ATP release due to Thy-1–integrin binding induces P2X7-mediated calcium entry required for focal adhesion formation

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Summary

Thy-1, an abundant mammalian glycoprotein, interacts with αvβ3 integrin and syndecan-4 in astrocytes and thus triggers signaling events that involve RhoA and its effector p160ROCK, thereby increasing astrocyte adhesion to the extracellular matrix. The signaling cascade includes calcium-dependent activation of protein kinase Cα upstream of Rho; however, what causes the intracellular calcium transients required to promote adhesion remains unclear. Purinergic P2X7 receptors are important for astrocyte function and form large non-selective cation pores upon binding to their ligand, ATP. Thus, we evaluated whether the intracellular calcium required for Thy-1-induced cell adhesion stems from influx mediated by ATP-activated P2X7 receptors. Results show that adhesion induced by the fusion protein Thy-1-Fc was preceded by both ATP release and sustained intracellular calcium elevation. Elimination of extracellular ATP with Apyrase, chelation of extracellular calcium with EGTA, or inhibition of P2X7 with oxidized ATP, all individually blocked intracellular calcium increase and Thy-1-stimulated adhesion. Moreover, Thy-1 mutated in the integrin-binding site did not trigger ATP release, and silencing of P2X7 with specific siRNA blocked Thy-1-induced adhesion. This study is the first to demonstrate a functional link between αvβ3 integrin and P2X7 receptors, and to reveal an important, hitherto unanticipated, role for P2X7 in calcium-dependent signaling required for Thy-1-stimulated astrocyte adhesion.

Key words: ATP, Calcium, Cell adhesion, Integrins, P2X7-R, Thy-1

Introduction

Thy-1, an abundant mammalian glycoprotein described initially 50 years ago (Barker and Hagood, 2008; Morris, 1992), is expressed in T cells, activated endothelial cells and neurons (Lee et al., 1998; Williams, 1976), and acts in trans as a ligand for αvβ3 integrin and syndecan-4 on astrocytes (Avalos et al., 2004; Avalos et al., 2009; Leyton et al., 2001). Binding of Thy-1 to both αvβ3 integrin and syndecan-4 in astrocytes triggers tyrosine phosphorylation of focal adhesion (FA) proteins, activation of protein kinase Cα (PKCα), RhoA and its effector p160ROCK, thereby increasing astrocyte adhesion to the extracellular matrix (Avalos et al., 2004; Avalos et al., 2002; Avalos et al., 2009; Hermosilla et al., 2008; Leyton et al., 2001). These signaling events require the interaction of the RLD integrin-binding sequence and the heparin-binding domain of Thy-1 with αvβ3 integrin and syndecan-4 on DI TNC1 astrocytes, respectively (Avalos et al., 2009; Leyton et al., 2001).

Integrin-mediated cell adhesion is known to trigger calcium transients required for subsequent signaling events in endothelial cells (Schwartz, 1993). Indeed, participation of the calcium-dependent PKC isoform PKCα, suggests this should also be the case following Thy-1 binding to αvβ3 integrin on astrocytes (Avalos et al., 2009). Although the elevation of intracellular calcium ($[Ca^{2+}]_i$) frequently results from both inositol trisphosphate [Ins(1,4,5)P_3]-mediated calcium release from endoplasmic reticulum and the calcium influx through plasma membrane calcium channels, the origin of the $[Ca^{2+}]_i$ and the events leading to intracellular increments of this second messenger downstream of integrins remains poorly defined.

Astrocytes are known to respond to various stimuli through calcium-dependent mechanisms and, interestingly, ATP is now appreciated as an important gliotransmitter associated with calcium signaling (Hamilton et al., 2010; Verderio and Matteoli, 2001). Non-lytic ATP release from cells to the extracellular medium following mechanical or biochemical stimulation has been observed in many different types of cells, including those from the nervous system. Although the relevance of extracellular ATP to cell signaling remains frequently controversial, ATP release has been suggested to participate in a number of processes, including platelet aggregation, cell proliferation, migration, death and neuronal excitability (for reviews, see Bodin and Burnstock, 2001; Hamilton and Attwell, 2010).

Extracellular ATP is viewed as a primary messenger because it stimulates P2 purinergic receptors at the cell surface of the same (autocrine) or neighboring (paracrine) cells. Members of the two-purinergic receptor families, P2Y and P2X, are present in astrocytes, as well as neurons, and are important in communication between these cells. Additionally, P2X7 receptors have been detected in microglia and are known to form large, non-selective cation pores with prolonged activation upon binding of extracellular ATP (for a review, see Fields and Burnstock, 2006).

Because ATP is a relevant extracellular mediator of intercellular signaling and activation of astrocytes (Anderson et al., 2004), we investigated whether Thy-1-induced ATP release was required for astrocyte responses to Thy-1. Here, we show that upon astrocyte stimulation with Thy-1, ATP released to the extracellular medium stimulates calcium influx through P2X7 receptors. Furthermore, ATP-dependent elevation of $[Ca^{2+}]_i$ is required for FA formation. Finally, binding of Thy-1 to αvβ3 integrin is implicated because Thy-1(RLD)-Fc, but not Thy-1 mutated in the integrin-binding domain (RLD to RLE), increases extracellular ATP. Thus, these results uncover an ATP-mediated, functional association between αvβ3 integrin and P2X7 receptors that is required for astrocyte adhesion.

Results

Thy-1-induced ATP release is required for FA formation in astrocytes

The presence of extracellular ATP and its function in FA formation in DI TNC1 astrocytes stimulated with soluble Thy-1 was studied. Indeed, ATP release was apparent 5 minutes following the addition of Thy-1(RLD)-Fc–Protein A complexes (Fig. 1A). Maximal ATP accumulation (2.5-fold basal levels) in the extracellular medium was detected 10 minutes after adding Thy-1. Moreover, ATP accumulation (Fig. 1B), as well as Thy-1-induced FA formation (Fig. 1C) were totally abolished by Apyrase (1 UI/ml), an enzyme that hydrolyzes ATP. The latter was corroborated by quantification of FA number (Fig. 1D, white bars) and size (Fig. 1D, black bars). Thus, augmented extracellular ATP was required for Thy-1-induced FA formation in astrocytes.

