Journal of Trace Elements in Medicine and Biology 33 (2016) 81–86

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Potentiation of endothelin-1-induced prostaglandin E_2 formation by Ni^{2+} and Sr^{2+} in murine osteoblastic MC3T3-E1 cells

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ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form
30 September 2015
Accepted 10 October 2015

Keywords:
Nickel
Strontium
Prostaglandin
Endothelin
Osteoblast
MC3T3-E1

ABSTRACT

Cation recognition mechanisms beyond calcium-sensing receptors are still largely unexplored and consequently there is surprisingly little information on linking of this primary event to key metabolic features of different cell systems, such as arachidonic acid metabolism. However, information on the modulatory role of extracellular cations in cellular function is scarce.

In this study we have demonstrated, that Ni^{2+} and Sr^{2+} potentiate endothelin-1 induced prostaglandin E_2 formation in the osteoblastic cell line, MC3T3-E1, even in the absence of extracellular calcium. The effect is strictly dependent of receptor-mediated signal transduction processes evoked by endothelin-1 and arachidonate release involves cytosolic phospholipase A_2 activity. The ligation sites, at least for Ni^{2+} are extracellular. The data suggest a novel activation mechanism for arachidonate release and subsequent prostaglandin formation that does not require calcium.

1. Introduction

Extracellular calcium-sensing receptors (CaR) provided a new paradigm in signal transduction in which an extracellular ion, Ca^{2+}, serves as an agonist for a cell-surface receptor [1,2]. Besides that other local mechanisms of calcium sensing have been proposed, because of differences in response to cations with respect to CaR. In osteoclasts, candidates include another PLC-coupled receptor [3], a voltage-gated calcium channel and a receptor-operated transient receptor potential channel [4]. Zaidi et al. detected by immunohistochemistry a type II ryanodine receptor located in the plasma membrane and postulated that it might sense extracellular calcium and regulate its influx [5]. Recent evidence suggests that a cation sensing entity in osteoblasts which is activated by Sr^{2+}, may be functionally different from other CaRs [6]. It has been shown that Sr^{2+} is an agonist of the osteoblast CaR, but with lower affinity than calcium [7]. Being in competition with high Ca^{2+}, the physiological effects of Sr^{2+} are likely to occur via novel ligation sites yet to be discovered. Additionally, the presence of calcium-sensing in CaR-deficient osteoblasts indicates the existence of a novel cation-recognizing mechanism in osteoblasts as also strongly suggested by several studies [8,9].

Cation recognition mechanisms beyond CaR are still largely unexplored and consequently there is surprisingly little information on linking of this primary event to key metabolic features of different cell systems, such as arachidonic acid (AA) metabolism. We have previously described a unique cation-sensing mechanism probed with Ni^{2+} in osteoblasts that does not evoke metabolic cell responses *per se*, but strikingly amplifies agonist (bradykinin, bk) -evoked prostaglandin E_2 (PGE_2) synthesis, a key modulator of osteoblast and osteoclast function. Amplification of paracrine signaling between bone cells via PGE_2 by extracellular cations could thus reveal unknown sites for possible pharmacological intervention. However, information on the modulatory role of extracellular cations in cellular function is scarce. Cadmium has a stimulatory effect on osteoblastic PGE_2 synthesis, an effect that might be at least partially contributed to its cytotoxicity [9,10]. Sr^{2+} can stimulate phosphinositide breakdown in platelets leading to thromboxane formation and 5-hydroxytryptamine release [11,12]. Besides Ca^{2+} and Mn^{2+} it can activate phospholipase A_2 and hence cause PGE_2 synthesis in kidney medulla slices [13]. More recently, activation of phospholipase C (PLC) and protein kinase C (PKC) (leading most likely to PGE_2 synthesis) in osteoblastic MC3T3-E1 cells has been achieved with Ca^{2+} and the effect attributed to the CaR or some other Ca^{2+}-sensing mechanism [14]. Furthermore, extracellular Ca^{2+} is a potent inducer of prostaglandin-endoperoxide synthase 2 (PGHS-2) in murine primary osteoblasts [15]. Ni^{2+} exposure also induces PGHS-2 expression at transcriptional and protein levels in

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http://dx.doi.org/10.1016/j.jtemb.2015.10.001
0946-672X/

human bronchoepithelial cells and murine embryonic fibroblasts [16,17]. It was shown to induce annexin II expression in human HaCaT keratinocytes [18], a known regulatory protein of phospholipase A_2 (PLA_2) activity and hence prostaglandin formation [19]. Taken together, a large number of physiological effects have been described for the abovementioned cations, which cannot or at least only in part be attributed to the CaR. It is thus conceivable to assume other ligation sites that result in specific metabolic cell responses.

We have previously described mechanisms of PGE_2 formation after stimulation of osteoblastic MC3T3-E1 cells with endothelin-1 (ET-1) [20,21]. The aim of the present study was therefore to investigate the effects of these cations on osteoblastic PGE_2 formation and their modulatory role after ET-1 stimulation.

