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Annexin V disruption impairs mechanically induced calcium signaling in osteoblastic cells

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Abstract

The mechanical environment of the skeleton plays an important role in the establishment and maintenance of structurally competent bone. Biophysical signals induced by mechanical loading elicit a variety of cellular responses in bone cells, however, little is known about the underlying mechanotransduction mechanism. We hypothesized that bone cells detect and transduce biophysical signals into biological responses via a mechanism requiring annexin V (AnxV). AnxV, a calcium-dependent phospholipid binding protein, has several attributes, which suggest it is ideally suited for a role as a mechanosensor, possibly a mechanosensitive ion channel. These include the ability to function as a Ca^{2+} selective ion channel, and the ability to interact with both extracellular matrix proteins and cytoskeletal elements. To test the hypothesis that AnxV has a role in mechanosensing, we studied the response of osteoblastic cells to oscillating fluid flow, a physiologically relevant physical signal in bone, in the presence and absence of AnxV inhibitors. In addition, we investigated the effects of oscillating flow on the cellular location of AnxV. Oscillating fluid flow increased both $[Ca^{2+}]_i$ levels and c-fos protein levels in osteoblasts. Disruption of AnxV with blocking antibodies or a pharmacological inhibitor, K201 (JTV-519), significantly inhibited both responses. Additionally, our data show that the cellular location of AnxV was modulated by oscillating fluid flow. Exposure to oscillating fluid flow resulted in a significant increase in AnxV at both the cell and nuclear membranes. In summary, our data suggest that AnxV mediates flow-induced Ca^{2+} signaling in osteoblastic cells. These data support the idea of AnxV as a Ca^{2+} channel, or a component of the signaling pathway, in the mechanism by which mechanical signals are transduced into cellular responses in the osteoblast. Furthermore, the presence of a highly mobile pool of AnxV may provide cells with a powerful mechanism by which cellular responses to mechanical loading might be amplified and regulated.

Keywords: Osteoblast; Annexin; Mechanotransduction; Calcium; Shear stress

Introduction

The mechanical environment of the skeleton is known to play an important role in the establishment and maintenance of structurally competent bone. However, the mechanisms by which biophysical signals exert such biological effects are unclear. It has been proposed that bone cells are ideally situated to detect and transduce mechanical signals and alter the composition of the bone matrix accordingly. Consequently, many cell culture models have been established to investigate the influence of mechanical loading on bone cells and as a result many physical stimuli have been shown to affect bone cell metabolism including direct cell deformation [1], fluid-induced shear stress [2–4], and bioelectric fields [5]. Such studies have shown that bone cells respond in a variety of ways to applied mechanical/biophysical perturbation. For example, fluid shear stress stimulates the production of intracellular and extracellular messengers such as intracellular calcium [2,3,6,7], IP_3 [8], cAMP [9] and PGE_2 [10], activation of G-proteins [11], NO production [4], alterations in the cytoskeleton [12], and gene

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expression [6,12–14]. Experiments such as these clearly show that bone cells are sensitive to their mechanical environment. It is unknown, however, how these physical signals are transduced into cellular responses.

The identity of a ''mechanoreceptor'' has, thus far, proved elusive. Membrane ion channels that open and close in response to mechanical force have been suggested to be ideal candidate mechanoreceptors. While these channels have been identified electrophysiologically to exist in a variety of cell types including bone cells [15–22], little is known about their molecular structure or how these channels function. In this study, we proposed to investigate the role of annexin V (AnxV), a putative ion channel, in bone cell mechanotransduction. AnxV has previously been proposed as a candidate for a mechanoreceptor, possibly as a mechanosensitive channel [23,24]. This suggestion was raised because recombinant AnxV has been shown to have the ability to bind to collagens in vitro, including collagen I [25,26] and, therefore, might be stimulated by deformation in the extracellular matrix. Interestingly, there is also evidence that AnxV may associate with cytoskeletal elements in addition to extracellular proteins and might therefore be regulated by changes in or contribute to cytoskeletal structure [27,28].

