Modulation of intracellular calcium signaling and mitochondrial function in cultured osteoblastic cells by dexamethasone and celecoxib during mechanical stimulation

S. Schuchmann, M. Wiontzek, G.-R. Burmester, F. Buttgereit

Medizinische Klinik mit Schwerpunkt Rheumatologie und Klinische Immunologie, Universitätsklinikum Charité, Humboldt Universität Berlin, Germany.

Abstract

Objective
Evaluation of potentially therapeutically relevant effects of dexamethasone and celecoxib on crucial parameters of bone physiology during and following mechanical stimulation in cultured osteoblasts.

Methods
An in vitro mechanical stimulation model based on the rat osteogenic cell line UMR-106 was developed to investigate glucocorticoid (dexamethasone) and selective COX-2 inhibitor (celecoxib) induced changes in the intracellular calcium concentration ([Ca$^{2+}$]$_{i}$) and mitochondrial membrane potential ($\Delta$Ψ$_{m}$). Microfluorometric techniques were applied to monitor [Ca$^{2+}$]$_{i}$ (Fura-2 AM) and $\Delta$Ψ$_{m}$ (rhodamine 123) online as the main parameters of the actual cellular metabolism.

Results
Basal [Ca$^{2+}$]$_{i}$ was found to be 92.2 ± 3.7 nM and increased up to 711 ± 27 nM during mechanical stimulation under controlled conditions. Addition of 100 nM dexamethasone or 10 µM celecoxib for 24 h suppressed the increase in [Ca$^{2+}$]$_{i}$ significantly to 530 ± 33 nM and 546 ± 39 nM, respectively. Dexamethasone significantly reduced, but celecoxib significantly increased the spread velocity of the mechanically induced intracellular calcium wave. Furthermore, the effects induced by dexamethasone were amplified during the inhibition of gap junction coupling and diminished following enlarged gap junction coupling.
In contrast, the modulation of gap junction coupling exerted only a minor influence on the celecoxib-induced effects. Short-term application of dexamethasone (5 min) caused significantly reduced mechanically induced depolarization of the mitochondrial membrane, but long-term application (24 h) did not. In contrast, only the long-term application (24 h) of celecoxib caused such depolarization.

Conclusion

The observed effects of dexamethasone and celecoxib on mechanically induced changes in [Ca$^{2+}$]$_{i}$ and $\Delta$Ψ$_{m}$ are suggested to result from short-term changes in membrane characteristics and long-term changes in protein synthesis. This indicates an influence of these drugs on cell-to-cell communication and metabolism that may be therapeutically relevant.

Key words
Osteoblast, mechanical stimulation, intracellular Ca$^{2+}$ concentration, mitochondrial membrane potential, gap junction, glucocorticoid, celecoxib.
Introduction

Destructive alterations of articular cartilage and bone tissue in rheumatic diseases are based on a disturbed balance between aggressive and protective factors as mediated by different cytokines and growth factors. Direct cell-to-cell communication by osteoblasts are considered to be a critical component of this process, since osteoblastic gap junctions have been shown to be involved in the perception of extracellular signals and mechanical transmission (1, 2), induction of cytokines (3), hormonal responsiveness (4) and the modulation of gene expression (5). The bone-forming osteoblastic multicellular network is based on gap junctions formed by connexin 43 and connexin 45 (6-8). Disruption in gap junction coupling induced by inflammation, but also by pharmacological intervention may therefore take part in the disturbance of bone remodelling and the destruction of bone tissue. With regard to the effect of therapeutic agents, recently concerns have been raised about the use of nonsteroidal anti-inflammatory drugs in patients with skeletal injury. These drugs inhibit cyclooxygenases that have been suggested to play a possible role in bone repair. In this study we posed the question as to whether drugs with effects on the COX-2 enzyme such as glucocorticoids and selective COX-2 inhibitors may also interfere with gap junction coupling.

Glucocorticoids are known to inhibit bone formation on the basis of a decrease in osteoblastic cell replication and differentiation, and an increase in the apoptosis of mature osteoblasts (for an overview, see 9). They have also been shown to stimulate differentiated functions of osteoblasts (10) and to induce the promotion of osteoblastic phenotype and matrix mineralization in osteoblastic cell cultures (11, 12). However, the influence of glucocorticoids on essential gap junction regulated processes during cell differentiation is still poorly understood. Investigations on cultured hepatocytes showed a glucocorticoid-induced increase in gap junction expression, and changes in gap junction signaling and growth control (13-15). Studies on astrocytes confirm a potent modulation of intracellular Ca$^{2+}$ signaling by glucocorticoids, but could not demonstrate a change in gap junction function (16,17). Investigations on osteoblasts showed the correlation of cellular coupling in the osteoblastic network with the level of gap junctions protein expression (18). In this study, we used dexamethasone, which is known to produce strong genomic, but also intense rapid nongenomic glucocorticoid effects. Moreover, we designed our study to distinguish between these different glucocorticoid effects on mechanically induced changes in osteoblasts.

