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Interleukin-1 β and 6-Induced Calcium Channel Current Modulation in MC3T3-E1 Osteoblast Cell Line

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Abstract

Interleukin-1 β (IL-1 β) and -6 (IL-6) are inflammatory cytokines that are involved in bone resorption under pathological conditions. The cytokines are also involved in bone remodeling under physiological conditions. Voltage sensitive Ca²⁺ channels (VSCCs) serve as crucial mediators of membrane excitability and many Ca²⁺-dependent functions such as growth of bone, regulate proliferation, differentiation, enzyme activity and gene expression. The effects of IL-1 β and -6 on VSCCs in osteoblast cell line (MC3T3-E1) were investigated using patch-clamp recording. Our results showed that application of 50 pM-50 nM IL-1 β facilitated VSCCs current (I_{Ca}) carried by Ba²⁺ (I_{Ba}). Application of 50 pM-5 nM IL-6 facilitated I_{Ba}. In contrast, 50 nM IL-6 inhibited I_{Ba} in MC3T3-E1 cells. Treatment with MAPK inhibitor, PD98059, attenuated the 5 nM IL-6-induced facilitation of I_{Ba}. Treatment with STAT3 inhibitor, staflific, attenuated the 50 nM IL-6-induced inhibition of I_{Ba}. Treatment with PD98059 also attenuated the 50 nM IL-6-induced inhibition of I_{Ba}. These results suggest that 5 nM IL-6 facilitates VDCCs involving MAPK pathways. In addition, 50 nM IL-6 inhibits VDCCs involving STAT3 and MAPK pathways in MC3T3-E1 cells.

Keywords: osteoblasts, Interleukin-1 β , Interleukin-6, calcium channels

1. Introduction

Interleukin-1 β (IL-1 β), a potent inflammatory cytokine, is upregulated during inflammation mainly produced by activated monocytes and macrophages (Dinarello, 1997; Stylianou et al., 1998). It stimulates bone resorption in several cell types, including osteoblasts (Dinarello, 1988). IL-6 is a multifunctional cytokine which has diverse effects on bone metabolism. IL-6 has been shown to affect growth and differentiation in osteoblasts (Fang et al., 1991; Hughes et al., 1992; Ishimi et al., 1990).

Osteoblasts are known to play a major role in the bone formation. It is well accepted that osteoblasts express IL-1 β and -6 receptors and therefore it plays an important role in bone remodeling (Blanchard et al., 2009; Shen et al., 1990).

Voltage sensitive Ca²⁺ channels (VSCCs) serve as crucial mediators of membrane excitability (Miller, 1987) and many Ca²⁺-dependent functions such as growth of bone (Duriez et al., 1993), regulate proliferation (Loza et al., 1994), differentiation (Wen et al., 2012), enzyme activity (Reuter 1983) and gene expression (Murphy et al., 1991). It also has been demonstrated that osteoblasts express VSCCs (Duncan et al., 1998; McDonald, 2004). Several reports demonstrated that IL-1 β and -6 modulated VSCCs in neuronal cells (Ma et al., 2012; Zhou et al., 2006). However, the mechanism of IL-1 β and -6 effects on VSCCs in osteoblasts has been extensively studied, but remains unclear and even controversial.

Consequently, in this study we investigated the effect of IL-1 β and -6 on VSCCs in osteoblast cell line (MC3T3-E1).

2. Materials and Methods

2.1 Cell Culture

Murine osteoblastic MC3T3-E1 cells were purchased from Summit Pharmaceuticals International Corporation (Tokyo, Japan). Cells were cultured at 37°C in a 5% (v/v) CO₂ atmosphere with α -modified minimal essential medium (α -MEM; Gibco BRL, Grand Island, NY, U.S.A.). Unless otherwise specified, the medium contained 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cell culture medium was changed every 2-3 days. For patch-clamp experiments, cells were harvested using a 0.05% trypsin/0.02% EDTA solution, when cells reached confluence. Cells were plated at very low density in 35 mm tissue culture dishes. Prior to recordings, the cells were washed at least three times with Krebs solution of the following composition (in mM): 136 NaCl; 5 KCl; 2.5 CaCl₂; 0.5 MgCl₂; 10.9 glucose; 11.9 NaHCO₃ and 1.1 NaH₂PO₄. The pH was 7.3-7.4. Cell culture reagents were purchased from Sigma (Tokyo, Japan). Our recordings were performed on 10-30 μ m in diameter, since they were well suited for patch clamp recordings.

