Biochimica et Biophysica Acta 1793 (2009) 1868–1875

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Adenosine A_1 receptors selectively target protein kinase C isoforms to the caveolin-rich plasma membrane in cardiac myocytes

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ARTICLE INFO

Article history:
Received 5 June 2009
Received in revised form 14 October 2009
Accepted 21 October 2009
Available online 30 October 2009

Keywords:
Adenosine receptor
Protein kinase c
Caveolae
Caveolin-3
Cardiac myocyte

ABSTRACT

Adenosine is a naturally occurring nucleoside that has been shown to regulate a variety of functions in the cardiovascular system. However, the mechanisms in adenosine receptor signaling are not completely understood. Given that adenosine receptors have been linked to protein kinase C (PKC) in cardioprotection and caveolae is critical for receptor signaling, we sought to determine whether activation of adenosine A1 receptors induces selective translocation of PKC isoforms to the membrane from the cytosol and whether activated PKC is targeted to the caveolin-rich plasma membrane microdomains. The freshly isolated adult rat cardiac myocytes were used to examine PKC isoforms including PKCα, PKCβ, PKCε, PKCδ and PKCζ. Immunoblot analysis revealed that the immunoreactivity for PKCε or PKCδ but not for PKCα, PKCβ or PKCζ increased significantly in the membrane fractions from cells pretreated with the selective adenosine A1 receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA, 100 nM) when compared with non-stimulated cells. The effect of CCPA on PKCε or PKCδ translocation was blocked by adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 nM). When Western blot was performed from the caveolin-enriched plasma membrane fractions, the immunoreactivity for PKCε or PKCδ but not PKCα, PKCβ or PKCζ was enhanced significantly by CCPA. Furthermore, PKCε and PKCδ were detected in the anti-caveolin-3 immunoprecipitates but not in the samples without primary antibody. Immunofluorescence staining further indicates increased colocalization of PKCε or PKCδ with caveolin-3 at cell peripheral region and T-tubular-like structures in response to adenosine A1 receptor activation. In conclusion, we demonstrate that activation of adenosine A1 receptors promotes the selective translocation of PKCε and PKCδ to the caveolin-enriched plasma membrane microdomains in cardiac myocytes.

1. Introduction

Adenosine is a natural nucleoside produced from the cardiovascular system and exerts a variety of functions including bradycardia, hypotension and coronary vasodilation [1]. Adenosine recognizes specific cell surface receptors including four adenosine receptor subtypes (A1, A2A, A2B and A3). All these receptor subtypes are coupled to guanine nucleotide binding proteins (G proteins). The most characterized mechanism is the effect on adenylate cyclase. In addition, another important pathway involving protein kinase C (PKC) has been postulated to play a key role in adenosine receptor signaling [2–4].

PKC exists as a family of at least 12 isoforms. PKC isoforms mediate distinct cellular functions by phosphorylating specific downstream target proteins. Substrate specificity is most likely associated with subcellular localization of activated PKC isozymes. In the heart, a variety of signaling molecules have been localized in caveolae [5,6]. Caveolae are small (50 to 100 nm) cholesterol and sphingolipid enriched "cave"-like invaginations of the surface membrane, very rich in many of the signaling molecules [7–11]. These microdomains may act to generate subcellular signaling compartments by recruiting interacting signaling molecules. Indeed, cardiac myocyte caveolae are the focal points for activated PKC isoforms and their downstream signaling molecules [10].

PKC can be activated by either exogenous PMA or endogenous receptor-mediated signaling. While PMA activates both classical and novel PKC isoforms, translocation of PKC by distinct receptor signaling is isoform selective. Adenosine A_1 receptor is well known to couple to inhibitory G proteins, and inhibitory G protein often couple to their effectors by PKC [2,12,13]. It has been shown that PKCε translocation to cardiac sarcolemma or T-tubular structures is involved in adenosine receptor-mediated responses [14–16]. Recent evidence suggests that adenosine A1 receptor activation can selectively modify phosphatase and mitogen-activated protein kinases (MAPK) activities in caveolin-rich cardiac membrane fractions [17]. However, it is not known whether adenosine receptor activation targets translocated PKC isoforms to the caveolin-rich plasma membrane microdomains. To determine whether activation of adenosine A1 receptors promotes translocation and caveolar targeting of specific PKC isoforms, we

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doi:10.1016/j.bbamcr.2009.10.007

employed the freshly isolated adult cardiac myocytes and examined whether activation of adenosine A_1 receptors by CCPA induces translocation of PKC isoforms to the cell membrane and whether translocated PKC isoforms by CCPA are targeted to caveolin-rich plasma membrane microdomains. We focused on examining six major isoforms of PKC that are known to be expressed in adult rat cardiomyocytes [18,19]. Our results demonstrate that activation of adenosine A_1 receptors increases immunoreactive membrane PKCε and PKCδ but not PKCα, PKCβ and PKCζ in the caveolin-rich microdomains.