The inhibition of COX-2 synthesis represents just one aspect of the therapeutic action of glucocorticoids. In contrast, selective COX-2 inhibitors such as celecoxib act almost exclusively by inhibiting this enzyme. COX-2 catalyzes the reactions of arachidonic acid to form prostaglandin E$_2$ (19), but its detailed role in bone physiology and remodelling is still unclear (19-21). Prostaglandins are known to have stimulatory as well as inhibitory effects on bone metabolism and therefore COX-2 may play a role in bone formation and resorption (22,23). It is clear however that COX-2 mediates the induction of bone formation in response to mechanical loading (24) and regulates mesenchymal cell differentiation into osteoblasts (25). Therefore, several studies suggest that COX-2 may play a critical role in bone repair and fracture healing (25, 26).

Different mechanical stimuli have been suggested to activate physiological osteoblastic cell responses (27). Studies have investigated the continuous mechanical load induced by continuous fluid flow (28-30) or biaxial stretching (31, 32), but have also analyzed acute, short-lived mechanical loading by centrifugation (33) or short fluid jet (27,34-36). Short-lived mechanical stimuli are preferable to study gap junction activation and intracellular Ca$^{2+}$ signaling (34). Therefore, we used here the short fluid jet model based on the rat osteogenic cell line UMR-106 to investigate the short- and long-term effects of a dexamethasone and a celecoxib during and following mechanical stimulation of osteoblasts. Microfluorometric techniques were applied to monitor online...
changes in intracellular calcium $[Ca^{2+}]$, and mitochondrial membrane potential ($\Delta \Psi_m$) as the main parameters of the actual cellular metabolism. In this set-up, TNF-$\alpha$ and IL-1$\beta$ were used to simulate inflammatory effects.

**Materials and methods**

**UMR-106 cell culture**

For the experiments presented here, we used the clonal osteoblastic cell line UMR-106. The UMR-106 cells were a generous gift from Dr. M. Wiemann (Institut für Physiologie, Universität Essen). The UMR-106 cell line was initially obtained from a $^{32}$P-induced rat osteosarcoma (37). Several studies have demonstrated the osteoblastic phenotype of the UMR-106 cell clone, including its responsiveness to calcitropic hormones and cytokines (38-40) and UMR-106 cells have been shown to possess typical osteoblastic gap junctions (6, 8). The cells were maintained in MEM (minimum essential medium; Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 $\mu$M glutamine, 100 $\mu$M Na-pyruvate and non-essential amino acids (all Gibco) at 37°C in a 5% CO$_2$ atmosphere. Standard antibiotics (10 units/ml penicillin, 10 $\mu$g/ml streptomycin, Gibco) were used during cultivation. Cells were grown to 80–90% confluence in 24-well plates (Nunc, Roskilde, Denmark) and on poly-D-lysine-coated 12 mm glass coverslips (5–6 x 10$^4$ cells/coverslip). Cells were treated with the compounds noted at the concentrations indicated and for various time periods (5–30 min, 12 h, 24 h, 48 h).

**Fluorescence measurements**

Microfluorometric experiments were carried out using an imaging system based on an inverse microscope (IX50, Olympus, Hamburg, Germany) with 10x, 40x and 60x objectives (numerical aperture 0.3, 1.15 and 1.20, respectively; Olympus), a xenon light source with a monochromator (Polychrome II; TILL-Photonics, Martinsried, Germany) and a charge-coupled device camera (Imago, TILL-Photonics). Image hardware was controlled by an IBM-compatible computer running commercial software developed by TILL-Photonics. Figure 1 shows a schematic representation of the imaging system.

Microfluorometric experiments were conducted on cultured UMR-106 osteoblastic cells using an inverse microscope coupled to an imaging system. Mechanical stimulation was applied using a short jet from a glass pipette (10-15 kPa for 2-3 s), which was placed approximately 100 µm above the cell monolayer. The emission fluorescence signals $\lambda_{em}$ were separated from the excitation wavelength $\lambda_ex (> \lambda_{em})$ by a suitable dichroic mirror and detected using a CCD camera.

