FEBS Letters 584 (2010) 4442–4448

journal homepage: www.FEBSLetters.org

Protein kinase Cμ mediates adenosine-stimulated steroidogenesis in primary rat adrenal cells

Yung-Chia Chen [a,1], Ying Chen [a], Shih-Horng Huang [b,c,*], Seu-Mei Wang [a,*]

[a] Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan
[b] Department of General Surgery, Far Eastern Hospital, Taiwan
[c] Yuan Ze University Graduate School of Biotechnology and Bioengineering, Taoyuan, Taiwan

ARTICLE INFO

Article history:
Received 22 July 2010
Revised 17 September 2010
Accepted 6 October 2010
Available online 13 October 2010

Edited by Robert Barouki

Keywords:
Adenosine
Steroidogenesis
Adenosine receptor
Protein kinase C
Steroidogenic acute regulatory protein
mRNA

ABSTRACT

Adenosine (Ado), an endogenous nucleoside, can stimulate corticosterone synthesis in adrenal cells via the A_{2A}/A_{2B} adenosine receptors (ARs). This study evaluated the contribution of protein kinase C (PKC) isoforms in Ado-induced steroidogenesis. The PKC inhibitor calphostin c blocked Ado-induced steroidogenesis, the mitogen-activated protein kinase (MEK)-extracellular signal-related regulated kinase (ERK)-cyclic AMP responsive element-binding protein cascade, and the mRNA expression of steroidogenic acute regulatory protein and CYP11B1. Further analyses revealed that PKCμ was indeed activated by Ado. Moreover, downregulation of PKCμ by small interfering RNA (siRNA) inhibited Ado-stimulated steroidogenesis and ERK phosphorylation. Finally, inhibition of either A_{2A}AR or A_{2B}AR led to the suppression of PKCμ phosphorylation. Together, these findings suggest that A_2AR-PKCμ-MEK signaling mediates Ado-stimulated adrenal steroidogenesis.

1. Introduction

Traditionally, corticosterone production in adrenal cells is controlled by protein kinase A (PKA) and protein kinase C (PKC) systems [1,2]. In addition, extracellular signal-related regulated kinase (ERK) and tyrosine kinase, including Janus kinase 2 (JAK2) and Src kinase, also regulate steroidogenesis in adrenal Y-1 cells and bovine adrenal glomerular cells [3,4]. The downstream targets of the activation of these specific kinases include hormone sensitive lipase, steroidogenic factor 1, cyclic AMP responsive-element binding protein (CREB), steroidogenic acute regulatory protein (StAR) proteins, and 11β-hydroxylase [5–8].

Adenosine (Ado) is a naturally occurring purine nucleoside derived from the breakdown of adenosine triphosphate. The actions of Ado in cells are operated via adenosine receptors (ARs), which are classified into four subtypes, A_1, A_{2A}, A_{2B}, and A_3 [9]. ARs are G protein-coupled receptors (GPCRs), with A_1AR and A_3AR interacting with Gi/o proteins to inhibit adenylyl cyclase, and A_{2A}AR and A_{2B}AR interacting with Gs, $G_{i/o}$ or G_{olf} [9] to induce the activation of various signaling mechanisms in different cell types.

One of the physiological functions of Ado is the stimulation of corticosterone production in adrenal cells, which is mediated by the activation of the A_2AR-JAK2 signaling pathway [10]. However, the JAK2 pathway is only responsible for about half of the Ado-stimulated adrenal steroidogenesis [10]. The nature of other signaling pathways leading to Ado-induced steroidogenesis remains to be explored. Since PKC is known to regulate adrenal steroidogenesis, we hypothesized that PKC might participate in the Ado-induced steroidogenesis. We tested this hypothesis in primary cultures of adrenal cells, and tried to identify the PKC isoforms involved in this event.

2. Materials and methods

2.1. Drugs

Ado and the PKCδ inhibitor rotterlin were purchased from the Sigma Chemical Co. (St. Louis, MO). The non-specific PKC inhibitor

Abbreviations: Ado, adenosine; AR, adenosine receptor; CREB, cyclic AMP responsive-element binding protein; ERK, extracellular signal-related regulated kinase; GPCRs, G protein-coupled receptors; JAK2, Janus kinase 2; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory protein

* Corresponding authors. Address: Department of General Surgery, Far Eastern Hospital, Taiwan (S.-H. Huang), Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, 1-1 Jen-Ai Road, Taipei 10051, Taiwan. Fax: +886 2 23915192 (S.-M. Wang).

E-mail address: smwang@mc.ntu.edu.tw (S.-M. Wang).

[1] Present address: Department of Anatomy, Kaohsiung Medical University, Kaohsiung, Taiwan.

0014-5793/$36.00
doi:10.1016/j.febslet.2010.10.001

calphostin c, the specific PKCε inhibitor εV_{1-2}, and the specific PKCαβ inhibitor Gö6976 were purchased from Calbiochem (La Jolla, CA). The PKCβ inhibitor LY333531 was from Enzo Life Sciences International Inc. (Plymouth Meeting, PA). The A_{2A}AR antagonist ZM241385 and the A_{2B}AR antagonist MRS1706 were from Tocris Cookson Inc. (Avonmouth, UK).

2.2. Cell culture

Adrenal cell cultures were obtained as described by Chen et al. (2005) [11]. Day 2–3 cultures were used in all experiments.

2.3. Corticosterone radioimmunoassay

The collected culture medium samples were assayed for corticosterone according to the procedure described by Chen et al. (2005) [12].