Thy-1(RLE)-Fc, the fusion protein mutated in the integrin-binding domain, does not interact with αxβ2 or αvβ3 integrins (Choi et al., 2005; Hermosilla et al., 2008) nor stimulates FA formation (Avalos et al., 2002; Hermosilla et al., 2008). In the present study, no ATP increment was observed when Thy-1(RLE)-Fc–Protein A was present for 15 minutes (Fig. 1B), suggesting participation of the integrin-binding domain of Thy-1 in ATP liberation.

P2X7-dependent calcium influx contributes to Thy-1-induced FA formation in astrocytes

Extracellular ATP reportedly acts through P2X7 receptors that are abundantly expressed in cells of the nervous system (Sperlagh and Illes, 2007). These receptors mediate calcium influx and transient elevation of $[Ca^{2+}]_i$ in a manner independent of voltage-gated channels. Therefore, we investigated whether ATP-activated P2X7 receptors participated in Thy1-induced FA formation by pre-incubating astrocytes with a P2X7 antagonist, oxidized ATP (oATP; 600 μM), with a calcium chelator (EGTA; 2 mM), or with the P2X7 agonist 3′-*O*-(4-benzoylbenzoyl) ATP (BzATP; 100 μM) (Fig. 2).

Thy-1(RLD)-Fc–Protein A complexes increased the number of FA in DI TNC1 cells (Fig. 2A). Interestingly, BzATP induced the formation of larger FA in a manner similar to that observed with Thy-1 (Fig. 2A). Quantification of both the number of FA per cell and the mean area of individual FA (Fig. 2B,C) corroborated that the differences shown in Fig. 2A were significant; however, the number of FA increased only slightly with BzATP alone and no synergy was observed between BzATP and Thy-1. Additionally, Thy-1-induced FA formation was significantly reduced in astrocytes following chelation of extracellular calcium with EGTA or pre-incubation for 30 minutes with oATP (Fig. 2B,C). Together, these

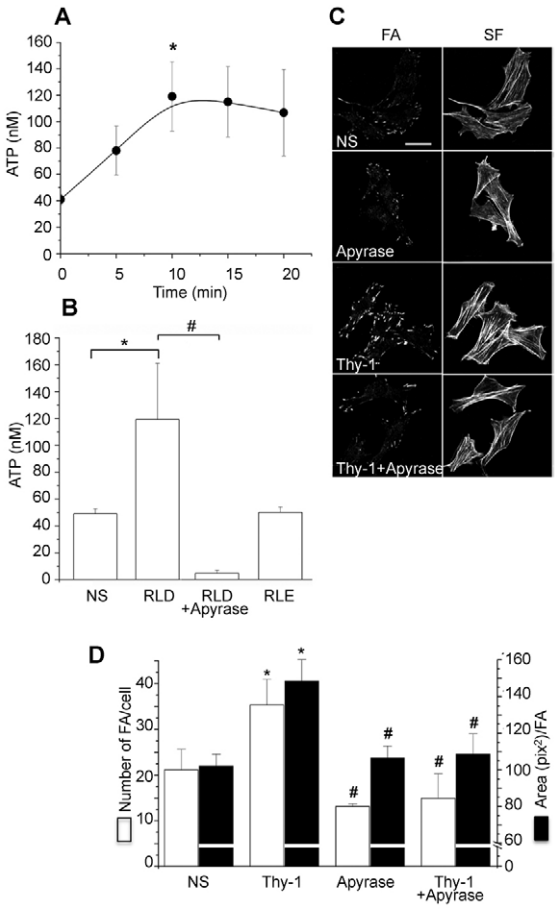

Fig. 1. Thy-1-induced ATP release depends on its interaction with integrin and is required for FA formation in DI TNC1 astrocytes. (**A**) The kinetics of ATP release from cells stimulated with Thy-1(RLD)-Fc–Protein A is shown; n=4; mean ± s.e.m.; *P<0.05, compared with time zero. (**B**) ATP concentrations in cell supernatants for non-stimulated astrocytes (NS) or following treatment for 15 minutes with Thy-1(RLD)-Fc–Protein A (RLD), pre-treatment with Apyrase (1 UI/ml; 30 minutes at 37°C) followed by Thy-1(RLD)-Fc–Protein A (RLD+Apyrase), or treatment with Thy-1(RLE)-Fc–Protein A (RLE); n=3, mean + s.e.m. P<0.05, compared to NS (*) or to RLD (#) condition. (**C**) Immunofluorescence analysis using anti-vinculin and rhodamine-conjugated phalloidin shows focal adhesions (FA) and stress fibers (SF), respectively. Astrocytes stimulated with Thy-1(RLD)-Fc–Protein A (Thy-1) or in cells pre-treated with Apyrase (as in B) followed by Thy-1(RLD)-Fc–Protein A (Thy-1+Apyrase) are shown. Scale bar: 25 μm. (**D**) Quantification of the number of FA per cell and the average area per FA for each condition tested in C. Confocal microscopy images were analyzed using ImageJ (plug-in: analyze particles). In each experiment, values for at least 50 different cells were averaged (n=3, mean + s.e.m.); P<0.05, compared to NS (*) or to Thy-1 (#) condition.

results suggest that ATP release and subsequent calcium influx through P2X7 receptors were required for Thy-1-induced FA formation in astrocytes.