**Fig. 1.** Schematic representation of the experimental setup. Microfluorometric experiments were conducted on cultured UMR-106 osteoblastic cells using an inverse microscope coupled to an imaging system. Mechanical stimulation was applied using a short jet from a glass pipette (10-15 kPa for 2-3 s), which was placed approximately 100 µm above the cell monolayer. The emission fluorescence signals $\lambda_{em}$ were separated from the excitation wavelength $\lambda_ex (> \lambda_{em})$ by a suitable dichroic mirror and detected using a CCD camera.
and saturating levels of Ca\(^{2+}\) and hence the minimum (R\(\text{min}\)) and maximum (R\(\text{max}\)) fluorescence ratios and also the ratio of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated fluorescence excited at 380 nm (\(\beta\)), required for the equation (43):

\[
[\text{Ca}^{2+}] = \frac{K_i \beta R - R_{\text{min}}}{R_{\text{max}} - R}
\]

The value of the in vitro dissociation constant \(K_i\) in the described system was close to the reported data (224 nM; [see 44]). The imaging recording frequency was adapted for the different experiments at between 0.5 and 30 images/s. The fluorescence signals (Fura-2 AM and Rhodamine 123) from single osteoblasts were analyzed offline by adjusting individual regions of interest (ROI) using NIH Image (version 1.61, http://rsb.info.nih.gov/nih-image).

Mechanical stimulation

Measurements of [Ca\(^{2+}\)], and the mitochondrial membrane potential during mechanical stimulation were performed on UMR-106 cells growing on glass coverslips. For the experiments, osteoblasts were carefully transferred from the media-containing culture dish to the recording chamber and flooded with 2 - 3 mm oxygenated Ringer solution above the coverslip. The recording chamber (volume 2.8 ml) was continuously perfused with oxygenated Ringer solution using a peristaltic pump (Minipuls; Gilson, Villiers, France) at a rate of 1.0 - 1.2 ml/min. The osteoblasts were kept under these conditions for at least 30 minutes until starting the experiments. During this time no change in the intracellular calcium concentration was observed. Subsequently, a small group of these cells (approximately 3 - 5) were mechanically stimulated using a short fluid jet (oxygenated Ringer solution, 10-15 kPa for 2-3 s), which was manually applied via a glass pipette and a connected injection system. The short fluid jet was controlled by a barometric measurement unit, which was connected to the injection system. Pipettes were produced from borosilicate glass capillaries (GC 150 TF, Harvard Apparatus, Edenbridge, U.K.) using a vertical electrode puller (P-87, Sutter Instruments Company, Novato, USA). At the top the outer diameter of the pipettes ranged from 1 to 2 µm and was controlled by electrical resistance measurements daily (45). The pipettes were filled with Ringer solution and positioned approximately 100 µm above the cell layer using a micromanipulator, obtaining a minimal step-width of 10 µm (Narashige, London, UK). The described system ensured the production of a precise and repeatable stimulus due to the constant diameter between the cells and the pipette tips, the velocity of the fluid jet and the constant angle of the pipette.

Drugs and solutions

To analyse the basal level and vitamin D (1.25-cholecalciferol) induced changes in [Ca\(^{2+}\)], dye-loaded cells were incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS. During the stimulation experiments, cells were continuously superfused with oxygenated (95\% O\(_2\), 5\% CO\(_2\)) Ringer solution, containing in mM: NaCl 124; KCl 3; NaH\(_2\)PO\(_4\) 1.25; MgSO\(_4\) 2; CaCl\(_2\) 2; NaHCO\(_3\) 26; glucose 10; pH 7.35. For a certain number of experiments nominal Ca\(^{2+}\)-free Ringer solution (in mM: NaCl 124, KCl 3, NaH\(_2\)PO\(_4\) 1.25, MgSO\(_4\) 1.8, NaHCO\(_3\) 26, glucose 10; pH 7.4) supplemented with the Ca\(^{2+}\)-chelator ethylene glycol-bis(aminomethylene)-N,N,N,N-tetracetic acid (0.5 mM EGTA) was used. For another number of experiments the following drugs were added to the culture media or Ringer solution: 100 µM carbonoxolone, 10 nM 1,25-cholecalciferol (vitamin D), 10 nM NH\(_4\)Cl, 50 nM thapsigargin (all: Sigma, Deisenhofen, Germany). In order to stimulate UMR-106 cells the inflammatory cytokines TNF-\(\alpha\) (100 ng/ml) and IL-1\(\beta\) (10 ng/ml) (both: Sigma, Deisenhofen, Germany) were added to the culture media according to similar experimental procedures as described by different authors (21, 46-48).

Unstimulated UMR-106 osteoblasts

Controls: For each data point at least 4 - 5 × 10\(^4\) UMR-106 osteoblasts out of eight separate cultures (8 wells for each culture) were studied. The basal [Ca\(^{2+}\)]\(\text{in}\) was found at 92 ± 4 nM in unstimulated UMR-106 cells, which was stable within in 48 h (30 min: 91 ± 4 nM; 48 h: 94 ± 4 nM).

