Sphingosine-1-phosphate receptors stimulate macrophage plasma-membrane actin assembly via ADP release, ATP synthesis and P2X7R activation

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Summary

Eukaryotic plasma membranes assemble actin filaments within seconds of activation of many receptors, especially during chemotaxis. Here, serum or sphingosine-1-phosphate stimulation of J774 and RAW macrophages released ADP within seconds into the extracellular medium, along with an adenylate kinase activity that converted ADP to ATP. ATP then activated the P2X7 receptor (P2X7R) that was necessary for a peak of plasma-membrane actin assembly within 5 to 10 seconds in P2X7R-expressing J774, RAW and primary macrophages. Neither actin assembly nor characteristic P2X7R channel activity was seen in response to ATP in P2X7R-knockout macrophages, as detected by patch-clamp analysis. Since P2X7R has been shown previously to form a macromolecular complex with actin we propose that it is involved in the membrane assembly of actin. Our data reveal a surprisingly rapid and complex relay of signaling and externalization events that precede and control actin assembly induced by sphingosine-1-phosphate. The overall model we present is strongly supported by the data presented in the accompanying paper that focuses on latex bead phagosomes.

Supplementary material available online at
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Key words: ATP, P2X7, Sphingosine-1-P, Actin, Macrophages, Phagosomes

Introduction

The plasma membrane of cells can assemble and disassemble actin filaments within seconds of adding ligands of receptors, such as those that bind EGF or thrombin (Peppelenbosch et al., 1993; Omann et al., 1987; Carson et al., 1986). In many cases this dynamic assembly and disassembly of actin has been linked to chemotaxis of cells such as *Dictyostelium*, neutrophils, T cells and macrophages (Goetzl et al., 2004; McRobbie and Newell, 1983). In this, and other processes where actin is nucleated and polymerized on membrane surfaces, the monomers are inserted at the membrane surface by a membrane-bound machinery (Tilney and Cardell, 1970). In many systems these rapid changes in actin are correlated with transient increases in phosphatidylinositol-(4,5)-bisphosphate (PIP2) (Yin and Janmey, 2003).

Here, we address the rapid assembly of actin at the plasma membrane of mouse RAW 264.7 and J774A.1 macrophage cell lines (Defacque et al., 2000), and bone marrow primary macrophages. Preliminary evidence showed that the addition of serum to these cells led to a rapid accumulation of extracellular ATP that coincided with the actin assembly process. Extracellular nucleotides such as ATP, ADP and UTP bind to distinct members of a family of 15 cell-surface purinergic receptors and induce a spectrum of signaling events (Burnstock, 2006). We focused here on the P2X7 receptor (P2X7R), which is expressed by cells of the monocyte lineage, including macrophages, and is one of the best-characterized purinergic receptors (North, 2002). A prominent feature of P2X7R is its response to very high ATP levels (0.1-10 mM) where, apart from opening an ion channel pore, it might induce the opening of large pores that allow the passage of molecules of up to 900 Da into macrophages (Steinberg et al., 1987; Steinberg and Silverstein, 1987). Less appreciated, but important for the present, and the accompanying study (Kuehnel et al., 2009), is the finding that P2X7 receptor has an additional high-affinity site that responds to low concentrations (1-10 μM) of ATP (Klapperstück et al., 2001); such levels of ATP have been constitutively detected on the surface of many eukaryotic cells (Hayashi et al., 2004; Yegutkin et al., 2006b). Particularly relevant to the current study was the fact that the P2X7 receptor could be detected in a complex with 12 proteins, including actin, phosphoinositide 4-kinase and α-actinin (Kim et al., 2001). In conjunction with the parallel study focused on latex bead phagosomes (Kuehnel et al. 2009), our data collectively argue that the P2X7 receptor is part of a membrane-bound machinery that assembles actin.

Results

Serum induces actin assembly and releases extracellular ATP within seconds

We modified a widely used quantitative assay for monitoring the rapid assembly and disassembly of actin at the plasma membrane in response to receptor activation; this is based on the amount of Rhodamine-phalloidin that binds to F-actin in fixed and permeabilized cells (Howard and Oresajo, 1985; Defacque et al., 2000). When medium containing fresh fetal bovine serum was added to either J774 or RAW macrophages, reproducible peaks of actin

