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BG60S dissolution interferes with osteoblast calcium signals

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Abstract We investigated the influence of extracellular calcium concentration, caused by the dissolution of a bioactive glass with 60% of silicon (BG60S), on intracellular calcium (Ca_i^{2+}) signals and expression of inositol 1, 4, 5-triphosphate receptors ($InsP_3R$) in primary culture of osteoblasts. We found that BG60S caused an increase in Ca_i^{2+} signals in this cell type. Additionally, osteoblasts preincubated in the presence of BG60S showed an increase in Ca_i^{2+} when cells were stimulated with vasopressin. On the other hand, a decrease in Ca_i^{2+} signals were observed in osteoblasts pre-treated with BG60S and stimulated with KCl. We furher found that in osteoblasts, the type I $InsP_3R$ is preferentially distributed in the nucleus while the type II $InsP_3R$ in the cytoplasm. Preincubation of osteoblasts with BG60S altered the receptor expression level, increasing the type I $InsP_3R$ in the nucleus and decreasing type II $InsP_3R$ in the cytosol. Together, our results showed that in osteoblasts, BG60S increased Ca_i^{2+}signals and altered Ca_i^{2+} machinery.

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1 Introduction

Intracellular calcium (Ca_i^{2+}) is a ubiquitous second messenger responsible for controlling numerous cellular processes including fertilization, mitosis, neuronal transmission, contraction and relaxation of muscles, gene transcription, and cell death [1]. In osteoblasts Ca_i^{2+} is known to control functions such as proliferation, differentiation and gene regulation [2]. Osteoblasts can trigger Ca_i^{2+} signals upon stimulation, and there are at least two mechanisms known to be involved in this process. One mechanism requires intracellular inositol 1, 4, 5-trisphosphate ($InsP_3$) generation and subsequent release of Ca_i^{2+} from intracellular stores [2]. The other mechanism involves depolarization of the plasma membrane with subsequent opening of membrane bound voltage-operated calcium channels [4]. It is known that extracellular calcium concentration (Ca^{2+}) can also modulate osteoblast functions. For instance, elevation in extracellular Ca^{2+} concentration increases osteoblast chemotaxis and proliferation [5, 6]. Extracellular Ca^{2+} is also known to alter the levels of expression of collagen and alkaline phosphatase by osteoblasts [7, 8]. Additionally, it was demonstrated that extracellular calcium concentration (Ca^{2+}) canalter Ca_i^{2+}fluctuations and modulates bone remodeling [9]. In our previous study, we showed that treating osteoblasts with the ionic products from the dissolution of bioactive glass with 60% of silicon (BG60S) caused an increase in cell proliferation and collagen production. We also showed, using Atomic Absorption Spectrometry, that BG60S dissolution has high Ca^{2+}concentration [8]. In this work, our aim was to investigate if the high extracellular Ca^{2+} from BG60S dissolution, could alter osteoblast Ca^{2+} signaling pattern.

2 Materials and methods

2.1 Culture of osteoblastic cells

Osteoblasts were obtained from calvaria of 1–5 days old neonatal Wistar rats [10]. The calvaria were dissected and freed from soft tissue, cut into small pieces and rinsed in sterile phosphate-buffered saline (PBS) (Dulbecco) without Ca^{2+} and Mg^{2+}. The calvaria pieces were incubated with 1% trypsin-EDTA (w/v) (GibcoBRL, NY, USA) for 5 min, followed by four sequential incubations with 2% collagenase (Boehringer Pharma, Biberach, Germany) at 37°C for 45 min each. The supernatant of the first collagenase incubation, which contain a high proportion of periosteal fibroblasts, were discarded. The supernatant of the other digestions produced a suspension of cells with high proportion of preosteoblast and osteoblasts that was centrifuged at $1000 \times g$ for 5 min. After centrifugation each pellet was ressuspended in 5 mL of RPMI-1640 (Sigma, St Louis, USA) medium supplement with 10% fetal bovine serum (FBS) (GibcoBRL, NY, USA), 1% antibiotic-antimycotic solution (GibcoBRL, NY, USA). The cells were seeded into 25 tissue culture flasks (Nunc products, Naperville, USA), and led to grow in a controlled 5% CO_2 95% humidified incubator at 37°C. Cells were used on passage 2.