AnxV belongs to a group of structurally related Ca^{2+} binding proteins called annexins, most commonly known for their ability to bind to phospholipid membranes in a calcium-dependent manner. AnxV is the most abundant annexin in bone cells [29–31]. The ion channel activity of AnxV was first described by Rojas et al. [32] who showed that purified human AnxV interacted with phospholipid bilayers at the tip of a patch clamp pipette to form Ca^{2+} selective channels. To investigate a possible role for AnxV in bone cell mechanotransduction, we performed experiments in which bone cell responses to oscillating fluid flow were quantified in the presence and absence of AnxV inhibitors. We chose oscillating flow as our mechanical stimulus as it has been proposed as an important biophysical signal in mechanotransduction in bone [2,33–35]. Indeed recently, we and others have shown that fluid flow effects may be more stimulatory to bone cells than direct cell deformation [7,10,34].

As our cellular responses we have chosen to look at both intracellular calcium (Ca_i^{2+}) and c-fos protein levels. Intracellular calcium (Ca_i^{2+}) responses are one of the earliest and most ubiquitous responses to mechanical load. Ca_i^{2+} plays a critical role in a diverse number of cellular processes as a second messenger and, therefore, could have a powerful role in mechanotransduction. c-fos is an activator protein-1 (AP1) transcription factor subunit, which has been identified as an important player in bone metabolism [36] and its expression increased in bone cells in response to biophysical signals in vivo and in vitro [12,37,38]. To disrupt AnxV activity, we used an anti-AnxV blocking antibody and K201 (JTV-519), a 1,4-benzothiazepine derivative [39]. K201 (JTV-519) has been shown to inhibit the Ca^{2+} transport properties of AnxV [39] and block Ca^{2+} influx into matrix vesicles by inhibition of AnxV [40]. In addition, we examined the effects of oscillating fluid flow on the cellular location of AnxV.

Materials and methods

Cell culture

The human osteoblastic cell line MG63 was cultured in minimal essential medium (MEM; Life Technologies, Inc.) containing 25 mM HEPES, 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% penicillin/streptomycin (Life Technologies, Inc.) and maintained in a humidified incubator at 37°C with 5% CO_2. Two days before experiments, cells were subcultured onto either glass slides (75 × 38 × 1.0 mm, 3×10^5 cells/slide) for c-fos and relocation studies or quartz glass slides (Friedrich and Dimmock, Inc., Millville, NJ; 76 × 26 × 1.6 mm, 1.5×10^5 cells/slide) for $[Ca^{2+}]_i$ studies.

Oscillating fluid flow

Cells were exposed to fluid flow as previously described [2,6,7]. Briefly we used two custom-designed parallel plate flow chambers of similar construction but varying capacity. For short-term Ca_i^{2+} imaging experiments, we used a vacuum sealed flow chamber with a flow channel of 38 × 10 × 0.28mm. For the longer duration, c-fos and annexin relocation studies we used a larger chamber with a flow channel of 60 × 24 × 0.28 mm. This chamber utilizes a mechanical clamp to achieve a tight seal and can be assembled in a sterile environment and kept in an incubator throughout the flow exposure period. To deliver flow, we used Hamilton glass syringes custom mounted into a servo pneumatic loading frame (EnduraTec, Eden, Prairie, MN). To verify the output of the syringes, we attached an ultrasonic flow meter (Model 106, Transonic Systems, Ithaca, NY) to the chamber inlet. We used this system to generate sinusoidally oscillating flow in each flow chamber at 1 Hz and a peak shear stress of 20 dyn/cm^2. Flow media was MEM with 2% FBS and 25 mM HEPES. Experiments were performed to determine the time course of the induction of c-fos protein. At 60 min, the response was maximal and was not increased further after 90 min (data not shown). Therefore, in subsequent experiments, c-fos was measured at the 60-min time point.

