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Research paper

The 2-arachidonoylglycerol effect on myosin light chain phosphorylation in human platelets

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ABSTRACT

In human platelets the endocannabinoid 2-arachidonoylglycerol (2-AG) stimulates some important pathways leading to thromboxane B_2 formation, calcium intracellular elevation, ATP secretion and actin polymerisation. The aim of the present study was to examine the 2-AG effect on myosin light chain (MLC) phosphorylation and to investigate the mechanisms involved. We demonstrated that 2-AG induced a rapid MLC phosphorylation, stimulating both the RhoA kinase (ROCK) and MLC kinase (MLCK) in a dose and time-dependent manner. In addition MLC phosphorylation was strengthened through the MLC phosphatase inhibition. MLC phosphatase inhibition was accomplished through the RhoA/ROCK and protein kinase C mediated phosphorylation of MLC phosphatase inhibiting subunits MYPT1 and CPI-17. The presence of CB1 receptor in human platelets and the involvement of CB1 receptor in MLC phosphorylation and MLC phosphatase inhibition was shown.

1. Introduction

Endocannabinoids are derivatives of arachidonic acid and belong to the eicosanoid family. The two main endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide are synthesized in response to increasing intracellular calcium concentrations by diacylglycerol lipase [1]. Endocannabinoids metabolism occurs through hydrolysing enzymes, notably fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [2]. It was proposed that 2-AG and anandamide are the main endogenous agonists of type-1 (CB1) and type-2 (CB2) cannabinoid receptors. In particular 2-AG seems to be the true physiological agonist of CB1 and CB2 [3]. Human platelets express CB1 receptor and at a lesser extent CB2 receptor [4]. In addition platelets have the tools to bind and metabolize endocannabinoids that have been proposed as physiological agonists of these cells [5,6]. Previously it was shown that in platelet-rich plasma micromolar concentrations of 2-AG activate platelets through a CB1/CB2 dependent mechanism [7]. In contrast Baldassarri et al. [8] excluded the presence of CB1 and CB2 receptors in platelets and suggested that platelet activation by 2-AG occurs through non-CB1/CB2 receptors. In response to specific agonists platelets undergo morphological alterations (shape change), secrete the content of their granules and aggregate. Phosphorylation of the 20 kDa myosin (MLC) is one of the primary steps in the activation of acto-myosin contractile events, which leads to reorganization of the cytoskeleton structure, shape change and secretion [9,10]. The time-course of MLC phosphorylation parallels that of association of myosin with the actin cytoskeleton [11]. MLC phosphorylation initiates myosin Mg^{2+}-ATPase activity, causing the binding of myosin to F-actin and subsequent acto-myosin fibre formation, folding of the cell membrane and centraling platelet secretory granules. In intact cells MLC is found to be phosphorylated on residues thr18 and/or ser19 by Rho kinase (ROCK) which can be activated by the small GTP binding protein RhoA and/or by the Ca^{2+}/CaM-dependent MLC kinase (MLCK) [12]. In human platelets both ROCK and MLCK pathways mediate MLC phosphorylation independently of each other as a function of different platelet stimuli [13,14]. The extent of MLC phosphorylation is regulated also by MLC phosphatase (MLCP) [15,16]. MLCP is composed of a 37 kDa catalytic subunit (PP1C) that is responsible for dephosphorylation of its highly specific substrate MLC and of two non catalytic subunits a 130 kDa regulatory myosin binding subunit (MYPT1) and a 20 kDa subunit (CPI-17). Phosphorylation of

Abbreviation: 2-AG, arachidonoylglycerol; CB1/2, type 1/2 cannabinoid receptor; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; MLC, p20 myosin light chain; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; ROCK, RhoA kinase.

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MYPT1 by ROCK results in loss of ability of MLCP to dephosphorylate MLC [16,17]. Another mechanism for inhibition of MLCP involves the subunit CPI-17 [18–20] whose inhibitory potency is increased by PKC-dependent phosphorylation at thr38 residue [18,19]. Thus both ROCK and PKC can contribute to the inhibition of MLCP. There is evidence that PKCα and RhoA translocate and associate with each other in response to stimulation with acetylcholine in colon smooth muscle [21]. Moreover in rabbit colon smooth muscle cells RhoA interacts directly with PKCα in vitro [22] and in human endothelial cells activation of ROCK by RhoA requires PKC activation [23]. In conclusion stimuli which activate ROCK and/or PKC have been shown to potentiate MLC phosphorylation through the inactivation of MLCP. Previously ATP secretion [8] and actin polymerisation [6] induced by 2-AG have been described. In contrast no information on 2-AG stimulated MLC phosphorylation is reported. In the present report we studied the 2-AG effect on MLC phosphorylation to clarify the mechanisms involved. We found that 2-AG stimulates MLC phosphorylation. The activation of both ROCK and MLCK signalling pathways and the inhibition of MLCP take part in these mechanisms.