Data suggesting that oATP is a weak and toxic P2X7 receptor antagonist (Evans et al., 1995; Peng et al., 2009) prompted us to additionally evaluate the effects of Coomassie Brilliant Blue G (BBG), an antagonist of higher selectivity and lower toxicity (Peng et al., 2009). This compound has been described as a specific P2X7 receptor antagonist when used at low concentrations (<5 μM) and incubated for short periods of time (<60 minutes) (Diaz-

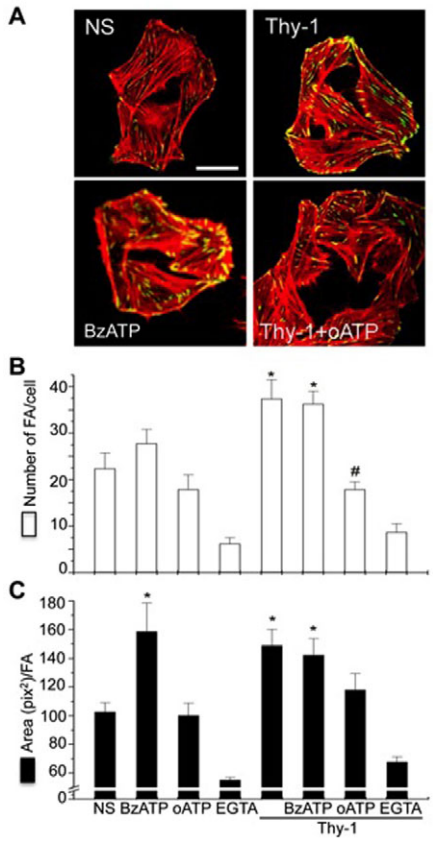

Fig. 2. P2X7 is involved in Thy-1-induced FA formation. (**A**) Confocal images of FA (green or yellow) and stress fibers (red). Non-stimulated astrocytes (NS) or astrocytes stimulated with either Thy-1(RLD)-Fc–Protein A (Thy-1) for 15 minutes, or incubated with BzATP (100 µM) for 15 minutes, or pre-incubated with oATP (600 µM) and then stimulated with Thy-1(RLD)-Fc–Protein A for 15 minutes (Thy-1+oATP) are shown. Scale bar: 25 µm. (**B**,**C**) Results from A, which also included treatment with 2 mM EGTA for 15 minutes (only shown in B and C) were quantified (n=3). The number of FA per cell (B) and the area per FA (C) are shown. In each experiment, values for at least 50 different cells were averaged per condition (mean + s.e.m.); P<0.05 compared to control (*) or to Thy-1-stimulated samples (#).

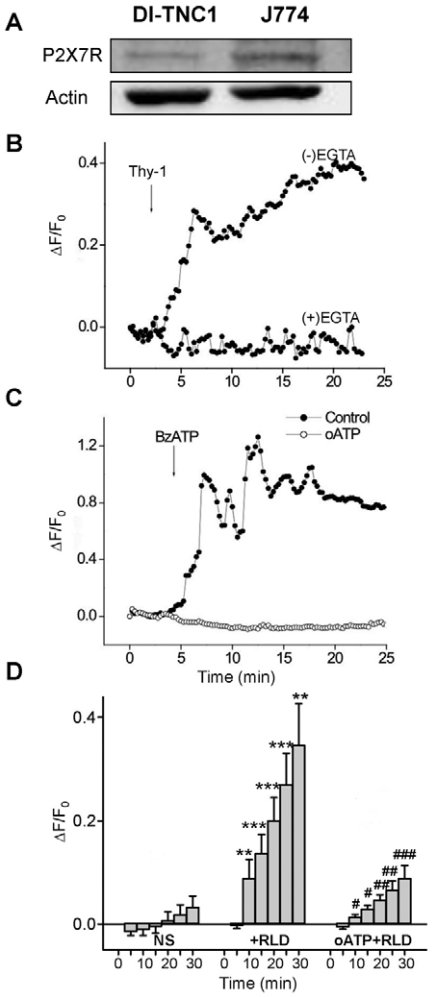

Fig. 3. Thy-1 stimulation augmented $[Ca^{2+}]_i$ by promoting calcium influx. (**A**) Cell lysates (50 µg per lane) analyzed by western blotting reveal the presence of P2X7 receptors (~69 kDa). Actin is shown as a control. Astrocytes were loaded for 30 minutes with Fluo-3 AM (2 µM) at 37°C. Then, cells were stimulated with Thy-1-Fc–Protein A and changes in the $[Ca^{2+}]_i$-dependent fluorescence were evaluated for 25–30 minutes. (**B**) Basal $[Ca^{2+}]_i$ levels were measured for 2–3 minutes, then Thy-1-Fc was added (arrow) in the absence (–) or presence (+) of 2 mM EGTA. Increased fluorescence ($\Delta F/F_0$) was monitored in an inverted microscope. (**C**) Effect of BzATP (100 µM), with or without pre-incubation with oATP (600 µM, for 30 minutes). Fluorescence ($\Delta F/F_0$) transients are representative of at least three independent experiments in each case. (**D**) Effect of Thy-1(RLD)-Fc stimulation with or without a pre-treatment with oATP (600 µM, 30 minutes). Increased fluorescence ($\Delta F/F_0$) was monitored in a microplate reader. Values from three independent experiments performed in triplicates are shown (mean + s.e.m.). Statistically significant differences are indicated: P<0.05 compared to its respective Thy-1-stimulated condition (#); P<0.01 compared to its respective non-stimulated (NS) control (**) or to Thy-1-stimulated condition (##); P<0.001 compared to NS control (***) or to Thy-1-stimulated samples (###).

Hernandez et al., 2008). In our experiments, astrocytes incubated for 30 minutes with BBG at concentrations of 1, 5 and 10 µM inhibited FA formation induced by CAD cells, a neuron-like cell line derived from cathecolaminergic neurons (Qi et al., 1997) (supplementary material Fig. S1). These cells express high levels of Thy-1 on their surface and are as effective as the soluble Thy-1-Fc–Protein A complexes in stimulating FA formation in DI TNC1 cells (Avalos et al., 2004; Hermosilla et al., 2008). Thus, these results support our conclusion that the effects observed are due to the inhibition of P2X7 receptors and not other P2X molecules.

