Ionomycin induces prostaglandin E2 formation in murine osteoblastic MC3T3-E1 cells via mechanisms independent of its ionophoric nature

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**Ionomycin induces prostaglandin E₂ formation in murine osteoblastic MC3T3-E1 cells
via mechanisms independent of its ionophoric nature**

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Short title: Ni²⁺ and Sr²⁺ potentiate osteoblastic prostaglandin formation

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Abstract

Ionomycin and A23187 are divalent cation ionophores with a marked preference for calcium. Studies using these ionophores have been almost exclusively interpreted their results in the light of calcium elevation. It was the aim of this study to investigate effects of ionomycin in osteoblastic MC3T3-E1 cells that are not attributable to its ionophoric properties. Thus, we have found that, in contrast to A23187, ionomycin shows similar effects on prostaglandin E2 formation as bradykinin and endothelin-1, being potentiated by extracellular nickel and inhibited by cholera toxin and pertussis toxin. Our data strongly suggest that ionomycin, at least in part, exerts its effects via specific binding to a G-protein coupled receptor, thereby evoking downstreams cellular events like arachidonate release with subsequent prostaglandin formation.

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Keywords: ionomycin; A23187; prostaglandin; nickel; osteoblast; MC3T3-E1

1. Introduction

The polyether carboxylic acid class antibiotics ionomycin and A23187 are divalent cation ionophores with a marked preference for calcium (Liu and Hermann 1978; Liu et al. 1978; Westley 1977) by forming complexes with 1:1 (ionomycin) or 2:1 (A23187) stoichiometry and electroneutral exchange (Kauffman et al. 1980; Taylor et al. 1993). Numerous studies have been performed using these ionophores as a pharmacological tool to elevate intracellular calcium levels, and observed effects have been almost exclusively interpreted in the light of calcium elevation. Especially for ionomycin, however, there are some studies suggesting activity beyond the classical ionophoric activation, such as prostaglandin (PG) release (Erlj et al. 1986) or effect on membrane conductance, thereby underlining the difficulties in attempting to separate ionophoric and other activities (Ito and Dulon 2002).

In previous studies we have described the effect of Ni^{2+} on PGE_2 synthesis in MC3T3 cells. Ni^{2+} enhances prostaglandin release after stimulation with bradykinin (BK) and endothelin-1 (ET-1) by a hitherto unknown calcium-independent mechanism that requires activation of G-protein coupled receptors (GPCR) (Leis and Windischhofer 2016; Leis et al. 1994). Ca^{2+} -ionophores are believed to cause arachidonic acid (AA) release and subsequent PG formation in many cell systems by elevation of intracellular calcium levels and hence activation of phospholipase (PL) A_2 .

It was the aim of this study to investigate the mechanisms that lead to PGE_2 formation after ionomycin treatment of MC3T3-E1 cells. As the stability constants of A23187- and ionomycin- Ni^{2+} complexes are markedly higher compared to Ca^{2+} , we have used Ni^{2+} as a pharmacological tool to distinguish between calcium transport-related and other effects of the ionophores.

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 Böhrringer (Mannheim, FRG). L-glutamine was from Serva (Vienna, Austria). A23187, ionomycin, EGTA, HEPES, ET-1 and BK were purchased from Sigma-Aldrich (Vienna, Austria). Pentafluorobenzyl bromide (PFBBBr), N-methy-N-(trimethylsilyl) trifluoroacetamide (MSTFA), silylation grade pyridine, acetonitrile and O-methoxyamine hydrochloride (MOX), were from Pierce Chemical Co. (Rockford, USA). Culture dishes were from Falcon via Szabo (Vienna, Austria). MC3T3-E1 cells were kindly donated by Dr. Klaushofer (Vienna, Austria)). Deuterated PGE₂ was obtained through MSD Isotopes via IC Chemikalien GmbH. 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 $\mu\text{g mL}^{-1}$ ascorbate and L-glutamine (0.584 g L^{-1}) in a humidified atmosphere of 5% CO₂ in 80 cm² flasks (initial plating density 2×10^4 cells cm⁻²) and transferred to 4 cm² 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 hours prior to stimulation to avoid serum induction of PGHS-2 (Pilbeam et al. 1993). 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).