2.2 Whole-Cell Patch-Clamp Recordings

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Fabricated recording pipettes (2-3 M Ω) were filled with the internal solution of the following composition (in mM): 150 CsCl; 5 EGTA; 10 D-glucose and 10 HEPES. The pH was adjusted to 7.3 with CsOH. After the formation of a giga seal, in order to record VSCCs current (I_{Ca}) carried by Ba²⁺ (I_{Ba}), the extracellular solution was replaced from Krebs solution to a solution containing the following (in mM): 115 BaCl₂ and 20 HEPES. The pH was adjusted to 7.4 with tetraethylammonium hydroxide. Command voltage protocols were generated with a computer software pCLAMP version 10 (Axon Instruments, Union City, CA, U.S.A.) and transformed to an analogue signal using a DigiData1440A interface (Axon Instruments, Union City, CA, U.S.A.). The command pulses were applied to cells through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and a computer software pCLAMP10 acquisition system. Access resistance (< 15 M Ω) was determined by transient responses to voltage commands. L-1 β and -6 were applied by perfusion pressure ejection from perfusion tubes with a tip diameter of 1 mm placed within 10 mm of the cell surface. Although the concentration of IL-1 β and -6 actually seen by the cell is certainly lower than that contained in the stock solution, we applied the IL-1 β and -6 using same perfusion system. L-1 β and -6 were applied by perfusion pressure ejection from perfusion tubes with a tip diameter of 1 mm placed within 10 mm of the cell surface.

2.3 Chemicals

IL-1 β , IL-6 and PD98059 were purchased from Sigma (Tokyo, Japan). SQ22536 was purchased from Biomol Research Laboratories (Plymouth, PA, U.S.A.). 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Staffic was purchased from Tocris (Bristol, U.K.). All drugs except GF109203X and staffic were dissolved in distilled water. GF109203X and Staffic were dissolved in DMSO. All drugs were diluted to the desired final concentration in the external solution just before use. The final concentration of DMSO was < 0.01%, which had no effect on the I_{Ba}.

2.4 Statistical Analysis

All data analyses were performed using the pCLAMP10 acquisition system. Values in text and figures are expressed as mean \pm S.E.M. Statistical analysis was done using Student's *t* test for comparisons between pairs of groups and one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability (p) values of less than 0.05 were considered significant. Modulation of I_{Ba} was calculated with the following formula:

Modulation of I_{Ba} (%) = After Application peak current / Before Application peak current

Table 1. Statistical mean \pm S.E.M. at each concentration levels

	IL-1 β	n	IL-6	n
50 pM	22.8 \pm 4.0	5	15.8 \pm 2.7	10
500 pM	21.8 \pm 2.0	6	23.0 \pm 4.5	7
5 nM	34.1 \pm 3.9	6	35.6 \pm 4.0	8
50 nM	56.7 \pm 5.2	3	-21.4 \pm 2.5	3

Table 2. Statistical mean ± S.E.M. for IL-6 treated with each inhibitors.

	IL-1β	n	IL-6	n
Control	35.6 ± 4.0	8	-21.4 ± 2.5	4
SQ22536	30.0 ± 3.6	4	-21.4 ± 1.3	5
GF109203	36.9 ± 6.4	4	-22.6 ± 1.4	5
Staffic	35.2 ± 4.8	4	-3.5 ± 0.6	5
PD98059	7.5 ± 1.1	4	-4.0 ± 0.9	5

3. Results

3.1 IL-1β Facilitated I_{Ba}

Figure 1a and b shows that, in the presence of high external Ba²⁺, 5 nM IL-1β facilitated I_{Ba} as shown with raw current traces obtained at +10 mV from -80 mV in the absence and presence of IL-1β. I_{Ba} was evoked every 5 s with a 100 ms depolarizing voltage step to -10 mV from a holding potential of -80 mV. As shown in Figure 1a, application of 5 nM IL-1β facilitated I_{Ba} from -161 pA to -225 pA (39.7% facilitation) in this cell.

To investigate the voltage dependency of facilitation of I_{Ba} by IL-1β, we analyzed the current-voltage relations in the absence and presence of IL-1β. The current-voltage relations measured before and during application of IL-1β are shown in Figure 1c. From a holding potential of -80 mV, I_{Ba} was activated with a peak current amplitude at 0 mV. IL-1β did not alter the current-voltage relationship. These results suggest that 5 nM IL-1β facilitated I_{Ba} in MC3T3-E1 cells.