DI TNC1 astrocytes express functional P2X7 receptors responsible for Thy-1-induced intracellular calcium transients

Astrocytes reportedly express P2X7 receptors (Fields and Burnstock, 2006). To determine whether these purinergic receptors are also present in DI TNC1 astrocytes, cell extracts were analyzed by western blotting (Fig. 3A). A band of ~69 kDa was detected both in DI TNC1 and J774 macrophages (positive control) lysates.

To confirm that extracellular calcium was required and that calcium transients were induced upon Thy-1 stimulation, relative fluorescence changes of the Fluo-3-AM probe were measured following Thy-1(RLD)-Fc addition, in the presence or absence of EGTA (2 mM). Stimulation with Thy-1–Protein A complexes induced an increase in fluorescence that was not observed when

using calcium-free medium (Fig. 3B, ±EGTA). As expected, the non-hydrolyzable ATP analog, BzATP, induced calcium transients with kinetics similar to those detected with Thy-1 (Fig. 3C, closed circles) that were blocked by pre-incubation with oATP (Fig. 3C, open circles). Taken together, these results indicate that the calcium transients detected upon Thy-1 addition required the presence of extracellular ATP, possibly to activate P2X7 receptors. To corroborate this interpretation, cells were incubated with oATP prior to addition of Thy-1(RLD)-Fc–Protein A complexes. Only a small calcium increase was triggered by Thy-1 in the presence of this ATP antagonist (Fig. 3D), indicating that Thy-1-induced responses require active P2X7 receptors. Thus, DI TNC1 astrocytes possess functional P2X7 receptors at the plasma membrane that are activated following ATP release induced by Thy-1–integrin interaction.

Signal transduction downstream of integrin and P2X7 receptors in DI TNC1 astrocytes involves Ins(1,4,5)P_3 receptor and RhoA activation

Binding of Thy-1 to αvβ3 integrin in astrocytes induces integrin receptor clustering and activation of PKCα and RhoA, leading to increased astrocyte adhesion (Avalos et al., 2004; Avalos et al., 2009; Hermosilla et al., 2008; Leyton et al., 2001). To test whether P2X7 receptors were upstream of RhoA in this signaling pathway, the effect of a P2X7 receptor agonist on RhoA activation was tested. To this end, cells were stimulated with 100 µM BzATP. Interestingly, BzATP induced ~2.5-fold activation of RhoA, which was inhibited by pre-incubation for 30 minutes with 5 µM BBG (Fig. 4A), indicating that this small GTPase is activated in the signaling pathway downstream of P2X7 receptors.

According to the findings presented here, $[Ca^{2+}]_i$ elevation is also part of the signaling cascade downstream of P2X7 (Fig. 3). However, in ATP-treated rat cortical type-I primary astrocytes, such calcium elevation is biphasic with an early, large transient component, probably due to release from intracellular stores, and a second, smaller sustained calcium rise that involves calcium influx through P2X7 receptors (Nobile et al., 2003). In our experiments, the early calcium transient was not observed, presumably due to the low time resolution (15 seconds) of $[Ca^{2+}]_i$ measurements. To evaluate the possibility that such an early calcium transient due to Ins(1,4,5)P_3 receptor activation might be involved, effects of the Ins(1,4,5)P_3 receptor inhibitor (2-aminoethyl diphenyl borate, 2APB) were tested. Indeed 2APB, but not 0.1% methanol used as a control, blocked Thy-1-induced RhoA activation (Fig. 4B) and FA formation (Fig. 4C) in astrocytes, confirming that, as reported for ATP-treated primary astrocytes, an initial calcium transient from intracellular stores is likely to participate in the response observed here. It should be noted that for experiments shown in Fig. 4, different modes of astrocyte stimulation were employed. For the affinity precipitation assays (Fig. 4B), Thy-1-Fc coupled to Protein A-Sepharose beads were used because this permits quick removal of the stimulus. Additionally, induction of FA formation was evaluated following stimulation with CAD cells (Fig. 4C).

Decreased P2X7 receptor presence reduced Thy-1-induced responses in astrocytes

To further explore the role of P2X7 receptors in FA formation, P2X7 receptor levels were decreased in DI TNC1 astrocytes by transfection with specific short interfering siRNA (siRNA). In astrocytes co-transfected with a plasmid encoding GFP (pEGFP-C1) and P2X7-specific siRNA, P2X7 receptor mRNA levels were reduced (Fig. 5A). Silencing of the P2X7 protein was not readily