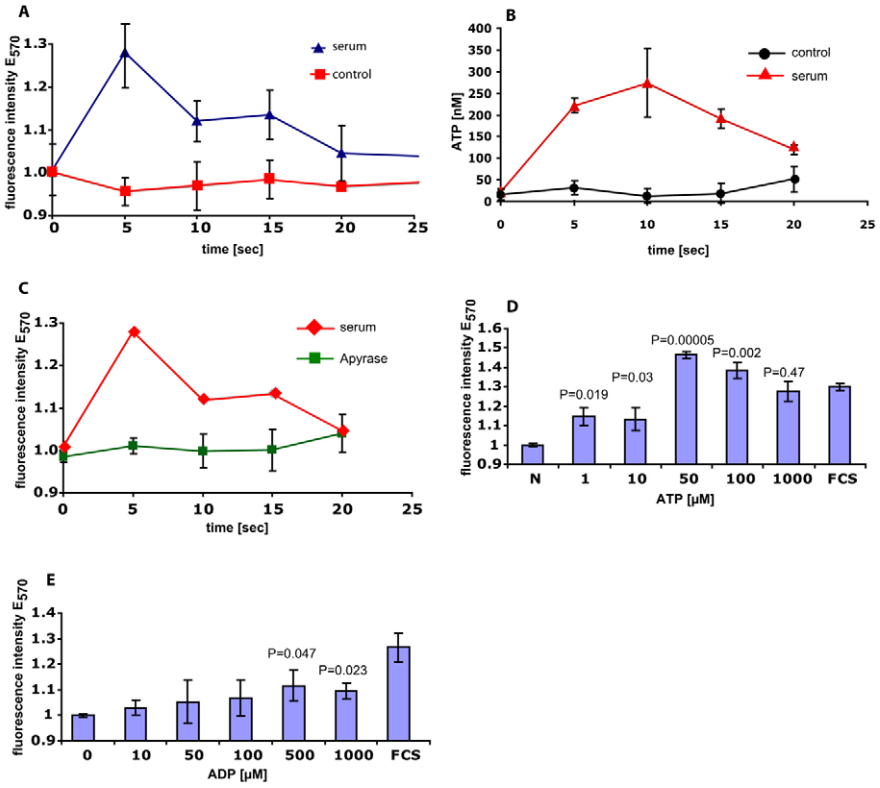

Fig. 1. (A) Quantification of F-actin changes in RAW cells stimulated with depleted or 'old' (control) or fresh fetal calf serum (serum). (B) Corresponding concentration of ATP in the supernatant of RAW cells estimated with the luciferase assay. (C) Effect on the F-actin assembly induced by medium containing fresh serum or serum plus 30 U/ml apyrase. (D,E) F-actin levels 5 seconds after stimulation in response to the indicated amounts of ATP (D) or ADP (E). Statistics: *P* values for significant effects are given for experiments done in triplicate. See Table 1 for a summary of the effects of different effectors on actin assembly by RAW macrophages 5 seconds after stimulation.

assembly were seen after 5 seconds in RAW cells (Fig. 1A) and after 10-15 seconds in J774 cells (supplementary material Fig. S1A). This effect is not the result of a physical perturbation of the system, which might, for example, activate stretch receptors or other receptors, because the addition to cells of depleted medium that had been incubated with cells overnight failed to stimulate actin assembly (Fig. 1A). When the extracellular levels of ATP were measured using the luciferase-luciferin-based assay the initial results showed the appearance of extracellular ATP in the same time-scale as the assembly of actin, for both cell types (Fig. 1B) (for J774 cells, see supplementary material Fig. S1). The maximum levels of ATP free in the extracellular fluid after S1P stimulation for 5 seconds, was 1-1.5 μM, estimated using quantitative HPLC analysis. Such levels of bulk ATP are likely to reflect significantly higher ATP levels on the plasma-membrane surface (Yegutkin et al., 2006b; Okada et al., 2006; Pellegatti et al., 2005; Yegutkin et al., 2006a; Beigi et al., 1999).

We asked whether ATP accumulation preceded, and perhaps induced actin assembly, or whether actin assembly occurred first. This issue could be resolved by showing that the addition of apyrase to medium containing fresh serum, that rapidly degrades ADP and ATP, completely blocked the ability of serum to activate the 5 second peak of actin assembly (Fig. 1C). This strengthened the hypothesis that the binding of serum factors to macrophages induced a receptor-mediated transmembrane signaling that led to active release of ATP, as supported by many other studies (Burnstock, 2006; North, 2002).

We now hypothesized the following scenario: (1) Binding of a serum ligand 'X' to its receptor initiates a transmembrane signal that leads to ATP release, and (2) binding of the extracellular ATP to an ATP receptor leads to a second transmembrane signaling event towards the machinery that assembles actin. In agreement with this hypothesis the addition of as little as 1 μM ATP to cells under serum-free conditions induced the 5 second activation of actin by RAW cells; the maximum signal was seen with 50 μM ATP (Fig. 1D). By contrast, ADP failed to significantly induce the actin assembly; a slight stimulation was seen only at 500 μM ADP (Fig. 1E). This result made it unlikely that a plasma-membrane-bound adenylate kinase activity was present that could convert ADP to ATP. AMP (1-100 μM) also had no effect (data not shown).

The serum factor responsible for assembling actin is S1P

We next addressed the identity of the putative serum ligand 'X' that was responsible for inducing the 5 second peak of actin in RAW macrophages. Serum complement was unlikely to be important since it had been destroyed by heat. Mouse IgG, lysophosphatidic acid (LPA), albumin, epidermal growth factor (EGF) and tumor necrosis factor-α (TNF-α) had no effect (Table 1). However, the addition of 10 nM sphingosine-1-phosphate (S1P) gave a reproducible 5 second peak of actin assembly in RAW macrophages (Fig. 2A). This treatment also induced a parallel accumulation of extracellular ATP in the same time-scale as the actin polymerization (Fig. 2B).