Dexamethasone: In order to sample dexamethasone induced short- and long-term changes in basal [Ca\(^{2+}\)], 100 nM dexamethasone were added to the culture medium. A significant increase in [Ca\(^{2+}\)], up to 133 ± 4 nM (dexamethasone vs. control p = 0.009) was observed in UMR-106 osteoblasts within 30 min after application. However, a significant decrease in [Ca\(^{2+}\)] in UMR-106 osteoblasts was found within 48 h in the presence of dexamethasone compared with control condition (61 ± 7

Statistical analysis

Values are given as means ± standard deviation (SD). Statistical differences were assessed by ANOVA and Bonferroni/Dunn contrast. P values < 0.05 were accepted as significant.

Results

Changes in basal [Ca\(^{2+}\)], in the presence of dexamethasone and celecoxib

To analyse the baseline concentration and changes in the intracellular calcium concentration ([Ca\(^{2+}\)]) in UMR-106 osteoblasts following TNF-\(\alpha\)/IL-1\(\beta\) stimulation, cells were cultured in 24-well plates containing 1 ml culture medium. Following incubation with the calcium indicator Fura-2 AM under sterile conditions, unopened 24-well plates were placed on the experimental setup and [Ca\(^{2+}\)], was directly measured using the microfluorometric technique (see Fig. 1). Figure 2 illustrates the [Ca\(^{2+}\)], in unstimulated (Fig. 2 A, C) and TNF-\(\alpha\)/IL-1\(\beta\)-stimulated (Fig. 2 B, D) UMR-106 osteoblasts in the presence or absence of dexamethasone or celecoxib.

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Mechanically induced changes in $[\text{Ca}^{2+}]$, and mitochondrial membrane potential ($\Delta \Psi_m$) in UMR-106 osteoblasts

Intracellular calcium waves and the resulting depolarisation of the mitochondrial membrane potential ($\Delta \Psi_m$) were elicited by mechanical stimulation of single osteoblasts in monolayer of cultured UMR-106 cells in a precise and repeatable manner (see Materials and Methods). Fluorescence imaging technique enables the measurement of $[\text{Ca}^{2+}]$, and $\Delta \Psi_m$ from direct mechanically stimulated cells as well as indirectly activated cells. In Figure 3 images from typical experiments show the spreading out of an intracellular calcium wave and the resulting changes in $\Delta \Psi_m$ following mechanical stimulation by a short jet via a glass pipette. Individually adjusted ROIs from two single cells with a distance of approximately 100 µm were used to analyse the changes in $[\text{Ca}^{2+}]$, and $\Delta \Psi_m$.

nM, vs. control $p = 0.024$).

Celecoxib: An initial $[\text{Ca}^{2+}]$ increase after 30 min was also observed in the presence of 10 µM celecoxib, which dropped back to $[\text{Ca}^{2+}]$ under control conditions after 48 h (30 min: 118 ± 5 nM, vs. control $p = 0.028$; 48 h: 94 ± 8 nM).

$\text{TNF-}\alpha/\text{IL-1}\beta$ stimulated UMR-106 osteoblasts

Controls: The inflammatory cytokines TNF-α (100 ng/ml) and IL-1β (10 ng/ml) were added for 4 hours to the culture media to stimulate UMR-106 cells. Under these conditions, the $[\text{Ca}^{2+}]$, increased up to 119 ± 4 nM (vs. unstimulated control $p = 0.017$) and decreased significantly within 48 h to 104 ± 4 (vs. stimulated control $p = 0.037$).

Dexamethasone: The addition of dexamethasone elicited a rapid and long-lasting decrease in $[\text{Ca}^{2+}]$, in stimulated UMR-106 osteoblasts (30 min: 102 ± 4 nM, vs. stimulated control $p = 0.033$; 48 h: 61 ± 5 nM, vs. stimulated control $p = 0.001$).

Celecoxib: In contrast to the result with dexamethasone, $[\text{Ca}^{2+}]$, did not differ significantly following the application of celecoxib compared with stimulated control condition (celecoxib 30 min: 128 ± 4 nM, 48 h: 95 ± 4 nM).

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Fig. 2. Changes in $[\text{Ca}^{2+}]$, in the presence of vitamin D (1,25-cholecalciferol), dexamethasone and celecoxib.

A and B: Dexamethasone (100 nM) effected a short-term (30 min) increase in $[\text{Ca}^{2+}]$, which significantly decreased within 48 h compared to control conditions. Following stimulation with TNF-α (10 ng/ml) and IL-1β (100 ng/ml), basal $[\text{Ca}^{2+}]$, was slightly increased, but the short- and long-term effects of dexamethasone on $[\text{Ca}^{2+}]$, were unchanged.