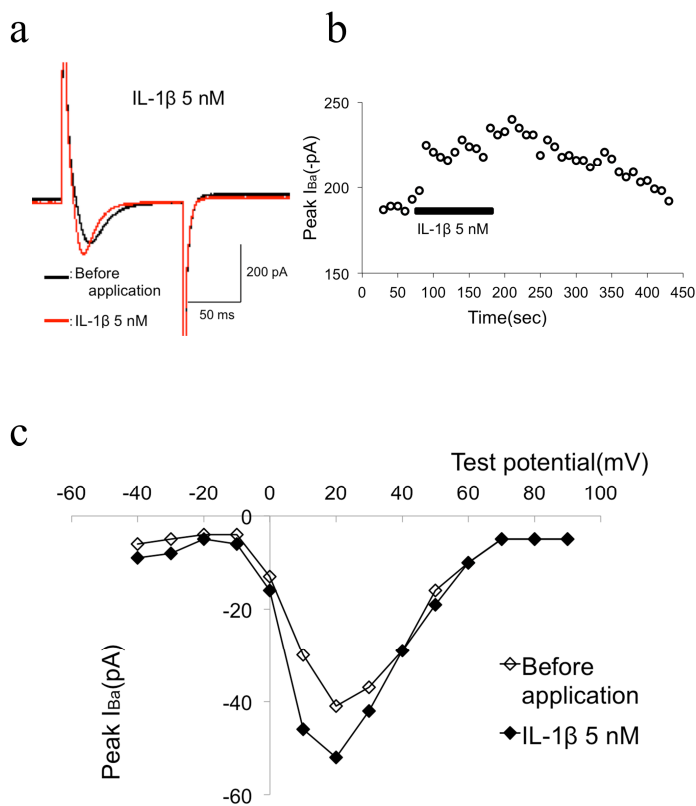


Figure 1. (a) Typical superimposed I_{Ba} traces according to the time course graph B. I_{Ba} was evoked from a holding potential of -80 mV by a 100 msec voltage step to -10 mV at 5 sec intervals. (b) Typical time course of 5 nM IL-1β-induced facilitation of I_{Ba}. IL-1β (5 nM) was bath-applied during the times indicated by the filled bar. (c) Current-voltage relationship of I_{Ba} evoked by a series of voltage steps from a holding potential of -80 mV to test potentials between -80 and +40 mV in +10 mV increments in the absence (opened circles) and presence (filled circles) of 5 nM IL-1β

3.2 IL-6 Facilitated I_{Ba}

Figure 2a and b shows that 5 nM IL-6 facilitated I_{Ba} as shown with raw current traces obtained at +10 mV from -80 mV in the absence and presence of IL-6. I_{Ba} was evoked every 5 s with a 100 ms depolarizing voltage step to -10 mV from a holding potential of -80 mV. As shown in Figure 2A, application of 5 nM IL-6 facilitated I_{Ba} from -75 pA to -103 pA (37.3% facilitation) in this cell.

To investigate the voltage dependency of facilitation of I_{Ba} by 5 nM IL-6, we analyzed the current-voltage relations in the absence and presence of 5 nM IL-6. The current-voltage relations measured before and during application of 5 nM IL-6 are shown in Figure 2c. From a holding potential of -80 mV, I_{Ba} was activated with a peak current amplitude at 20 mV. IL-6 did not alter the current-voltage relationship. These results suggest that 5 nM IL-6 facilitated I_{Ba} in MC3T3-E1 cells.