2.2 Preparation of ionic product from BG60S dissolution

BG60S (in weight %: silica 60, calcium 35 phosphate) was prepared using tetraethylorthosilicate (TEOS), triethylphosphate and tetrahydrated calcium nitrate as silica, phosphorus and calcium source respectively. The hydrolysis was performed in the presence of acid solution of HNO_3, 2N. The H_2O/TEOS molar ratio used was 12. Hydrofluoric acid (HF) was used in order to catalyze the gelation. BG60S powder (approximately particles size of 38 μm) was suspended in culture medium RPMI (Sigma, St Louis, USA), or in KRH medium (Krebs-Ringer-HEPES in mM: NaCl 124; KCl 5; $MgSO_4$ 1.25; $CaCl_2$ 1.45; KH_2PO_4 1.25; HEPES 25 and dextrose 0.9 g/L). For all the experiments we used the ionic product from the dissolution of BG60S. We dissolve 0.5 g of powder in 50 ml of medium [11] shaked for 5 h at 37°C and filtered twice. The second filtration was conducted in a 0.22 μm filter to sterilize the solution.

2.3 Ionic Coupled Plasma/Atomic Emission Spectrometry (ICP/AES)

ICP-AES is based on the excited atoms that emit electromagnetic radiation at a wavelength characteristic of a particular element. The intensity of this emission is indicative of the concentration of the element within the sample. In plasma emission spectroscopy, a sample solution is introduced into the core of an inductively coupled argon plasma (ICP) at a temperature of approximately 8000°C. At this temperature all elements become thermally excited and emit light at their characteristic wavelengths. This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its constituent wavelengths. Within the spectrometer, this diffracted light is then collected by wavelength and amplified to yield an intensity measurement that can be converted to an elemental concentration by comparison with calibration standards. ICP/AES was performed using a Spectroflam-ICP analyzer (Spectrocompany, UK) to quantify calcium, silicon and phosphate in the medium containing the ionic product from BG60S dissolution.

2.4 Intracellular Ca^{2+} measurements

Osteoblasts were plated at $5 \times 10^4/cm^2$ cell density onto glass coverslips and experiments were performed 72 h later. To investigate the influence of BG60S on $Ca_i{}^{2+}$ signal, osteoblasts were loaded with 6 μM Fluo/4-AM and transferred to a perfusion chamber on the stage of a BioRad MRC-1024 confocal microscope (Hercules, CA). $Ca_i{}^{2+}$ images were monitored by time lapse confocal microscopy. The cells were kept in a KRH-buffered solution during the experiments and were observed using a 63X, 1.4 numerical aperture objective. The 488 nm line of a krypton/argon laser was used to excite the dye, and emission signals between 505 and 550 nm were collected. Osteoblasts were stimulated with 100 mM KCl, 2 mM vasopressin, or BG60S, and images were acquired at a rate of 2–10 frames/s. All conditions the pH was adjusted to 7.4. Neither autofluorescence nor background signals were detectable at the machine settings used.

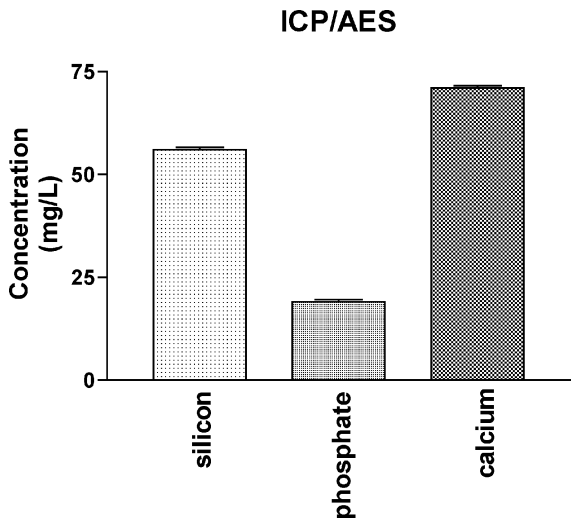

Fig. 1 ICP/AES analysis of the medium containing ionic product from the dissolution of BG60S. Calcium concentration was around 71 mg/L