C and D: Celecoxib (10 µM) also effected a short-term (30 min) increase in $[\text{Ca}^{2+}]$, which returned to values comparable to control conditions within 48 h. No significant change was observed in the presence of the inflammatory cytokines TNF-α (10 ng/ml) and IL-1β (100 ng/ml).

Fig. 3. Mechanically induced intracellular calcium wave in the UMR-106 cell monolayer.

Transmission images show the UMR-106 cell monolayer with two marked osteoblasts (top, left; scale bar 10 µm) and the position of the glass stimulation pipette (top, middle). The marked osteoblast next to the top of the pipette (single asterisk) represents the directly stimulated cell (1st cell), and the second marked osteoblast (double asterisk) the indirect stimulated cell (2nd cell). The distance between the two osteoblasts was approximately 100 µm.

A. The plots indicate the changes in $[\text{Ca}^{2+}]$, following mechanical stimulation (top, right). Grayscale images indicate the spread of the induced intracellular calcium wave (the time points provided correspond to the time after mechanical stimulation).

B. The plots indicate the changes in mitochondrial membrane potential following mechanical stimulation (top, right). Grayscale images indicate the site and extent of mitochondrial membrane depolarization (the time points indicated correspond to the time after mechanical stimulation).
Mechanically induced changes in \([\text{Ca}^{2+}]_{i}\), and \(\Delta\Psi_{m}\) in the presence of dexamethasone and celecoxib 
\([\text{Ca}^{2+}]_{i}\); Figure 5 summarizes the changes in \([\text{Ca}^{2+}]_{i}\), following mechanical stimulation in the presence of 100 nM dexamethasone or 10 µM celecoxib in UMR-106 cells at a distance of approximately 100 µm (1st and 2nd cells). Application for short periods (5 min) of either dexamethasone or celecoxib were found to slightly elevate the mechanical induced \([\text{Ca}^{2+}]_{i}\), increase (dexamethasone: 1st cell 757±35 nM, 2nd cell 744±42 nM; celecoxib: 1st cell 784±40 nM, 2nd cell 769±48 nM).

A significant delay in the \([\text{Ca}^{2+}]_{i}\), increase was measured in the 2nd cell in the presence of dexamethasone, but not celecoxib for 5 minutes compared to control conditions (14.4±2.3 s, vs. control p = 0.042; 11.3±3.4 s, vs. control p = 0.423). The observed changes in the delay in \([\text{Ca}^{2+}]_{i}\), increase were found to be more marked after the addition of dexamethasone and celecoxib for 24 h to the medium. Dexamethasone effectuated a prolonged delay, i.e. a reduction in the velocity of the intracellular calcium wave (18.6±3.8 s, vs. control p < 0.001, vs. 5 min p = 0.044). In contrast, a significantly reduced delay in the \([\text{Ca}^{2+}]_{i}\), increase was found in the 2nd cell with a distance of 100 µm in the presence of celecoxib (8.7±2.7 s, vs. control p = 0.048, vs. 5 min p = 0.041). Furthermore, generally reduced \([\text{Ca}^{2+}]_{i}\), increases were measured after the 24 h application of dexamethasone (1st cell 530±33 nM, 2nd cell 477±35 nM) and celecoxib (1st cell 546±39 nM, 2nd cell 523±41 nM) compared with control conditions.

Figure 4. Mechanically induced changes in \([\text{Ca}^{2+}]_{i}\), are dependent on intracellular calcium stores, but not on the extracellular calcium concentration.

Application of 50 nM thapsigargin, an inhibitor of calcium-ATPase of the endoplasmic reticulum, for 30 minutes induced the depletion of intracellular calcium stores. Mechanical stimulation caused significantly reduced \([\text{Ca}^{2+}]_{i}\), increases (black bars) compared to control conditions (white bars). The extracellular calcium concentration had no influence on the mechanically induced \([\text{Ca}^{2+}]_{i}\), increases (grey bars).
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33 nM, vs. control p < 0.001; celecoxib 211±22 nM, vs. control p < 0.001).

This clearly demonstrates that mechanically induced [Ca\textsuperscript{2+}] increases require calcium release from intracellular stores in cultured UMR-106 osteoblasts.

Relevance of gap junction function for mechanically induced changes in [Ca\textsuperscript{2+}]

Controls: In order to investigate the relevance of gap junction function for mechanically induced intracellular calcium waves in cultured UMR-106 cells, gap junctions were pharmacologically modulated. To test whether mechanically induced intracellular calcium waves in osteoblasts travel via gap junctions, the gap junction blocker carbenoxolone (100 µM) was applied (54). Reduced gap junction coupling effectuated in the UMR-106 cell monolayer a reduction in the [Ca\textsuperscript{2+}], increase at a distance of 100 µm from the stimulation site (1st cell 688±39 nM, 2nd cell 438 ± 37 nM; p < 0.001; Fig. 6A). Moreover, a significant delay in the [Ca\textsuperscript{2+}], increase was observed during gap junction blockade (19.8 ± 3.1 s, vs. control p < 0.001; Fig. 6A).

