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The immunomodulator FTY720 is phosphorylated and released from platelets

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Abstract

The novel immunomodulator FTY720 causes lymphocytes from peripheral blood to accumulate in lymphoid tissues. *In vivo*, FTY720 is phosphorylated to FTY720-P, which binds to the sphingosine 1-phosphate receptor S1P₁. So far, it has been unclear where FTY720-P is produced. We demonstrate that platelets efficiently convert FTY720 to FTY720-P and release it into the extracellular space. This release is mostly independent of platelet activation, but is slightly increased upon thrombin stimulation. These results suggest that platelets are a major source of plasma FTY720-P, and that FTY720-P release is mediated by two different transporters.

Keywords: FTY720; Sphingosine 1-phosphate; Sphingolipid; Immunomodulator; Platelet

1. Introduction

The sphingolipid metabolite sphingosine 1-phosphate (S1P) is a bioactive lipid molecule that regulates several cellular processes (proliferation, regulation of cell migration, actin cytoskeletal rearrangement, and adherens junction assembly) through binding to its cell surface receptors (Kihara et al., 2007; Sanchez and Hla, 2004). To date, five mammalian S1P receptors (S1P₁/Edg1, S1P₂/Edg5, S1P₃/Edg3, S1P₄/Edg6, S1P₅/Edg8) have been identified.

S1P is abundant in plasma and is physiologically important, especially in the vascular and immune systems and through its binding to S1P₁. The importance of S1P in the immune system has been demonstrated using a novel immunomodulating agent, FTY720 (fingolimod; 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol). FTY720, an effective agent against transplantation rejection and autoimmune diseases, does not impair lymphocyte activation, proliferation, or effector function, but rather reduces circulating lymphocyte numbers (especially those of mature T cells) (Budde et al., 2006). Treatment causes accumulation of lymphocytes in secondary lymphoid organs such as lymph nodes and Peyer's patches (Chiba et al., 1998). FTY720 is phosphorylated *in vivo* by sphingosine kinase and binds to all S1P receptors except S1P₂ (Brinkmann et al., 2002; Mandala et al., 2002).

S1P₁ is highly expressed in mature T cells and endothelial cells, and is the most important S1P receptor in the immune system. S1P and the S1P₁ receptor have pivotal functions in the egress of lymphocytes from secondary lymphoid organs and thymus (Allende et al., 2004; Matloubian et al., 2004). Two models have been proposed for the molecular mechanism of FTY720-induced peripheral lymphopenia (Rosen and Goetzl, 2005). In the first, phosphorylated FTY720 (FTY720-P) causes prolonged internalization of S1P₁ on lymphocytes. Since S1P is a potent chemoattractant for lymphocytes, loss of cell surface

S1P₁ results in reduced responsiveness to the S1P gradient between lymphoid organs and plasma. In the second model, FTY720-P binds to S1P₁ on endothelial cells of the lymphoid organs and stimulates adherens junction assembly. As a result, lymphocytes become unable to pass through the cell junctions, and accumulate in the lymphoid organs.

Two major blood cells, platelets and erythrocytes, provide plasma S1P (Hänel et al., 2007; Ito et al., 2007; Yatomi et al., 1997a; Yatomi et al., 1997b). Most, but not all, of the S1P release from platelets is dependent on stimuli such as thrombin and protein kinase C activation (Yatomi et al., 2000; Yatomi et al., 1997b), whereas erythrocytes release S1P constitutively (Ito et al., 2007; Yang et al., 1999). In contrast, it has been unclear where FTY720-P is produced. Therefore, we tested the involvement of plasma and related cells (endothelial cells, platelets, and erythrocytes) in the production of FTY720-P. We found that only platelets can produce, store, and release FTY720-P into plasma. This release was largely independent of stimuli, in contrast to S1P release.

2. Materials and methods

2.1. Preparation of erythrocytes, platelets, and plasma

Wister ST rats (7-14 weeks old, male) were anesthetized with diethyl ether and pentobarbital (50 mg/kg rat weight), and whole blood was collected from their hearts. This study was carried out in accordance with the Declaration of Helsinki and/or with the guide for the Committee on the Care and Use of Laboratory Animals of Hokkaido University.

Erythrocytes, platelets, and plasma were prepared as described elsewhere (Ito et al., 2007; Yang et al., 1999). Blood (~ 10 ml) was collected in 2 ml anticoagulant ACD (0.8% citric acid, 2.2% sodium citrate, and 2.45% glucose) then separated by centrifugation (120 x g for 15 min) into pellet and supernatant (platelet-rich plasma). The pellet (~ 5 ml) was suspended in 25 ml buffer A (20 mM HEPES/NaOH (pH 7.5), 138 mM NaCl, 3.3 mM NaH₂PO₄, 2.9 mM KCl, 1 mM MgCl₂, 1 mg/ml glucose, and 1% fatty acid-free BSA), mixed with 5 ml ACD, and centrifuged as above. The resulting pellet was then suspended in 5 ml buffer B (buffer A without BSA). Erythrocytes were isolated from leukocytes and platelets by passage through a column of α -cellulose and cellulose powder (Sigmacell Cellulose Type 50) (1:1, w/w).

