cAMP-dependent inhibition is dominant in regulating superoxide production in the bone-resorbing osteoclasts

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Abstract

Calcitropic hormones such as parathyroid hormone (PTH) and calcitonin have been shown to have stimulatory and inhibitory effects respectively on superoxide anion (O_2^-) generation by osteoclasts, but the exact intracellular signalling mediating these pathways has not been investigated. In order to elucidate the intracellular pathways controlling O_2^- generation, we have carried out a systematic study of the effect of different agents on O_2^- production in osteoclasts cultured on bovine cortical bone. Dibutyl cAMP and cholera toxin, while having no effect on the basal level of O_2^- production in bone-resorbing osteoclasts, were, however, found to completely block the stimulation of free radical production by PTH, pertussis toxin and ionomycin. The stimulation of O_2^- production was found to be independent of protein kinase C-dependent pathways since the presence of bisindolyl-

maleimide (GF109203X) (1 μ M) did not block stimulation by PTH and pertussis toxin. Interestingly, while exposure to bisindolylmaleimide at this concentration did not have any effect on the basal level of O_2^- production, exposure to a higher concentration (10 μ M), which is known to inhibit both protein kinase C and A, produced significant stimulation. These *in vitro* findings suggest that in the bone-resorbing cells, cAMP-dependent protein kinases prevent further stimulation of NADPH oxidase by agents such as PTH and pertussis toxin. The increase in cAMP has also been recently demonstrated to be associated with down-regulation of the oxidative burst in adherent neutrophils; and the findings reported here suggest a similar role for cAMP in O_2^- generation in osteoclasts cultured on bone.

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Introduction

The osteoclast, a multinucleate cell, is unique among the cells of macrophage/monocytic lineage in its ability to carry out extracellular bone dissolution, a process of resorption involving the secretion of hydrolytic enzymes and protons and the generation of superoxide anions (O_2^-). Osteoclastic bone resorption is inhibited by calcitonin which leads to increased levels of cytosolic cAMP and calcium via two different G-proteins (Zaidi *et al.* 1990). It is generally accepted that the modulation of osteoclast activity by other major hormones, such as parathyroid hormone (PTH), 1,25-dihydroxycholecalciferol and oestrogens is mediated indirectly via cells of osteoblast lineage. However, a number of studies have recently demonstrated that osteoclasts do in fact possess receptors for PTH (Agarwala & Gay 1992, Tong *et al.* 1995, May & Gay 1997), 1,25-dihydroxycholecalciferol (Mee *et al.* 1996) and oestrogens (Kameda *et al.* 1997). The exposure of osteoclasts to PTH was found to directly stimulate hydrogen ion production via a G-protein-coupled receptor (May & Gay 1995, 1997). One possible explanation for the failure of earlier studies to identify

direct acute modulatory effects of these major hormones on the osteoclast is their dependence on the *in vitro* bone-resorption assays, which are known to be quite insensitive and imprecise (Boyde & Jones 1991). Recent studies, employing O_2^- production by the osteoclast as an index of osteoclast function, have also demonstrated an instantaneous O_2^- burst in isolated osteoclasts following exposure to PTH (Datta *et al.* 1996).

Both indirect and direct measurements have shown that osteoclasts produce O_2^- (Garret *et al.* 1990), the production of the anion being inhibited by calcitonin and elevated extracellular calcium, and stimulated by bone matrix and PTH (Datta *et al.* 1995, 1996). The results of these studies suggest that the agents that inhibit and activate osteoclastic bone resorption have corresponding effects on O_2^- production by these cells, implying a link between O_2^- production and osteoclast activity. The importance of free radicals in osteoclast function was first suggested on observation of the abolition of cell resorptive activity by superoxide dismutase (Albanese *et al.* 1991) and nitric oxide (MacIntyre *et al.* 1991). In view of the important role of O_2^- in osteoclastic bone resorption, it is vital to understand the precise intracellular signalling that

is involved in the regulation of free radical production. Like neutrophils and macrophages, osteoclasts also express NADPH oxidase (Steinbeck *et al.* 1994). O_2^- produced by leucocytes, including neutrophils and macrophages, plays an important role in the destruction of microorganisms. These cells are known to produce O_2^- by activating the NADPH oxidase system after exposure to bacteria, which is an essential step in the destruction and phagocytosis of the pathogens. In contrast, O_2^- produced by osteoclasts has been shown to be up-regulated by components of the extracellular matrix and divalent cations (Datta *et al.* 1995). The present investigation was designed to elucidate the role of intracellular second messengers in the regulation of O_2^- production in osteoclasts.