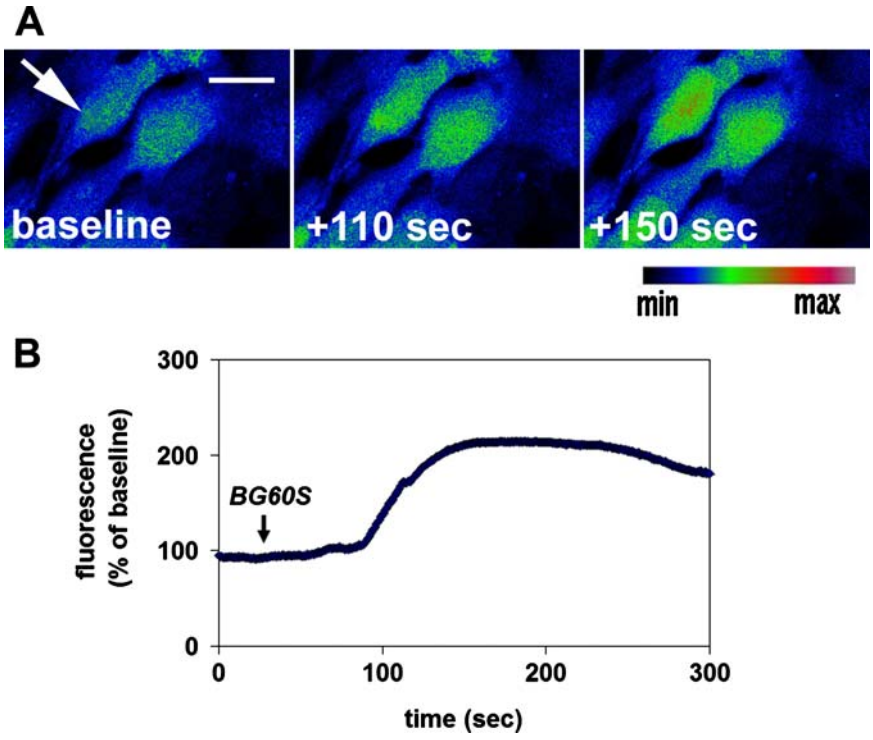

Fig. 2 BG60S increases Ca_i^{2+}signals in osteoblasts. (A) Shown are serial confocal images of osteoblasts loaded with Fluo/4-AM, before and during BG60S stimulation. The images are pseudocolored according to the color scale on the right. (B) Tracing of the Fluo/4-AM fluorescence over time, of the cell pointed by the arrow. Scalibar = 10 μm. Results are representative of those seen in 3 separate experiments ($n = 35$ cells)