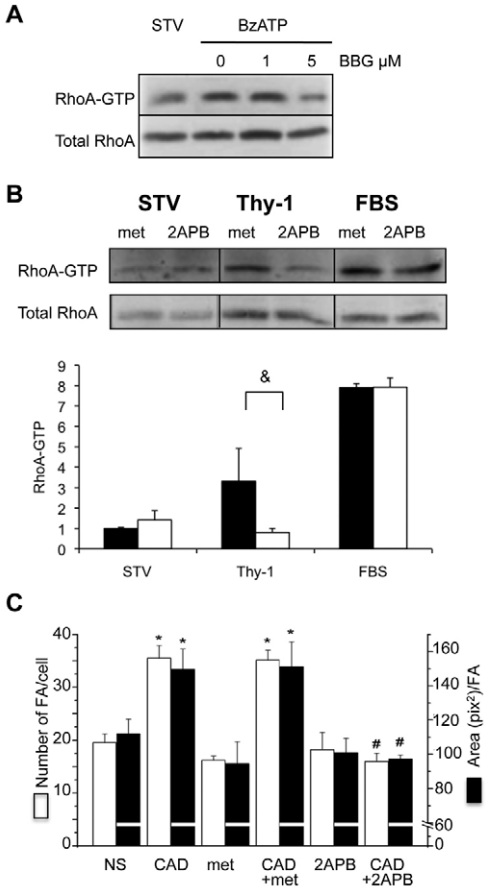

Fig. 4. Signaling events triggered by integrin and P2X7 receptor engagement involves Ins(1,4,5)P_3 receptor and RhoA activation in astrocytes. (**A**) DI TNC1 cells were incubated overnight in serum-free medium (STV), and then treated with 100 µM BzATP for 15 minutes, in the presence or absence of the P2X7 receptor antagonist BBG at 1 or 5 µM (pre-incubated for 30 minutes). Cells were then lysed, and affinity-precipitated active RhoA (RhoA-GTP) or total RhoA from whole cell lysates were visualized by immunoblotting with anti-RhoA monoclonal antibodies. A representative blot is shown. (**B**) DI TNC1 cells were serum-starved overnight. Then, the astrocytes previously treated with 0.1% methanol (met) used as a vehicle or with 2APB (10 µM, 30 minutes at 37°C) in serum-free medium were left as is (STV), treated with Thy-1-Fc beads for 5 minutes (Thy-1), or with 3% fetal bovine serum for 3 minutes (FBS, positive control). Cell extracts were prepared, and affinity-precipitated to detect active RhoA as indicated in A. A representative blot is shown. Numerical values obtained by scanning densitometry were averaged from three independent experiments and the fold increase in active RhoA normalized to total protein is shown in the graph. Statistical difference is indicated, $^{\&}P$=0.05. (**C**) DI TNC1 astrocytes pretreated with 2APB (10 µM, 30 minutes at 37°C) or with 0.1% methanol (met), were stimulated for 10 minutes with Thy-1-containing CAD cells. Non-stimulated cells (NS) were used as controls. After the different treatments, cells were washed, fixed and permeabilized to evaluate the number and size of FA as indicated in Fig. 1D. The average number of FA per cell + s.e.m. (white bars), as well as the average area per FA + s.e.m. (black bars), were determined for at least 50 cells in each experimental condition (n=3); $^{*}P$<0.05 for CAD-stimulated cells compared to NS cells; $^{\#}P$<0.05 for CAD-stimulated cells in the presence of 2APB (CAD+2APB) compared with CAD-stimulated cells in the absence of 2APB (CAD+met).

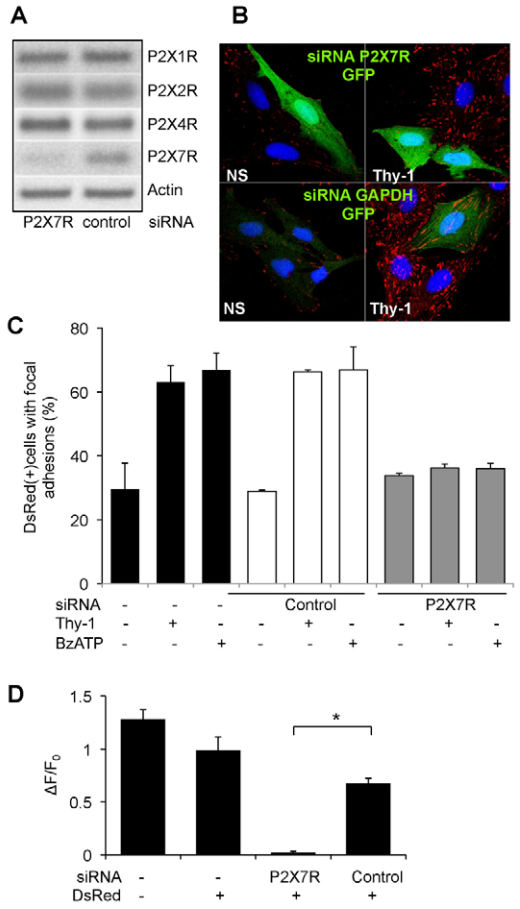

Fig. 5. P2X7 silencing precludes Thy-1-triggered FA formation and BzATP-induced calcium influx. (**A**) Levels of mRNA encoding P2X and actin were analyzed by semi-quantitative RT-PCR in samples obtained from cells transfected with siRNAs targeting either GAPDH (control) or P2X7R. Actin was used as internal control. Levels found for P2X1, P2X2, P2X4 and P2X7 are shown. (**B**) Astrocytes were co-transfected with pEGFP-C1 (green) and either GAPDH-specific siRNA or P2X7-specific siRNA. After 48 hours, cells were stimulated with Thy-1(RLD)-Fc–Protein A (Thy-1) for 15 minutes. FA were stained with anti-vinculin antibodies (red), and the nuclei with Syto Blue. (**C**) Same as B, but co-transfection was with pDsRed and either a random siRNA sequence or P2X7-specific siRNA. Stimulation was performed as indicated in B for Thy-1, or for 15 minutes with 100 μM BzATP. In addition, FA were stained using Alexa-Fluor-488-conjugated secondary antibody. The percentage of DsRed-positive cells that were stimulated in each indicated condition is shown. In cells scored as stimulated, elongated FA and at least a 1.5-fold increase in the number of FA per cell were detected. Values represent the mean + s.d. estimated from 50 DsRed-positive cells per condition (n=2). (**D**) The effect of 100 μM BzATP for 10 minutes in cells co-transfected or not with siRNA targeting P2X7 receptors (or control siRNA) and pDsRed. Increased fluorescence ($\Delta F/F_0$) was monitored in a microplate reader. Values from three independent experiments performed in duplicates are shown (mean + s.e.m.). Only the statistically significant difference found with the siRNA transfection control is indicated (*P<0.05); however, data for P2X7R-specific siRNA were significantly different from results for all controls tested.

detected because transfection efficiency was generally 40% or less. It should be noted that DI TNC1 cells possess all known P2X receptors; however, only P2X7 receptor expression levels were decreased by the P2X7-specific siRNA transfection (shown for P2X1, P2X2, P2X4 and P2X7, Fig. 5A).