Table 1. Summary of the effects of different effectors on actin assembly by RAW macrophages 5 seconds after stimulation

Substance	Concentration	Effect on actin assembly
IgG	1-5 g/ml	No
EGF	100 and 500 ng/ml	No
LPA	1 and 10 M	No
TNFα	10-500 μM	No
Albumin	1-10 mg/ml	No
LPS	10-100 ng/ml	No
S1P	10 nM	Positive

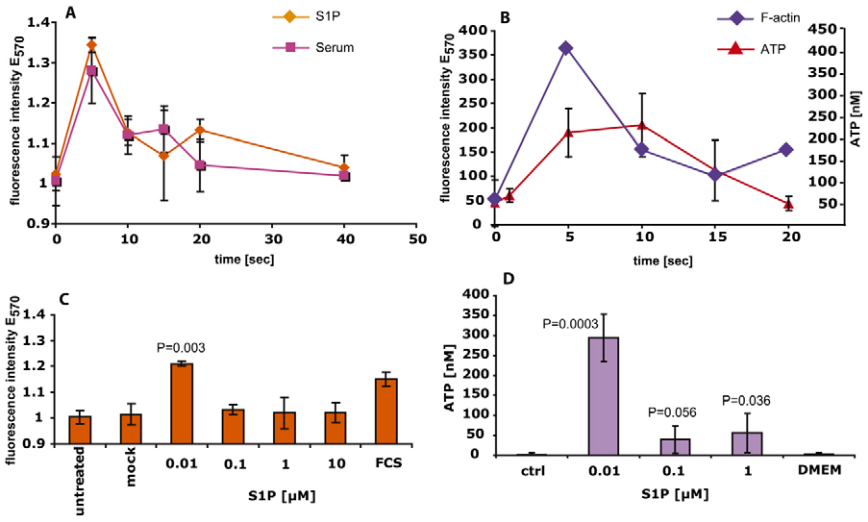

Fig. 2. (A) Comparison of F-actin assembly in RAW cells in response to fresh serum or 10 nM S1P. (B) F-actin levels and extracellular ATP concentration in RAW cells in response to 10 nM S1P. (C,D) Effects of different concentration of S1P on actin assembly (C) and ATP release (D) in RAW cells as seen by luciferase fluorescence assay. Mock, solvent control without lipid. Statistical significance for differences relative to the mock treatment analysed using the Student's *t*-test are indicated.

Both the kinetics and magnitude of the response to S1P on actin assembly was very similar to that seen with serum in RAW cells (Fig. 2A) and in J774 cells (supplementary material Fig. S1).

Although a relatively low concentration of S1P (10 nM) stimulated both the extent of ATP accumulation and the actin assembly process, it was striking that higher concentrations of the sphingolipid failed to stimulate either process (Fig. 2C,D). In fact, these data agree with many studies showing that S1P induces opposite signaling effects in T cells, depending on its concentration, with 1-10 nM stimulating cell motility and chemotaxis, whereas concentrations above 100 nM inhibited these processes (Rosen and Goetzl, 2005; Graler and Goetzl, 2002). Since S1P is also known to be a chemotactic signal in macrophages, we speculate that the receptor response to S1P that we observed is part of the earliest response of the chemotactic process. Of the five known S1P receptors, S1PR1 and S1PR2 are expressed in macrophages (Goetzl et al., 2004). In agreement with this, we could detect S1PR1 and S1PR2, but not S1PR3, S1PR4 or S1PR5 in RAW macrophages by RT-PCR (results not shown).

ADP is released in response to S1P or serum and rapidly converted to ATP by an adenylate-kinase-like activity

We sought definitive proof that ATP was released in response to S1P. For this, we monitored using HPLC, the release of labeled ATP, as well as AMP and ADP from RAW or J774 cells that had been metabolically labeled for 1 hour with [^{3}H]adenosine (Anderson et al., 2004). Within 2-3 seconds of adding S1P, a reproducible signal for labeled AMP and ADP could be detected: a time point when no ATP was detectable by HPLC (Fig. 3). At this, technically earliest possible time to make measurements, ATP was also not detectable by the luciferase assay (Fig. 1B), although it could be detected after 3-5 seconds by both assays (Fig. 3A, Fig. 1B). Unexpectedly, the kinetics of release shows that AMP and ADP were released first in both RAW and J774 cells, with ATP accumulating subsequently, in response to serum or S1P (Fig. 3A; supplementary material Fig. S2). In cells treated with medium free of S1P or serum, no ADP or AMP was detected at the 3 second time point, in contrast to the cells treated with serum or S1P. A delayed, transient peak of ADP and AMP was seen at around 5 seconds, significantly later than after lipid treatment (Fig. 3). Under this condition however, no ATP was detected.

We collected medium from RAW cells that had been stimulated for 5 or 10 seconds with serum or S1P. Incubation of this medium at room temperature in the presence of [^{3}H]ADP induced a conversion of ADP to ATP and AMP (Fig. 3B). No detectable conversion of [^{3}H]ADP to ATP was detected from cell supernatants to which serum-free medium was added without S1P (Fig. 3B, control). The co-incubation of the medium with diadenosine

