Biochimica et Biophysica Acta 1863 (2016) 2175–2188

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Integrin-mediated transactivation of P2X7R via hemichannel-dependent ATP release stimulates astrocyte migration

Alvaro Alvarez [a,c,1], Raúl Lagos-Cabré [a,c], Milene Kong [a,c,2], Areli Cárdenas [a,c], Francesca Burgos-Bravo [a,c], Pascal Schneider [d], Andrew F.G. Quest [a,b,c], Lisette Leyton [a,b,c,*]

[a] Cellular Communication Laboratory, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, 838-0453 Santiago, Chile
[b] Advanced Center for Chronic Diseases, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, 838-0453 Santiago, Chile
[c] Biomedical Neuroscience Institute, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, 838-0453 Santiago, Chile
[d] Department of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland

ARTICLE INFO

Article history:
Received 6 January 2016
Received in revised form 29 April 2016
Accepted 23 May 2016
Available online 25 May 2016

Keywords:
Cell migration
Thy-1
ATP
Calcium
Connexins
Pannexins

ABSTRACT

Our previous reports indicate that ligand-induced $\alpha_V\beta_3$ integrin and Syndecan-4 engagement increases focal adhesion formation and migration of astrocytes. Additionally, ligated integrins trigger ATP release through unknown mechanisms, activating P2X7 receptors (P2X7R), and the uptake of Ca^{2+} to promote cell adhesion. However, whether the activation of P2X7R and ATP release are required for astrocyte migration and whether $\alpha_V\beta_3$ integrin and Syndecan-4 receptors communicate with P2X7R via ATP remains unknown. Here, cells were stimulated with Thy-1, a reported $\alpha_V\beta_3$ integrin and Syndecan-4 ligand. Results obtained indicate that ATP was released by Thy-1 upon integrin engagement and required the participation of phosphatidylinositol-3-kinase (PI3K), phospholipase-C gamma (PLCγ) and inositol trisphosphate (IP3) receptors (IP3R). IP3R activation leads to increased intracellular Ca^{2+}, hemichannel (Connexin-43 and Pannexin-1) opening, and ATP release. Moreover, silencing of the P2X7R or addition of hemichannel blockers precluded Thy-1-induced astrocyte migration. Finally, Thy-1 lacking the integrin-binding site did not stimulate ATP release, whereas Thy-1 mutated in the Syndecan-4-binding domain increased ATP release, albeit to a lesser extent and with delayed kinetics compared to wild-type Thy-1. Thus, hemichannels activated downstream of an $\alpha_V\beta_3$ integrin-PI3K-PLCγ-IP3R pathway are responsible for Thy-1-induced, hemichannel-mediated and Syndecan-4-modulated ATP release that transactivates P2X7Rs to induce Ca^{2+} entry. These findings uncover a hitherto unrecognized role for hemichannels in the regulation of astrocyte migration via P2X7R transactivation induced by integrin-mediated ATP release.

1. Introduction

Integrins are heterodimers of α and β subunits, both of which contain short intracellular domains that lack enzymatic activity and interact with many enzymes, scaffolding proteins, and adaptors to form large protein complexes known as focal adhesions (FAs) [1,2]. FAs are elongated structures that connect the extracellular matrix (ECM) proteins to the cytoskeleton via integrins [3] and thereby mediate cell adhesion to the ECM, an essential event for cell migration [4–6].

Migration is a cyclic process that involves cell polarization, membrane protrusion at the leading edge, FA and stress fiber formation, cell contraction, and retraction of the trailing edge to allow cells to move forward [4,7]. Strong cell adhesion through FAs reduces migration, whereas increased turnover of FAs enhances cell motility. From data available on the composition of the integrin adhesome, a database that has mapped the interactions between 180 or more proteins present in integrin signaling complexes [8,9], it is clear that many receptors and signaling proteins control cell adhesion and migration. One of these proteins is the transmembrane proteoglycan, Syndecan-4, which is involved in promoting anchorage to ECM substrates [10] and binding to other cells [11]. Additionally, in fibroblasts, the binding of Syndecan-4 to fibronectin allows the association of Syndecan-4 with protein kinase C-alpha (PKCα), which activates RhoG and triggers endocytosis of β_1 integrin and increased FA turnover [12]. Thus, Syndecan-4 has been suggested to regulate the transition from strong cell adhesion to cell migration.