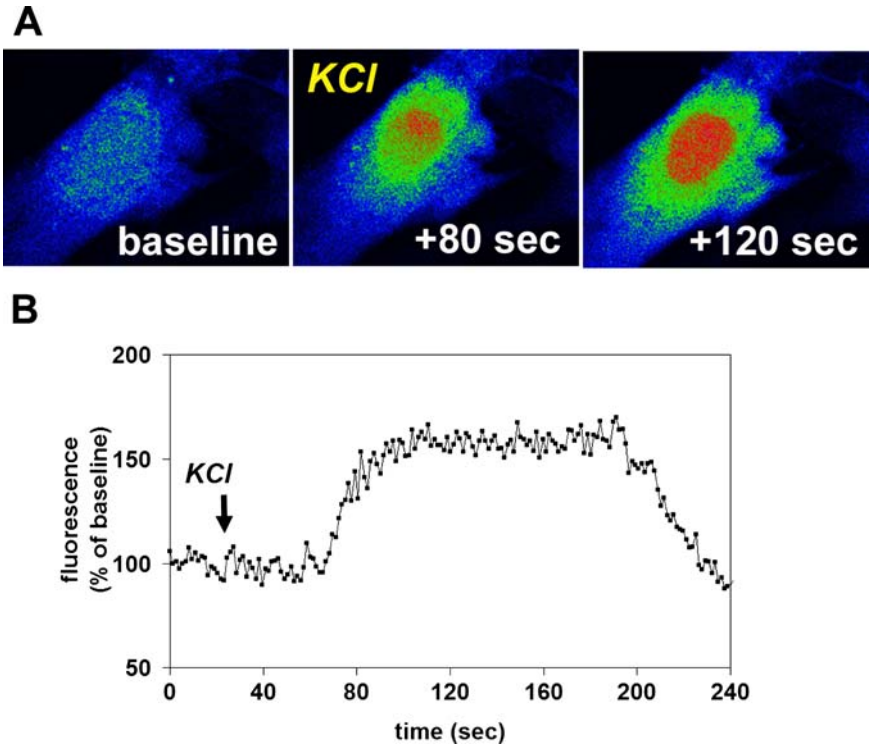

Fig. 3 Osteoblast intracellular calcium increase after stimulation with KCl. (A) Shown are serial confocal images of osteoblasts loaded with Fluo/4-AM, before and during KCl stimulation. (B) Tracing shows the pattern of Ca_i^{2+}increase in osteoblast stimulated with KCl. Black arrow in the graphic shows the moment of starting the perfusion with KCl. Results are representative of three separate experiments ($n = 60$ cells). (C) Shown are serial confocal images of BG60S pre-incubated osteoblasts loaded with Fluo/4-AM, before and during KCl stimulation. (D) Tracing shows the pattern of Ca_i^{2+}increase in BG60S pre-incubated osteoblast stimulated with KCl. Black arrow in the graphic shows the moment of starting the perfusion with KCl. Results are representative of three separate experiments ($n = 60$ cells) (E) Summary of Ca_i^{2+}increase when osteoblasts were stimulated with KCl. Stimulation with 100 mM KCl caused in BG60S pre-treated osteoblasts an increase 125 ± 14% lower than in control osteoblasts, $p < 0.05$)

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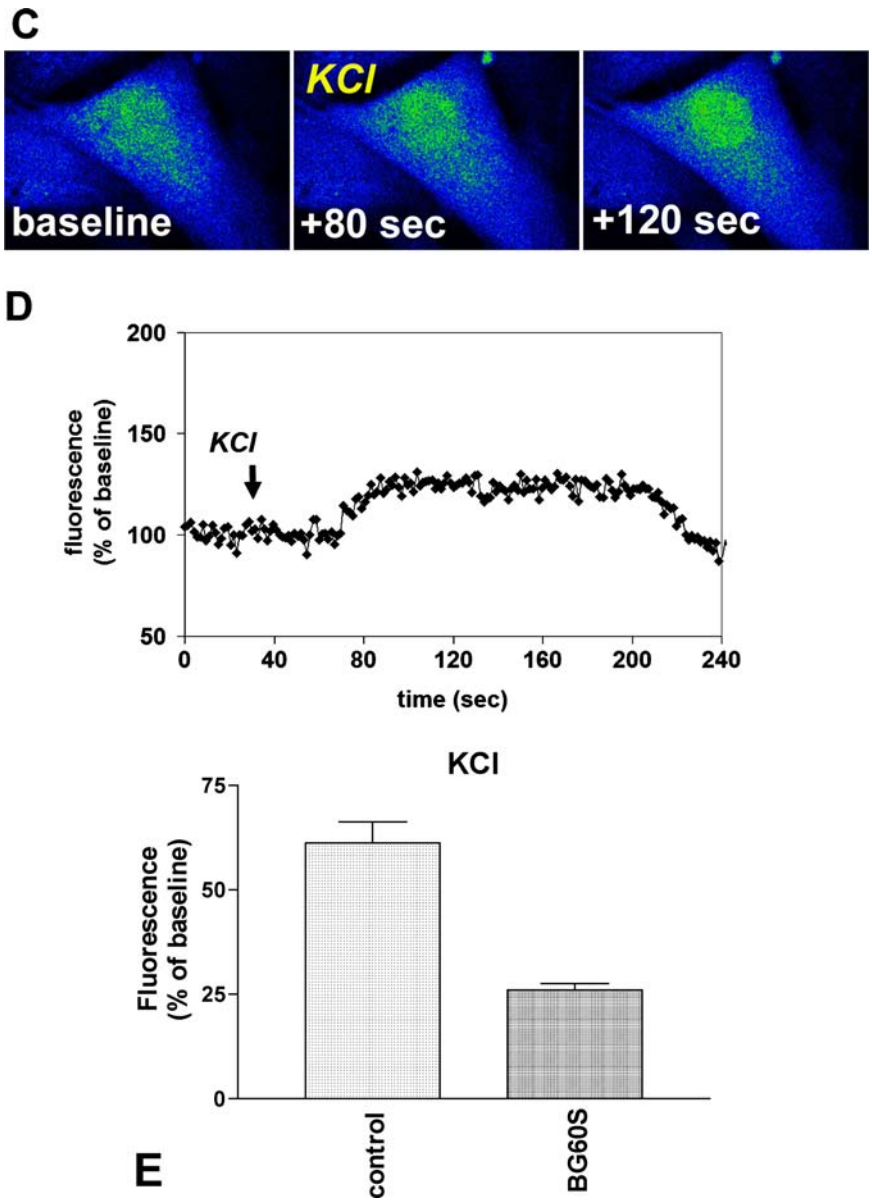
Fig. 3 (*Continued*)