Materials and Methods

Reagents

Cytochrome *c* from horse heart, bovine liver superoxide dismutase, dibutyl cAMP, pertussis toxin, PTH, fetal calf serum and other organic and inorganic chemicals were obtained from Sigma (Poole, Dorset, UK). Forskolin was from ICN (Thame, Oxfordshire, UK); bisindolylmaleimide (GF109203X) was from Calbiochem-Novabiochem (Nottingham, UK) and 3,3'-dithiobis(sulphosuccinimidylpropionate) (DTSSP) was supplied by Pierce and Warriner (Chester, Cheshire, UK). $NaClO_4$ and $MgCl_2$ were supplied by BDH (Poole, Dorset, UK). Salmon calcitonin was a gift from Dr Tudor Arvinte (Ciba, Horsham, W Sussex, UK). Medium 199 with Hepes was supplied by Gibco Life Technologies (Paisley, Renfrewshire, Scotland, UK).

Osteoclast bone culture

Devitalized bovine cortical bone slices were used as substrata for osteoclast culture. In brief, the devitalized bone was sliced into circular discs of 13 ± 1 mm in diameter and approximately 1 mm thick. The bone was then cleaned in distilled water by sonication, followed by a final wash with ethanol and distilled water. Newborn Wistar rats (2–3 days old) were killed by cervical dislocation and their long bones removed and freed from any tissues, curetted and desegregated into prewarmed Medium 199 containing 10% fetal calf serum ($37^\circ C$). The cell suspension was then left for 90 min at $37^\circ C$ in a humidified 5% CO_2 incubator, allowing the osteoclasts to adhere to the bone slices. Cellular debris was removed by washing the preparation vigorously with a prewarmed phosphate buffer ($37^\circ C$), comprising 145 mM NaCl, 4.5 mM KCl, 5 mM NaH_2PO_4 , 6 mM glucose, 10 mM Hepes and 1 mM $MgCl_2$ and adjusted to pH 7 with NaOH, leaving the osteoclasts attached to the substratum. Experiments were carried out in wells containing 0.5 ml

phosphate buffer and an individual slice of bone to which osteoclasts were attached. For each slice, osteoclasts from one neonatal rat were used.

Osteoblast and osteoclast co-culture

Our previous studies have shown that ROS17/2.8 produce an undetectable basal level of O_2^- and no detectable response to PTH (Datta *et al.* 1996). Therefore, in order to exclude an indirect contribution of osteoblast-like cells to the production of O_2^- by the bone-resorbing osteoclasts, a series of experiments was performed in which osteoblast-like osteosarcoma cells (ROS17/2.8) were co-cultured with osteoclasts. In these experiments osteoclasts were co-cultured with low (5–10%), intermediate (30–50%) or high (>70%) confluence of ROS17/2.8 cells, and the effect of these cells on basal and PTH-stimulated O_2^- generation by the osteoclasts was determined in order to detect the effect on the kinetics and magnitude of the response.

Assessment of the purity of the osteoclast culture

The contaminating non-osteoclast cells were removed by performing separate experiments in which osteoclast–bone slice cultures were exposed for 3 min to trypsin/EDTA and then washed vigorously with prewarmed phosphate buffer ($37^\circ C$; the composition of the buffer is given above) (Chambers 1978, Sahni *et al.* 1996). The purity of the osteoclast preparation was checked by microscopy, and the presence of osteoblasts was determined by histochemical staining for alkaline phosphatase (Procedure 85; Sigma). This process was found to produce an osteoclast population of very high purity ($93 \pm 4\%$, mean $\pm$ S.E.M.).

Characterization of osteoclasts on bone slices

In order to characterize the cells after the experiments, bone slices were stained with tartrate-resistant acid phosphatase (TRAP) using an established protocol (kit no. 386; Sigma). Briefly, the cells were fixed on the bone slice in citrate/acetone solution for 30 s and the slices were then rinsed in deionized water and allowed to air dry for a minimum of 15 min. The slices were then incubated in an acetate/tartrate solution for 1 h at $37^\circ C$ in the dark, washed for 3 min in deionized water and stained in acid/haematoxylin solution for 5 min. The number and density of the osteoclasts on well-rinsed and dried slices was ascertained using a Nikon TMS microscope ($\times 400$ magnification).