In cells co-transfected with pEGFP-C1 and siRNA specific either for the P2X7 receptor or for GAPDH as a control, the capacity to form FA in response to stimulation with Thy-1–Protein A complexes was evaluated. GFP-positive astrocytes, co-transfected with P2X7-specific siRNA, did not respond to Thy-1 stimulation. Alternatively, those transfected with either pEGFP-C1 alone, or pEGFP-C1 and GAPDH-specific siRNA were stimulated in the presence of Thy-1 (Fig. 5B). The number of GFP-positive cells for which Thy-1-stimulated FA were observed was evaluated as indicated in Materials and Methods. GFP-positive cells, co-transfected or not with GAPDH-specific siRNA were stimulated by addition of Thy-1–Protein A complexes (60±16% and 62±4%, respectively). However, in those GFP-positive cells co-transfected with the P2X7-specific siRNA, Thy-1 did not stimulate FA formation (23±4%, mean ± s.e.m., P<0.05, compared to Thy-1-stimulated control condition) beyond the normal basal levels observed in non-transfected cells (28±4%). In order to test the effect of BzATP in these experiments, results previously obtained were repeated by co-transfecting pDsRed with the siRNA targeting P2X7 receptors or with a random siRNA sequence as a negative control. Again, in the presence of Thy-1–Protein A complexes, ~65% of the cells appeared stimulated in both non-transfected and control transfected cells. The same was found when treating with 100 μM BzATP. However, the effects of either Thy-1 or BzATP were not observed in cells transfected with P2X7-specific siRNA (Fig. 5C).

Similarly, BzATP-induced Ca^{2+} influx (Fig. 3B) was not observed in cells co-transfected with both pDsRed and P2X7-specific siRNA, indicating that decreased levels of P2X7 receptors render astrocytes refractive to the P2X7 receptor agonist BzATP (Fig. 5D). These results confirm the importance of P2X7 receptors in Thy-1-induced FA formation, and also that the elevation of $[Ca^{2+}]_i$ levels requires active P2X7 receptors, as suggested by the previous experiments using a pharmacological approach. Importantly, BzATP had no effect on $[Ca^{2+}]_i$ levels in cells lacking P2X7 receptors, corroborating the specificity of this agonist-receptor interaction.

Discussion

Integrins are known receptors for extracellular matrix-mediated cell adhesion. They activate signaling pathways that involve the non-receptor tyrosine kinases, FA kinase (FAK) and Src; tyrosine phosphorylation of a number of proteins including paxillin, p130Cas, FAK and Src; activation of small GTPases; and a cascade of events that results in the formation of a supramolecular complex containing over 100 proteins (Burridge et al., 1997; Burridge and Wennerberg, 2004). Despite the wealth of information available in the literature concerning integrin-mediated adhesion, and the previously reported links between purinergic receptors, Ca^{2+} flux and integrins (Altieri et al., 1990; Crowley and Horwitz, 1995; Kaczmarek et al., 2005; Nebe et al., 1996), the possibility that ATP released as a consequence of ligand-mediated integrin engagement might represent a requirement for integrin signaling to induce adhesion had not been addressed. Here, we demonstrate that Thy-1 engagement of αvβ3 integrin stimulates ATP release required for subsequent calcium influx through P2X7 receptor activation. These events are essential for Thy-1-induced FA formation.

In astrocytes, ATP is released in response to receptor stimulation by noradrenaline, UTP, glutamate and low calcium solution. Relevant sources of extracellular ATP include exocytosis, ATP transporters triggered by activation of various receptors, mechanical stress, cell swelling and lysis (Fields and Burnstock, 2006; Hamilton

and Attwell, 2010). Identifying specific mechanisms important for ATP release from Thy-1-stimulated astrocytes will require additional studies beyond the scope of the current manuscript. However, cell lysis and mechanical stress could be excluded for at least two reasons: first, no lactate dehydrogenase release was detected when cells were treated with Thy-1(RLD)-Fc (data not shown); and second, no ATP accumulation was detected in the medium of untreated cells or in cells treated with Thy-1(RLE)-Fc (Fig. 1B).

The inhibition of FA formation by EGTA was more substantial than that observed with oATP (Fig. 2B,C) or Apyrase (Fig. 1D). Taking into account that Thy-1-stimulated cells also receive adhesion signals from the extracellular matrix, the possibility exists that EGTA affects both matrix-, as well as Thy-1-induced cell adhesion, whereas ATP antagonism or depletion only affects Thy-1 stimulation. Therefore, integrin signaling events triggered by Thy-1 through ATP release, appear exclusively related to cell–cell adhesion.

The significant effect of BzATP on FA size, but not number of FA, in cells not stimulated with Thy-1 (Fig. 2B) is intriguing, and suggests that ATP signaling might participate in the maturation of FA rather than formation of new one FA downstream of integrin receptors. In agreement with the results obtained with BzATP, oATP did not affect basal formation of FA and only blocked those stimulated by Thy-1 treatment. Presence of oATP was expected to reduce more substantially Thy-1-induced FA size. The relatively modest effect obtained with this P2X7 antagonist might be due to interactions with other reported targets (Di Virgilio, 2003). Consistent with this interpretation, effects observed after treatment with the highly specific antagonist BBG were more significant.

