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Intracellular calcium dynamics dependent on defined microtopographical features of titanium

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ABSTRACT

Detailed insights into the complex cellular behavior at the biomaterial interface are crucial for the improvement of implant surfaces with respect to their acceptance and integration. The cells perceive microtopographical features and, in consequence, rearrange their adhesion structures like the actin cytoskeleton and adaptor proteins. But little is known about whether these altered cellular phenotypes have consequences for intracellular calcium signaling and its dynamics. To elucidate if an artificial, geometrical microtopography influences calcium ion (Ca^{2+}) mobilization in osteoblasts, human MG-63 cells were stained with the calcium dye Fluo 3-acetoxymethyl ester and set on defined silicon–titanium (Ti) arrays with regular pillar structures (P5, 5 × 5 × 5 μm) and compared with planar Ti. To induce an immediate calcium signal, cells were stimulated with adenosine 5′-triphosphate (ATP). Interestingly, osteoblasts on micropillars expressing a shortened actin cytoskeleton were hampered in their calcium mobilization potential in signal height as well duration. Even the basal level of the intracellular Ca^{2+} concentration was reduced, which was accompanied by a disturbed fibronectin synthesis. The expression of the voltage-sensitive calcium channels $Ca_v1.2$, $Ca_v1.3$ (L-type) and $Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$ (T-type) as well as the signaling proteins phospho-AKT and phospho-GSK3α/β remained unaffected on pillars. The topography-dependent calcium dynamics observed here provide new insights into how topographical cues alter cell functions – via the intracellular Ca^{2+} signaling.

1. Introduction

Cells are able to perceive the physico-chemical properties of their surroundings and to pass these signals into the cell to modulate adhesion [1], organization of adhesion structures [2], proliferation [3] or production of extracellular matrix proteins accordingly [4–6]. However, these complex cellular interactions at the interface to a biomaterial are not yet fully understood. Adhesion receptors like integrins are mechanotransducers [7] and interact specifically with extracellular matrix proteins [8,9]. These initiate the formation of an intracellular focal contact [10,11] which connects the actin cytoskeleton with integrins [12,13]. This stage is characterized by an active process of actin polymerization [14] (Fig. 1). The actin cytoskeleton is responsible for both the morphological and functional cellular response [15] and is essential in transduction of external signals and forces into the cell [16,17]. This integrin-actin interaction triggers signal transduction [7,18], for example cascades of different signaling molecules [19] like the activation of Serine/Threonine (Ser/Thr) protein kinases (Akt; protein kinase B) and the GSK3α/β (glycogen synthase kinase 3) component of the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway (Fig. 1): This component is involved in cellular processes such as migration, proliferation and differentiation [20–22]. Also, intracellular calcium ions (Ca^{2+}) are responsible, as second messengers, for the transduction of signals [19,23,24] and act on different cellular processes such as the regulation of cytoskeleton components [25,26], bone remodeling [27,28], proliferation [29] or expression of extracellular matrix proteins [19,30,31]. The basal intracellular concentration of Ca^{2+} in the cell (10^{-7} M) is regulated within a very narrow range [32]. The short-term repetitive increase in free Ca^{2+} in the cytoplasm is induced by a variety of external factors such as mechanical stress [19], ATP molecules [23] or inositol-3,4,5-triphosphate (IP3) [32,33]. In intracellular calcium mobilization, different transport molecules in the cell membrane

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like gap junctions [1,34], ligand-gated ion channels (G-protein receptors) or voltage-sensitive calcium channels (VSCC) [27,30] are involved (Fig. 1). The heteromultimers VSCCs are represented in many cell types and usually consist of 5 subunits [27,35], where the main α_1-subunit forms the channel and possesses the voltage sensor [36]. Based on their electro-physiological and pharmacological properties, 10 different α_1-subunits [37] are known and enable the classification into high-voltage activated- (HVA; P/Q, N, R and L-types) and low-voltage activated channels (LVA, T-types) [37,38]. The Ca^{2+} influx is mediated by the tiny and transient (T)-type VSCCs: $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}), $Ca_v3.3$ (α_{1I}) and the large and long lasting (L)-type VSCCs: $Ca_v1.1$ (α_{1S}), $Ca_v1.2$ (α_{1C}), $Ca_v1.3$ (α_{1D}), and $Ca_v1.4$ (α_{1F}) [35,39]. The $Ca_v1.2$ primarily allows the calcium influx in proliferating osteoblasts [40]. VSCCs are associated with the actin cytoskeleton [30], are even dependent on the stage of actin polymerization [31] and require integrin-actin interaction [8]. Intracellular calcium mobilization via G-protein receptors is carried out by the activation *inter alia* of ATP [23,34]. Here, the phospholipase-C pathway (PLC) is activated, wherein the cleavage product IP3 diffuses to IP3-receptors (IP3-R) in the membrane of the endoplasmic reticulum (ER) [34,41] (Fig. 1). These induce the release of intracellular Ca^{2+} from this intracellular store [37,38,42].

The transmission of a topographical stimulus into the cell and in consequence intracellular signaling are not fully elucidated. The use of a uniformly textured material surface provides the advantage of a surface description with few characteristics and facilitates the analysis of the direct affect of the topography on the cell physiology [6,43,44]. Previous studies on defined microstructures showed the influence on cell morphology and formation of actin cytoskeleton which adapts strictly to the underlying pattern (mimicry) [43,45–47]. Matschegewski et al. [43] were able to determine an intense cell architecture-cell function dependency due to alterations in actin formation. However, the intracellular molecular processes leading to altered cell functions could not be further elucidated. In this study we will focus on how the microtopographic stimulus triggers signal transduction, mainly calcium signaling with its high regulatory importance in human osteoblasts.