2. Materials and methods

2.1. Materials

Mouse IgG directed against caveolin-3 and rabbit IgG directed against PKCε, PKCβ1, PKCβ2 or PKCδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG directed against PKCα was purchased from Cell Signaling Technology (Danvers, MA). Rabbit IgG directed against PKCζ was purchased from Santa Cruz Biotechnology or Sigma (St. Louis, MO). The phorbol ester phorbal-12-myristate-13-acetate (PMA), its inactive congener 4α-PMA, adenosine A1 receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA) and adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were supplied from Sigma. PMA, 4α-PMA, CCPA and DPCPX were all used at a final concentration of 100 nM. DPCPX was applied 5 min before and during addition of CCPA to cell suspension. All drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.

2.2. Isolation of cardiomyocytes

Adult rat ventricular myocytes were isolated from adult Sprague–Dawley rats (250 to 300 g) by enzymatic dissociation [20,21]. In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O2) Tyrode's solution containing (in mM) NaCl 126, KCl 5.4, CaCl2 1.0, MgCl2 1.0, NaH2PO4 0.33, HEPES 10 and glucose 10 at 37 °C. The perfusate was then changed to a Tyrode's solution that is nominally Ca2+ free but otherwise has the same composition. The hearts were perfused with the same solution containing collagenase for 20 min. Softened ventricular tissues were removed, cut into small pieces and mechanically dissociated by trituration. The digested cells were initially stored in a solution containing (in mM) KCl 20, KH2PO4 10, glucose 10, potassium glutamate 70, β-hydroxybutyric acid 10, taurine 10, mannitol 5 and EGTA 5, along with 1% albumin, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with or without PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min at 37 °C prior to homogenization for subsequent biochemical and immunofluorescence experiments.

2.3. Purification of caveolin-enriched membrane fractions

Caveolin-rich fractions from adult rat cardiomyocytes were prepared by using a previously described detergent-free method with some modification [22]. Briefly, freshly isolated cardiomyocytes were pretreated without or with PMA or CCPA for 5 min at 37 °C. Cells were then spin down and resuspended in 0.5 M sodium carbonate and homogenized. The homogenate was adjusted to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM Mes, pH 6.5/0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (in MBS containing 250 mM sodium carbonate) was formed above by overlaying with 4 ml of 35% sucrose (prepared in MBS with 250 mM sodium carbonate) and then 4 ml of 5% sucrose (again prepared in MBS with 250 mM sodium carbonate). Tubes were centrifuged at 39,000 rpm for 18–20 h in an SW41 rotor. Twelve 1-ml fractions were collected from the top to the bottom of the gradient for subsequent analysis by Western blot. Caveolin-rich fractions (fractions 4–6) which contain caveolin but exclude most other cellular proteins were centrifuged at 40,000×*g* for 2 h to pellet caveolae, which was then suspended in lysis buffer and sonicated. Samples were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane and analyzed by probing with various antibodies.

2.4. Membrane fractionation and western blotting

Freshly isolated cardiac myocytes were incubated in the presence and absence of PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min prior to homogenization. Cell lysates were first centrifuged at 1000×*g* to get rid of unbroken cells and nucleus. Particulate and cytosolic fractions were prepared by centrifugation at 27,000×*g* for 1 h or 45,000×*g* for 30 min. Immunoblot analysis was carried out as described previously [23,24]. Briefly, the cytosolic and particulate fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and electrophoresed on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4) and incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies in TBS, 0.1% Tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system.

2.5. Co-immunoprecipitation

Immunoprecipitation experiments were performed as reported previously [22,25]. Cells were pretreated with or without CCPA prior to homogenization. The cell lysate was incubated with or without antibody against caveolin-3 for 2 h at 4 °C. Antigen–antibody complexes were captured with r-protein-A agarose (4 °C, 30 min). Agarose beads were washed 4 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane and analyzed by probing with antibodies against PKCε, PKCδ or caveolin-3.

2.6. Immunofluorescence confocal microscopy

As described previously [22], after pretreated cells with or without CCPA, the cells were fixed with 4% formaldehyde in PBS for 30 min, blocked, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min) and labeled with primary antibody for 2 h. Cells were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 h. Immunofluorescence was visualized with confocal laser scanning microscopy. All images were analyzed using a background subtraction method offline.

3. Results

3.1. Effect of PMA on translocation of PKC isoforms from the cytosol to the particulate fraction

To investigate whether activation of adenosine receptors induces translocation of PKC isoforms from the cytosol to the particulate (membrane) fraction, we first examined the subcellular localization of a representative isoform from each PKC subgroup, i.e. PKC-α (the classic PKC), -ε (the novel PKC) and -ζ (the atypical PKC) following PMA (100 nM) treatment [26]. It is known that PMA activates the classical and novel but not the atypical PKC isoforms. As expected, Western blot analysis of cytosolic and membrane fractions separated by high speed centrifugation from rat cardiomyocytes detected all