Biochimie 93 (2011) 149–159

Contents lists available at ScienceDirect

Biochimie

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

Research paper

PKCdelta is a positive regulator of chondrogenesis in chicken high density micromass cell cultures

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ARTICLE INFO

Article history:
Received 28 June 2010
Accepted 3 September 2010
Available online 17 September 2010

Keywords:
Rottlerin
MEK-ERK
Sox9
shRNA
Cartilage formation

ABSTRACT

We aimed to elucidate the role of the Ca-independent PKC isoenzyme PKCdelta in the regulation of spontaneous *in vitro* chondrogenesis occurring in a 6-day-long culturing period in chicken limb bud-derived high density cell cultures (HDC). PKCdelta expression and activity were detectable throughout the entire culturing period with a peak on days 2 and 3, when most of the chondroblasts differentiate. To inhibit the activity of PKCdelta, either the natural compound rottlerin was transiently applied to the culture medium of HDC in 2.5, 5 or 10 μM concentrations, or gene silencing was performed by using PKCdelta shRNA. Rottlerin significantly reduced the overall PKC activity in enzyme activity assays of cell-free samples of untreated control HDC, probably via the inhibition of PKCdelta. On the contrary, we were unable to detect any consistent change of PKC enzyme activity assayed in samples of HDC treated with rottlerin during culturing. PKCdelta gene silencing resulted in a significantly lower PKC activity. Both rottlerin and PKCdelta shRNA caused a severe reduction in cartilage formation, furthermore protein and phospho-protein levels of Sox9, the key transcription factor of chondrogenesis, were also significantly decreased. Rottlerin lowered, while PKCdelta gene silencing elevated the phosphorylation status of ERK1/2. Our data suggest that PKCdelta stimulates chondrogenesis via influencing Sox9 and ERK1/2 phosphorylation, but the inhibition of cartilage formation in the rottlerin-treated HDC is probably PKCdelta independent and rottlerin might have different effects when applied to cells or to an *in vitro* enzyme activity assay.

1. Introduction

During vertebrate limb development, at the onset of the multi-step process of endochondral bone formation, undifferentiated chondroprogenitor mesenchymal cells first undergo a condensation phase characterised by rapid proliferation of cells. As a result, chondrogenic mesenchymal cells become closely packed, which initiates a complex and a yet not fully understood signalling mechanism that governs differentiation of cells within these condensations into chondroblasts and then mature chondrocytes [1]. Among a number of signalling molecules, members of the phospholipid-dependent serine/threonine protein kinase C (PKC) family are known regulators of *in vitro* chondrogenesis [2,3].

PKCs, present in almost all cell types, are involved in the regulation of various cellular processes. All known 11 PKC isoforms are divided into three subgroups based on their N-terminal regulatory domains and mechanism of activation: classical PKCs (cPKC; PKCalpha, betaI, betaII and gamma), novel PKCs (nPKC; PKCdelta, epsilon, eta and theta) and atypical PKCs (aPKC; PKCzeta, iota/lambda and mu or PKD) [4]. The activation of PKCdelta also requires lipid second messengers (*e.g.* DAG) or tumour-promoting phorbol esters (*e.g.* PMA) without the requirement of Ca^{2+}. Moreover, PKCdelta exhibits tyrosine-phosphorylation sites, which are targets

Abbreviations: BMP, bone morphogenic protein; BSA, bovine serum albumin; CaM-KIII, calcium/calmodulin dependent protein kinase III; CMF–PBS, calcium/magnesium free PBS; DAG, diacyl-glycerol; DMMB, dimethyl-methylene blue; dNTP, deoxy nucleotide triphosphate; DTT, 1,4-dithio-threitol; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; HDC, high density culture; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein kinase; PAGE, polyacrilamide gel electrophoresis; PBS, phosphate buffered saline; PBST, phosphate buffered saline and Tween-20; PI, propidium iodide; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethylsulphonyl-fluoride; PRAK, p38-regulated/activated protein kinase; RT, reverse transcription; SDS, sodium-dodecyl-sulphate; TAE, TRIS-acetate-EDTA buffer.

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doi:10.1016/j.biochi.2010.09.005

for Src family kinases. Upon phosphorylation on tyrosine residues, PKCdelta can act as a lipid-independent enzyme [5]. One of its distinguishing characteristics is that unlike other PKC isoforms, PKCdelta activity is also involved in negative regulation of various cellular processes, *e.g.* suppression of proliferation and survival [6]. PKCdelta is also a crucial component of the cellular stress response, since it is required for apoptotic processes, however, some data suggest that it can also negatively influence apoptosis [7].

To identify the physiological substrates and multiple roles of various PKC isoenzymes in cellular processes, several protein kinase inhibitors have been developed with a variable specificity for individual kinases. The bisindolylmaleimide GF109203X is a general PKC inhibitor with a more potent effect on cPKC. The indolocarbazole Gö6976 inhibits both cPKC and nPKC [8]. However, the specificity of these pharmacological inhibitors is a rather controversial issue [9]. Some protein kinase inhibitors exhibit variable degrees of specificity for different enzymes at distinct concentrations. A polyphenolic compound [5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene], rottlerin, isolated from a common Indian rain forest tree, *Mallotus philippinensis*, is reported to inhibit several PKC isoforms, and is thought to selectively inhibit PKCdelta 5–30-fold stronger than other PKCs at 3–6 μM concentration [4]. However, according to the findings of an *in vitro* enzyme activity measurement campaign conducted by the group of Davies [9], in which the specificity of various protein kinase inhibitors were assayed, rottlerin was found to inhibit many protein kinases (*e.g.* PRAK, MAPKAP-2) much more potently than PKCdelta, and in fact it failed to inhibit *in vitro* PKCdelta activity. Rottlerin was also described to inhibit some other kinases as Akt/PKB and CaM-KIII at 500 nM [10]. Moreover, in a recent review analysing data of publications describing controversial results gained by the application of rottlerin in order to inhibit PKCdelta, it is concluded that rottlerin can be considered as a mitochondrial uncoupler rather than a direct inhibitor of this enzyme [11].

