin, ○, parathyroid hormone 10 nM. (B) Effect of indomethacin on forskolin-induced bone resorption. Neonatal mouse calvarial bones were incubated with forskolin 1 μM ± indomethacin 1 μM. Values represent pooled data from three separate experiments with six bones/group in each experiment, and are presented as means ± S.E.M. of % stimulation with control values set to 100%. ***Significantly different from control, $P < 0.001$; §, significantly different from treatment with forskolin alone, $P < 0.05$, as determined by ANOVA. (C) Effect of A23187 on forskolin-induced bone resorption. Neonatal mouse calvarial bones were incubated with forskolin 1 μM ± A23187 10 nM. Values represent pooled data from three separate experiments with six bones/group in each experiment, and are presented as means ± S.E.M. of % stimulation with control values set to 100%. **Significantly different from untreated control, $P < 0.01$; NS: not significantly different from forskolin treatment only, as determined by Student's *t*-test for unpaired data.

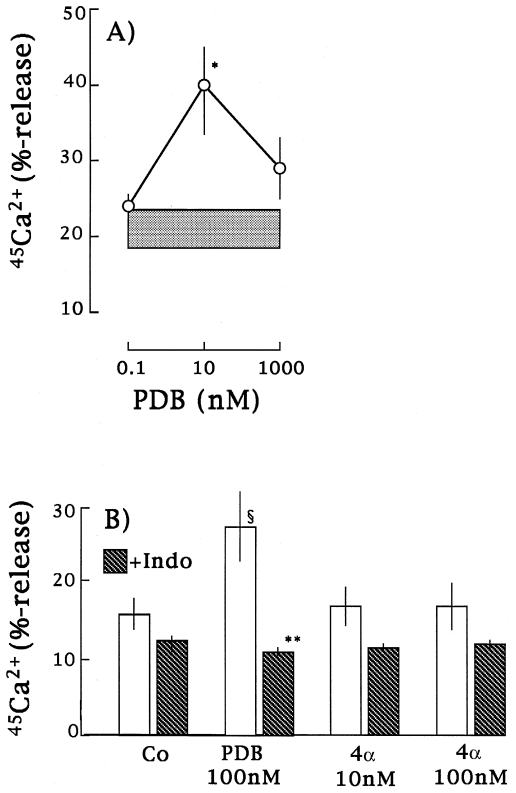

Fig. 4. Effect of PDB on bone resorption. A $^{45}Ca^{2+}$ release was assayed as described in legend to Fig. 1. (A) Dose-response of PDB-induced bone resorption. Values represent means ± S.E.M. for six bone fragments. Shaded area represents mean ± S.E.M. for untreated controls. * Significantly different from untreated controls, $P < 0.05$, as determined by ANOVA. (B) Effect of indomethacin on PDB-induced bone resorption. Values represent means ± S.E.M. for six bone fragments. §, Significantly different from untreated controls, $P < 0.05$, ** significantly different from PDB-treated group, $P < 0.01$, as determined by Student's *t*-test for unpaired data. The 4α represents the inactive phorbol ester, 4α-phorbol 12,13 didecanoate.

calvariae with the inactive phorbol ester, 4α-phorbol 12,13 didecanoate (Fig. 4B).

4. Discussion

The Ca^{2+}-ionophore, A23187, stimulates bone resorption in fetal rat bones (Dziak and Stern, 1975, 1976). This finding was one of the bases for the theory that an increase in $[Ca^{2+}]_i$ may be involved as a second messenger in, e.g., parathyroid hormone-induced bone resorption, previously believed to be mediated by cyclic AMP (Vaes, 1968; Klein and Raisz, 1971). The results of cloning of the parathyroid hormone receptor in rat were published in 1992, and the receptor was shown to consist of a seven-membrane spanning G-protein, hypothesised to activate both the adenylate cyclase and the phospholipase C second messenger pathways (Abou-Samra et al., 1992). Several groups have suggested that parathyroid hormone uses both cyclic AMP and $[Ca^{2+}]_i$ in rat osteosarcoma cells to increase bone resorption (Löwik et al., 1985; Reid et al., 1987; Abou-Samra et al., 1992). However, we now present evidence that stimulation of bone resorption by an increase in $[Ca^{2+}]_i$ or by activation of protein kinase C in bone cells is mediated via prostaglandin formation, whereas, parathyroid hormone induced bone resorption is independent of prostaglandin formation. Other agents known to increase $[Ca^{2+}]_i$ in osteoblasts, such as bradykinin and thrombin (Ljunggren et al., 1991a,b), also stimulate bone resorption via enhanced local prostaglandin formation (Lerner et al., 1987a; Gustafson and Lerner, 1983). This view is also supported by the fact that treatment of cells with Ca^{2+} ionophores is known to cause enhanced prostaglandin formation (Ljunggren et al., 1991a; Knapp et al., 1977), possibly due to a direct regulatory effect of Ca^{2+} on phospholipase A_2 (Irvine, 1982; Chang et al., 1987). Parathyroid hormone, however, stimulates bone resorption via a pathway that is independent of local prostaglandin formation (Lerner et al., 1987b; present study). It is well established that parathyroid hormone enhances cyclic AMP formation in osteoblasts (Wong, 1986). Furthermore, bone resorption in vitro is stimulated by agents that enhance cyclic AMP formation, e.g., forskolin (Lerner et al., 1986) or cholera toxin (Ransjö and Lerner, 1987). Prostaglandin E_2 generates cyclic AMP, and prostaglandin E_2-induced bone resorption is abolished by the protein kinase A inhibitor, Rp-cAMPS (Kaji et al., 1996). We have previously reported that bone resorption induced by parathyroid hormone is blocked by Rp-cAMPS (Ljunggren and Ljunghall, 1993). The fact that forskolin-induced bone resorption is not abolished by indomethacin (Lerner et al., 1986 and present study) therefore, further supports the view that cyclic AMP is involved as a second messenger in parathyroid hormone-induced bone resorption.

The results in this report argue against a role for $[Ca^{2+}]_i$ as a second messenger in parathyroid hormone-induced bone resorption. There is further evidence from our previous findings that parathyroid hormone may stimulate the formation of cyclic AMP without enhancing $[Ca^{2+}]_i$, and that no increase in $[Ca^{2+}]_i$ by parathyroid hormone treatment was detectable in several osteoblastic cell lineages that reacted to parathyroid hormone with cyclic AMP formation (Ljunggren et al., 1992, 1993). Other groups have reported similar findings, noting cyclic AMP production in response to parathyroid hormone, but no, or faint, Ca^{2+} spikes (Boland et al., 1986; Schöfl et al., 1991). The experiments in which Ca^{2+} spikes are detected are often performed with cell suspensions rather than adherent single cells (Babich et al., 1997). Furthermore, Bringhurst et al. (1989) found that parathyroid hormone responsiveness was inhibited in clonal osteoblasts expressing a mutated cAMP-dependent protein kinase.

In summary, bone resorption induced by a Ca^{2+}-ionophore is abolished by indomethacin. This observation indicates that bone resorbing agents known to increase $[Ca^{2+}]_i$ subsequently enhance local prostaglandin formation. Furthermore, bone resorption induced by the protein kinase C

activator, PDB, is also abolished by indomethacin. On the other hand, agents stimulating adenylate cyclase, such as forskolin, induce bone resorption that is unaffected by indomethacin. Parathyroid hormone-induced bone resorption is also unaffected by indomethacin. These data indicate that cyclic AMP, but not $[Ca^{2+}]_i$, is involved as a second messenger in parathyroid hormone-induced bone resorption.

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