2.5 Confocal immunofluorescence microscopy

For immunofluorescence, we used BG60S preincubated osteoblasts and control osteoblasts, without BG60S. The cells were fixed with 4% paraformaldehyde (Merk, Brazil) in phosphate buffered saline (PBS) for 10 min, and washed three times in PBS. The cells were incubated in blocking solution (PBS, 1% bovine serum albumin, 5% normal goat serum, 0.5% triton-X) and then incubated with one of the following primary antibodies: anti $InsP_3R$-I, anti $InsP_3R$-II and anti $InsP_3R$-III [12, 13]. Monoclonal antibody against $InsP_3R$-III was obtained from Transduction Laboratories (Lexington, UK), while polyclonal antibody against $InsP_3R$-I was purchased from Affinity BioReagents (Golden, USA). Polyclonal antibody against $InsP_3R$-II was prepared in our laboratory as previously described [14]. The cells were washed three times in PBS and incubated for 1 h in PBS 1% BSA containing secondary antibody conjugated to Alexa 488 (diluted 1:500). Immunofluorescence images were obtained with Zeiss CLSM510 confocal microscope.

3 Results

Since the Ionic Coupled Plasma analysis showed that Ca^{2+} concentration on medium containing the ionic products from the BG60S dissolution (BG60S) was around 70 mg/L (Fig. 1), our first aim was to investigate if the enhanced extracellular Ca^{2+} concentration could alter $Ca_i{}^{2+}$ signals on osteoblasts. When we stimulated osteoblasts with BG60S we observed a sustained $Ca_i{}^{2+}$ increase during the perfusion ($n = 60$ cells, from 7 different experiments), (Fig. 2). Since $Ca_i{}^{2+}$