2. Materials and methods

2.1. Materials

2-AG, apyrase, benzamidine, Colorburst™ electrophoresis markers, dithiothreithol, leupeptin, β-mercaptoethanol, okadaic acid, PGE_1 (prostaglandin E_1), PMSF, protease inhibitor cocktail and all chemicals were from Sigma–Aldrich, USA. Calyculin A, ML-7, Y27632 and ROCK activity EIA kit, were purchased from Merck Biosciences, Germany. URB597 and URB754 was from Alexis Biochemicals, USA. SR141716 and SR144528 were from Cayman Chemical. H1152, GSK429286, LY320135 and AM251 were from Tocris Bioscience, UK. Inhibitors were diluted in saline from a stock DMSO solution immediately before each experiment. Anti-CB1 and anti-CB2 were from Abcam Plc, UK. Anti-phospho-MLC (thr18/ser19) was from Cell Signaling Technology, USA. Anti-phospho-MLCK (ser234), anti-β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, USA. Anti-phospho-CPI-17(thr38), anti-phospho-MYPT1(thr696) and anti-MYPT1 were from Millipore, USA. [^{32}P] Phosphoric acid was from PerkinElmer Life and Analytical Sciences, USA. RhoA activity G-LISA kit was from Cytoskeleton U.S.A. ECL® system and protein G-sepharose were from GE Healthcare, USA. Nitrocellulose membranes (pore size 0.45 μm) were purchased from Bio-Rad Laboratories, USA. EnzChek® phosphate assay kit was from Molecular Probes, USA. BCA protein assay kit was from Pierce Biotechnology USA.

2.2. Blood collection and preparative procedures

Freshly drawn venous blood from healthy volunteers of the "Centro Trasfusionale, Ospedale San Martino" in Genoa was collected into 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors claimed to have not taken drugs known to interfere with platelet function during two weeks prior to blood collection, and gave their informed consent. Washed platelets were prepared centrifuging whole blood at 100 × *g* for 25 min. The obtained platelet-rich plasma was then centrifuged at 1100 × *g* for 15 min in the presence of 2 mU/mL apyrase and 4 μM PGE_1. Pellet was washed once with pH 5.2 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose), centrifuged at 1100 × g for 15 min and then resuspended in calcium-free 10 mM HEPES buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose (pH 7.4).

2.3. Immunoblotting analysis of proteins

Platelet suspensions (1.0×10^9 platelets/mL), prewarmed with saline or additions at 37 °C, were stimulated with 2-AG. Incubation was stopped by adding 2× Laemmli-SDS reducing sample buffer. Samples, heated for 5 min at 100 °C, were separated by 5–7.5% or 5–10% SDS-PAGE, and transferred to nitrocellulose membranes. Running was performed in the presence of Colorburst™ Electrophoresis weight markers. Blots were blocked in 5% BSA dissolved in TBST (Tris buffer saline, pH 7.6, containing 10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) at 37 °C for 30 min, and then incubated overnight at 4 °C with anti-CB1, anti-CB2, anti-phospho-MLC (thr18/ser19), anti-phospho-MYPT1(thr696), anti-phospho-CPI-17(thr38) (1:1000 dilutions) or anti-phospho-MLCK(ser234) (1/200 dilution). Membranes were extensively washed and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody. After further washings, blots were developed using the ECL® system. Finally nitrocellulose membranes, stripped by incubation with 62.5 mM Tris/HCl (pH 6.7), 2% SDS, 100 μM β-mercaptoethanol for 30 min at 50 °C, were reprobed with anti-β-actin.

2.4. MLC phosphorylation detection by [^{32}P] phosphoric acid loaded platelets

Washed platelets (2.5×10^9 platelets/mL) were incubated at 37 °C for 60 min with 1 mCi [^{32}P] phosphoric acid under gentle shaking, centrifuged, washed once with pH 7.4 HEPES buffer and finally resuspended to 5.0×10^8 platelets/mL in the same buffer. Labelled-platelets were preincubated at 37 °C with saline or additions, then 2-AG was added. Incubation was stopped by adding suitable aliquots of 2× Laemmli-SDS reducing sample buffer containing 10% β-mercaptoethanol. Samples were boiled for 5 min and proteins were separated by 5–10% SDS-PAGE. Gels were dried and the [^{32}P] phosphorylated bands, revealed using the Cyclone Storage Phosphor System® (PerkinElmer Life And Analytical Sciences, Inc., USA), were quantified with the related software package. Running was carried out in the presence of standard molecular weight markers.

2.5. Immunofluorescence confocal microscopy

The presence of CB1/CB2 receptors was assayed by immunofluorescence confocal microscopy as previously described [24]. Briefly washed platelets (1.0×10^9 platelets/mL), preincubated at 37 °C with saline or SR141716 and then incubated with 2-AG as indicated, were fixed with 2% paraformaldehyde-PBS and permeabilized with 0.05% Triton X-100-PBS. After washing with PBS containing 1% BSA, the preparations were treated overnight at 4 °C with anti-CB1 or anti-CB2 as primary antibody (1:1000 dilution). After further washing with PBS containing 1% BSA, samples were incubated 60 min at room temperature with Alexa 488 fluorescent secondary antibody (1:500 dilution). Finally, samples were mounted on coverslips and sealed with nail polish. Images were collected by confocal microscopy using a Bio-Rad MRC1024 instrument (krypton/argon laser, Bio-Rad, USA) attached to a Nikon Diaphot 200 inverted microscope (Nikon Inc., USA), using a planapochromat ×60 oil-immersion objective with N.A.1, 4. FluoroGuard anti-fade reagent was used before analysis.

2.6. RhoA activation assay

Washed platelets (1.0×10^9 platelets/mL), preincubated at 37 °C with saline, were stimulated with 2-AG at 37 °C for 15 s under mild shaking without stirring. Incubation was stopped by putting