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Binding of Osteopontin to the Osteoclast Integrin $\alpha_v\beta_3$

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Abstract. Occupancy of the chicken osteoclast $\alpha_v\beta_3$ integrin stimulates immediate cell signals. Peptides from osteopontin containing Arg-Gly-Asp and peptides from the osteopontin and bone sialoprotein sequences containing Arg-Gly-Asp stimulated immediate reductions in osteoclast cytosolic Ca^{2+}. The changes in cytosolic Ca^{2+} required the Arg-Gly-Asp sequence, and were blocked by LM609, a monoclonal antibody to the $\alpha_v\beta_3$ integrin. Osteoclast stimulation by the proteins through the integrin did not require immobilization since soluble peptides produced changes in cytosolic Ca^{2+} and inhibited osteoclast binding to bone particles and bone resorption. The decrease in cytosolic Ca^{2+} stimulated by osteopontin and related peptides was due to activation of a plasma membrane Ca^{2+}-ATPase. Thus, the data suggest that ligand binding to the osteoclast $\alpha_v\beta_3$ integrin results in a reduction in cytosolic Ca^{2+} which participates in regulation of osteoclast function.

Introduction

Osteoclasts, specialized cells responsible for bone resorption, are in contact with mineralized bone matrix at two modified areas of the cell surface. These areas, referred to as the 'clear zone' and the 'ruffled border' [1,2], represent polarization of osteoclast morphology related to its function in bone resorption. At the point of cell–bone contact the plasma membrane of the clear zone contains numerous protrusions organized in rows called podosomes. Podosomes contain β_3 integrin [3] associated with talin and vinculin and surrounding the microfilament cytoskeleton core [4,5]. The mechanism of osteoclast attachment to bone has not been clearly determined, but the integrin $\alpha_v\beta_3$ is thought to be a key mechanism of attachment to matrix proteins [3,6,7].

The bone matrix proteins recognized by the vitronectin receptor ($\alpha_v\beta_3$) of the podosome include [8] matrix protein containing a functional Arg-Gly-Asp (RGD) cell-binding sequence [9,10]. We have recently shown that osteopontin plays a key role in anchoring the osteoclast to the bone surfaces [1], and that another candidate protein for a function similar to that proposed for osteopontin is bone sialoprotein, another RGD-containing bone matrix protein.

Thus, the purpose of this study was to determine whether osteopontin and bone sialoprotein generate immediate cell signals similar to those previously shown to participate in the organization of the osteoclast necessary for resorptive activity. The experimental approach utilized osteoclasts cultured in serum-containing media. The cells were adherent to glass and were incompletely polarized, thus potentially affording soluble peptides access to integrins. After brief deprivation of serum, which contains vitronectin, we found that osteopontin and peptides from the osteopontin and bone sialoprotein sequences containing RGD rapidly stimulated Ca^{2+} efflux from osteoclast by activating the plasma membrane Ca^{2+}-ATPase by a calmodulin-dependent mechanism. The process was inhibited by a monoclonal antibody against the integrin $\alpha_v\beta_3$, thus proving that the signal was transmitted via this membrane-bound heterodimer. These results demonstrate a new signal type from integrin stimulation, decreasing cytosolic Ca^{2+} ($[Ca^{2+}]_i$), and further support the critical nature of $[Ca^{2+}]_i$ regulation in osteoclast function.

Methods

Materials

The acetoxymethyl ester fura-2 (fura-2 AM) was purchased from Molecular Probes. The bone sialoprotein peptides,* an 18 mer BSP-A, an 8 mer BSP-2C, and an osteopontin peptide, a 20 mer OPN-A were kind gifts from Dr Frank Robey, Mineralized Tissue Research Branch NIDRR/ National Institutes of Health. Chicken vitronectin was the kind gift of Dr Robert Dunlay. Rat osteopontin was the kind gift of Dr William Butler. The RGD peptides were purchased from Peninsula Laboratories (Belmont, CA). Mouse monoclonal antibodies – LM609 directed against intact $\alpha_v\beta_3$ [11] and LM142 [12] against the α_v chain – were purified from ascites by chromatography on Protein-A Sepharose and used as solutions in phosphate-buffered saline. Ryanodine was purchased from Calbiochem (San Diego, CA); thapsagargin was purchased from LC Services (Woburn, MA).