Ca_i^{2+} imaging

Preconfluent cells were washed with phenol red-free MEM and 2% FBS solution at 37°C. Cells were then incubated with the dual excitation fluorescent Ca^{2+}-probe Fura-2 (acetoxymethyl ester 10 μM, Molecular Probes, Eugene, OR) for 30 min at 37°C. The cells were then

washed with fresh MEM and 2% FBS solution, the slide mounted on the parallel plate flow chamber and placed on an inverted fluorescence microscope (Nikon Diaphot 300) and left undisturbed for 30 min. Addition of 25 mM HEPES to the flow media to control for pH changes had no significant effect on the calcium response to flow ($P > 0.05$, $n = 5$).

The cells were illuminated as described previously [2,6,7,41,42]. A Metafluor imaging system (Universal Imaging, West Chester, PA) was used to sample and record the emitted light from the cells in the field of view once every 2 s (emission wavelength 510 nm) and Metafluor imaging software was used to subtract the background fluorescence from each image and to outline and calculate the 340:380 ratio of light emitted in response to excitation at 340 and 380 nm for each cell in the field of view, as this ratio reflects $[Ca^{2+}]_i$. A calibration curve was constructed by acquiring 340:380 values (background subtracted) for a series of solutions of known free Ca^{2+} concentration (0–39.8 μM, Molecular Probes) and 1 μM fura2 pentapotassium salt (Molecular Probes). This calibration curve was used to convert ratio values from individual cells into $[Ca^{2+}]_i$. To identify Ca^{2+} transients, we used a numerical procedure adapted from mechanical fatigue analysis, known as Rainflow Cycle Counting [43]. This simple algorithm reliably and automatically identifies and determines the amplitudes of spikes and transients in time history data even when superimposed over each other or in the presence of background noise [44]. We have previously used this algorithm to identify transients in Ca_i^{2+} in chondrocytes and bone cells [2,6,7,41,42]. We defined a response as a transient increase in Ca_i^{2+} of 80 nM or greater. Data were collected for 1 min at the start of each experiment before flow and then for a period of 3 min during flow.

AnxV inhibitors

To disrupt AnxV, bone cells were exposed to anti-AnxV antibody before flow (40 μg/ml for 24 h, rabbit polyclonal antibody, full-length AnxV, Santa Cruz, Santa Cruz, CA), control Ab (40 μg/ml for 24 h, COX -2, goat polyclonal antibody, carboxy terminus, Santa Cruz), or standard media for 24 h. All antibodies were prepared such that they were free of sodium azide. In other experiments, cells were exposed to K201 (JTV-519) before flow (100 μM for 30 min) to disrupt AnxV activity, DMSO (10 μl/100 ml for 30 min) as a vehicle control, or standard media for 30 min. K201 (JTV-519) was kindly supplied by Japan Tobacco Inc., Tokyo, Japan.

Cell fractionation

To determine the effects of OFF on AnxV relocation, MG63 cells were exposed to OFF at 1 Hz for either 1 or 3 h after which cell membrane, cytosolic, nuclear extract, and nuclear membrane fractions were isolated by differential centrifugation as previously described [45]. Control cells were mounted in flow chambers but not subjected to flow.

Briefly, cells were washed in homogenization buffer (10 ml of a salt solution containing 20 mM tetrasodium pyrophosphate, 20 mM sodium phosphate, 1 mM magnesium chloride, 0.1 mM EDTA, 300 mM sucrose, and 100 μl of a protease inhibitor cocktail containing 0.8 mM benzamidine, 1.0 mM iodoacetamide, 1.1 μM leupeptin, 0.7 μM pepstatin A, 23 μl of 0.23 mM PMSF, and 5 μl of 76.8 nM aprotinin), scraped from the glass surface and transferred to a dounce homogenizer. After 30–40 strokes with a loose fitting pestle, cells were centrifuged for 10 min at 700 × *g*. The pellet was resuspended in a sucrose solution (320 mM sucrose, and 3 mM $MgCl_2$) and used for nuclear fraction isolations. The supernatant was centrifuged for 15 min at 38,000 × *g* using a Beckman Ultracentrifuge (Sw55Ti, Beckman Instruments Inc., Palo Alto, CA) to separate the cell membrane (pellet) from the cytosol (supernatant). The cell membrane pellet was resuspended in 50 μl of homogenization buffer [45].