2.3. PGE₂ analysis

Incubations with test compounds or vehicle were carried out for the indicated time periods. The incubation medium was removed and PGE₂ measured by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) (Leis et al. 1987). Briefly, PGE₂ was converted to its PFB ester-trimethylsilyl ether-O-methyloxime derivative. Quantitation

was carried out by use of tetradeuterated PGE₂. 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. Synthesis of ionomycin methyl ester

A solution of a given concentration of ionomycin free acid in methanol was evaporated under nitrogen and the residue reconstituted with 3 mL of methanolic HCl (200 µL of acetyl chloride added to 6 mL methanol). After an incubation time of 15 minutes at room temperature the solvent and the excess reagent were evaporated under nitrogen. The residue was dissolved in methanol and purity checked by TLC and reversed phase HPLC.

2.5. Statistical analysis

Statistical analysis was performed with student's t-test for unpaired samples, where appropriate. All results are shown as one representative dataset from at least three independent experiments to retain the variability of PGE₂ determinations. Statistical data were calculated with SigmaPlot software version 13.0.

3. Results and discussion

3.1. Ionophore-induced PGE₂ formation

PGE₂ formation in MC3T3-E1 cells is very sensitive to culture conditions (cell density, passage number, etc.) and hence may vary considerable among different experiments. Nevertheless, this is true only for the amount of PGE₂ formed. In any case, the observed effects (IC₅₀ values, percentage-inhibition, etc.) were qualitatively and quantitatively reproducible, despite differences in absolute amounts of PGE₂ formation. Thus, one representative dataset from at least three independent experiments is shown in the figures, and

calculated parameters (IC₅₀ values, etc.) are presented from the pooled data of the different experiments.

When MC3T3-E1 cells were challenged with A23187 and ionomycin (2 μ M) in the presence of various amounts of Ni²⁺ a completely different PGE₂ response was observed for the two ionophores, as shown in Fig. 1. Expectedly Ni²⁺ blocks A23187-induced PGE₂ production with an IC₅₀ of 5.6 ± 0.3 μ M (from three independent experiments), reaching complete inhibition above 10 μ M, an effect independent of extracellular calcium concentration, as almost identical kinetics were obtained in the presence or absence of extracellular Ca²⁺ (see Fig. 2). This can be correlated to the stability constants of the Ni²⁺-A23187 complex and the Ca²⁺-A23187 complex that differ by three orders of magnitude (log K [Ni²⁺] = 7.54; log K [Ca²⁺] = 4.50) (Leis and Windischhofer 2016; Taylor et al. 1993). Thus, A23187-induced prostaglandin formation can be attributed to its ionophoric properties. The situation changes completely when ionomycin was used. At low Ni²⁺ Ionomycin also stimulates PGE₂ formation, but this effect is not blocked by increasing Ni²⁺. In contrast, PGE₂ levels rise with increasing Ni²⁺, the effect starting at 20 μ M and being strikingly prominent at 4 mM. As stability constants for the ionomycin-Ni²⁺ complex are even four orders of magnitude higher compared to Ca²⁺-ionomycin (log K [Ni²⁺] = 10.25; log K [Ca²⁺] = 6.27) (Taylor et al. 1993) we would expect a similar behaviour as with A23187: Ni²⁺-ionomycin should freely cross membranes, but shall not release Ni²⁺ nor bind Ca²⁺ for release from internal stores and consequently show no prostaglandin formation. It is thus obvious that ionomycin activates PGE₂ formation in MC3T3-E1 cells by mechanisms beyond the classical ionophoric effects. This effect of Ni²⁺ and ionomycin is virtually identical to the response of BK and ET-1 with Ni²⁺ in these cells (Leis and Windischhofer 2016; Leis et al. 1994), where the cation potentiates the agonist-evoked PG formation in a similar manner. From the abovementioned studies it is evident, that activation of GPCRs is necessary to produce the effect. We thus

suggest that activation of a hitherto unknown GPCR is responsible for the strikingly different activation of PGE₂ formation by A23187 and ionomycin in these cells.