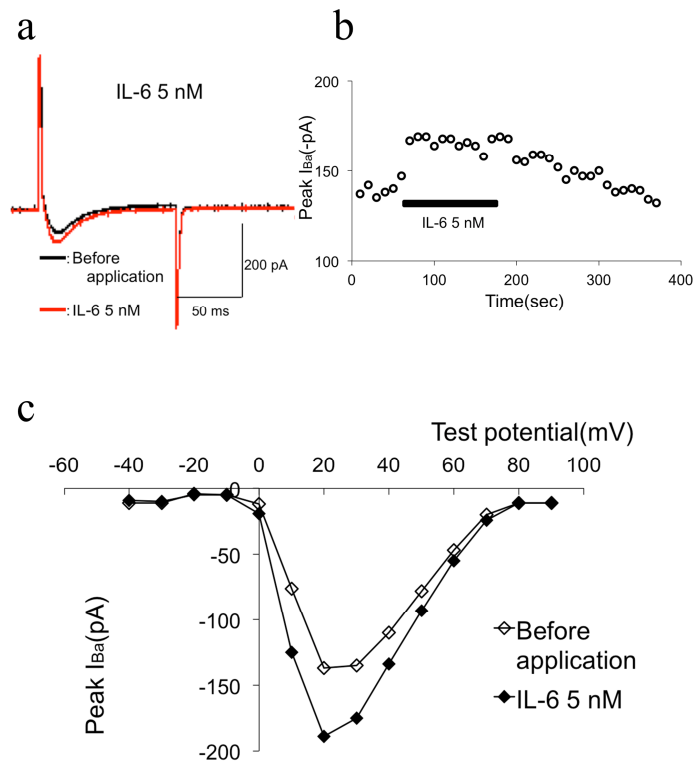


Figure 2. (a) Typical superimposed I_{Ba} traces according to the time course graph B. I_{Ba} was evoked from a holding potential of -80 mV by a 100 msec voltage step to -10 mV at 5 sec intervals. (b) Typical time course of 5 nM IL-6-induced facilitation of I_{Ba} . IL-6 (5 nM) was bath-applied during the times indicated by the filled bar. (c) Current-voltage relationship of I_{Ba} evoked by a series of voltage steps from a holding potential of -80 mV to test potentials between -80 and +40 mV in +10 mV increments in the absence (opened circles) and presence (filled circles) of 5 nM IL-6

3.3 IL-6 Inhibited I_{Ba}

In addition to facilitation, IL-6-induced inhibition of I_{Ba} could be observed. Figure 3a and b shows that 50 nM IL-6 inhibited I_{Ba} as shown with raw current traces obtained at +10 mV from -80 mV in the absence and presence of IL-6. I_{Ba} was evoked every 5 s with a 100 ms depolarizing voltage step to -10 mV from a holding potential of -80 mV. As shown in Figure 3a, application of 50 nM IL-6 inhibited I_{Ba} from -328 pA to -236 pA (28.0% inhibition) in this cell.

To investigate the voltage dependency of facilitation of I_{Ba} by 50 nM IL-6, we analyzed the current-voltage relations in the absence and presence of 50 nM IL-6. The current-voltage relations measured before and during application of 50 nM IL-6 are shown in Figure 3c. From a holding potential of -80 mV, I_{Ba} was activated with a peak current amplitude at 20 mV. IL-6 did not alter the current-voltage relationship. These results suggest that 50 nM IL-6 inhibited I_{Ba} in MC3T3-E1 cells.

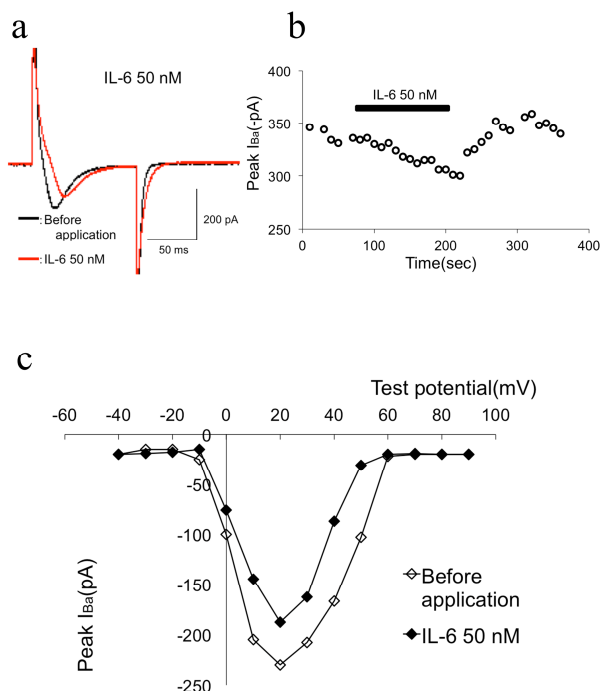


Figure 3. (a) Typical superimposed I_{Ba} traces according to the time course graph B. I_{Ba} was evoked from a holding potential of -80 mV by a 100 msec voltage step to -10 mV at 5 sec intervals. (b) Typical time course of 50 nM IL-6-induced inhibition of I_{Ba} . IL-6 (50 nM) was bath-applied during the times indicated by the filled bar. (c) Current-voltage relationship of I_{Ba} evoked by a series of voltage steps from a holding potential of -80 mV to test potentials between -80 and $+40$ mV in $+10$ mV increments in the absence (opened circles) and presence (filled circles) of 50 nM IL-6