Osteoclast Preparation

Osteoclasts were prepared as previously reported [13]. Between days 3 and 6 of culture pure preparation of multinucleated cells were formed that expressed osteoclast characteristics. The level of cell polarization expressed by these cells was such that a few podosomes are seen by staining of F-actin, but organization of densely packed podosomes into rows at the cell periphery typical of resorbing osteoclasts was absent.

Measurement of Cytosolic Calcium

$[Ca^{2+}]_i$ was measured in single cells using the fluorescent calcium indicator fura-2. Osteoclasts cultured on coverslips were loaded for 1 h at 25°C in Krebs–Ringer–Hepes buffer containing 125 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2 mmol/l $CaCl_2$, 25 mmol/l Hepes, 6 mmol glucose, pH 7.4 and 10 μmol/l fura-2-AM. Fluorescence was measured in single cells excited with 340 and 380 nm light selected by two monochromators and directed through the stage of a Nikon inverted microscope equipped with a × 100 fluor objective. Emitted light was collected at 505 nm and monitored photometrically (SPEX Industries, Edison, NJ). Calibration of fura-2 fluorescence was performed as previously reported [14,15].

*Peptide sequences used in the studies reported here:

OPN-A	TVDTYDGRGDSVVYGLRSKS
OPN-2	GGGRGDS
BSP-A	YESENGEDRGDNYRAYED
BSP-2C	GGERGDN
BSP-1C	YNDGYEIYESENGED

Results

Effects of Osteopontin and Bone Sialoprotein Peptides on $[Ca^{2+}]_i$

Exposure of chicken osteoclast cultures on proteins that bind to $\alpha_v\beta_3$, intact osteopontin or vitronectin, produced prompt decreases in $[Ca^{2+}]_i$ (Fig. 1) in 100% of cells tested. A 20 amino acid osteopontin peptide containing the RGD sequence (OPN-A) produced results generally similar to osteopontin. In 66% of cells, OPN-A produced a reduction in $[Ca^{2+}]_i$ in which 56.3% of the cells tested with OPN-A produced a rapid decrease in $[Ca^{2+}]_i$ was followed by a rapid dose-dependent, with the half-maximal effect at 100 μg/ml (Fig. 2). In approximately 15% of cells studied OPN-A produced an increase in $[Ca^{2+}]_i$, and 19% of cells did not respond to OPN-A. It is unknown what caused a proportion of cells to exhibit an increase in $[Ca^{2+}]_i$ in

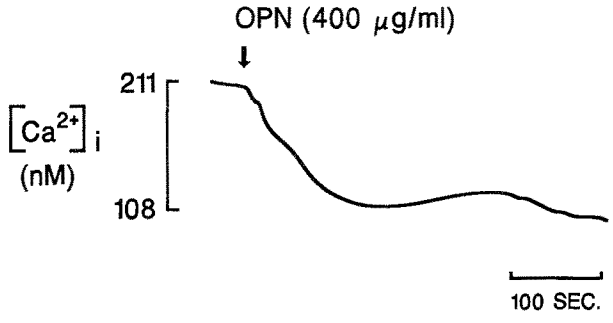

Fig. 1. Effect of intact osteopontin and related peptides from the osteopontin and bone sialoprotein sequences on osteoclast $[Ca^{2+}]_i$, determined in fura-2 loaded cells as described in methods. Osteopontin (OPN) produced immediate reductions in $[Ca^{2+}]_i$ which persisted for variable periods.