The nuclear pellet that had been resuspended in a 320 mM sucrose/3 mM $MgCl_2$ solution was split in two with half being used for nuclear extract isolation and half for nuclear membrane isolation. To obtain the nuclear extract, the sample was centrifuged at 700 × *g* and the pellet resuspended in 150 μl of a low-salt buffer solution (20 mM HEPES, pH 7.9, at 4°C, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). One hundred fifty microliters of a high-salt buffer was added to the solution in a dropwise fashion (20 mM HEPES, pH 7.9, at 4°C, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). The solution was then allowed to extract for 30 min while gently mixing. Following the extraction, the solution was centrifuged for 30 min at 25,000 × *g*, and the supernatant recovered as the nuclear extract. For the nuclear membrane isolation, the 320 mM sucrose/3 mM $MgCl_2$ solution was centrifuged at 700 × *g* for 10 min and the cell pellet was resuspended in 100 μl of 0.1 mM $MgCl_2$ and 50 μg/ml deoxyribonuclease I (DNase-I), then 400-μl digestion buffer [10 mM Tris–HCl, pH 8.5, containing 0.1 mM $MgCl_2$, 5 mM B-mercaptoethanol, and 10% sucrose (w/v)] was added and incubated at room temperature for 15 min. The reaction was terminated with an equal volume of ice-cold water added to the solution and centrifuged for 15 min at 38,000 × *g*. Subsequently, the pellet was resuspended in 500-μl digestion buffer containing 10 μg/ml of DNase I and incubated at room temperature for 20 min. This second reaction was terminated with an equal volume of ice-cold water and the solution was centrifuged for 15 min at 38,000 × *g*. The resulting pellet was taken as the nuclear membrane and was resuspended in phosphate-buffered saline (PBS). AnxV levels in each fraction were assessed by Western blot. Densitometry was used to quantify differences in

AnxV levels between flow and no flow control cell fractions.

Western blot analysis for AnxV and c-fos

Before electrophoresis, protein concentration was determined using a commercial assay, based on the Lowry method (Bio-Rad). Equal amounts of protein (5 μg) were loaded onto 7.5% SDS-polyacrylamide gels, resolved by electrophoresis, and transferred to nitrocellulose membranes that were blocked in 5% nonfat milk in Tris-buffered saline [1× TBST (Bio-Rad) with 0.05% Tween-20] for 1 h at room temperature. The membranes were then incubated for 2 h at room temperature with a 1:200 dilution of rabbit anti-AnxV polyclonal antibody (Santa Cruz) in blotto (5% non-fat milk in TBST), or for 1 h at room temperature with a 1:500 dilution of rabbit anti-c-fos polyclonal antibody (Santa Cruz) in blotto. The membranes were washed three times in TBST and incubated with goat anti-rabbit IgG linked to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min diluted 1:3000 for AnxV and for 1 h diluted 1:2500 for c-fos. After three additional washes with TBST and one wash in 1× TBS, the membranes were soaked in ECL detection reagents for 1 min (Amersham, UK). The sheet was then exposed to X-ray film.

Data analysis

From the Rainflow analysis of the calcium response data, the mean ± SE was computed for each treatment. To compare observations of control, control Ab, and anti-AnxV Ab, a repeated-measures ANOVA was utilized with Bonferroni/Dunn post hoc testing. Similarly, an ANOVA was utilized to compare control, DMSO control, and K201 (JTV-519)-treated groups. ANOVAs were used to compare both the percentage of cells responding and mean amplitude of responding cells.

To compare c-fos levels between flow and no flow conditions and to assess differences in AnxV protein levels in the cell fractions, paired *t* tests were performed. Data were normalized to the no flow control for each treatment studied or for each cell fraction and expressed as mean ± SE. Optical densities, as determined using densitometry, were used to quantify protein levels.