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ATP, adenosine 5′-triphosphate; BBG, Brilliant Blue G; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; ECM, extracellular matrix; FAs, focal adhesions; FAK, focal adhesion kinase; FBS, fetal bovine serum; FGF, fibroblast growth factor; HBD, heparin-binding domain; IP3, inositol trisphosphate; IP3R, IP3 receptors; P2X7R, P2X7 receptors; PBS, phosphate-buffer saline; PI3K, phosphatidylinositol-3-kinase; PLCγ, phospholipase-C gamma; PKCα, protein kinase C-alpha; p160ROCK, Rho-associated kinase; s.e.m., standard error of the mean.

* Corresponding author at: Programa de Biología Celular y Molecular, Facultad de Medicina, Universidad de Chile, Santiago, Chile.
E-mail address: lleyton@med.uchile.cl (L. Leyton).
[1] Present address: Faculty of Science, Universidad San Sebastian, Santiago, Chile.
[2] Present address: Department of Biomedicine, Faculty of Health Sciences, University of Antofagasta, Chile.

http://dx.doi.org/10.1016/j.bbamcr.2016.05.018
0167-4889/

Our results indicate that the neuronal glycoprotein Thy-1 interacts with both $\alpha_V\beta_3$ integrin and Syndecan-4 in astrocytes to promote cell adhesion and migration. Ligand-receptor interactions occur through two different domains of the Thy-1 protein: 1) a region containing the arginine-leucine-aspartic acid (RLD) tripeptide, and 2) a heparin-binding domain (HBD), respectively [11,13,14]. Other reports have also confirmed such ligand-receptor binding [15–17]. Thy-1–integrin/Syndecan-4 interactions increase astrocyte adhesion to the ECM [11] and require the recruitment and aggregation of $\alpha_V\beta_3$ integrin. Integrin clustering activates focal adhesion kinase (FAK), Rho-associated kinase (p160ROCK), and RhoA [18]. Integrin signaling also involves PKCα, and possibly inositol trisphosphate (IP3) receptor (IP3R) activation because IP3R inhibitors reduce RhoA activity and astrocyte adhesion induced by Thy-1 [11]. Moreover, prolonged Thy-1 stimulation induces the polarization and migration of DITNC1 astrocytes via the activation of FAK, phosphatidylinositol-3-kinase (PI3K), and the small GTPase Rac1 [19].

Strong astrocyte adhesion induced by Thy-1 also requires increased extracellular adenosine 5′-triphosphate (ATP), activation of the purinergic P2X7 receptor (P2X7R), and Ca^{2+} entry into the cell [20]. Whether this molecular mechanism is also required for cell migration and how ATP is released from the cells to the extracellular media are questions that remain to be explored. Moreover, how these molecular events temporarily connect to the integrin-induced signaling pathways requires further studies.

Under physiological conditions, two general mechanisms for ATP release have been proposed. The first one is exocytic [21,22] and the second one involves the hemichannel proteins [23–30], connexins or pannexins, which are present in astrocytes [31,32]. Connexin hemichannels are well-established gap junction constituents that allow individual cells to communicate with the extracellular medium by permitting the passage of a variety of molecules, such as sugars, small peptides, glutamate, and ATP [33–36]. Pannexins are transmembrane channels that, like connexins, participate in the liberation of ATP and other small molecules through the plasma membrane. Likewise, pannexins also form gap junctions in vitro [29,37,38]. Interestingly, through the release of ATP, hemichannels are known to participate in cell migration events. In fibroblasts, the *C*-terminal domain of Connexin-43 can interact with and modify tubulin and cytoskeletal dynamics [39]. Furthermore, Connexin-43 also impacts on cell polarity [39]. In astrocytes, Connexin-43 is required for cell migration under pro-inflammatory conditions and it interacts with many cytoskeletal proteins, such as β-actin and glial fibrillary acidic protein [40,41]. Conversely, pannexins regulate motility of different cell types, such as neutrophils or leucocytes, tumor cells, venous fibroblasts, and neurons, among others [42–44]. Given these observations, we evaluated whether these hemichannels participate in Thy-1-induced migration of astrocytes by mediating ATP release and P2X7R activation.

Here we show that P2X7R activation is important for cells to migrate in response to Thy-1. Additionally, both Connexin-43 and Pannexin-1 participate in Thy-1-induced ATP release downstream of $\alpha_V\beta_3$ integrin, leading to P2X7R transactivation. We also provide evidence demonstrating that after Thy-1 stimulation, hemichannel opening is modulated by Syndecan-4 and requires PI3K/phospholipase C gamma (PLCγ)-mediated IP3R activation, which leads to the subsequent release of Ca^{2+} from intracellular stores. Together, these findings reveal a novel mechanism linking integrin function to P2X7R transactivation and astrocyte migration via ATP release through hemichannels.