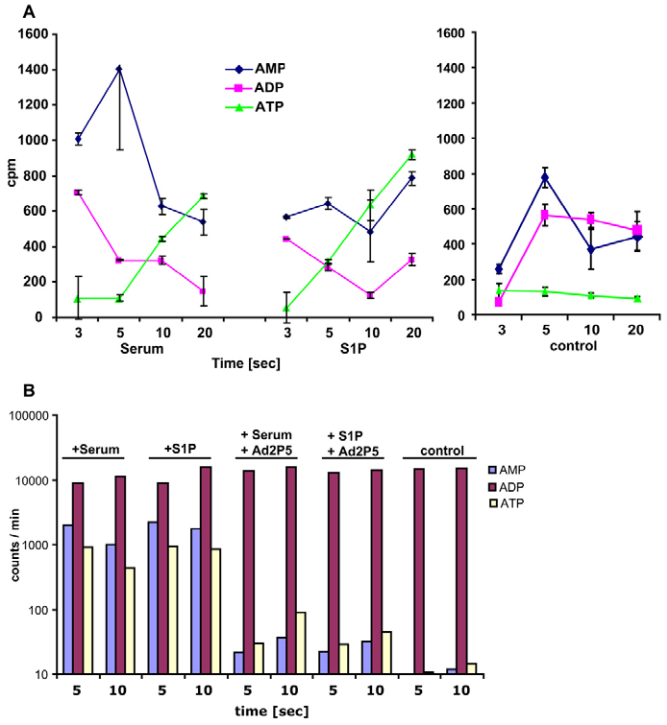

Fig. 3. (A) HPLC quantification of ^{3}H-labeled AMP, ADP and ATP released into supernatants of RAW cells after stimulation with serum or 10 nM S1P compared with cells treated with medium without serum or S1P (control). (B) Cell supernatants were collected at indicated times and incubated in the presence of ~15,000 c.p.m. [^{3}H]ADP. After 1 minute of incubation, nucleotides were detected by HPLC. Controls are supernatants without serum, S1P or Ad_2P_5.

pentaphosphate (Ad_2P_5), an inhibitor of adenylate kinase (Quillen et al., 2006) (25 μM) significantly inhibited the conversion of ADP to ATP and AMP. These data indicate that an adenylate-kinase-like activity is released either from the cell surface or from intracellular sources in response to activation of S1P receptors.

The P2X7 receptor transmits the signal from external ATP to the membrane actin assembly machinery

We next addressed the identity of the macrophage receptor that binds to extracellular ATP. In supplementary material Table S1 we provide pharmacological data on RAW cell actin assembly that resemble the known characteristics of the P2X7R, such as inhibition by oxidized ATP and Coomassie blue (North, 2002). Since the different ATP-binding receptors have characteristic electrophysiological 'signatures' (North, 2002), more definitive experiments were undertaken using patch-clamp analysis, an approach widely used to characterize P2X receptors. Fig. 5A demonstrates typical currents evoked by application of ATP to RAW macrophages. A small current could be detected with as little as 1 μM ATP^{4-} (free, uncomplexed form of ATP). As ATP concentration increased, a significant channel activity was detected that remained open as long as ATP was applied, arguing against the activation of desensitizing P2X1, P2X3 or P2X4 receptors but in favor of non-desensitizing P2X2 or P2X7 receptors. The large concentrations of ATP^{4-} needed to reach maximal effects are consistent with the activation of P2X7 receptors (North, 2002). The small current seen at low ATP^{4-} is probably due to the activation at the high affinity ATP^{4-} binding site that was recently described for human P2X7 receptors (Klapperstück et al., 2001). Modeling of the ATP^{4-} concentration-response curve was also consistent with the P2X7 receptor having two distinct binding sites (supplementary material Fig. S3).

Further evidence favoring the predominant expression of the P2X7R in RAW cells was its activation by benzoyl ATP (BzATP), with a several-fold higher potency than ATP (Fig. 4B) (North, 2002). The block of ATP-induced currents by oxidized ATP (Fig. 4C) or Mg^{2+} (Fig. 4D) is also consistent with the known behavior of P2X7R (North, 2002). To further investigate the possibility that the currents activated by low μM ATP concentrations might be due to activation of non-desensitizing P2X2 receptors or slowly desensitizing P2X4 receptors, the effect of adding extracellular Zn^{2+} was tested. Co-application of 10 μM Zn^{2+} with ATP^{4-} decreased the purinergic receptor-dependent currents, in comparison with the Zn^{2+}-free control (Fig. 4E). This is typical for P2X7 receptors (Virginio et al., 1997), but not for P2X4- or P2X2-receptor-dependent ion currents, which are potentiated by Zn^{2+} (Xiong et al., 1999). Furthermore, incubation of the cells in 30 U/ml apyrase did not lead to resensitization of desensitizing currents (0 of 7 cells tested; results not shown), as was observed for P2X1R-dependent ion currents in HL60 cells (Buell et al., 1996). These data strongly suggest the involvement of P2X7R in the low-ATP-dependent channel activity.