Measurement of O_2^- production

Fabrication of the gold electrode A gold wire (5 mm in length and 1 mm in diameter) was soldered to a copper wire before insertion of the whole into a capillary glass

tube 2 mm in diameter. The gold was placed near one end of the capillary tube (Goodfellow, Cambridge, UK). The wire, including the solder joint, was then potted in an epoxy resin (Araldite) and allowed to dry for 12 h. The cross-section of the gold wire was exposed by grinding the electrode on a polishing wheel. The gold surface was then polished with successively finer grades of diamond paste and/or alumina.

Immobilization of cytochrome *c* at a surface-modified gold electrode The electrode was made as previously described (McNeil *et al.* 1989). Briefly, it was cleaned by leaving it for 1 min at +1 V and 1 min at −1 V against an Ag/AgCl electrode in a 0.1 M HCl solution. After being rinsed with distilled water, the electrode was immersed in a fresh aqueous solution of 50 mM DTSSP for 1 min and immediately afterwards in 125 µl 2 mM cytochrome *c* solution prepared in 100 mM phosphate buffer containing 20 mM NaClO₄ and incubated at 4 °C for about 20 h. The electrodes were found to be stable for up to 6 h.

Calibration of the electrode O₂[−] was generated as an intermediate during the oxidation of xanthine by xanthine oxidase (XOD) in 20 mM NaH₂PO₄ buffer (pH 7.4) containing NaCl (150 mM) and NaClO₄ (100 mM) at room temperature. A stock solution of xanthine (30 mM) in KOH (0.1 M) was prepared and added to the electrochemical cell containing the phosphate buffer to produce a final concentration of 3 mM. XOD was added to give a final concentration of 0.8 µM, and the rate of current production at +100 mV vs Ag/AgCl during the reoxidation of reduced cytochrome *c* was measured using a low-current potentiostat.

Instrumentation The electrochemical instrumentation consisted of a locally constructed high-sensitivity potentiostat (Electronics Workshop, Department of Chemistry, University of Newcastle upon Tyne) interfaced to a 486DX 25 MHz PC via an analogue interface board (DAS1602; Keithley Metrabyte, Reading, Berks, UK). The software was written in MS Visual Basic and was used to control the potentiostat, emulate a *Y–t* chart recorder and transfer the current–time data to a spreadsheet for graphing and analysis.

The currents measured at millimetre sized superoxide electrodes in our experiments were of the order of 1–100 pA and therefore an earthed aluminium box was used as a Faraday cage to contain the experiment culture well and the electrodes were connected to the potentiostat via guarded inputs. The largest available feedback resistors, i.e. 0.1–100 GΩ, were used to maximize the signal-to-noise ratio. An isolated power supply and software-enabled RC filter with time constant of 1 s were also used. The analogue output of the potentiostat was digitized at about 250 Hz, and values of current were averaged over intervals

Figure 1 Typical multinucleate TRAP-positive osteoclasts cultured on bone slices for the investigations (magnification × 200).

of 0.5 s or greater and plotted against time on the PC screen.

The position of the electrode could be controlled either manually or by using two-stepper motor-driven translation stages with micron resolution (MFN08-PP; Newport, Newbury, Berks, UK) mounted in an X-Z configuration. The motors were interfaced to the PC via a driver card (Motor Technology; Stockport, UK) and a programmable stepper motor-controller interface (MSTEP-5; Keithley Metrabyte).

Results

The purity and morphology of the osteoclast culture on the bone slices was determined by TRAP staining, and a typical multinucleate TRAP-positive cell cultured on a bone slice is shown in Fig. 1 (magnification × 400). The staining revealed that over 90% of the cells were TRAP-positive multinucleate cells. The co-culture of osteoclasts with different confluent levels of osteoblast-like cells (ROS17/2.8) was found to have no effect on the kinetics or the magnitude of O₂[−] generation stimulated by PTH.

The specificity of the modified cytochrome *c* gold electrode to O₂[−] is illustrated by its response to *in situ* generation of O₂[−] using xanthine/XOD (Fig. 2a). An immediate increase in current is seen when XOD is added to the solution, and addition of superoxide dismutase to the incubating solution produced an immediate fall in the current related to the decrease in the production of O₂[−] (Fig. 2b). In the experiments, the electrode was positioned between 0.5 and 1 mm above the slice of bone on to which osteoclasts were fixed.

The acute response of osteoclasts cultured on devitalized cortical bone to PTH (100 nM) and pertussis toxin (1 ng/ml) is shown in Fig. 3a and b respectively. The two agents showed similar response time, peak height and decay of the response. Osteoclast response to ionomycin (10 µM) was also stimulatory, but the kinetics of the O₂[−]

Figure 2 A typical instantaneous response of cytochrome c electrode to *in situ* generation of O_2^- using xanthine/XOD (a). The addition of superoxide dismutase (SOD) shows that the electrode current is solely from O_2^- generation (b).