2. Results

2.1. The P2X7 receptor is required for Thy-1-induced astrocyte polarization and migration

We previously demonstrated that the P2X7R is required to increase Ca^{2+} uptake and cell adhesion to the underlying matrix [20]. Here, we set out to study whether P2X7R is also required for Thy-1-stimulated migration of astrocytes. We observed that incubation of DITNC1 astrocytes with the nucleotidase enzyme, Apyrase, precluded Thy-1-induced migration (Fig. 1A), indicating a requirement for ATP. We then tested the effects of different BzATP concentrations (0.1, 1, 5, 10, and 100 μM) on cell migration. BzATP, a non-hydrolyzable ATP analogue, was found to induce cell migration at 10 and 100 μM (control vehicle, 1.0 ± 0.04; [BzATP] = 10 μM, 1.37 ± 0.05, $p < 0.05$; [BzATP] = 100 μM, 1.6 ± 0.1, $p < 0.001$). The latter concentration was used in subsequent experiments (unless indicated otherwise) and coincides with that employed in our previous study [20]. Moreover, migration stimulated by either Thy-1-Fc fusion protein or BzATP was inhibited by two P2X7R antagonists, Brilliant Blue G (BBG) and A438079 (Fig. 1A). The negative control for the Fc-portion of the Thy-1 fusion proteins used in these experiments (Trail-R2-Fc; see Methods) behaved similarly to the control with serum-free medium (Fig. 1A).

To confirm the role of the P2X7R in wound closure, cell migration was evaluated following knockdown of this receptor. Importantly, the siRNA for P2X7R used in these experiments decreases P2X7R mRNA levels without affecting the mRNA levels of other P2X receptors [20]. Here, we demonstrate that diminished P2X7R protein levels (Fig. 1B, insert) blocked migration of astrocytes induced by Thy-1-Fc but had no effect on Trail-R2-Fc-treated cells (Fig. 1B).

The role of P2X7R was also tested in a cell polarization assay. Reorientation of the Golgi apparatus towards the leading edge was evaluated as previously described [19,45]. The increased number of polarized DITNC1 cells induced by either Thy-1-Fc or BzATP was reduced by BBG and A438079 (Fig. 1C). Therefore, P2X7R-mediated events required for astrocyte adhesion to the underlying matrix [20] are also essential for cell polarization and migration induced by Thy-1.

2.2. Thy-1 induces ATP release and cell migration dependent on integrin and Syndecan-4 engagement

To investigate the mechanism involved in ATP release upon Thy-1 addition, extracellular ATP was measured at different time points following the stimulation of astrocytes with various previously described Thy-1 mutants [11,13]. First, SDS-PAGE and Coomassie Blue staining were employed to assess the quality of the Thy-1 mutants. The results revealed the presence of a single band in all cases (Fig. 2A). In addition, Thy-1-Fc induced astrocyte migration, whereas neither the single-domain Thy-1 mutated molecules [Thy-1(RLE)-Fc or Thy-1(AEAAA)-Fc] nor the double mutant [Thy-1(RLE/AEAAA)-Fc] induced astrocyte migration [19] (Fig. 2A, bottom panel). Moreover, to demonstrate the functionality of different Thy-1 proteins, single-domain mutants were combined and migration was then evaluated. When both mutant proteins were added in combination, the increase in migration observed was similar to the effect obtained with the wild-type Thy-1-Fc molecule (Fig. 2A, bottom panel), indicating that the individual mutations did not disrupt the Thy-1 structure.

Subsequently, ATP levels were measured upon treating astrocytes with each of the four different Thy-1 molecules. Thy-1 mutated in the HBD [Thy-1(AEAAA)-Fc] (Fig. 2B, squares) led to a delayed release of less ATP compared to non-mutated Thy-1-Fc protein (Thy-1-(RLD)-Fc; Fig. 2B, circles). Upon stimulation of astrocytes with Thy-1 mutated in the integrin-binding domain [Thy-1(RLE)-Fc], which possesses an intact HBD that permits binding to Syndecan-4, no changes in extracellular ATP levels were observed (Fig. 2B upright triangles). In addition, Thy-1 mutated in both binding sites [Thy-1(RLE/AEAAA)-Fc] did not result in ATP release (Fig. 2B, inverted triangles). These findings, together with results indicating that BzATP added at concentrations >10 μM suffice to induce cell migration (Fig. 1A), led us to hypothesize that the molecular changes leading to ATP release in Thy-1-stimulated DITNC1 cells are triggered by $\alpha_V\beta_3$ integrin engagement, whereas signaling pathways downstream of Syndecan-4 do not trigger ATP release, but rather appear necessary to modulate integrin-induced ATP release. To test this hypothesis, cells were treated with BzATP at