Interestingly, previous studies indicate that stretch- or stab-induced damage causes the activation of a G-protein-coupled P2Y nucleotide receptor in astrocytes, thereby activating extracellular-signal-regulated kinase (ERK), glial fibrilar acidic protein (GFAP) expression and astrocyte proliferation (Franke et al., 1999; Neary et al., 2003). Other studies have investigated the role of the P2Y2 nucleotide receptor (P2Y2R) in the activation of astrocytes. This ATP/UTP-activated receptor contains an RGD motif that enables P2Y2Rs to interact with αvβ3 and αvβ5 integrins (Erb et al., 2001), thereby leading to increased astrocyte migration mediated by signaling pathways that include phosphoinositide 3-kinase (Wang et al., 2005). Despite the evidence linking purinergic receptors to integrins, their role in cell adhesion remains unclear. One important possibility, currently being explored in our laboratory, is that the calcium peak might be generated by αvβ3 integrin engagement and coupling of P2Y2R to αvβ3 or αvβ5 integrins, activation of PLC and Ins(1,4,5)P_3 generation, and subsequent calcium release from the endoplasmic reticulum mediated by Ins(1,4,5)P_3 receptor activation. We propose that the $[Ca^{2+}]_i$ increase generated by calcium influx through ATP-activated P2X7 receptors is equivalent to the second, sustained calcium signal observed by others (Nobile et al., 2003).

P2X7 receptors are known to increase $[Ca^{2+}]_i$ levels in osteoclasts. Importantly, P2X7 receptor activation was recently implicated in calcium-dependent translocation of PKCα and PKCβI, but not PKCδ, to the basolateral membrane of osteoclasts or differentiated RAW264.7 cells. Such P2X7–PKCα coupling was further corroborated by showing that PKCα activation did not occur in P2X7(–/–) cells stimulated with BzATP (Armstrong et al., 2009). These findings are of interest because they support the hypothesis that increments of $[Ca^{2+}]_i$ triggered by binding of Thy-1 to integrin and mediated by ATP-gated P2X7 receptors might help maintain PKCα at the plasma membrane once activated downstream of integrin engagement. We have previously shown that FA formation requires the combined association of the αvβ3 integrin–syndecan-4 receptor pair with Thy-1 and the activation of PKCα and RhoA-dependent pathways (Avalos et al., 2009). Membrane-bound active PKCα is expected to participate in formation of the ternary complex with phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and Thy-1-stimulated syndecan-4. Thus, by triggering the cascade integrins/ATP release/P2X7 activation/calcium influx, elevated cytosolic calcium might help retain active PKCα at the plasma membrane, thereby permitting formation of the syndecan-4–PtdIns(4,5)P_2–PKCα ternary complex and the subsequent activation of RhoA to stabilize FA. Interestingly, our results indicate that BzATP activates P2X7 receptors, induces calcium influx, activates RhoA and leads to the formation of FA. This sequence of events was not observed in cells lacking P2X7 receptors, suggesting that RhoA activation occurs downstream of these receptors. Therefore, because PKCα and RhoA reportedly participate in a linear signaling pathway to promote adhesion in embryo fibroblasts (Dovas et al., 2006) and astrocytes (Avalos et al., 2009), it is likely that the P2X7–PKCα coupling also exists in astrocytes to activate RhoA. Further research is underway to elucidate the signal transduction mechanisms involved.

In summary, our data identify ATP release, P2X7 receptor activation and calcium influx as novel, unanticipated intermediates in the sequence of events that links engagement of αvβ3 integrin in astrocytes to augmented cell adhesion and changes in cell shape. These astrocyte responses are initiated by in trans interaction with Thy-1, a molecule abundantly expressed by neurons in the adult brain. The findings might be relevant in brain injury, where astrocytes undergo morphological changes associated with increased adhesion and actin contraction (Sofroniew, 2009). Interestingly, following spinal cord trauma in mice, high levels of extracellular ATP, which induce death of neurons and oligodendrocytes but not astrocytes, have been detected in the peri-traumatic area (Wang et al., 2005). Moreover, injury to the CNS reportedly increases the expression of several proteins in reactive astrocytes, including P2X7 receptors (Narcisse et al., 2005), αvβ3 integrin and αvβ5 integrin (Wang et al., 2005), further highlighting the potential importance of our observations to inflammatory events following brain damage.

Materials and Methods

Cell cultures

The rat astrocytic cell line DI TNC1 (ATCC CRL-2005) and J774 macrophages were maintained in RPMI medium 1640 (GIBCO) containing 5% serum (FBS, HyClone), 0.1 mM 2-mercaptoethanol (GIBCO) and 100 U/ml penicillin plus 100 μg/ml streptomycin (GIBCO), in 5% CO_2 at 37°C.

Thy-1 stimulation

Thy-1-Fc–Protein A complexes were formed by pre-incubating mouse Thy-1(RLD)-Fc or Thy-1(RLE)-Fc, obtained as reported (Leyton et al., 2001), and Protein A (Sigma) in a 10:1 ratio for 30 minutes at 4°C. Cells were serum-deprived for 30 minutes prior to addition of Thy-1-Fc–Protein A complexes (4 μg:0.4 μg) to a 24-well plate or (1 μg:0.1 μg) to a 96-well plate in serum-free medium. The time- and concentration-dependent effects of these Thy-1 complexes in astrocytes are shown here for the first time (supplementary material Fig. S2A,B). Other modes of stimulation included the addition of Thy-1-Fc–Protein A-Sepharose or the neuronal-like CAD cells, which were used as previously reported (Hermosilla et al., 2008).