Figure 3 The effect of PTH (100 nM), pertussis toxin (1 ng/ml) and ionomycin (10 μ M) on O_2^- generation by rat osteoclasts cultured on devitalized bovine cortical bone. The agents were added to the culture after baseline production of the radical was established. Similar responses are observed with PTH and pertussis toxin (a and b), but ionomycin was found to have a more sustained effect (c). The addition of superoxide dismutase (SOD) shows an instantaneous decline in the rate of O_2^- production (d). Each profile represents an individual experiment carried out on a separate bone slice, and, in each case, at least four to six replicate experiments were carried out; however, for the sake of clarity not all profiles are displayed.

response were quite different from those obtained with PTH or pertussis; a sustained stimulation by the ionophore was evident (Fig. 3c). Although the increase in O_2^- production by PTH and pertussis toxin produced was transient, the final level of production of the free radical remained higher than the initial basal levels. That this increase in current was solely due to O_2^- production was confirmed by exposing the osteoclast culture to superoxide dismutase, which immediately abolished the observed rise in current (Fig. 3d). In fact, exposure to the enzyme was associated with a decrease in the current to below that of the starting level, confirming that osteoclasts cultured on bone produce a constant but sustained basal level of O_2^- .

We then tested dibutyl cAMP (200 μ M), cholera toxin (5 μ g/l) and bisindolylmaleimide (1 μ M) on the osteoclast culture but found that none of these agents had any effect on the basal level of O_2^- production (Fig. 4a, b and c). Interestingly, exposure to a higher concentration of bisindolylmaleimide (10 μ M, Fig. 4d) produced a sustained increase in free radical production with a similar type of response curve to that seen with ionomycin (Fig. 3c).

The action of all the stimulatory agents, i.e. PTH, pertussis toxin and ionomycin, was blocked when the cells were exposed to dibutyl cAMP (200 μ M) (Fig. 5a, b and c). Cholera toxin, like dibutyl cAMP, had no effect on

Figure 4 The effect of dibutyl cAMP (200 μ M), cholera toxin (5 μ g/l) and bisindolylmaleimide (1 and 10 μ M) on O_2^- generation. Three representative examples of each different experiment are shown. As can be seen there was no effect on the rate of free radical production with dibutyl cAMP (a), cholera toxin (b) or bisindolylmaleimide (1 μ M) (c). At a concentration of 10 μ M, bisindolylmaleimide produced a sustained increase in O_2^- production (d).

the basal level of the free radical production, but unlike dibutyl cAMP, the toxin failed to block the stimulation by PTH (Fig. 5d). However, cholera toxin treatment of the osteoclast culture was found to markedly decrease the extent of stimulation by PTH.

In order to assess the importance of protein kinase C in mediating the PTH-induced burst of oxygen free radicals, we exposed the cultured osteoclasts to bisindolylmaleimide (1 μ M) before the addition of PTH (Fig. 6a) (Toullec *et al.* 1991). Bisindolylmaleimide, a protein kinase C inhibitor, failed to block the stimulation of O_2^- production by both PTH (100 nM) and pertussis toxin (1 μ g/l) when used at 1 μ M (Fig. 6a and b). Interestingly, exposure of the cells to bisindolylmaleimide at a concentration known to inhibit both protein kinase C and A (10 μ M) resulted in the prolonged stimulation of radical production (Fig. 4d).

We then ascertained the sensitivity of O_2^- production by exposing the cells to salmon calcitonin (10 nM), which

was found to suppress the anion production (data not shown); similar inhibition was also observed with forskolin (10 μ M) (Fig. 7).

Discussion

We found that the kinetics and quantitative aspects of O_2^- production in osteoclasts cultured on bone matrix and stimulated with PTH were identical in purified osteoclast (>90% osteoclast cells as indicated by TRAP staining) and osteoclast–osteoblast co-culture (Datta *et al.* 1996). Determination of O_2^- production in the osteoclasts alone and osteoclast and osteoclast–ROS17/2.8 co-culture has shown that the kinetics and magnitude of the stimulation of free radical generation by PTH is unaffected by the presence of osteoblast-like cells (Datta *et al.* 1996).