Gap junctions are known to be sensitive to changes in intracellular pH (55). Therefore, gap junction coupling is enhanced during intracellular alkalosis (55) and is decreased during intracellular acidosis (56). In the present study 10 mM NH\textsubscript{4} was applied to increase the intracellular pH (alkalosis). Under this condition, similar [Ca\textsuperscript{2+}] increases were measured in the directly stimulated cell as well as in the 2nd cell (1st cell 706 ± 44 nM, 2nd cell 698 ± 44 nM). The delay in [Ca\textsuperscript{2+}] increase was significantly reduced compared with control conditions, indicating an amplified intracellular calcium wave (8.2 ± 1.3 s, p = 0.043). Finally, during intracellular alkalosis the [Ca\textsuperscript{2+}] increase was prolonged compared with control conditions (see Fig. 6B).

In a further step, we investigated the influence of gap junction coupling in the presence of dexamethasone or celecoxib on the intracellular calcium wave.

Dexamethasone: In the presence of dexamethasone, blocking gap junctions caused a drastic reduction in the mechanically induced [Ca\textsuperscript{2+}] increase in the 2nd cell and decelerated the spread of the intracellular calcium wave compared with control conditions (312 ± 26 nM, vs. control p = 0.033; 27.2 ± 3.4 s, vs. control p=0.023). Enhanced gap junction coupling on the other hand partly compensated for the dexamethasone induced reduction in the [Ca\textsuperscript{2+}] increase and enabled a nearly normal velocity in the intracellular calcium wave compared with control conditions (588 ± 25 nM; 11.8 ± 1.8 s).

Celecoxib: In the presence of celecoxib, only minor effects during both decreased and increased gap junction coupling were measured. Thus, the [Ca\textsuperscript{2+}] increases during gap junction blockade and activation were comparable with control conditions (carbenoxolone: 1st cell 578±35 nM, 2nd cell 565±32 nM; NH\textsubscript{4}: 1st cell 582 ± 32 nM, 2nd cell 604...
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Celecoxib stabilized the spread of intracellular calcium waves and caused a delay in the \([\text{Ca}^{2+}]_{\text{i}}\) increase comparable with control conditions also in the presence of the gap junction blocker or activator (carbenoxolone 12.3 ± 2.2 s, \(\text{NH}_4\) 10.2 ± 2.0 s). These results indicate that dexamethasone amplifies, whereas celecoxib equalizes, the gap junction effects on intracellular calcium waves.

Cytokine stimulation induced a significant reduction in mechanically induced \([\text{Ca}^{2+}]_{\text{i}}\) increase (control 711 ± 27 nM, TNF-\(\alpha/\text{IL-1}\beta\) 602 ± 31 nM, \(p = 0.039\); see Fig. 7 A). This reduction in the mechanically induced \([\text{Ca}^{2+}]_{\text{i}}\) increase following TNF-\(\alpha/\text{IL-1}\beta\) stimulation was unaffected by changes in gap junction coupling (carbenoxolone: control 688 ± 39 nM, TNF-\(\alpha/\text{IL-1}\beta\) 512 ± 38 nM; \(\text{NH}_4\): control 706 ± 44 nM, 588 ± 32 nM; see Fig. 7 A). The delay in the \([\text{Ca}^{2+}]_{\text{i}}\) increase was significantly enlarged following cytokine stimulation under control conditions and during gap junction blockade using carbenoxolone (control: 10.8 ± 1.7 s vs. TNF-\(\alpha/\text{IL-1}\beta\) 15.2 ± 2.4 s, \(p = 0.009\); carbenoxolone: 19.8 ± 3.1 s vs. TNF-\(\alpha/\text{IL-1}\beta\) 41.3 ± 8.6 s, \(p < 0.001\); \(\text{NH}_4\): 8.2 ± 1.3 s vs. TNF-\(\alpha/\text{IL-1}\beta\) 10.3 ± 4.7 s, \(p = 0.224\); see Fig. 7 D).

In the presence of either 100 nM dexamethasone (24 h) or 10 \(\mu\)M celecoxib (24 h) no significant difference in the \([\text{Ca}^{2+}]_{\text{i}}\) increase was observed between TNF-\(\alpha/\text{IL-1}\beta\)-stimulated and control osteoblasts, even during gap junction modulation (see Fig. 7 B and C). Likewise, dexamethasone had no significant influence on the delay in the \([\text{Ca}^{2+}]_{\text{i}}\) increase, except during during gap junction blockade using carbenoxolone (27.2 ± 3.4 s vs. TNF-\(\alpha/\text{IL-1}\beta\) 44.8 ± 6.8 s, \(p < 0.001\); see Fig. 7 E). In contrast, celecoxib effected comparable delays in the \([\text{Ca}^{2+}]_{\text{i}}\) increase under all investigated conditions (see Fig. 7 F).