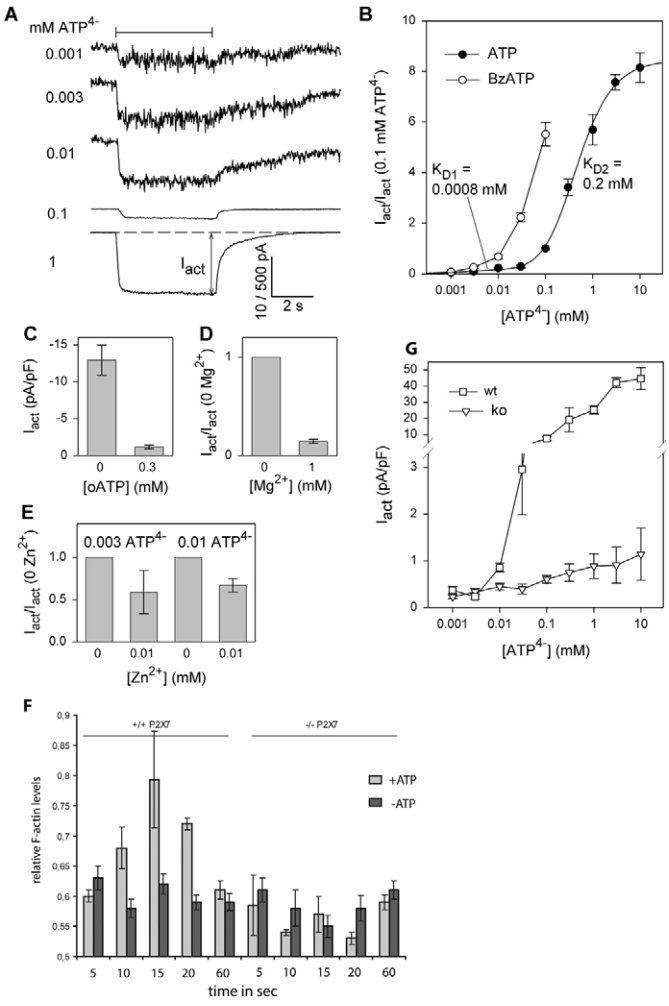

Fig. 4. Functional expression of P2X7 receptors in mouse macrophages. (A) Currents induced by ATP (time indicated by horizontal bar) in a RAW macrophage. (B) Normalized concentration-response for P2X7 receptor agonists ATP^{4-} and $BzATP^{4-}$. (For approximation, see supplementary material Fig. S3.) (C) Effect of oxidized ATP. Currents were activated by application of 1 mM ATP^{4-} for 3 seconds and normalized to the cell capacitance. (D) Effect of extracellular Mg^{2+}. Currents were activated by 0.6 mM total ATP without or with additional 1 mM $MgCl_2$ (0.1 or 0.035 mM free ATP^{4-}, respectively). (E) Effect of co-application of Zn^{2+} on ATP^{4-}-dependent ion currents. Current amplitudes were normalized to the respective values before Mg^{2+} or Zn^{2+} application. Means and s.d. from 5-50 cells are given. (F) F-actin assembly in response to 50 μM ATP in wild-type (+/+) and P2X7R-knockout (–/–) primary bone marrow macrophages. Columns represent means of three individual experiments with s.d. Statistical significance was determined by the Student's *t*-test. (G) Electrophysiological currents induced by 50 μM ATP in wild-type and P2X7R-knockout bone marrow macrophages. Means and s.d. from 5-50 cells are given.

P2X7R-knockout bone marrow macrophages are defective in ATP-stimulated actin assembly and ion currents

To test the hypothesis that the P2X7R is essential for the ATP-dependent currents and rapid actin assembly that occurs in response to S1P or ATP in a second cell type, we used primary bone marrow macrophages from wild-type and P2X7R-knockout mice (Solle et al., 2001). In contrast to J774 and RAW macrophages, neither type of primary macrophages showed an elevation of actin assembly in response to serum or S1P within 20 seconds, presumably because they lack functional S1P receptors on their plasma membranes. However, the wild type did respond to ATP; 50 μM ATP was able to transiently induce actin assembly in these cells within 10-20 seconds (Fig. 4F). By contrast, the P2X7R-knockout cells failed to induce actin assembly in response to ATP.

The signaling of both cell types in response to ATP was then tested by patch-clamp analysis (Fig. 5G). At very low μM ATP concentrations, a detectable current could be seen in both P2X7R-knockout and wild-type macrophages. We believe this is a response