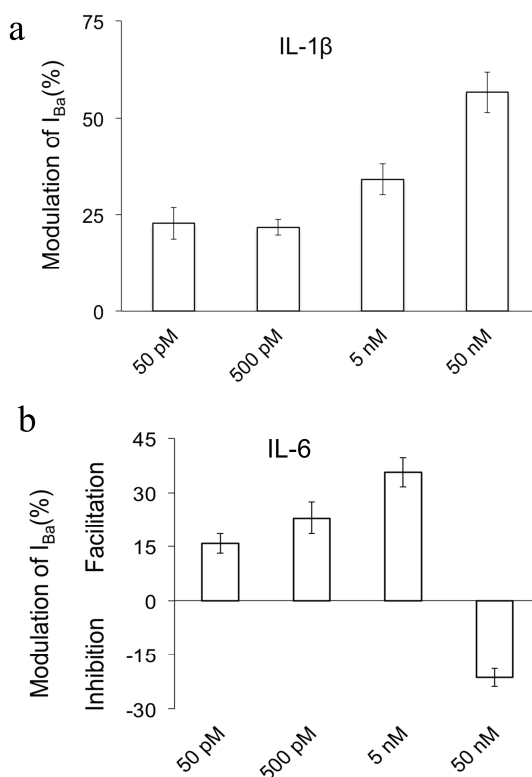


Figure 4. (a) Histogram demonstrating the degree of I_{Ba} modulation by 50 pM-50 nM IL-1 β . (b) Histogram demonstrating the degree of I_{Ba} modulation by 50 pM-50 nM IL-6

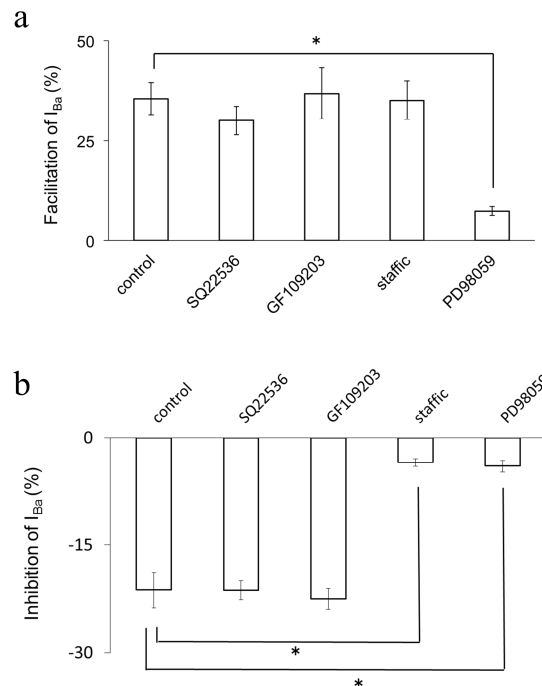


Figure 5. (a) Summary of IL-6-induced facilitation of I_{Ba} under various conditions. Histogram demonstrating the degree of I_{Ba} facilitation by 5 nM IL-6 in control (untreated cells), after SQ22536 (an AC inhibitor), after GF109203 (a PKC inhibitor), after staffic (a STAT3 inhibitor), and PD98059 (a MAPK inhibitor). (b) Summary of IL-6-induced inhibition of I_{Ba} under various conditions. Histogram demonstrating the degree of I_{Ba} inhibition by 50 nM IL-6 in control (untreated cells), after SQ22536 (an AC inhibitor), after GF109203 (a PKC inhibitor), after staffic (a STAT3 inhibitor), and PD98059 (a MAPK inhibitor). * <0.05 compared with control

3.4 IL-1 β and -6 Modulated I_{Ba} Depends On Concentration

The concentration-response relationship in the IL-1 β and -6-induced modulation of I_{Ba} is shown in Figure 4a and b, respectively.