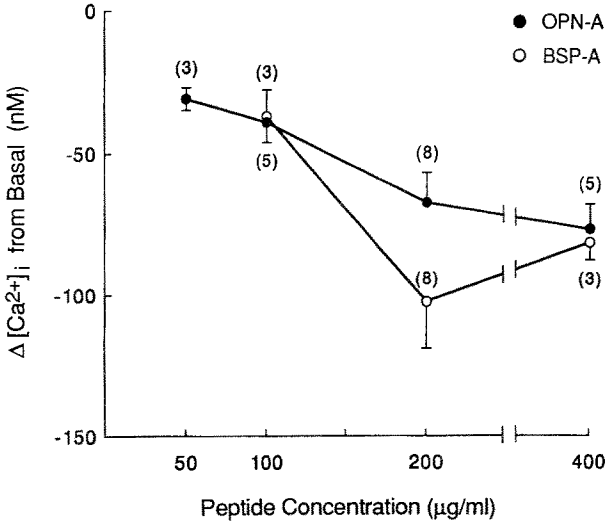

Fig. 2. Dose response of $[Ca^{2+}]_i$ to OPN-A and BSP-A peptides added to the media of chicken osteoclasts on glass coverslips as in Fig. 1. The peak reduction in $[Ca^{2+}]_i$ (nanomoles) is related to peptide concentration. The number of experiments for each point is given brackets.

response to OPN-A. The increase in $[Ca^{2+}]_i$ was never observed in experiments with intact osteopontin and vitronectin. Basal levels of $[Ca^{2+}]_i$ did not predict the type of response to OPN-A. Heterogeneity of response was limited to the small RGD-containing peptides and fibronectin, which binds to a different integrin. The effects of fibronectin were not affected by the monoclonal antibody LM609 (see below), suggesting that the increases in $[Ca^{2+}]_i$, stimulated by fibronectin and occasionally by RGD peptides were through an integrin other than $\alpha_v\beta_3$. The variability of the basal $[Ca^{2+}]_i$ was similar to that previously reported and was unrelated to the type of response observed.

The bone sialoprotein peptides BSP-A and BSP-2C produced effects on $[Ca^{2+}]_i$ similar to those of OPN-A (Fig. 3E and F). The effects of BSP-A on $[Ca^{2+}]_i$ tended to be more transient and consistent in stimulating a decrease than was OPN-A. Fig. 3F also demonstrates that submaximal stimulation of the decrease in $[Ca^{2+}]_i$ did not produce desensitization to a second stimulus after an interval of 5–10 min. The effects of the BSP-A were also dose-dependent, and the dose-response curve was similar to that of OPN-A (Fig. 2). BSP-IC which does not contain RGD, had no effect on $[Ca^{2+}]_i$.

The effects of osteopontin, OPN-A, BSP-A and BSP-2C on osteoclast $[Ca^{2+}]_i$ was inhibited by preincubation on the cells with LM609, the monoclonal antibody to the $\alpha_v\beta_3$ human vitronectin receptor (Fig. 3). The antibody itself exhibited an effect on $[Ca^{2+}]_i$ similar in direction but smaller in magnitude to osteopontin, OPN-A and BSP-A (Fig. 3A–C) in 57% of cells tested ($n = 14$). In the other experiments there was no effect. LM142, a monoclonal antibody to the α subunit of the same integrin (Fig. 4D), and a fragment of an irrelevant IgG (CD4b) (Fig.4E) not containing an RGD sequence were ineffective in either affecting $[Ca^{2+}]_i$ or inhibiting the effect of the OPN-A and BSP-A. The chicken integrin β_3 has been shown to react with LM609 [16], and LM609 has been shown by immunoprecipitation to surface-labeled chicken osteoclasts to recognize an $\alpha_v\beta_3$-like protein (Ross et al., unpublished data). In contrast LM142 recognizes an epitope not present in chicken osteoclast $\alpha_v\beta_3$ (Ross et al., unpublished data). The specificity of the effects of osteopontin and bone sialoprotein peptides on $[Ca^{2+}]_i$ was established by the failure of fibronectin to effect $[Ca^{2+}]_i$ in the osteoclasts.