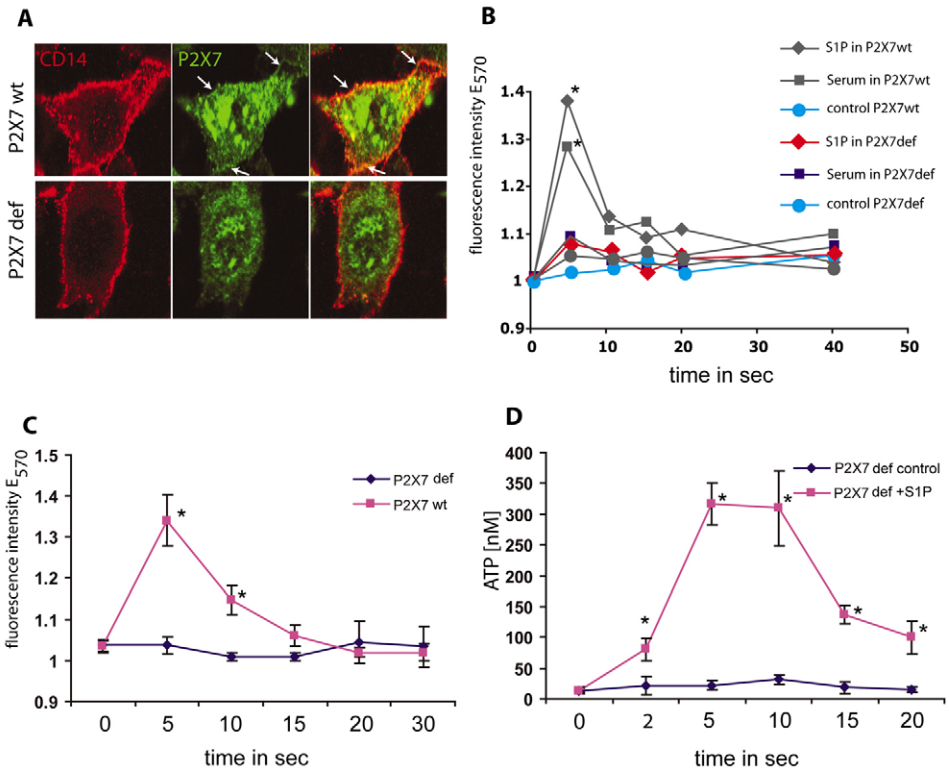

Fig. 5. (A) Immunofluorescence localization of P2X7R and the membrane marker CD14 in wild-type and P2X7R-knockout (def) J774 cells. Arrows indicate the prominent plasma-membrane labeling for both markers only in the wild-type cells. (B) F-actin assembly in P2X7R wild-type and P2X7R knockout (def) J774 cells, with and without S1P or serum stimulation. (C) F-actin assembly in the two J774 cell lines in response to 50 mM ATP. (D) Luciferase assay quantification of extracellular ATP in the two J774 cell lines in response to S1P. Values represent means and s.d. of three individual experiments. **P*<0.01.

due to few functionally expressed P2X2 and/or P2X4 receptors. Above 3 μM ATP^{4-} concentration, a reproducible pattern of additional channel activity could be seen in the wild-type cells, which was completely absent in the cells lacking P2X7 receptors. This provides compelling evidence that the main channel activity detected in response to ATP is due to the P2X7R.

J774 cells functionally deficient in P2X7R do not assemble actin in response to serum, S1P or ATP

Since even the wild-type bone marrow macrophages failed to respond to S1P in the above experiments we took advantage of a cloned J774 cell line that has very low amounts of functionally active P2X7R on their plasma membrane; these cells have been desensitized to ATP by maintaining them for long periods in ATP (Steinberg and Silverstein, 1987; Steinberg et al., 1987), as was also shown for a RAW cell line (Hiken and Steinberg, 2004). Immunofluorescence labeling for P2X7R (in green) confirmed that whereas the control cells show a prominent plasma-membrane labeling for P2X7R, the mutant cells gave only weak labeling at the plasma membrane; both cell types showed a prominent intracellular labeling in vesicular structures (Fig. 5A). In this experiment, the plasma membrane was highlighted by additionally double-labeling the cells with an antibody against CD14, which is enriched on the cell surface of macrophages (Fig. 5A, red).

When the two cell types were compared for their response to serum, S1P or ATP, the control cells gave a prominent peak of actin assembly, as expected. However, the P2X7R-defective J774 cells failed to respond to any of these effectors (Fig. 5B,C). Nevertheless these P2X7R-defective cells were capable of responding to S1P because they showed an S1P-dependent accumulation of extracellular ATP in response to S1P (Fig. 5D). This demonstrates that, in contrast to the BMM cells, the P2X7R-defective J774 cells have functional S1P receptors. However, since they lack the P2X7R at the plasma membrane, they are unable to transmit the signal from S1P downstream to the actin assembly machinery.

Discussion

In this study, we showed that the addition of serum or S1P to the surface of RAW or J774 macrophages induced an observable cascade of three separate transmissions of signals across the plasma membrane within seconds. The (still unknown) S1P receptor signals inwards (step 1) to induce an immediate ADP transport out, along with release of an adenylate kinase activity (step 2) that rapidly converts ADP to ATP. The latter then binds to the P2X7 receptor, hypothesized to be linked (upstream) to the actin assembly machinery (see below), which is turned on transiently after 5-10 seconds, in RAW macrophages (step 3).

In the primary bone marrow macrophages, there was no response to S1P, but the wild-type cells did respond to ATP, which induced rapid actin assembly. By contrast, the cells lacking P2X7R failed to show either the ATP-induced actin response or the characteristic P2X7R channel activity detected in the wild-type cells. We additionally took advantage of the availability of J774 cells that are depleted in P2X7R at the plasma membrane (see Steinberg and Silverstein, 1987). Whereas the wild-type parental J774 cells corresponding to this cell line showed a rapid actin assembly in response to serum, S1P and ATP, as expected, the defective cells failed to respond. Nevertheless, these cells showed an early signaling response in response to S1P, because they could induce the rapid accumulation of extracellular ATP, which was similar to what we observed with RAW macrophages and our clone (A1) of J774 cells.