The results indicate that dexamethasone and celecoxib suppress a cytokine induced reduction in the \([\text{Ca}^{2+}]_{\text{i}}\) increase in UMR-106 osteoblasts. Moreover, celecoxib, but not dexamethasone prevents a cytokine-induced elevation in the delay of the \([\text{Ca}^{2+}]_{\text{i}}\) increase during gap junction blockade.

**Discussion**

In these studies we found that the glucocorticoid dexamethasone and the
selective COX-2 inhibitor celecoxib at therapeutically relevant concentrations influence the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) and the propagation of mechanically induced intracellular calcium waves in cultured UMR-106 osteoblastic cells. The results demonstrate an effect on gap junctional coupling, which was stronger due to dexamethasone compared with celecoxib. The different influence on gap junctions was enhanced following cytokine stimulation using TNF-\(\alpha\) and IL-1\(\beta\). Finally, mechanically induced depolarization of the mitochondrial membrane was found to be changed in the presence of dexamethasone and celecoxib, which indicates alterations in mitochondrial function in these cells.

Bone matrix deformation in response to mechanical load is known to be an important regulator of bone turnover. The mechanically induced signal cascade in osteoblasts has not been completely clarified, although cell to cell communication via gap junctions has been proposed to play a central role (57). Furthermore, several intracellular signal pathways have been suggested to be involved in the response of mechanical load, like stretch-activated ion channels (58), calcium release from intracellular stores (59), the increased production of prostaglandin and nitric oxide (60) and activated extracellularly regulated kinase (ERK) (61).

The effects of mechanical loading on osteoblastic activation and differentiation can be simulated in osteoblastic cell cultures (60, 62). In order to investigate alterations in the mechanically induced signal cascade in the presence of dexamethasone and celecoxib, we studied changes in [Ca\(^{2+}\)]\(_i\) and the mitochondrial membrane potential during mechanical stimulation in cultured UMR-106 cells. UMR-106 cells have been shown to predominantly express the gap junction protein connexin 45, which has been suggested to allow the passage only of small ions (7). The fast calcium waves following mechanical stimulation in these cells requires calcium release from intracellular stores, as demonstrated by Jørgensen et al. (35) and in this study (see Figs. 3 and 5). Jørgensen and co-workers suggested that gap junctional communication modulates calcium wave propagation, which requires the activation of ATP-sensitive purinergic receptors (35). Nevertheless, mechanical stimulation has been shown to induce ATP release from osteoblastic cells (27). Therefore, mechanically induced intracellular calcium waves were sustained by both gap junctional communication and the activation of purinergic receptors by increased ATP release (see also 36).

In the present study, application of dexamethasone (24 h) decreased mechanically induced [Ca\(^{2+}\)]\(_i\) and reduced the propagation velocity of intracellular calcium waves. The dexamethasone dosage used (100 nM) is therapeutically relevant (49) and has been shown to enhance osteoblastic differentiation, but reduce the number of cells (63). Since dexamethasone has been demonstrated
not to influence ATP release and KCl induced \([\text{Ca}^{2+}]\), (64), we suggest that the reported changes were affected by decreased gap junction communication. This hypothesis is supported by the observation that these effects were enlarged following the blockade of gap junction coupling and equalized after the activation of gap junction coupling. Oscillatory mechanical stimulation using fluid flow has been demonstrated to enlarge gap junctional communication via increased prostaglandin release and COX-2 expression (65,66). Furthermore, the effect of mechanical stimulation on gap junctions was blocked in the presence of a COX inhibitor (65). In accordance with these studies, we found reduced \([\text{Ca}^{2+}]\) increases following mechanical stimulation in the presence of celecoxib. In contrast, the celecoxib induced acceleration of intracellular calcium waves propagation was not affected by modulation of gap junction and therefore may result from a direct activation of purinergic receptors on UMR-106 cells.

The inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) are known to influence second messenger systems such as intracellular calcium and accordingly cellular activity (67). Therefore, the inhibitory effect of cytokines on \([\text{Ca}^{2+}]\), and gap junctional communication result partly from similar targets. Our data show this close relationship between the inhibitory effect of cytokines on the mechanically induced \([\text{Ca}^{2+}]\) increase and intracellular calcium propagation. In contrast to dexamethasone, selective COX-2 inhibition was able to block the cytokine-induced escalation, which has also been demonstrated for very low celecoxib concentrations (68). The results suggest that TNF-\(\alpha\) and IL-1\(\beta\) induced effects have no influence on gap junction communication, but require prostaglandin synthesis. A concentration of 10 \(\mu\)M celecoxib has been shown to inhibit prostaglandin synthesis, but has no influence on COX-2 expression (69).