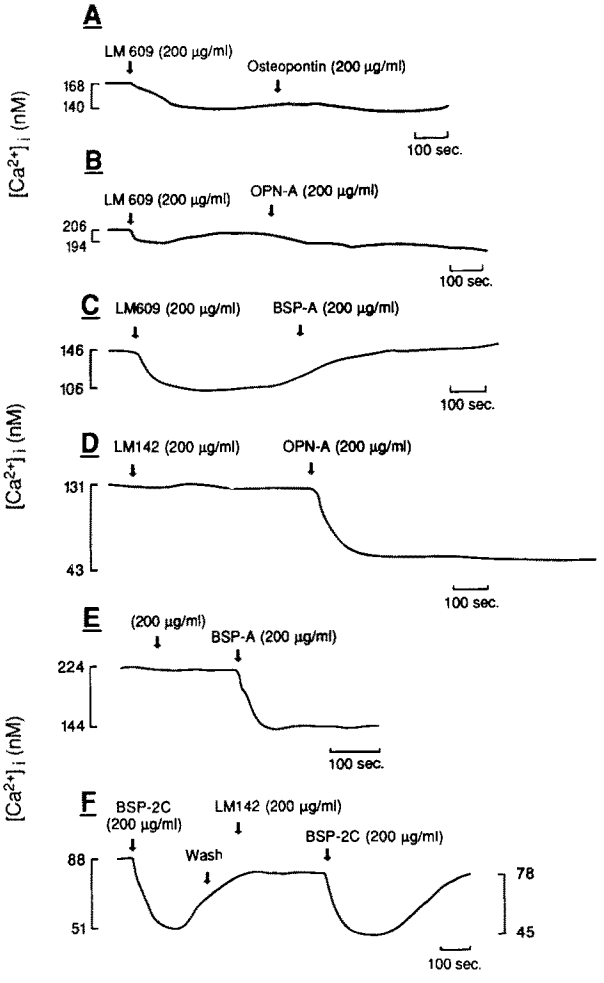

Fig. 3. Effect of the monoclonal antibody LM609 on changes in $[Ca^{2+}]_i$ induced by osteopontin and bone sialoprotein peptides. LM609 (antivitronectin receptor) completely inhibited the effects of intact of osteopontin (**A**), OPN-A (**B**) and BSP-A (**C**). However, the monoclonal antibody, LM142 (**D**,**F**) had no effect on changes in $[Ca^{2+}]_i$ stimulated by OPN-A or BSP-2C. CD4ß, from an irrelevant IgG, Has also failed to affect the changes in $[Ca^{2+}]_i$ produced by osteopontin (not shown) and bone sialoprotein peptides (**E**). The representative tracings demonstrate results observed with each antibody at least five times.

Effects of Osteopontin and Bone Sialoprotein Peptides on Bone Attachment and Bone Resorption

When osteoclasts were incubated in media containing osteopontin, OPN-A or BSP-A, binding of added ^{3}H-labeled bone particles was decreased. This suggests that occupancy of the $\alpha_v\beta_3$ integrin decreases binding of osteoclasts to bone. In addition, these proteins also decreased resorption of the added bone particles during a 24 h incubation period.

Mechanism of Osteopontin and Bone Sialoprotein Peptide Induced Decrements in Osteoclast $[Ca^{2+}]_i$

Incubation of osteoclasts with vanadate blocked the effect of the BSP-A and BSP-2C peptides on $[Ca^{2+}]_i$. This inhibitor of plasma membrane CA^{2+}-ATPase was previously shown to be effective in inhibiting proton-stimulated calcium efflux from osteoclasts [15]. When osteoclasts were stimulated with ryanodine, a substance known to block the muscle sarcoplasmic reticulum CA^{2+} channel in the open state [17], an increase in osteoclast $[Ca^{2+}]_i$, occurred as expected with release of intracellular Ca^{2+} stores, but the decrease in cytosolic calcium stimulated by BSP-A was unaffected. Similarly, addition of