Indirect immunofluorescence

Astrocytes grown to 60–70% confluency on coverslips were stimulated in 24-well plates for 15 minutes with Thy-1 or BzATP (100 μM; Sigma). In some experiments, a pre-incubation for 30 minutes with oATP (600 μM; Sigma), BBG (1 and 5 μM; G-250, American Bioanalytical, Natick, MA), or a concomitant addition of EGTA

(2 mM; Sigma) and Thy-1 stimulus were used. Concentrations of BzATP, BBG and oATP used were selected on the basis of dose–response curves obtained for BzATP (1–100 μM) and BBG (1–100 μM) as well as for oATP (100 and 600 μM) using the Lucifer Yellow incorporation assay (Faria et al., 2005) (supplementary material Figs S3 and S4). Additionally, a dose–response curve was employed to define for oATP (50–800 μM), the concentration required to inhibit FA formation triggered by CAD cells (supplementary material Fig. S5). The formation of FA and stress fibers were evaluated using anti-vinculin antibodies (Sigma) and Rhodamine-labeled phalloidin (Sigma) as previously reported (Avalos et al., 2009). To test the effect of the Ins(1,4,5)P_3 receptor inhibitor 2APB (Sigma), astrocytes were pretreated for 30 minutes with 10 μM of 2APB in 0.1% methanol in serum-free medium or vehicle only. Then, neuronal CAD cells were added to astrocytes in the same medium to stimulate FA formation for 10 minutes. These mouse neuronal cells express large amounts of Thy-1 on their surface and have been shown to stimulate FA formation in astrocytes in a Thy-1-dependent manner (Hermosilla et al., 2008). Astrocytes were also transiently co-transfected with the plasmid pEGFP-C1 or pDsRed (Clontech Laboratories, Mountain View, CA) and either P2X7-specific siRNA (Dharmacon, Lafayette, CO) or the indicated negative siRNA control, using the siPORT Amine transfection reagent (Ambion, Foster City, CA). Samples, stained with anti-vinculin followed by goat anti-mouse IgG conjugated to Alexa Fluor 546 or to Alexa Fluor 488, and Syto blue (Molecular Probes) were evaluated with a Carl Zeiss Axiovert-135M confocal microscope (LSM Microsystems). The number of FA per cell was quantified as previously described (Avalos et al., 2004). In the siRNA experiments, due to the low efficiency of transfection (<40%), the number of GFP-positive cells (or DsRed-positive cells) that contained elongated FA and presented at least a 1.5-fold increase in the number of FA compared to non-stimulated cells were scored as GFP (or DsRed)-positive cells with FA (Avalos et al., 2009).

Affinity precipitation of active RhoA

RhoA activity was measured by affinity precipitation assay (Avalos et al., 2004). After overnight serum deprivation, astrocytes were incubated for 30 minutes with the Ins(1,4,5)P_3 receptor inhibitor 2APB (10 μM) or vehicle (0.1% methanol) in serum-free medium. Cells in 6-cm plates at 80% confluency were then stimulated during 5 minutes with 40 μg Thy-1(RLD)-Fc bound to Protein A-Sepharose beads (Avalos et al., 2004). The effect of the P2X7 receptor agonist BzATP (15 minutes with 100 μM), in the presence or the absence of the P2X7 antagonist BBG at 5 μM (30 minutes pre-incubation), on RhoA activity was also assessed.

Western blots

Whole cell lysates were prepared in ice-cold lysis buffer as described (Leyton et al., 1999). Protein extracts (30 μg per lane) were separated by SDS-PAGE in 10% minigels and transferred to PVDF membranes (Millipore). Membranes were blocked with TBS containing 5% gelatin and incubated with goat anti-P2X7 antibody (Santa Cruz Biotechnology) followed by a horseradish peroxidase (HRP)-coupled secondary antibody (Sigma). To monitor RhoA activation, western blots were performed as reported (Avalos et al., 2004). The peroxidase activity was detected by enhanced chemiluminescence EZ-ECL (Biological Industries, Beit-Haemek, Israel) using a Discovery 12iC model Ultralum detection system (Claremont, CA. USA).

ATP measurements

Extracellular ATP content was measured using a luminescence assay (Cell-Titer Glo Kit, Promega). Briefly, DI TNC1 cells plated in 96-well plates were incubated with Thy-1-Fc–Protein A complexes. Once the time course was established (see Fig. 1A), the effects of Thy-1(RLD)-Fc and Thy-1(RLE)-Fc were tested at 15 minutes. For inhibition experiments, cells were preincubated for 30 minutes with or without the ATP hydrolyzing enzyme Apyrase (1 UI/ml at 37°C; Sigma). Resulting luminescence was measured in a Synergy HT Multi-Mode Microplate Reader (Biotek).

Intracellular calcium measurements

DI TNC1 cells were loaded with 2 μM Fluo-3-AM (Invitrogen) and incubated for 30 minutes at 37°C in serum-free RPMI medium. After washing twice, the medium was replaced by Normal Ringer solution or 2 mM EGTA-Ringer solution in the absence of $CaCl_2$. Changes in fluorescence at 526 nm for up to 30 minutes following addition of either Thy-1(RLD)-Fc–Protein A complexes or BzATP (100 μM) were monitored in an inverted microscope (model IM 35, Zeiss) equipped with a 63× NA 1.3 oil immersion objective lens, or in a Synergy HT Multi-Mode Microplate Reader. In inhibition experiments, oATP (600 μM) was added 30 minutes prior to the stimulus. P2X7-silenced cells obtained by co-transfecting P2X7-specific siRNA with pDsRed were also used. The images from the inverted microscope, acquired at 15-second intervals using a charge-coupled device camera (model SPOT 2, Instruments Diagnostic, Sterling Heights, MA), were processed and analyzed using NIH ImageJ and Origin software. The results were expressed as $\Delta F/F_0$, where ΔF is the fluorescence change and F_0 is the basal fluorescence.

Silencing of P2X7 receptors

siGENOME SMARTpool for rat P2X7 receptor (10 or 100nM, ThermoScientific) was co-transfected, with either pEGFPC1 or pDsRed to identify transfected cells, using Silencer siRNA transfection kit, siPORT Amine (Ambion). Silencing was evaluated by RT-PCR using primers for all P2X receptors as previously reported (Buvinic et al., 2009). Silencer Negative Control #1 siRNA (random sequence, Ambion) or GAPDH-specific siRNA were used as controls for specificity.

Statistical analysis

Results were compared by non-parametric Mann–Whitney analysis. Statistical significance is indicated in each figure.

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