Results

Role of AnxV in OFF-induced Ca_i^{2+} responses

As we have previously shown [2,6,7,41,42,46], osteoblastic cells respond to OFF with a robust and transient increase in Ca_i^{2+} when flowed in standard media (Ctrl) (Figs. 1A, 2A). This response was attenuated in the presence of AnxV blocking antibodies (AnxV Ab) or K201 (JTV-519) (Figs. 1A, 2A). The response was unaffected in the presence of control antibodies (Ctrl Ab) or a DMSO control (DMSO Ctrl) (Figs. 1A and 2A); 75.5 ± 3.9% of the cells in standard media responded to fluid flow with an increase in Ca_i^{2+} and 63.6 ± 12.8% responded in the presence of a control antibody (Fig. 1B). However, in the presence of AnxV blocking antibodies, the response was significantly reduced to 27.3 ± 11.3% (Fig. 1B). Similarly, 72.4 ± 6.9% and 69.1 ± 8.1% of cells responded in control and DMSO treated cells, whereas in the presence of K201 (JTV-519), the response was significantly reduced to 30.4 ± 8.3% (Fig. 2B). There was no significant difference with regards to mean Ca_i^{2+} response amplitudes under the conditions tested. Mean Ca_i^{2+} response amplitudes were 152 ± 23, 190 ± 16, and 133 ± 30 nM for control, control Ab, and blocking Ab, respectively (Fig. 1B). Mean Ca_i^{2+} response amplitudes were

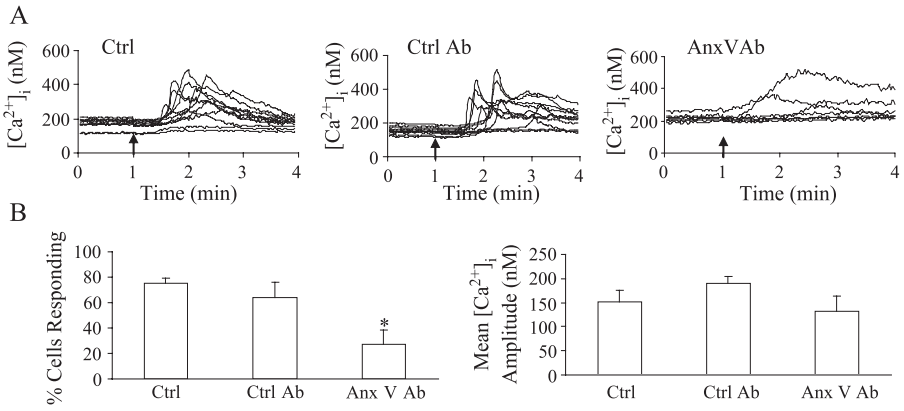

Fig. 1. (A) Effect of OFF (20 dyn/cm^2, 1 Hz) on Ca_i^{2+} in MG63 cells in standard media (Ctrl), the presence of a control antibody (Ctrl Ab), and AnxV blocking antibody (AnxV Ab). Each line represents the Ca_i^{2+} signal from a single cell and the arrow indicates the onset of flow. (B) Mean percentage of cells showing a Ca_i^{2+} response and mean amplitude of the response to OFF (20 dyn/cm^2, 1 Hz) in control media (Ctrl, $n = 6$ slides), the presence of a control antibody (Ctrl Ab, $n = 4$ slides), or anti-AnxV antibody (AnxV Ab, $n = 6$ slides). All bars represent the mean percentage of cells responding ± SEM, or mean Ca_i^{2+} response amplitude of those cells responding ± SEM. *$P < 0.05$, statistically significant difference from control.