thapsagargin, an inhibitor of the Ca-ATPase of the endoplasmic reticulum [18], produced an increase in $[Ca^{2+}]_i$ which slowly returned towards baseline after several minutes; the effects of OPN-A and BSP-A on $[Ca^{2+}]_i$ were not affected by thapsagargin. We found the calmodulin antagonist W_{13} to be effective in blocking the effects of BSP-A on OPN-A when osteoclasts were preincubated for 30 min with the antagonist. The Ca^{2+}-ATPase responsible for Ca^{2+} efflux from many cells is regulated both by calmodulin and by protein kinase A [19]. Thus, we also tried inhibiting stimulation by the BSP-A and OPN-A peptides with H_8, and inhibitor of protein kinase. H_8 had no effect on the stimulation of Ca^{2+} efflux.

Discussion

Our results demonstrate that osteopontin, vitronectin, and peptides synthesized from the human osteopontin and bone sialoprotein sequences, stimulate immediate reductions in cytosolic calcium of osteoclasts attached to glass coverslips – an effect previously associated with stimulation of podosome expression and bone resorption [15]. Since addition of these peptides in soluble form cannot be construed as equal to recognition of matrix proteins of bone surfaces, we sought functional assays to test the effects of adding matrix proteins in solution. We found that soluble osteopontin, OPN-A, and BSP-A decreased binding of added bone particles to osteoclasts similar to the effect of LM609. Thus, the soluble proteins mimicked the action of the monoclonal antibody LM609, competing with bone matrix for adhesion receptors (the $\alpha_v\beta_3$ integrin). Furthermore, the occupancy of receptors by soluble peptides decreased bone resorption, but to a lesser degree than did LM609, the monoclonal antibody to $\alpha_v\beta_3$. This might have been due to a greater decrease in $[Ca^{2+}]_i$ produced by osteopontin and related peptides than by LM609. This in turn could have increased the resorptive activity derived from vitronectin receptors occupied by proteins in the bone matrix.

The effects of osteopontin and related peptides to reduce $[Ca^{2+}]_i$ are specific in that they are completely inhibited by the LM609, the monoclonal antibody to $\alpha_v\beta_3$. They were not produced by matrix proteins without binding properties to the vitronectin receptor, such as fibronectin. A monoclonal antibody to human α_v, LM142, failed to exhibit any influence on the response to OPN-A or BSP-A, and irrelevant proteins, such as CD4β, also failed to influence the response to these peptides. The effects of OPN-A, BSP-A, BSP-2C and short RGD-containing sequences generally mimicked that of osteopontin, but occasionally stimulated an effect in the opposite direction. The results with BSP-1C demonstrated that the RGD sequence was necessary to stimulate the decrease in cytosolic calcium. Shorter peptides, GRGESP and GRGES, confirmed the results with BSP-1C, although some variability with GRGESP was observed.

The mechanism of calcium efflux further appeared to represent activation of the Ca^{2+}-ATPase, in that W_{13}, an inhibitor of calmodulin (a known regulator of the Ca^{2+}-ATPase), blocked the effect of OPN-A and BSP-A, while H_8, an inhibitor of protein kinase A, failed to block the effect of OPN-A. Additionally, inhibition of Ca^{2+} sequestration or release from intracellular stores had no effect on OPN-A or BSP-A stimulated reductions in $[Ca^{2+}]_i$.

The role of a decrease in $[Ca^{2+}]_i$ in osteoclast function requires clarification. Our previous studies [15] indicate that reduced $[Ca^{2+}]_i$ favors organization of actin filaments in podosomes and formation of these adhesion sites. Thus, a reasonable experimental model of osteoclast reorganization can be constructed beginning with recognition of bone matrix by specific integrins, leading to cytoskeletal reorganization and podosome formation, promoting establishment of the clear zone and ruffled border area. The role of immediate signals generated by integrin binding in contributing to these processes required further study. However, this is one of the first reports demonstrating a reduction in $[Ca^{2+}]_i$ associated with integrin binding, and the effects on osteoclast function appear to be important.

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