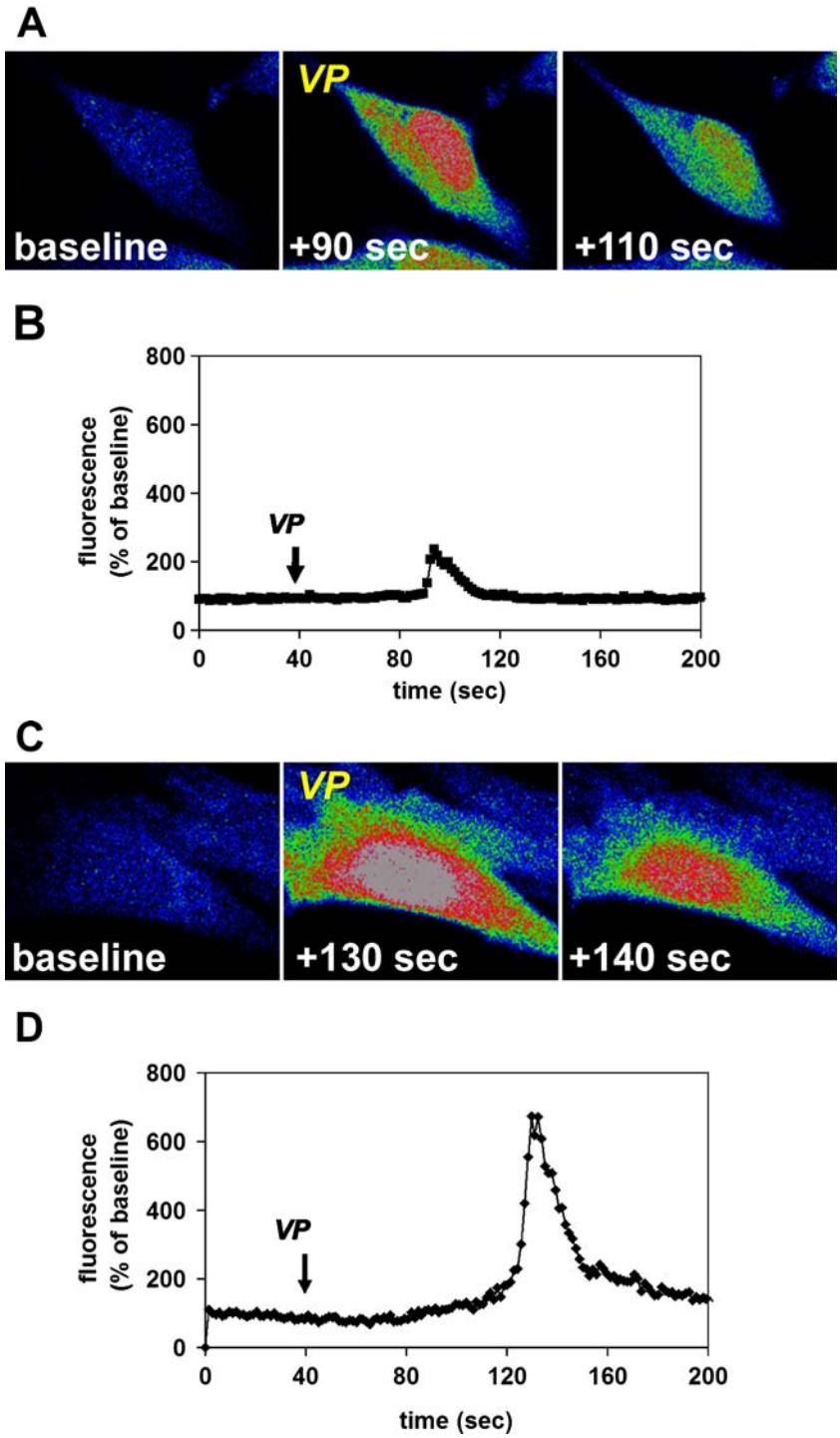


Fig. 4 Osteoblast intracellular calcium increase after stimulation with vasopressin. (A) Shown are serial confocal images of osteoblasts loaded with Fluo/4-AM, before and during vasopressin stimulation. (B) Tracing shows the pattern of Ca_i^{2+}increase in osteoblast stimulated with vasopressin. Black arrow in the graphic shows the moment of starting the perfusion with vasopressinl. Results are representative of three separate experiments ($n = 60$ cells). (C) Shown are serial confocal images of BG60S pre-incubated osteoblasts loaded with Fluo/4-AM, before and during vasopressin stimulation. (D) Tracing shows the pattern of Ca_i^{2+}increase in BG60S pre-incubated osteoblast stimulated with vasopressin. Black arrow in the graphic shows the moment of starting the perfusion with vasopressin. Results are representative of three separate experiments ($n = 60$ cells) (E) Summary of Ca_i^{2+}increase when osteoblasts were stimulated with vasopressinl. Stimulation with 2 mM vasopressin caused in BG60S pre-treated osteoblasts an increase 185 ± 15% higher than in control osteoblasts, $p < 0.05$)

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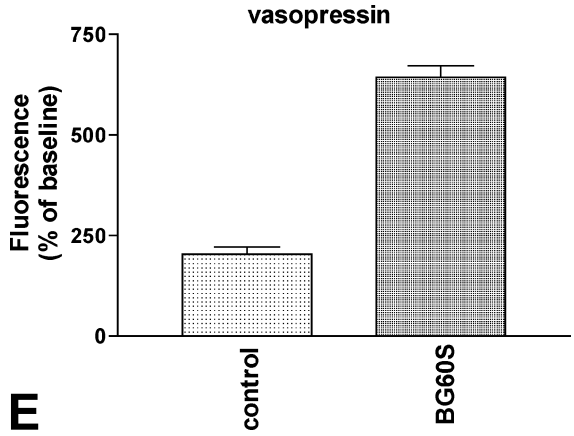