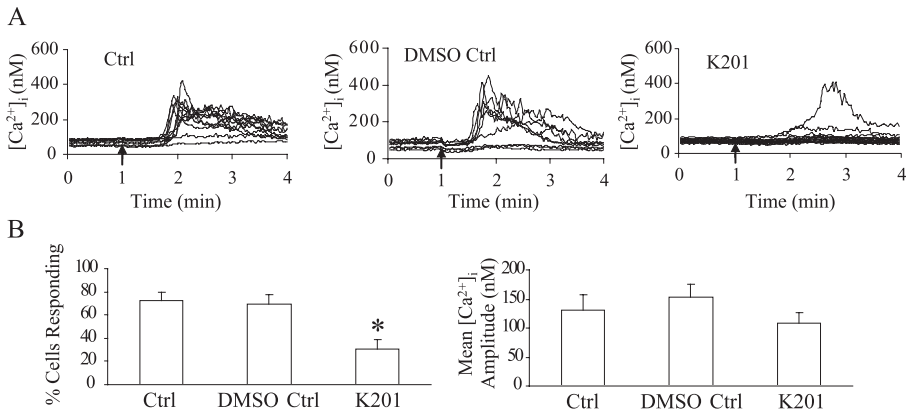

Fig. 2. (A) Effect of OFF (20 dyn/cm^2, 1 Hz) on Ca_i^{2+} in MG63 cells in standard media (Ctrl), the presence of DMSO as a vehicle control (DMSO Ctrl), and K201 (JTV-519). Each line represents the Ca_i^{2+} signal from a single cell and the arrow indicates the onset of flow. (B) Mean percentage of cells showing a Ca_i^{2+} response and mean amplitude of the response to OFF (1 Hz, 20 dyn/cm^2) in control media (Ctrl, $n = 7$ slides), DMSO (DMSO Ctrl, $n = 6$ slides), or K201 (JTV-519) (K201, $n = 5$ slides). All bars represent the mean percentage of cells responding ± SEM, or mean Ca_i^{2+} response amplitude of those cells responding ± SEM. *$P < 0.05$, statistically significant difference from control.

132 ± 26, 154 ± 22, and 108 ± 18 nM for control, DMSO control, and K201 (JTV-519), respectively (Fig. 2B).

Role of AnxV in OFF-induced c-fos protein level increases

c-fos protein levels were significantly increased in cells exposed to OFF for 60 min compared to no flow controls when cells were in standard media (Ctrl) or exposed to control antibodies (Ctrl Ab). This response was inhibited, however, in cells exposed to AnxV blocking antibodies (AnxVAb) ($P > 0.05$) (Fig. 3). Normalized protein levels, expressed as optical densities, were 1.51 ± 0.10 and 2.14 ± 0.27, in cells exposed to OFF in standard media and in the presence of control Ab, respectively (Fig. 3, the data were normalized to no flow control levels). In the presence of anti-AnxV Ab, cells exposed to flow demonstrated significantly decreased c-fos levels, 1.05 ± 0.06 (Fig. 3). Oscillating flow-induced increases in c-fos protein expression were also significantly attenuated in cells pre-incubated for 30 min before flow with 100 μM K201 (JTV-519) (data not shown).

AnxV relocation in response to OFF

There was no significant difference between the 1- and 3-h data for AnxV location, and therefore, these data were combined. With the data normalized to no flow control, AnxV protein levels significantly increased in cells exposed to OFF vs. no flow controls at both the cell and nuclear membranes ($n = 7$) (Fig. 4). There was no significant increase in AnxV protein levels in either the cytosol or nuclear extract in OFF-stimulated compared to no flow controls ($n = 7$) (Fig. 4).

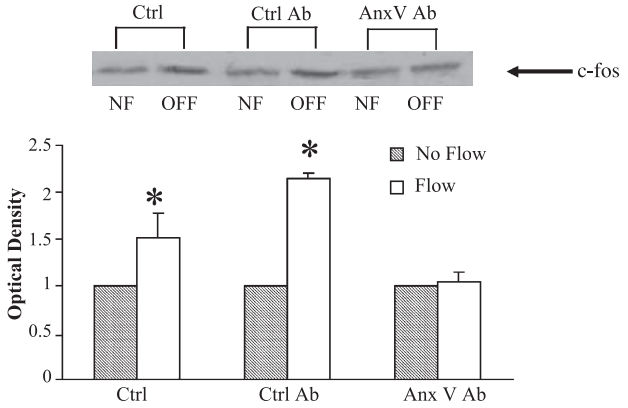

Fig. 3. Optical densities and representative Western blot for c-fos protein levels in cells exposed to standard media (Ctrl, $n = 5$), control antibody (Ctrl Ab, $n = 3$), or anti-AnxV antibody (AnxV Ab, $n = 3$) before 60 min of no flow (NF) or OFF (1 Hz, 20 dyn/cm^2). All bars represent the mean optical density normalized to no flow under the same conditions ± SEM. *$P < 0.05$, statistically significant difference from no flow.