2. Materials and methods

2.1. Microstructured surfaces

2.1.1. Material preparation

Geometrically microstructured samples were fabricated using deep reactive-ion etching technology (DRIE) (Center for Microtechnologies ZFM, Chemnitz University of Technology, Germany) on silicon wafers and coated with an additional 100 nm titanium (Ti) layer as previously described [45]. The fabricated arrays feature sharp-edged regular cubic pillars with the dimension of 5 × 5 × 5 µm (length × width × height) and a pitch width of 10 µm (P5) (Fig. 2). Unstructured planar references served as controls (Ref). The single wafer size was 10 × 10 mm. Before use in the experiments, the specimens were sterilized with 70% ethanol for 10 min and rinsed in phosphate-buffered saline (PBS) (PAA Laboratories, Pasching, Austria). The quality of the sample surface was examined by field-emission scanning electron microscopy (FE-SEM Supra 25, Carl Zeiss, Jena, Germany) at 3 kV using secondary electron (SE2) detectors.

2.1.2. Surface roughness measurement

Surface roughness measurements were performed with a JPK Nanowizard II AFM (Atomic force microscope; JPK Instruments, Berlin, Germany) in standard tapping mode using silicon cantilevers with a tip radius of less than 8 nm (42 N/m nominal force constant; NCH, NanoWorld, Neuchâtel, Switzerland). Roughness statistics were derived from the 1 × 1 µm topographic AFM image (n = 5). The R_a value was determined from the mean of five 2D measurements. The R_a value was calculated from 2D profiles that were obtained from the 3D scan. Roughness evaluation was conducted with Gwyddion freeware (version 2.31, copyright D. Nečas and P. Klapetek, http://www.gwyddion.net).

2.1.3. Contact angle measurement

The surface wettability of the samples was studied measuring the sessile drop contact angle of distilled water (0.5 µl, p.a., Carl Roth GmbH + CO. KG, Karlsruhe, Germany) with a contact angle meter (OCA 15EC, DataPhysics Instruments GmbH, Filderstadt, Germany). Drop images were acquired with the digital camera of the OCA and the contact angles were assessed with the attached software (SCA 20, V.4.1.11 build 1018) (n = 5/10).

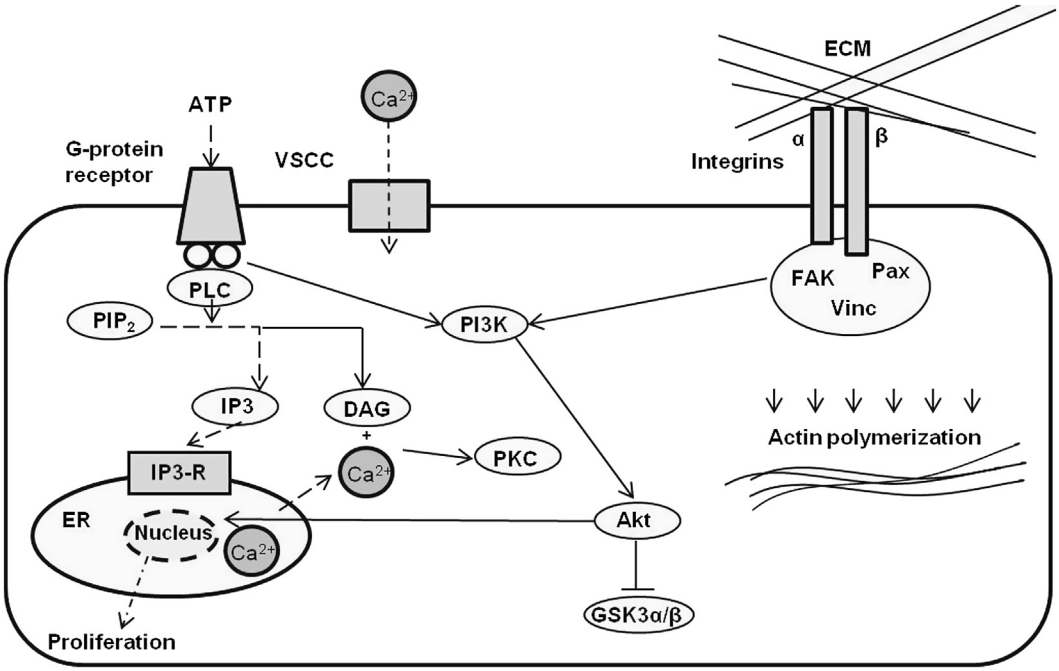

Fig. 1. Scheme of molecular mechanisms in signal transduction. The regulation of intracellular calcium signaling occurs through voltage-sensitive calcium channels (VSCC) or ligand-gated calcium channels (G-protein receptor). The G-protein-mediated signaling pathway triggered by ATP (adenosine triphosphate) initiates the phospholipase-C (PLC) pathway. Subsequently, inositol triphosphate (IP3) binds to IP3 receptors (IP3-R) in the membrane of the endoplasmic reticulum (ER) and causes the release of calcium ions (Ca^{2+}). Akt: Protein kinase B, DAG: Diacylglycerol, ECM: Extracellular matrix, FAK: Focal adhesion kinase, GSK: Glycogen synthase kinase, Pax: Paxillin, PI3K: Phosphatidylinositol 3-kinase, PIP2: Phosphatidylinositol 4,5-bisphosphate, PKC: Protein kinase C, Vinc: Vinculin.