Rottlerin modulates a great variety of cellular processes in both malignant and non-malignant cells, including apoptosis in lung cancer, breast cancer, leukaemia and myeloma cells, proliferation in glioma cells, secretory activity of pancreatic acinar cells [12–14], and it is also known to regulate tumour cell migration [15]. Rottlerin has also been described to interfere with the differentiation process of various cell types of mesenchymal origin, exerting its effects at least partially by inhibiting the activity of PKCdelta [16–18]. In a recent study, Choi and his co-workers reported that rottlerin altered the migration of prechondrogenic mesenchymal cells in chicken limb bud high density cultures by modulating integrin β1-signalling at focal adhesion complexes via a PKCdelta-independent mechanism [10].

In this study we applied the same *in vitro* chondrogenesis model, in which high density cell cultures are established from chondrogenic mesenchymal cells isolated from limb buds of chicken embryos. In HDC, formation of cartilage starts with the condensation of chondroprogenitor mesenchymal cells on the first day, that after nodule formation differentiate into chondroblasts and chondrocytes predominantly on the second and third days of culturing [19]. Steps of this differentiation process are regulated by numerous growth factors and other soluble morphogens [20] and differentiating cells start to secrete cartilage-specific extracellular matrix components, such as collagen type II and aggrecan on the third day of culturing [21]. Expression of cartilage-specific matrix molecules is regulated by Sox9, a high-mobility-group domain containing transcription factor, which is started to be expressed as soon as mesenchymal cells become committed toward the chondrogenic lineage [22]. Detection of the mRNA and protein expression level and the phosphorylation status of Sox9, as well as monitoring the expression of the core protein of aggrecan and collagen type II are reliable markers of *in vitro* chondrogenesis.

Here we provide evidence that PKCdelta is expressed by cells of chondrifying chicken limb bud high density cultures throughout their entire differentiation process. Administration of rottlerin to cells of HDC resulted in a time and concentration dependent inhibition of metachromatic cartilage matrix production and caused a marked decrease in the phosphorylation of both Sox9 and ERK1/2, but we failed to unambiguously demonstrate inhibition of PKCdelta activity with this compound. PKCdelta gene silencing significantly lowered the activity of PKC, abolished cartilage matrix production and decreased the level of phosphorylated Sox9, but elevated the phosphorylation of ERK1/2. Our results indicate that PKCdelta acts as a positive regulator of *in vitro* chondrogenesis via modulation of the ERK1/2 and Sox9 pathways. Nevertheless, the chondrogenesis-inhibiting effect of rottlerin is probably exerted via a PKCdelta-independent manner, therefore we do not recommend administration of rottlerin for PKCdelta inhibition in high density cell culture systems.

2. Materials and methods

2.1. Cell culturing

Distal parts of the limb buds of Ross hybrid chicken embryos (Hamburger–Hamilton stages 22–24) were removed and primary micromass cultures of chondrifying mesenchymal cells were established from cell suspensions with a density of 1.5×10^7 cells/mL. 100–100 μL droplets of the suspension were inoculated into plastic Petri dishes (Orange Scientifique, Braine-l'Alleud, Belgium). Day of inoculation is considered as day 0. After 2 h, colonies were nourished with Ham's F12 medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Gibco, Gaithersburg, MD, USA) and were kept at 37 °C in the presence of 5% CO_2 and 80% humidity in a CO_2 incubator. The medium was changed on every second day.

2.2. Transient gene silencing and pharmacologic inhibition of PKCdelta

PKCdelta shRNA (GenScript USA Inc., Piscataway, NJ, USA) was cloned into GeneSwitch™, the inducible protein expression system from Invitrogen (Invitrogen, Carlsbad, CA, USA). Plasmids were amplified using competent *E. coli* bacteria from One Shot chemical transformation kit (Invitrogen). Ampicillin or hygromycin resistant bacteria were grown on LB agar, and plasmids were isolated using MaxiPrep kit (QIAGEN, Valencia, CA, USA) according to the protocol of the manufacturer. Plasmids were delivered into cells of chondrifying cell cultures by using Lipofectamine 2000 (Invitrogen) transfection reagent. Lipofection delivery system protocol was performed on freshly isolated cell suspensions with a density of 1.5×10^7 cells/mL by decreasing the volume of the transfection reagent by 25%. 100 or 30 μL mixtures of the transfection reagent and cell suspension were inoculated into Petri dishes and into 24-well plates, respectively. After 2 h of transfection colonies were nourished with Ham's F12 medium supplemented with 10% fetal calf serum. On day 2 of culturing, 1 μM mifepristone was added to the culture medium for 24 h for the induction of GeneSwitch™ System. Activity of PKCdelta was inhibited by applications of 2.5, 5 or 10 μM rottlerin (Sigma) for 4 h on different days of culturing.

2.3. Light microscopic morphology

High-density cultures established from 30 μL droplets of chondrogenic limb bud mesenchymal cells of different experimental