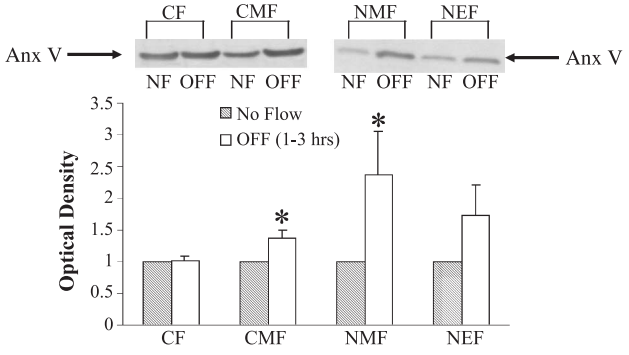

Fig. 4. Optical densities and representative Western blot for no flow control (NF) cell fractions and cell fractions exposed to OFF (1 Hz, 20 dyn/cm^2) for 1–3 h; cytosolic fraction (CF), cell membrane fraction (CMF), nuclear membrane fraction (NMF), and nuclear extract fraction (NEF). Data were normalized to the no flow control for each cell fraction. All bars represent the mean optical densities ± SEM. *$P < 0.05$, statistically significant difference from control.

Discussion

Our data confirm that osteoblast-like cells are sensitive to biophysical signals, and in addition, suggest that AnxV is involved in the mechanism by which mechanical signals elicit intracellular responses such as an increase in $[Ca^{2+}]_i$ and changes in c-fos protein levels. The mechanism, or mode of action of AnxV in the mechanosensory pathway, however, is less clear.

Although much is known of the structure of AnxV, significantly less is known about its cellular functions. Annexins as a family have been associated with a wide variety of functions including membrane organization, exocytosis, endocytosis, ion channel regulation, ion channel activity, and membrane to cytoskeleton linkage [47]. One possibility, therefore, is that AnxV is acting as a calcium influx pathway in a role as an ion channel. Indeed, there is accumulating evidence to support the role of AnxV as a calcium selective channel. The ion channel activity of AnxV was first described by Rojas et al. [32] who showed that purified human AnxV interacted with phospholipid bilayers at the tip of a patch clamp pipette to form Ca^{2+} selective channels. These channels were blocked by the lanthanide ion, La^{3+}, but were unaffected by the voltage-sensitive L-type Ca^{2+} channel blocker nifedipine [32]. Subsequently, electrophysiological studies have shown that ion channel activity of purified AnxV was very similar to channel activity observed when AnxV-rich matrix vesicles were fused with lipid bilayers [48]. Further confirmation that AnxV may act as a Ca^{2+} entry pathway in the cell membrane came from Ca^{2+} influx studies using large unilamellar vesicles (LUV). AnxV, added to suspensions of LUV, resulted in an influx of Ca^{2+} ions as measured using fura-2, which increased as the concentration of AnxV was raised [49]. Furthermore, neither denatured AnxV nor an AnxV mutant lacking the N-terminal domain elicited a Ca^{2+} influx [49]. Both Zn^{2+} and a novel 1,4-benzothiazepine derivative, K201 (JTV-519), were subsequently shown to block this Ca^{2+} influx [48,50]. In addition, AnxV also mediated Ca^{2+} influx into liposomes, which was inhibited by anti AnxV antibodies and by zinc and cadmium [51]. This evidence strongly suggests that AnxV forms Ca^{2+} channels in vitro. Subsequently, AnxV has been shown to mediate Ca^{2+} influx into lymphocytic B-cells [52]. In that study, Kubista et al. [52] demonstrated that peroxide induced AnxV membrane insertion and that peroxide-induced Ca^{2+} influx in B-cells was inhibited in cells lacking AnxV. These data, taken together, support the idea that AnxV may play a role as a calcium channel in vivo. If AnxV is acting as a channel in bone cells then this raises questions as to how the channel is activated.