Fig. 4 (*Continued*)

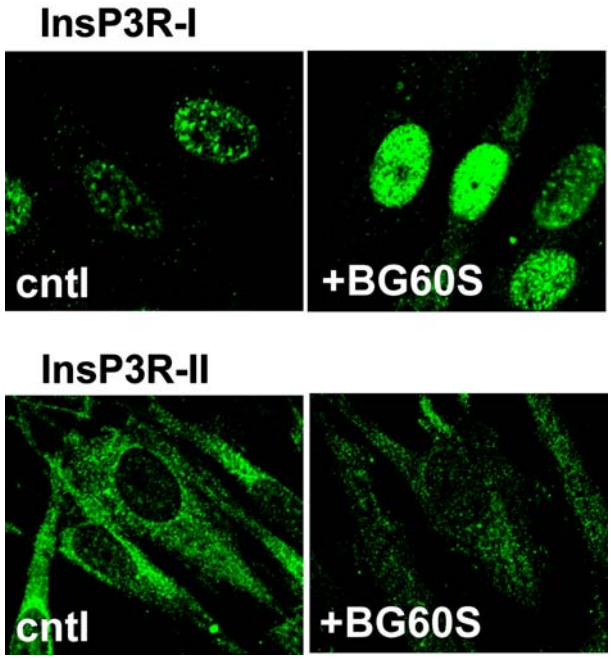

Fig. 5 Immunofluorescence for type-I and type-II InsP3R. (A) The type-I InsP3R are more concentrated in the nucleus of osteoblasts, but osteoblasts incubated with BG60S showed an increase in fluorescence. (B) The type-II InsP3R is more concentrated in cytoplasm of osteoblasts, and when the cells where incubated with BG60S there was a slightly decrease in fluorescence. Images are representative of three different experiments

increase could be due to Ca^{2+} influx and/or Ca^{2+} release from intracellular compartments, we investigated the influence of 100 mM KCl and 2 mM vasopressin on $Ca_i{}^{2+}$ signals in BG60S-pretreated osteoblasts. Stimulation of osteoblasts with KCl caused, in BG60S-pretreated cells, $Ca_i{}^{2+}$ increase of $26 \pm 4\%$ (mean $\pm$ SEM) above baseline ($n = 60$, $p < 0.05$), while control cells showed $Ca_i{}^{2+}$ increase of $55 \pm 8\%$ above baseline ($n = 60$ cells, $p < 0.05$), (Fig. 3). On the other hand, stimulation of osteoblast with 2 mM vasopressin, induced in BG60S-pretreated osteoblasts, $Ca_i{}^{2+}$ increase of $600 \pm 55\%$ above baseline ($n = 60$ cells, $p < 0.05$) while control cells showed $Ca_i{}^{2+}$ increase of $225 \pm 16\%$ above baseline ($n = 60$ cells, $p < 0.05$), (Fig. 4). Together, these data show that, in BG60S-pretreated osteoblasts, $Ca_i{}^{2+}$ increase from Ca^{2+} compartments is more effective than from Ca^{2+}influx. Vasopressin is known to activate G-protein coupled receptors, generating $InsP_3$ that can bind to $InsP_3R$ present in the endoplasmic reticulum, triggering $Ca_i{}^{2+}$ increase [15]. There are three isoforms of the $InsP_3R$, each of which has a different affinity for $InsP_3$ [16] and distinct functional properties at the single channel level [17, 18]. Therefore, we investigated the expression of the three isoforms of $InsP_3R$ in osteoblasts. We found that osteoblasts express the type I $InsP_3R$ and the type II $InsP_3R$. The type III $InsP_3R$ is not present in this cell type (Fig. 5). Additionally, we found that $InsP_3R$-I was mainly localized in the nucleus and that its distribution is increased in osteoblasts that were pre-incubated with BG60S. The type II $InsP_3R$ is localized mainly in the cytoplasm and its distribution is slightly decreased in BG60S treated cells (Fig. 5).