To harden this hypothesis the following experiments have been conducted. Fig. 2 shows the combined activity of Ni²⁺ (4 mM), Ca²⁺ (1.8 mM), ionomycin (1 μM), A23187 (1 μM) and ET-1 (50 nM) on PGE₂ formation in MC3T3-E1 cells at both, presence and absence of extracellular calcium. A23187 shows only a minor increase of prostaglandin synthesis in the presence of extracellular calcium (and without Ni²⁺) suggesting that the ionophore liberates calcium mainly from intracellular stores, whereas the difference is significantly more pronounced with ionomycin under the same conditions. This can be rationalized by already described alternate mechanisms of ionomycin, such as store-operated calcium entry or calcium-induced calcium release (Galitzine et al. 2005; Kochegarov et al. 2000; Kochegarov et al. 2001; Muller et al. 2013). Co-stimulation with A23187 and ionomycin results in a synergistic effect in the absence of Ca²⁺, but shows the striking potentiation of PGE₂ production in with Ni²⁺. This confirms or previous findings, that Ni²⁺ is not released intracellularly from A23187 and the effect of blunted PGE₂ formation in the presence of Ni²⁺ is due to strong complexation rather than intracellular inhibitory effects (Leis and Windischhofer 2016; Leis et al. 1994). Basically the same applies to the synergism of A23187 and ET-1. ET-1 and ionomycin behave qualitatively identical in this context, thus strengthen the suggestion for a receptor-operated activity of ionomycin.

In order to investigate whether the observed effect is dependent on cation chelation we have synthesised the methyl ester analogue of ionomycin (iono-ME), as cation binding relies strongly on the carboxylic acid function (Toeplitz et al. 1979). The results of PGE₂ measurement after incubating with iono-ME are shown in Fig. 3. Expectedly, there is no stimulation in the absence of Ni²⁺, as also Ca²⁺ - binding is abolished and there is no ionophoric activity. This was confirmed by the lack of stimulating activity of Iono-ME in the presence of extracellular calcium (results not shown). On the other hand, there is a weak

stimulation in the presence of Ni^{2+} , reaching a maximal rate of 12.7% of unmodified ionomycin. This can be attributed to a non-ionophoric interaction, suggesting that iono-ME is a weaker stimulant of receptor-mediated activation than ionomycin..

To gain further evidence for a GPCR-related action of ionomycin we have studied the contribution of G_i - and G_s -proteins with their specific inhibitors pertussis toxin (PTX) and cholera toxin (CTX), respectively. The results are shown in Fig. 4. PGE_2 formation induced by A23187 is not significantly different from unblocked controls, as expected. With ionomycin, however, Ni^{2+} -potentiated PGE_2 production is markedly reduced by 38.9 % (PTX) and 51.7% (CTX). These data are in line with our previous findings that ET-1 mediated prostaglandin synthesis in Ni^{2+} - stimulated cells is inhibited by 63.6% (CTX) and 43.6% (PTX). This responsiveness to G-protein mediated transduction events clearly demonstrates a specific receptor-dependent effect rather than unspecific interactions.