Interestingly, AnxV has been proposed as a candidate for a mechanosensitive channel [23,24]. This suggestion was raised because recombinant AnxV has been shown to have the ability to bind to collagens in vitro, including collagen I [25,26] and, therefore, might be stimulated by deformation in the extracellular matrix. For extracellular components to play a role, AnxV must function as a transmembrane protein or be present extracellularly. Indeed, there is evidence that AnxV can form a hexameric transmembrane complex [53] and AnxV has been found localized on the surface of chick chondrocytes and fibroblasts [25,54,55]. AnxV has also been shown to be released extracellularly by bone cells [29], though whether it functions as a transmembrane complex or extracellularly is unknown. There is evidence that AnxV can bind to cytoskeletal elements. For example, AnxV was shown to interact and co-localize with actin in human platelets [27] and an AnxV-containing complex, which was also recognized by antibodies against actin, was extracted from detergent-solubilized platelet membranes [27]. A recent study has also shown that AnxV binds to the β_5 integrin subunit [28]. Evidence that AnxV may associate with cytoskeletal elements in addition to extracellular proteins suggests that AnxV may function most effectively in a mechanosensing apparatus as a transmembrane complex. An ion channel with both extracellular and intracellular binding components, which would serve to effectively transmit force to the channel protein, might be an effective configuration for a mechanotransduction apparatus. Such an apparatus has been suggested to operate in mammalian inner ear hair cells, where deflection of apical stereocilia, which are connected to one another via extracellular tip links, opens ion channels [56–58]. In addition, the channel is thought to be linked to the actin cytoskeleton via a molecular complex composed of myosin [59].

In this study, we showed that oscillating fluid flow increased both $[Ca^{2+}]_i$ and c-fos protein levels in osteoblasts and that disruption of AnxV decreased both responses. It is possible, therefore, that both responses may be part of the same signal transduction pathway. Indeed, there is strong evidence to suggest a link between Ca^{2+} and c-fos in many cell types [60]. c-fos induction has been shown to be blocked by external Ca^{2+} chelation, specific inhibitors of L-type Ca^{2+} channels, and calmodulin agonists [61–64]. Membrane depolarization has been shown to lead to c-fos expression by the activation of L-type voltage-sensitive calcium channels [63,65]. Additionally, intracellular calcium levels have also been shown to stimulate c-fos mRNA expression [66] as has a Ca^{2+} ionophore [67,68].

In addition, our data suggest that mechanical signals also modulate the cellular location of AnxV. Interestingly, annexins are not permanently associated with phospholipid membranes, but exist in dynamic equilibrium between the cytosol and both nuclear and plasma membranes. Studies have shown that a rise in Ca_i^{2+} causes relocation of AnxV to both the nuclear and plasma membranes in many cell types including bone cells, fibroblasts, and neuroblastoma cells [29,69–71]. While these studies used supraphysiological levels of Ca^{2+} to induce relocation, both physiological levels of Ca^{2+} and the physiological agonist thrombin were shown to induce relocation and binding of AnxV to platelet membranes [72,73] and ATP caused the relocation of AnxV

to both nuclear and plasma membranes in bone cells [29]. Experiments have shown that the recruitment of AnxV to membranes is rapid, occurring after less than 10 s of Ca_i^{2+} being elevated in bone cells and fibroblasts [29,69]. Of particular interest to this study is that annexins have been shown to relocate to the plasmalemma in response to mechanical stimulation in parenchyma cells of *Bryonia dioica* plants [74]. The presence of a highly mobile pool of AnxV may provide cells with a powerful mechanism by which mechanically induced Ca^{2+} signaling might be amplified and regulated.

In summary, our data support the idea of AnxV functioning as a Ca^{2+} channel, or a component of the Ca^{2+} signaling pathway, in the mechanism by which mechanical signals are transduced into an appropriate cellular response in the osteoblast.

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