The dexamethasone and celecoxib induced effects on \([\text{Ca}^{2+}]\), and \(\Delta\Psi_m\) demonstrated in this study depend partly on the duration of application. We measured significant effects of dexamethasone within 5 minutes. This is much too rapid to be explained by the classical (genomic) mechanism of action. The genomic effects require the binding of a glucocorticoid to its cytosolic receptor and it is reckoned that up to 30 minutes elapse before significant changes occur on the level of the regulator proteins. However, regulator proteins also need time to cause the respective, mostly therapeutically relevant changes on the cellular, tissue, organ and organism levels (70). There is no doubt that some effects of the glucocorticoids occur much more rapidly. This fact has been observed repeatedly in several different cellular systems and also in our study here. Which mechanisms mediate these rapid glucocorticoid effects? At present, it is assumed that rapid glucocorticoid effects can be mediated: (1) by specific interactions with the cytosolic glucocorticoid receptor such as the quick release of small molecules; (2) by non-specific (physicochemical) interactions with cellular membranes; and (3) by specific interactions with membrane-bound glucocorticoid receptors.

Therefore, the rapid dexamethasone induced increase in \([\text{Ca}^{2+}]\), may result from a facilitated calcium release from the endoplasmatic reticulum (and perhaps by mitochondria) due to a dexamethasone-induced increase in membrane permeability for calcium. This increased ion permeability may well also be the reason for the observed decreased depolarization of the mitochondrial membrane. Thus, glucocorticoid-induced apoptosis has been demonstrated to be controlled by mitochondrial functions such as mitochondrial transition pores (71). In contrast, a delayed spread of the intracellular calcium wave may result from a dexamethasone induced reduction in functional gap junction coupling and/or from different direct effects on the plasma membrane, respectively. In a previous study we showed that the glucocorticoid methylprednisolone increases the permeability of the mitochondrial membrane for protons, but decreases the permeability of the plasma membrane for calcium ions (72). Alternatively, other non-genomic glucocorticoid effects such as the activation of membrane-bound receptors or the rapid release of small proteins from the cytosolic glucocorticoid-receptor complex after glucocorticoid binding should be discussed as being responsible for the effects we describe here. In addition to the short-term effects, in this study we describe long-term effects as induced by dexamethasone following application for hours. These effects clearly reflect genomic consequences, i.e. changes in the synthesis of regulatory proteins (see above). Under these conditions, dexamethasone induced effects mainly result in disturbances in the intracellular calcium homeostasis, as indicated by the reduction in basal \([\text{Ca}^{2+}]\).

Comparable with dexamethasone, celecoxib related effects showed a duration dependence on \([\text{Ca}^{2+}]\), and \(\Delta\Psi_m\), but with different consequences. The short-term application of celecoxib has been demonstrated to increase \([\text{Ca}^{2+}]\), in a dose-dependent manner (73). A rapid and significant increase, however, requires 5- to 10-fold higher celecoxib concentrations compared with the concentration used in the present study. Accordingly, we found no significant celecoxib induced short-term effects following mechanical stimulation. Different results followed the long-term application (> 24 h) of celecoxib. Apart from a reduction in the mechanically induced \([\text{Ca}^{2+}]\), increase, an elevation in the spread of the intracellular calcium wave was detected. The observed changes in \(\Delta\Psi_m\) presumably result from the changes in \([\text{Ca}^{2+}]\). Nonsteroidal anti-inflammatory drugs, and particularly COX-2 inhibitors, have been suggested to possess anti-cancerous effects (for an overview see 74). Changes in \([\text{Ca}^{2+}]\), and modulations in \(\Delta\Psi_m\) are proposed to play a central role in these anti-cancerous effects. Thus, selective COX-2 inhibition has been shown to inducing apoptosis via mitochondrial pathways (75). Furthermore, celecoxib has been demonstrated to inhibit endoplasmic reticulum Ca\(^{2+}\)-ATPase (51). Therefore, the long-term application of celecoxib may disturb \([\text{Ca}^{2+}]\), even at very low concentrations. Finally, in contrast to the TNF-\(\alpha\)/IL-1\(\beta\) induced effects, celecoxib related changes in \([\text{Ca}^{2+}]\), and
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$\Delta \Psi_m$ seem to take place irrespective of COX-2 expression, a finding which has also been demonstrated for celecoxib induced apoptotic cell death (52).

Overall, this study indicates an influence of the glucocorticoid dexamethasone and the COX-2 inhibitor celecoxib on osteoblastic cell communication and metabolism and may help to elucidate their complex roles in bone physiology.

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