2. Materials and methods

2.1. Materials

Alpha-minimum essential medium (α-MEM) and fetal calf serum (FCS) were obtained from Sera-lab (Vienna, Austria). Trypsin/ethylene diamine tetraacetic acid were purchased from Böhringer (Mannheim, FRG). L-glutamine was from Serva (Vienna, Austria). A23187, EGTA and HEPES were purchased from Sigma-Aldrich (Vienna, Austria). Ovine PGHS-1 and PGHS-2 were from Cayman via Biomol (Vienna, Austria). Pentafluorobenzyl bromide (PFBBr), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), silylation grade pyridine, acetonitrile, O-methoxyamine hydrochloride (MOX), and ET-1 were from Pierce Chemical Co. (Rockford, USA). Negative control siRNA and Flexitube siRNAs for cPLA2 (set of four siRNAs) were from QUIAGEN (Vienna, Austria). OligofectamineTM was from Invitrogen (Vienna, Austria). Culture dishes were from Falcon via Szabo (Vienna, Austria). MC3T3-E1 cells were kindly donated by Dr. Klaushofer (Vienna, Austria). Deuterated PGE_2 was obtained through MSD Isotopes via IC Chemikalien GmbH. Fura-2 acetoxymethylester (Fura-2 AM) was from Lambda Probes and Diagnostics (Graz, Austria). All other chemicals, solvents and reagents were from Merck (Darmstadt, FRG) or Sigma (Vienna, Austria).

2.2. Cell culture

MC3T3-E1 cells (passage number 10–30) were cultured routinely in α-MEM containing 5% FCS, 50 $\mu g\,mL^{-1}$ ascorbate and L-glutamine (0.584 $g\,L^{-1}$) in a humidified atmosphere of 5% CO_2 in 80 cm^2 flasks (initial plating density 2×10^4 cells cm^{-2}) and transferred to 4 cm^2 12-well culture dishes before experiments. Experiments were carried out at confluency (day 6 of culture). For prostaglandin measurements cells were cultured under starving conditions (0.2% FCS in α-MEM) for 24 h prior to stimulation to avoid serum induction of PGHS-2 [22]. Incubations with various agonists were carried out in the abovementioned medium or incubation buffer (D-glucose, 5.5 mM; KCl, 5.3 mM; NaCl, 136.8 mM; HEPES, 20.0 mM, ascorbate, 0.28 mM). Cells were pre-incubated with cations at various concentrations (0–15 mM for high-range concentration kinetics; 0–100 μM for low-range concentration kinetics; 1–2–4 mM for other experiments; concentrations were chosen to cover ranges from physiological to supernutritional) for ten minutes, followed by stimulation with ET-1 (50 nM).

2.3. PGE_2 analysis

Incubations with test compounds or vehicle were carried out for the indicated time periods. The incubation medium was removed and PGE_2 measured by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) [23]. Briefly, PGE_2 was converted to its PFB ester-trimethylsilyl ether-*O*-methyloxime derivative. Quantitation was carried out by use of tetradeuterated PGE_2. An ISQ GC–MS system (Thermo) was used. GC was performed on a 15 m DB-5MS fused silica capillary column (Thermo). The temperature of the splitless injector was kept at 290 °C, initial column temperature was 160 °C for 1 min, followed by an increase of 40°/min to 310 °C. NICI was carried out in the single ion recording mode with methane as a moderating gas.

2.4. PGHS-1 and PGHS-2 enzyme assays

Enzyme inhibitor potencies were elucidated in Tris/HCl buffer (30 mM, pH 8.0) containing glutathione (0.49 mM), epinephrine (1 mM) and hematine (1 μM). 1 u PGHS and 1 μM AA were used. Pre-incubation with cations was carried out for 10 min at room temperature. Incubation with AA was performed for 30 min at 37 °C. The reaction was terminated by the addition of ice-cold formic acid (0.2%) and PGE_2 measured.

2.5. Measurement of cytosolic Ca^{2+}

For $[Ca^{2+}]_i$ measurements, MC3T3 E1 cells, incubated with serum-free a-MEM, were loaded with 2 mM Fura-2 AM at 37 °C. After 30 min the loading medium was replaced by serum-free a-MEM without dye for another 15 min. Cells were then washed twice with a HEPES-buffered salt solution (HBSS; 20 mM HEPES, 136 mM NaCl, 5 mM KCl, 10 mM glucose; pH 7.3, 37 °C), and trypsinized. After harvesting, cells were resuspended in HBSS and kept at room temperature until use (10–60 min). For measurements cells were transferred to a stirred thermoregulated cuvette (37 °C). Fluorescence was monitored on a luminiscence spectrometer (Model LS-50B PerkinElmer) by continuously recording excitation signals at wavelengths of 340 and 380 nm and emission signals at 500 nm at 0.4 sec intervals. During the experiments, $[Ca^{2+}]_{ex}$ was adjusted to a final concentration as appropriate. Maximal and minimal fluorescence values were obtained by adding ionomycin and EGTA. Data were calculated according to the method of Grynkiewicz et al. [24].

2.6. $cPLA_2$ siRNA transfection

MC3T3-E1 cells were cultured in 6-well plates in α-MEM containing 5% fetal calf serum FCS without antibiotics. At 30–50% confluency cells were transfected with two different $cPLA_2$-specific siRNAs (100 nM) using the lipid transfection reagent oligofectamine™ according to the manusfacturer's instructions. A scrambled siRNA was used as a negative control. $cPLA_2$ silencing was monitored by Western Blotting (results not shown). At confluency, medium was exchanged with incubation buffer and pretreated with cations for 10 min. Cells were then stimulated with ET-1 and PGE_2 formation measured.

3. Results and discussion

3.1. Cations and ET-1-induced PGE_2 formation

In a previous study we have investigated ET-1-induced PGE_2 formation in osteoblast-like MC3T3-E1 cells and the calcium signaling events associated with it [20,21]. Thus, immediate prostaglandin response can be attributed to the action of PGHS-1 following liberation of arachidonic acid (AA) from phospholipid stores by phospholipases. A well acknowledged concept is that elevation of intracellular calcium results in activation of group IV cytosolic calcium-dependent phospholipase A_2 ($cPLA_2$) by effecting its translocation to the plasma membrane. Intracellular calcium ($Ca^{2+}{}_i$) signals evoked by ET-1 stimulation are a composite of