4 Discussion

Although it is known that extracellular Ca^{2+} is capable of producing a transient rise in $Ca_i{}^{2+}$ [19], the signal transduction mechanisms triggered by extracellular Ca^{2+} elevation have yet to be defined. Multiple cell types, including osteoblasts, sense extracellular Ca^{2+}, leading to specific cellular responses [20–22]. We found that in osteoblasts, BG60S increases $Ca_i{}^{2+}$. This could be explained by our results from the ionic coupled plasma/atomic emission spectrometry analysis that demonstrated that BG60S has 70 mg/L of Ca^{2+}. Our observation of lower $Ca_i{}^{2+}$ increase when BG60S-preincubated osteoblasts were stimulated with KCl, could be explained by previous findings which showed that elevation of extracellular Ca^{2+} hyperpolarizes osteoblasts, altering the voltage sensing Ca^{2+} channel opening [23]. Since Ca^{2+} channels are activated by membrane depolarization, prior membrane hyperpolarization would prevent or delay the channel openning [24]. In central nervous system, high extracellular Ca^{2+} directly enhances vasopressin inducing $Ca_i{}^{2+}$ increase [25]. This is in accord with our finding of higher level of $Ca_i{}^{2+}$ increase induced by vasopressin in BG60S-preincubated osteoblasts. This suggests that mechanisms involved in hormone stimulation described to central nervous system may be occurring in bone as well.

Cytosolic and nuclear Ca^{2+} signals have been reported to have distinct effects on a specific cellular function and are related to intracellular Ca^{2+} channels [13, 26]. We found that two isoforms of the $Ca_i{}^{2+}$ channel, $InsP_3Rs$, are present in osteoblasts and have distinct subcellular localization. The type-I $InsP_3R$ is more expressed in the nucleus, while the type-II $InsP_3R$ is more expressed in the cytosol. Moreover, we found that pre-incubation of osteoblasts with BG60S

can alter the expression level of the $InsP_3Rs$, decreasing the cytosolic type-II $InsP_3R$, and increasing the nuclear type-I $InsP_3R$. Most mammalian cell types express multiple $InsP_3R$ isoforms in distinct and overlapping intracellular pattern. The expression can alter during different stages of cell development, in response to different extracellular stimuli, as well as during disease state [27–29]. We were expecting to find an increase in the expression of type-II $InsP_3R$, due to a higher Ca_i^{2+} signals observed when BG60S-pretreated osteoblasts were stimulated with vasopressin. However, BG60S-preteated cells showed lower expression of type-II $InsP_3R$ compared to control. On the other hand, we found that the type-I $InsP_3R$ had an increased expression in the nuclear region in BG60S pretreated osteoblasts. Although each of three $InsP_3R$ isoforms acts as an $InsP_3$-gated channel, the isoforms are not uniformly sensitive to $InsP_3$. The relative order of affinity is type II, type I and type-III [16]. Because $InsP_3Rs$ have multiple $InsP_3$-binding sites [30, 31], the decreased expression of type-II $InsP_3R$ will increase net binding of $InsP_3$ to type-I, eventhough the type-II has a greater affinity for $InsP_3R$. Our results suggest that in BG60S-pretreated osteoblast nuclear expression of the type-I $InsP_3R$ is sufficient to trigger Ca_i^{2+} signal induced by vasopressin.

Ca^{2+} is able to influence the secretion of many hormones in osteoblasts [32] and it was already suggested that Ca^{2+} concentration above physiological conditions could stimulate bone metabolism [33]. Our results reinforce that better understanding of the interactions between Ca_i^{2+} and bioceramics may improve the development of tissue engineering and its application on healing mechanisms.

5 Conclusion

We found that BG60S sustained Ca_i^{2+} increase in osteoblasts. Pre-incubation of osteoblasts with BG60S stimulated Ca_i^{2+} signal induced by vasopressin although it decreased Ca_i^{2+} signals induced by KCl. The type-I and type-II $InsP_3Rs$ are present in osteoblasts and pre-incubation of these cells with BG60S altered expression level of the $InsP_3Rs$. Together, our results indicate that BG60S can influence Ca^{2+} signals in osteoblasts. Further investigations are necessary to study how the observed Ca^{2+} increase can modulate specific osteoblast functions.

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