Application of 50 pM-50 nM IL-1 β facilitated I_{Ba} . To generate a concentration-response curve, IL concentrations were applied randomly, and each MC3T3-E1 cells were exposed to only a single concentration. Figure 4a shows that progressive voltage-dependent facilitations in IL-1 β concentrations resulted in a progressively greater facilitation of I_{Ba} .

In addition, application of 50 pM-5 nM IL-6 facilitated I_{Ba} . In contrast to above results, 50 nM IL-6 inhibited I_{Ba} in MC3T3-E1 cells.

3.5 Characterization of Second Messengers in 5 nM IL-6-Induced Facilitation of I_{Ba}

To evaluate the possible contribution of adenylate cyclase (AC) to the IL-6-induced facilitation of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with SQ22536 (an AC inhibitor) were investigated. Treatment with SQ22536 (10 μ M for 30 min) did not attenuate the IL-6-induced facilitation of I_{Ba} .

To evaluate the possible contribution of protein kinase C (PKC) to the IL-6-induced facilitation of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with GF109203X (a selective PKC inhibitor) were investigated. Treatment with GF109203X (10 μ M for 30 min) did not attenuate the IL-6-induced facilitation of I_{Ba} .

To evaluate the possible contribution of STAT3 to the IL-6-induced facilitation of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with staffic (a selective signal transducers and activators of transcription 3, STAT3, inhibitor) were investigated. Treatment with staffic (20 μ M for 2 min) did not attenuate the IL-6-induced facilitation of I_{Ba} .

To evaluate the possible contribution of MAPK to the IL-6-induced facilitation of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with PD98059 (a selective MAPK inhibitor) were investigated. Treatment with PD98059 (10 μ M for 2 min) attenuated the IL-6-induced facilitation of I_{Ba} .

These results suggest that IL-6 facilitates VDCCs involving MAPK pathways in MC3T3-E1 cells.

3.6 Characterization of Second Messengers in 50 nM IL-6-Induced Inhibition of I_{Ba}

To evaluate the possible contribution of AC to the IL-6-induced inhibition of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with SQ22536 (an AC inhibitor) were investigated. Treatment with SQ22536 (10 μ M for 30 min) did not attenuate the IL-6-induced inhibition of I_{Ba} .

To evaluate the possible contribution of protein kinase C (PKC) to the IL-6-induced inhibition of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with GF109203X (a selective PKC inhibitor) were investigated. Treatment with GF109203X (10 μ M for 30 min) did not attenuate the IL-6-induced inhibition of I_{Ba} .

To evaluate the possible contribution of STAT3 to the IL-6-induced inhibition of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with stafluric acid (a selective STAT3 inhibitor) were investigated. Treatment with stafluric acid (20 μ M for 2 min) attenuated the IL-6-induced inhibition of I_{Ba} .

To evaluate the possible contribution of MAPK to the IL-6-induced inhibition of I_{Ba} , the effects of IL-6 on I_{Ba} in cells treated with PD98059 (a selective MAPK inhibitor) were investigated. Treatment with PD98059 (10 μ M for 2 min) attenuated the IL-6-induced inhibition of I_{Ba} .

These results suggest that IL-6 inhibits VDCCs involving STAT3 and MAPK pathways in MC3T3-E1 cells.

4. Discussion

The present study investigated the effects of IL-1 β and -6 on I_{Ba} in MC3T3-E1 cells. Application of 50 pM-50 nM IL-1 β facilitated I_{Ba} . Application of 50 pM-5 nM IL-6 facilitated I_{Ba} involving MAPK pathways. In contrast, 50 nM IL-6 inhibited I_{Ba} involving STAT3 and MAPK pathways in MC3T3-E1 cells. In this study, we used 50 pM-50 nM IL-1 β and IL-6 to modulate I_{Ba} . These IL concentrations are quite high. In normal state, serum IL-1 β concentration is 0.9 pg/ml approximately (Chang et al., 2013). In the electrophysiological study and the immunocytochemistry study, however, several study demonstrated that high concentration, i.e. 0.57 nM and 4.5 nM, of IL was used to modulate ion channel activity and protein expression in single neuron (Zhou et al., 2006; Islam et al., 2009). IL-1 β and -6 were applied by perfusion pressure ejection from perfusion tubes with a tip diameter of 1 mm placed within 10 mm of the cell surface. Although the concentration of IL-1 β and -6 actually seen by the cell is certainly lower than that contained in the stock solution, we applied the IL-1 β and -6 using same perfusion system. We also observed that I_{Ba} were recovered to values of peak I_{Ba} after IL wash out using time course graph (Figures. 1b, 2b and 3b). Therefore, it can be considered that 50 pM-50 nM IL-1 β and IL-6 had no effect on the I_{Ba} .

