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Applicability of microplate assay coupled to Fiske–Subbarow reducer for the determination of phosphorous produced by in vivo human lymphocytes: PKC is probably cross talking with ecto 5′-nucleotidase

Alejandro Martínez-Martínez[a,*], Laura A. de la Rosa[a], Claudia A. Jímenez-Muñoz[a], Angel Gabriel Díaz-Sánchez[a], Jesús A. Araujo-González[a], José R. Peralta-Videa[b], Guadalupe de la Rosa[c], Jorge L. Gardea-Torresdey[b,c]

[a]*Departamento de Ciencias Básicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Cd. Juárez, 32310, Chih., México*
[b]*Department of Chemistry, University of Texas at El Paso, El Paso, Texas 79968-0513, USA*
[c]*Environmental Science and Engineering PhD Program, University of Texas at El Paso, El Paso, Texas 79968-0513, USA*

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Abstract

In this research, the phorbol ester, phorbol 12-myristate 13-acetate (PMA), was used to assess the effect of protein kinase C (PKC) activation on the specific activity of ecto-5′-nucleotidase (eNT) in human lymphocytes. PMA mimics the effects of diacylglycerol, a natural compound released by the hydrolysis of the glycosilphosphatidilinositol (GPI) moiety, in activating PKC. In order to evaluate the activity of eNT in living lymphocytes, a micro-assay method was established with a low detection limit for inorganic phosphate (Pi) of 0.94 nmol Pi assay^{-1}. The dephosphorylation of Adenosine monophosphate (AMP) by functional lymphocytes was evaluated and the contribution of the eNT activity was calculated by its inhibition with the specific eNT inhibitor α,β-methylene ADP (MADP) and the use of the broad spectrum phosphatases inhibitor (but not eNT), levamisole. Under the conditions tested, we obtained an AMPase value of 8.05±4.4 nmol Pi million cells^{-1} h^{-1}. The addition of MADP to the incubation media decreased the AMPase activity to 2.43±0.9 nmol Pi million cells^{-1} h^{-1} ($p<0.05$). On the other hand, when lymphocytes were incubated with PMA, an increase of 182% in the AMPase activity was observed. However, the addition of levamisole inhibited the AMPase activity by about 17%, while the co-incubation of cells with PMA and levamisole reduced only an 8% of the total PMA-increased AMPase activity. These results show that (1) a non-radioactive micro-method can be used to assess the Pi production in living cells; (2) the obtained data strongly suggest that eNT is the main ecto-enzyme present on the surface of circulating lymphocytes responsible for the hydrolysis of extracellular AMP; and (3) that PKC is cross talking with eNT.

Keywords: Ecto-5′-nucleotidase; 5′NT; Nucleotidase; CD73; Lymphocyte; PKC; α-β-MADP; PMA; Pi microassay

1. Introduction

Lymphocyte cluster of differentiation 73 (CD73) is an ecto-enzyme possessing ecto-5′-nucleotidase activity (EC 3.2.3.5, afterwards eNT/CD73), which catalyzes the dephosphorylation of purine and pyrimidine, ribo- and deoxyribonucleoside monophosphates to their corresponding nucleosides [1]. This enzyme has a molecular mass of 70-kDa, it is anchored to the plasma membrane by a glycosilphosphatidilinositol (GPI) bridge and is present on approximately 15% of peripheral blood lymphocytes (PBL). However, it is unevenly distributed among the different lymphocyte subsets, being expressed on the majority of $CD8^+$ T cells (51%) and B cells (70%), whereas a lesser percentage of $CD4^+$ T cells is $CD73^+$ (11%) [2,3].

It has been suggested that CD73 plays a role in regulating lymphocyte adhesion to endothelium, since antibodies (Ab)

* Corresponding author. Fax: +52 656 6881836.
E-mail address: amartine@uacj.mx (A. Martínez-Martínez).

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against CD73 block lymphocyte adhesion to cultured endothelial cells [4,5]. Similarly, an anti-CD73 Ab blocked aggregation of freshly isolated germinal center B cells and follicular dendritic cells [6].