Our investigations were carried out on osteoclasts cultured on bone matrix, since our previous studies have

Figure 5 The experiments show the complete blockage of the stimulatory effects of PTH (100 nM) (a), pertussis toxin (1 ng/ml) (b) and ionomycin (10 μ M) (c), by prior administration of dibutyryl cAMP (200 μ M). Treatment with cholera toxin (5 μ g/l) failed to block the stimulatory effect of PTH (d).

shown that O_2^- production in osteoclasts cultured in this way is far greater than in cells cultured on plastic (Datta *et al.* 1995), an effect probably mediated via integrin. We also report that this can be further increased not only by the addition of PTH as reported earlier (Datta *et al.* 1996), but also by pertussis toxin and ionomycin. Exposure to dibutyryl cAMP and cholera toxin showed that these agents were neither stimulatory nor inhibitory. However, pretreatment with dibutyryl cAMP completely abolished the stimulatory effects of PTH, pertussis toxin and ionomycin. The stimulation by ionomycin and other agents such as PTH and pertussis toxin was not altered by the presence of the protein kinase C inhibitor bisindolylmaleimide.

The stimulation of free radical production by PTH, pertussis toxin and ionomycin was inhibited in the presence of dibutyryl cAMP. These *in vitro* results suggest that the inhibitory effects of cAMP-dependent kinase are dominant over the stimulatory signalling in modulating NADPH oxidase in osteoclasts cultured on bone. Whilst stimulation of free radical production is blocked by cAMP-dependent pathways, extracellular matrix-mediated free radical production is not affected, suggesting that the up-regulation seen in the osteoclasts cultured on bone rather than plastic is likely to be mediated via integrins and is independent of modulation by cAMP. The importance of cAMP-dependent kinase is further underlined by the fact that, while 1 μ M bisindolylmaleimide had no effect,

higher levels of the agent (10 μ M) stimulated a free radical burst. In view of the fact that bisindolylmaleimide at 1 μ M is known to be a potent selective inhibitor of protein kinase C, and at higher concentrations also inhibits protein kinase A, this underlines the dominant inhibitory role of protein kinase A, and hence the inhibition of protein kinase A leads to stimulation of O_2^- production.

The mechanism of PTH-mediated stimulation is clearly independent of protein kinase C. The fact that pertussis toxin, which catalyses ADP-ribosylation of G_i , mimics the effect of PTH suggests that the pathway mediating the stimulatory effect of the hormone may involve receptor-coupled inhibition of adenylate cyclase. This contention is further supported by the fact that inhibition by both stimulants is completely blocked by dibutyryl cAMP. However, cholera toxin did not mimic this action of dibutyryl cAMP. One possible explanation for this observation is that ADP-ribosylation of $G_s\alpha$ by cholera toxin may not produce sufficiently vigorous stimulation of adenylate cyclase, and therefore produces only a relatively small rise in cytosolic cAMP.

Whilst dibutyryl cAMP and cholera toxin had no effect on the basal production of the free radical, both salmon calcitonin (10 nM) and forskolin (10 μ M) were found to decrease O_2^- production. These data suggest that the common inhibitory actions of the latter two agents are cAMP-independent and are probably mediated by the modulation of cation channels. Indeed, recent studies have

Figure 6 The protein kinase C inhibitor bisindolylmaleimide (GF109203X) (1 μ M) did not have any effect on PTH- (a) and pertussis toxin- (b) mediated stimulation of O_2^- generation. For the sake of clarity only three vs four out of six individual profiles are shown.

Figure 7 Forskolin (10 μ M) was found to suppress the baseline levels of O_2^- generation by osteoclasts.

shown a cAMP-independent direct effect of forskolin on voltage-dependent sodium channels (Ono *et al.* 1995).

Studies on neutrophils have implied a possible link between O_2^- production and the stabilization of the

membrane potential, with the displacement of charge due to the release of O_2^- being balanced by H^+ (derived from the oxidation of NADPH) translocated across the membrane (Henderson *et al.* 1987, 1988). However, this link is unlikely to be important, since, in osteoclasts, proton pump activation by PTH is via protein kinase C (May & Gay 1997), while PTH-induced O_2^- production has been shown to be mediated independently of the kinase.

Our observation demonstrating a dominant inhibitory role of cAMP-elevating agents on O_2^- burst in the osteoclasts seems to suggest the existence of similar intracellular regulatory pathways controlling O_2^- generation in the cells of macrophage/monocytic lineage, since intracellular cAMP has been shown to be associated with the down-regulation of the oxidative burst in adherent neutrophils (Ottonello *et al.* 1995). However, further studies are needed to identify the possibility of subtle differences in the intracellular pathways occurring within the cells of macrophage/monocytic lineage and osteoclast and osteoclast-like multinucleate cells.

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