At present we do not know the receptor(s) involved in this process. In a recent study, however, ionomycin has been identified as a ligand for the peroxisome proliferator-activated receptor γ (PPAR γ) (Zheng et al. 2013). Although not a GPCR, its classical ligands, thiazolidinedione drugs, exert anti-proliferative and anti-inflammatory properties by activating the free fatty acid receptor 1 (FFAR1; GPR40) (Tang et al. 2015), leading to arachidonate release and prostanoid formation (Tsukamoto et al. 2004). It remains to be elucidated if this also accounts for ionomycin and if the found effects are specific for the osteoblastic phenotype or an ubiquitous activation mechanism. This will be the focus of our ongoing studies. Additionally, PPAR isoforms are involved in the regulation of expression of prostaglandin endoperoxide-H synthase 2 (PGHS-2), although their role is still controversial, showing both, activation and inhibition of PGHS-2 expression (Aleshin et al. 2009; Grau et al. 2006; Luna-Medina et al. 2005; Zhang et al. 2006). Nevertheless, activation of PGHS-2 expression cannot account for the observed immediate response of PGE_2 formation in our study, as we have previously demonstrated that within 30 minutes PGE_2 formation is solely

due to PGHS-1 activity in MC3T3-E1 cells and PGHS-2 protein levels peak around three hours after induction. (Leis et al. 1998). Similarly, PPAR activation has dichotomous control on the expression levels of cytosolic and secretory phospholipase A₂ in astrocytes showing inhibition in untreated cells and enhancement in LPS-stimulated cells (Sergeeva et al. 2010). Again, this induction cannot account for the release of arachidonate in short-term PGE₂ formation. It should be mentioned in that context, that we can rule out an intracellular activating effect of Ni²⁺ on PGHS, as we have previously shown that even low concentrations of the cation are inhibitory to these enzymes (Leis and Windischhofer 2016). Finally, our data implicate that cell biological responses obtained with ionomycin must be interpreted with care and the correlation to intracellular calcium elevation distinguished from its receptor-mediated effects.

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Figure captions

Figure 1: Effect of Ni^{2+} on PGE_2 formation induced by ionomycin ($\square$) and A23187 ($\blacksquare$). MC3T3-E1 cells were cultured as described and pre-incubated with Ni^{2+} at the indicated concentrations for ten minutes in the absence of extracellular calcium. Ionophores were then added (2 μM) and PGE_2 was measured after 30 minutes of stimulation. Values are presented as means of triplicate determinations from one representative dataset out of three independent experiments.

Figure 2: Combined effect of Ni^{2+} (4 mM), Ca^{2+} (1.8 mM), ionomycin (1 μM), A23187 (1 μM) and ET-1 (50 nM) on PGE_2 formation in MC3T3-E1 cells. Cells were cultured as described and pre-incubated with Ni^{2+} or vehicle for ten minutes in the absence or presence of extracellular calcium. Stimulation with ionophores, ET-1 or vehicle at the indicated combinations was carried out for 30 minutes and PGE_2 was measured. Values are presented as means of triplicate determinations from one representative dataset out of three independent experiments. n.s. = statistically not significant ($p > 0.05$); * = statistically significant ($p < 0.05$).

Figure 3: Effect of Ni^{2+} (4 mM) on PGE_2 formation induced by iono-ME in MC3T3-E1 cells. Cells were cultured as described and pre-incubated with Ni^{2+} or vehicle for ten minutes in the absence of Ca^{2+} . Stimulation with iono-ME or vehicle at the indicated concentrations was carried out for 30 minutes and PGE_2 was measured. Values are presented as means of triplicate determinations from one representative dataset out of three independent experiments. * = statistically significant ($p < 0.05$).

Figure 4: Effect of PTX (250 ng/mL) and CTX (250 ng/mL) on Ni^{2+} -potentiated PGE_2 formation induced by ionomycin (2 μM) and A23187 (2 μM). MC3T3-E1 cells were cultured as described. Pre-incubation with PTX or CTX was done for 17 hours. Ni^{2+} (4 mM) was then added in the absence of extracellular calcium. After ten minutes ionophores were added and PGE_2 was measured after 30 minutes of stimulation. Values are presented as means of

triplicate determinations from one representative dataset out of three independent experiments. Percentual relations were calculated after normalization of absolute PGE₂ values to cell number (ng PGE₂/10⁶ cells). n.s. = statistically not significant (p>0.05); * = statistically significant (p<0.05).

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