In osteoblastic cells, several studies demonstrated that hormones modulated VSCCs, included in parathyroid hormone (PTH) (Green et al., 1991; Li et al., 1997; Yamaguchi et al., 1987), bradykinin (Tokuda et al., 1994), 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) (Bergh et al., 2006; Uchida et al., 2012; Zanello et al., 2006) and phalloidin, a stabilizer of actin filaments (Li et al., 2011). To our knowledge, the data presented here demonstrate for the first time that IL-1 β and -6 modulates VSCCs in osteoblasts. In contrast to our results, Green et al. reported that IL-6 did not alter basal intracellular Ca²⁺ concentration ([Ca²⁺]_i) but inhibited Ca²⁺ transient induced by PTH, prostaglandin E₂ and endothelin-1 in osteoblast (Green et al., 1994).

In this study, we have shown that 50 pM-50 nM IL-1 β facilitated I_{Ba} . It has been demonstrated that local treatment with IL-1 β (0.5 ng/h) for 72 h resulted in increased osteoblasts concentration (Olmedo et al., 1999). Furthermore, reports of IL-1 β promoting osteoblast proliferation during acute bone repair (Lange et al., 2009) and inducing differentiation of human mesenchymal stem cells into osteoblasts (Sonomoto et al., 2012). Increased DNA synthesis in response to IL-1 β in vitro has been observed in osteoblast (Evans et al., 1989) and especially MC3T3-E1 cells (Ikeda et al., 1998). In contrast, however, it has been reported that IL-1 β inhibits the stimulation of osteocalcin synthesis and has no influence on proliferation (Taichman et al., 1992). IL-1 β also increases both osteoprotegerin protein release and mRNA levels (Hofbauer et al., 1998; Hofbauer et al., 1999). Recent study demonstrated that treatment with IL-1 β resulted in biphasic effects on osteoblast differentiation. Short-term exposure (2 days) to IL-1 β early in culture induces differentiation. Longer term exposure (6 days) to IL-1 β inhibits osteoblast differentiation (Bellido et al., 1997).

In this study, we have shown that 50 pM-5 nM IL-6 facilitated I_{Ba} involving MAPK pathways. In contrast, 50 nM IL-6 inhibited I_{Ba} involving STAT3 and MAPK pathways. What is the physiological relevance of IL-6-induced dual modulation of VSCCs, i.e. both facilitation and inhibition? Several studies indicated that IL-6 enhanced in vitro differentiation on osteoblasts (Bellido et al., 1997; Bellido et al., 1998; Franchimont et al., 2005; Itoh et al., 2006; Liu et al., 2006; Wong et al., 2003). In contrast to above reports, other studies have shown an inhibitory effect of IL-6 on bone formation (Malaval et al., 2001; Malaval et al., 2005). It can be considered that such dual effects are depends on IL-6 receptor's intracellular signals transduction mechanisms. There are three major MAPK subgroups identified, including the extracellular signal-regulated kinases (ERK1/2), p38 MAPK and c-jun-NH₂-terminal

kinases (JNKs). The signal transduction induced by IL-6 is mediated by homo- or heterodimerization of the gp130 receptor, subsequent activation of JAK, and phosphorylation of STAT (Heinrich et al., 2003). It has been demonstrated that activation of STAT3 is necessary for osteoblast differentiation and bone formation induced by IL-6 (Itoh et al., 2006). On the other hand, PKC δ and ERK1/2 are implicated in IL-6's inhibitory effect on bone formation (Chipoy et al., 2004). STAT3 plays multiple roles depending on the context or condition of cells. In some conditions, STAT3 is involved in growth arrest and differentiation, and even in cell death (Nakajima et al., 1996; Hirano et al., 1997; Hirano et al., 2000). In other conditions, STAT3 is involved in proliferation and cell survival (Hirano et al., 1997; Hirano et al., 2000). It is also possible that these dual effects depend on the conditions or differentiation stage of the osteoblast. IL-6 would stimulate the first stages of differentiation but on more mature cells, they would prevent further stimulation (Malaval et al., 2001; Malaval et al., 2005). Interestingly, VSCCs serve as growth of bone, regulate proliferation and differentiation, as mentioned above.

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