Cell type specific regulation of the expressed eNT/CD73 is suggested because it is sequestrated from the lymphocyte surface after treatment with specific antibodies; however, the same treatment was ineffective for eNT/CD73 expressed in endothelial cells [7]. Moreover, engagement of lymphocyte eNT/CD73 to the Ab results in tyrosine phosphorylation and dephosphorylation of intracellular protein substrates, whereas eNT/CD73 on endothelial cells remains resistant to the Ab treatment [7]. These differences in regulation and function suggest that the specific physiological roles of eNT/CD73 vary with the tissue where it is studied, and that several mechanisms may be involved in the regulation of eNT/CD73 activity and expression [8–10]. There is evidence that eNT/CD73 participates in the clustering of the leukocyte integrin LFA-1 and in the consequent binding of lymphocytes to endothelial cells [6,11]. However, little is known about the biochemical paths that eNT/CD73 uses to cross-talk to different effectors in lymphocyte cells. Furthermore, because anti eNT/CD73 monoclonal antibodies (mAbs) can stimulate processes such as the production of T cells, secretion of IL-2 (Interleukin-2), and the expression of the IL-2R (Interleukin-2-receptor), it has been proposed that eNT/CD73 can act as a membrane receptor that transmits co-stimulatory signals for human T cell proliferation in vivo [12–15].

Phosphoinositides and diacylglicerol (DAG) are ubiquitous second messengers, which may be generated from the phospholipase C-mediated hydrolysis of the phosphatidylinositol moiety of GPI-anchored membrane proteins. In addition, since eNT/CD73 is a GPI-linked molecule, it can move rather freely on the cell surface and form clusters with other cell surface molecules most likely in lipid rafts, which are preformed modules enriched in signaling molecules [13–16]. Interestingly, certain GPI-linked proteins have been shown to co-cluster with β_2 integrins [17–19]. Furthermore, engagement with the urokinase receptor increases β_2 integrin-mediated binding of neutrophils and monocytes to the endothelium [20]. Thus, it is very likely that eNT/CD73 has the same properties on active cells such as lymphocytes. The activation of eNT/CD73 could be related to microenvironment localization of several molecules related to the phosphoinositides/DAG signaling cascade, such as phospholipase C and protein kinase C that is activated by DAG [21].

In this work, the possibility of cross talk between protein kinase C and eNT activity in living lymphocytes was studied through the use of the PKC activator PMA and the eNT/CD73 specific inhibitor α,β-methylene ADP (MADP). A sensitive spectrophotometric method for the determination of inorganic phosphate released by enzyme activity was used. The results are reported herein.

2. Materials and methods

2.1. Reagents

Fiske–Subbarrow reducer was purchased from Fluka, water was MQ grade, trace metal grade sulfuric acid was from Fisher Scientific, and the rest of the chemicals were purchased from SIGMA with the maximum purity available.

2.2. Procedures

2.2.1. Lymphocytes purification

Blood samples were obtained from the Instituto Mexicano del Seguro Social Clínica #6, Ciudad Juárez, Chihuahua, with ethical agreement of the Institution.

Lymphocytes were purified by centrifugation through a Percoll® density gradient according to a technique modified by de la Rosa et al. [22] after Boyum [23]. Briefly, total blood was diluted 1:1 using a purification buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM glucose, 14 mM Trizma, 1 mM $CaCl_2$, pH 7.4 with HCl). Four milliliter of this diluted blood was gently overlaid on 4 mL of 60% isotonic percoll (avoiding mixing of the solutions), and centrifuged at 3000 rpm for 25 min at room temperature (Fisher Scientific, 8K). Lymphocytes were obtained from the interface between the plasma and percoll (lymphocytes form a white band) and were washed twice by resuspension in 2 mL of purification buffer followed by centrifugation at 2000 rpm for 10 min at room temperature. Washed lymphocytes were resuspended in 2 mL of purification buffer. After a gentle homogenization with a disposable plastic Pasteur pipette, 100 μL aliquots were withdrawn for cell counting. Cell number and size were measured using a channel analyzer ADVIA (Hematology System, Bayer®). The viability of the lymphocytes was determined in a Neubauer chamber using trypan blue as exclusion colorant to verify the integrity of the plasma membrane.

2.2.2. Enzyme activity (AMPase)

The ecto AMPase activity was determined on living cells trough a temporal course of inorganic phosphate production in presence and absence of AMP as a substrate following a protocol described by Martinez-Martinez et al. [24]. Total AMPase was assayed in 3 mL assay medium (125 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 0.5 mM glucose, 14 mM Tris, 1 mM $CaCl_2$, pH 7.4 with HCl 1N) containing 9×10^6 lymphocytes mL^{-1} and incubated at 37 °C. AMPase activity was measured by adding 1 mM final AMP concentration, and negative controls were preformed in which no substrate was added to the incubation medium. Aliquots of 150 μL of the mixture were withdrawn at 0, 60, 120, and 180 min and immediately sonicated on ice for 30 s (50% power) to disrupt cells. Sixty microliter of 50% ice-cold trichloroacetic acid (TCA) was immediately added to stop the enzyme activity and to precipitate the protein. Subsequently, the