Collectively, the data lead to the model summarized in Fig. 6. The main aspects of this model describing signaling at the plasma membrane were also evident in our analysis of latex bead phagosomes (Kuehnel et al., 2009). The results from the two systems are highly complementary. The first detectable reaction in this

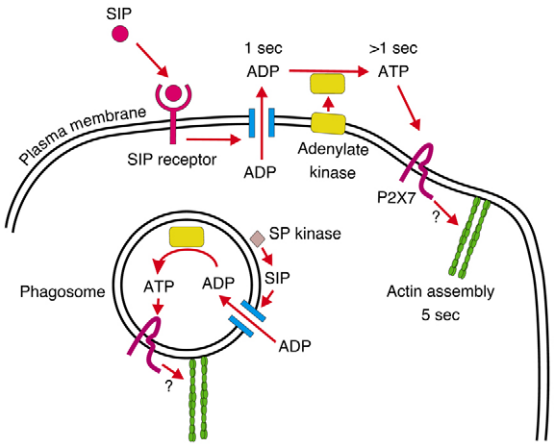

Fig. 6. Schematic model of the data presented in this study. On the plasma membrane we leave open the question as to whether the adenylate-kinase-like activity is exported from the cytoplasm or whether a plasma-membrane-bound form is activated or released by S1P receptor activation. It is also not clear whether ADP crosses the plasma membrane directly, as shown, or whether it crosses an intracellular vesicle membrane and is externalized by exocytosis. The summary scheme of the data from the phagosome system (Kuehnel et al., 2009) is also shown, for comparison.

scheme is the immediate release of ADP (and AMP, which has no effect on plasma-membrane actin assembly) into the cell medium within 3 seconds, in response to S1P receptor activation. Although ATP is thought to be more commonly released by cells (North, 2002), ADP can also be externalized by some cells, such as platelets (Kahner et al., 2006). Despite intensive investigation in this field, the mechanisms by which these nucleotides cross the plasma membrane or an intracellular vesicle membrane (being then secreted by exocytosis) are still unknown.

The ADP released by cells in less than 3 seconds in response to S1P is immediately converted to ATP by an adenylate kinase activity that was fully inhibited by the adenylate kinase inhibitor Ad_2P_5. Although extracellular adenylate kinase activity has been described attached to the plasma membrane (Yegutkin et al., 2006a; Picher and Boucher, 2003; Burrell et al., 2005; Fabre et al., 2006), our evidence argues that a pool of this enzyme is released into the extracellular fluid in response to S1P. Soluble adenylate kinase activity has also been described in a number of systems, but the molecular identity of this activity remains to be identified in any system (Donaldson et al., 2002; Dixon et al., 2003; Yegutkin et al., 2006b). All the five known adenylate kinases lack a signal sequence and are localized to either the cytoplasm or to mitochondria (Noma, 2005). How these become externalized (or enter the lumen of phagosomes) remains unknown. The analysis of the total ATP synthesis activity in the cell supernatants confirmed that significantly more ATP synthase activity was released from cells following treatment for 5 seconds with S1P, relative to the solvent-treated control cells. We tried to identify this protein by an affinity purification approach using ADP beads, but failed to detect any adenylate-kinase-like proteins in these cell supernatants by mass spectrometry, presumably because the putative protein is present at low concentrations and/or is quite labile (results not shown).

Within 5 seconds of SIP receptor activation, the ATP pool that is made up from the released ADP is sufficient to rapidly activate the P2X7R, which in RAW cells was the only receptor found to respond significantly to ATP in our patch-clamp analysis (Fig. 5). The presence in the extracellular medium of apyrase, an enzyme that breaks down ADP and ATP, completely blocked the ability of S1P to stimulate actin assembly, whereas as little as 3 μM ATP added exogenously showed a significant effect on actin assembly, which peaked at 50 μM (Fig. 1C).

Our patch-clamp analysis of RAW macrophages, in conjunction with the use of inhibitors in the electrophysiological experiments and in the actin assay, argued strongly that the main receptor in these cells that responds to ATP in the range of 3-100 μM was the P2X7R. Although even the wild-type bone marrow macrophages did not respond to serum or S1P, they did respond to 50 μM ATP, which both stimulated the rapid actin assembly response and gave a characteristic P2X7R-membrane cation-channel response to ATP. Significantly, both these responses were totally abrogated in the cells lacking P2X7R. These data argue that this receptor links the ATP-dependent signaling to the underlying actin assembly machinery. Such a mechanism would be consistent with the many links that have been made between the P2X7R and actin (Pfeiffer et al., 2004; Li and Wu, 2003; Li et al., 2003; Chen et al., 2006). Moreover, P2X7R has been found in a physical complex with actin, phosphoinositide 4-kinase, which is essential for actin assembly by latex bead phagosomes (Defacque et al., 2002), and ten other proteins (Kim et al., 2001).

The P2X7R has two distinct binding sites for ATP: a high-affinity site that responds to low μM [ATP], and a low affinity one that requires mM levels of ATP for activation (Klapperstück et al., 2001) (supplementary material Fig. S2). In addition to the actin assembly stimulated at low μM concentrations of ATP, a multitude of studies have focused on the P2X7R response to high (up to 10 mM) [ATP], especially the formation of large pores (North, 2002; Burnstock, 2006). Although prolonged ATP application can lead to apoptosis, transient treatment with high [ATP] also leads to an increase in phago-lysosome fusion and significant killing of pathogenic *Mycobacterium tuberculosis* in (non-apoptotic) macrophages (Lammas et al., 1997; Sikora et al., 1999; Kusner and Adams, 2000). These striking effects of activating the macrophage P2X7R at low or high ATP concentrations will justifiably be the continued focus of many laboratories in the near future.

Materials and Methods

Cells

RAW 264.7 and J774 A.1 mouse macrophages were grown as described (Anes et al., 2003). Wild-type J774.2 mouse macrophages and the matching clone defective in P2X7R (ATP-R B2) was described by Steinberg and Silverstein (Steinberg and Silverstein, 1987).