2.4. siRNA transfection

SiCONTROL cyclophilin B (Dharmacon Corporation, Lafayette, CO) was used to estimate the efficiency of siRNA silencing. SiRNAs targeting rat PKCμ coding sequences were custom-designed by Dharmacon Corp. (Lafayette, CO). Day 2 cultures were used for transfection, which was performed by incubation with DharmaFECT transfection reagent and cyclophilin B siRNA (100 nM) or PKCμ siRNA (100 nM) in culture medium for 48 h. The efficiency of silencing by cyclophilin B siRNA or PKCμ siRNA was verified by RT-PCR and downregulation of PKCμ protein by PKCμ siRNA was verified by Western blotting.

2.5. Western blot analysis

After various treatments, total cell homogenates were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose paper and Western blotting was performed as described previously [11]. Primary antibodies included mouse antibodies against phosphorylated ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Sigma), and rabbit antibodies against phosphorylated forms of mitogen-activated protein kinase kinase (MEK)$1/2^{ser217/221}$, CREB-^{ser133}, PKCμ^{ser916} or PKCμ$^{ser744/748}$ (Cell Signaling Technology Inc., Beverly, MA) diluted in Tris-buffered saline (150 mM NaCl, 50 mM Tris base, 0.1% Tween, pH 8.2). The densities of the bands on the nitrocellulose membrane were quantified by densitometry using Gel Pro 3.1 (Media Cybernetics, Silver Spring, MD). The band intensity of the control group was defined as 100% and the densities of the band in the test samples expressed as a percentage of this value.

2.6. Real-time quantitative polymerase chain reaction

Total cellular RNA was prepared using TRIzol reagent (Invitrogen, Paisley, UK) and 1 μg was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen, Paisley, UK) and the cDNA generated subjected to real-time quantitative polymerase chain reaction amplification using primers specific for rat StAR or CYP11B1 and β-actin as a housekeeper control. The primers for rat StAR, CYP11B1 and β-actin were designed using primer3 software and were: StAR forward, TTCGCTATCACCATCAGCACCG; StAR backward, CCTGGCACCTCACTCTCTTC; β-actin forward, GCCATTGTTACCAACTGGGACG; β-actin backward, TTGATGTCACGCACGATTTCC; CYP11B1 forward, CCCAAGACACTGAAGCCCTTTG; CYP11B1 backward, GCCCCATTTAGCAAGAACACAC. The SYBR green Advantage qPCR premix kit was purchased from Clonetech Laboratory, Inc. (Mountain View, CA) and the reaction time was according to the manufacturer's instructions.

2.7. Statistical analysis

All experiments were performed at least three times and the values are expressed as the mean ± S.D. Statistical differences were determined using the Kruskal–Wallis test and corrected using the Mann–Whitney test. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Ado-stimulated steroidogenesis is mediated by PKC

As shown in Fig. 1A, application of Ado led to a concentration-dependent increase in corticosterone production in adrenal cells. These effects were observed at as early as 3 h post treatments, and were much more prominent at 6 h post treatments. In all subsequent experiments, 30 μM Ado was used. In addition to PKA, ERK, or JAK2, it has been demonstrated that PKC also plays an

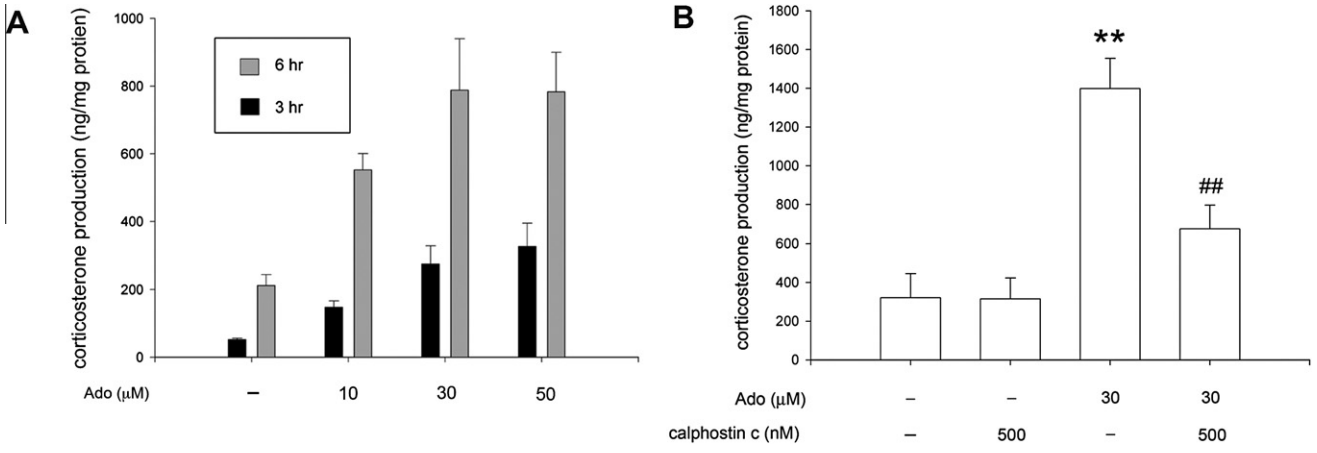

Fig. 1. Ado-induced steroidogenesis is time- and concentration-dependent and inhibited by calphostin C. (A) Time- and concentration-dependent effects of Ado on adrenal steroidogenesis. Adrenal cells were treated with 0.1% DMSO (vehicle) or 10, 30, or 50 μM Ado for 3 or 6 h, then the culture supernatants were assayed for corticosterone. Adrenal cells were pretreated for 30 min with the PKC inhibitor calphostin C (500 nM), then with 0.1% DMSO or 30 μM Ado for 6 h in the continued presence of the inhibitor, then the culture supernatants were assayed for corticosterone. (B) Effect of calphostin C. The results shown are the mean ± S.D. for three separate experiments. $^{**}P < 0.01$ compared to the control group. $^{\#\#}P < 0.01$ compared to the Ado group.