P2X7R-knockout mice

The primary P2X7R-knockout and wild-type bone marrow macrophages were isolated as described (Al-Haddad et al., 2001) from C57 BL/6 wild-type and P2X7R-knockout mice (Solle et al., 2001).

Actin assembly

This assay was a modification of the assay described (Defacque et al., 2000a). Cells were grown in 24-well plates and after the stimulant was added, cells were fixed with 3% formaldehyde then permeabilized with 0.1% Triton X-100 and the nuclei and F-actin stained simultaneously with DAPI and rhodamine-phalloidin (Defacque et al., 2000). Fluorescence intensities were measured using a Tecan multiwell plate reader and the total red actin signal was normalized to the total cell number represented by the DAPI signal.

Radioactive labeling of nucleotides

Macrophages were incubated for 1 hour with 5 μCi [^{3}H]adenosine in medium collected from RAW cells that had been incubated with fresh serum overnight (control). Following this, the cells were washed twice with 500 μl control medium and then incubated for different times with either control medium or medium containing fresh serum, or 10 nM S1P with or without Ad_2P_5 (Sigma, Germany). Samples were

collected at different times and rapidly frozen until analysis by HPLC. Radioactivity of fractions containing ATP, ADP or AMP was analyzed in a Beckman Coulter Counter LS 6500.

HPLC

The HPLC detection of ATP, ADP and AMP was done as described (Murphy et al., 1996).

ATP detection in macrophage culture supernatants

For detection of ATP in cell supernatants, J774A.1 or RAW264.7 macrophages were grown in six-well dishes until 70% confluency in 2 ml of culture medium. For testing effector molecules such as S1P, 1 ml of medium was taken out of each well and used to dissolve a stock solution of the drug and/or lipid and then added back to the cells. S1P (5 mM stock in ethanol; diluted with medium to be used at 100 nM). For testing the effect of serum, the cells were given new medium containing serum. The ATP concentration in supernatants was determined using the BioOrbit ATP detection kit and the BioOrbit 1250 luminometer, following the manufacturer's instructions as described (Gibbins and Mahaut-Smith, 2004). Briefly, samples were taken at indicated time points and immediately mixed with TCA to a concentration of 5% to inactivate enzyme activities. Samples were centrifuged at 14,000 ***g*** for 5 minutes; supernatants were retrieved and neutralized with NaOH. ATP estimation was done using a 50 µl sample volume.

Adenylate kinase assay in cell culture supernatants

Cells were incubated for different times with either medium that was in contact with these cells overnight (control) or medium containing fresh serum, as well as with or without S1P and/or Ad_2P_5. Supernatants were separated from the cells and incubated with ADP containing a ^{32}P label in the α position. After 1 minute of incubation, samples were frozen and subsequently analyzed by HPLC as described above.

Electrophysiology

Whole-cell currents were measured as described (Bretschneider et al., 1995; Lohn et al., 2001). Coverslips with macrophages were transferred to a perfusion chamber and rinsed with a bathing solution consisting of 140 mM NaCl, 0.5 $CaCl_2$, 10 mM HEPES and 10 mM glucose, pH 7.4, adjusted with NaOH. Patch pipettes were filled with a solution containing 65 mM Caesium aspartate, 65 mM CsCl, 3 mM EGTA, 3 mM BAPTA, 2 mM $CaCl_2$, 10 mM HEPES, 5 mM NaATP, 5.5 mM $MgCl_2$ and 10 mM glucose, pH 7.2 adjusted with CsOH. The free-Ca^{2+} concentration of this solution was calculated as 73 nM (Schubert, 1996). For activation of P2X7R-dependent currents, the bathing solution was quickly replaced for 3 or 6 seconds, by the U tube technique (Bretschneider and Markwardt, 1999), with bathing solution but containing free ATP^{4-} and/or Zn^{2+} as given in the figures and free 0.5 mM $CaCl_2$. For the experiments with oxidized ATP the cells were incubated for 3 hours in DMEM medium plus 0.3 mM oxidised ATP. The cells were clamped at –40 mV. All experiments were carried out at room temperature (22°C). Earlier studies have shown that all P2X receptors are fully functional at this temperature.

Immunofluorescence of P2X7R

J774 cells expressing wild-type P2X7R and J774 cells knocked down for P2X7R were fixed with 3% paraformaldehyde in PBS for 15 minutes and permeabilized with 0.1% saponin. After blocking with 1% BSA for 15 minutes, the cells were double labeled with a rat anti-CD14 (1:100; Becton Dickinson Transduction Laboratories) and a rabbit anti-P2X7R (1:200; kind gift from Blanche Schwappach, ZMBH, Heidelberg) antibody. Rabbits were immunized with the peptide: CRWRIRKEFPKSEGQYSGFKSPY from the C-terminal end of the human P2X7 receptor. The secondary antibodies used were anti-rat Cy3 (1:250; Dianova, Germany) and anti-rabbit Alexa Flour 488 (1:1000; Molecular Probes). All antibody incubations were done for 1 hour at room temperature.

Application of S1P

A stock solution of sphingosine-1-phosphate (S1P) was made (5 mM) in ethanol and used at 100 nM in the assays as described (Anes et al., 2003). Solvent without lipid was routinely tested and is referred to in the figures as the 'control'.

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