Platelets were prepared by treating platelet-rich plasma with apyrase (0.3 unit/ml) for 10 min at 37°C, then centrifuging at 1100 x g for 10 min at room temperature. The resulting pellets were suspended in buffer A and used as a platelet fraction. Blood cell numbers were determined using a COULTER Gen-S™ Hematology Analyzer (Beckman Coulter, Fullerton, CA).

2.2. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were grown in Endothelial Cell Basal Medium mixed with Endothelial Cell Growth Supplement. Prior to FTY720 treatment, the medium was changed to Endothelial Cell Defined Serum-Free Medium. All media were from Cell Applications (San Diego, CA).

2.3. Quantitative analysis of FTY720 and FTY720-P levels by HPLC

FTY720 was kindly provided by Sankyo Corporation (Tokyo, Japan). FTY720 and FTY720-P were measured by HPLC (Agilent 1100 series; Agilent Technologies, Palo Alto, CA) after derivatization with *o*-phthalaldehyde, essentially by lipid assays described elsewhere (Min et al., 2002).

2.4. Kinase assays

Platelets, erythrocytes, and HUVECs were suspended in buffer C (20 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 x protease inhibitor mixture (CompleteTM; Roche Diagnostics, Indianapolis, IN), and 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 1100 x g for 3 min at 4°C. The resulting supernatants were used as total cell lysates in further analyses. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL). Sphingosine kinase assays were performed essentially as described elsewhere (Billich et al., 2003). Total cell lysates (25 µg protein in 40 µl solution) were mixed with 50 µl 2X assay buffer (100 mM HEPES/NaOH (pH 7.5), 30 mM MgCl₂, 20 mM KCl, 10 mM NaF, 2 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 24 mM β-glycerophosphate, 1 mM 4-deoxyridine, EDTA-free CompleteTM, 2 mM DTT, and 2 mM PMSF), then with 10 µl of a

10X sphingosine solution (200 μ M sphingosine in 5% or 0.05% Triton X-100), 2.5 μ l 80 mM ATP, and 0.5 μ l [γ - 32 P]ATP (5.4 μ Ci; Institute of Isotopes, Budapest, Hungary). Kinase assays in which FTY720 as the substrate were performed using 20 μ M FTY720, 2 mM ATP, and 5.4 μ Ci [γ - 32 P]ATP. Samples were incubated at 37°C for 15 min, then lipids were extracted by the successive additions of 375 μ l chloroform/methanol/HCl (100:200:1, v/v), 125 μ l chloroform, and 125 μ l 1% KCl, with mixing. The resulting phases were then separated by centrifugation, and the organic phase was recovered, evaporated, and suspended in chloroform/methanol (2:1, v/v). Labeled lipids were resolved by TLC on Silica Gel 60 High Performance TLC plates (Merck) with 1-butanol/acetic acid/ water (3:1:1, v/v). Radioactivity associated with the SIP or FTY720-P band was quantified using a bioimaging analyzer, BAS-2500 (Fuji Photo Film, Tokyo, Japan).

2.5. Immunoblotting

Immunoblotting was performed as described previously (Kihara et al., 2003), using anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (2 μ g/ml; Ambion, Austin, TX) and HRP-conjugated anti-mouse IgG, F(ab')₂ fragment (diluted 1:10,000; GE Healthcare Bio-Sciences, Piscataway, NJ). Labeling was detected using an ECL kit (GE Healthcare Bio-Sciences).

3. Results

3.1. Platelets produce and release FTY720-P

We first investigated the ability of two major blood cells, platelets and erythrocytes, to produce and release FTY720-P. After platelets and erythrocytes were incubated with FTY720, cells and medium were separated by centrifugation. FTY720 and FTY720-P from each fraction were measured by HPLC. Almost no FTY720-P was detected in the erythrocyte fraction or its incubation medium (Fig. 1A). In contrast, FTY720-P was detected both in the platelet fraction and the incubation medium. About 60% of the added FTY720 was converted to FTY720-P, of which ~47% was released into the medium (Fig. 1A), indicating that platelets can efficiently convert FTY720 to FTY720-P and then release it. Since the total amount of FTY720/FTY720-P was nearly unchanged during the incubation, interconversion is likely the sole metabolic pathway for FTY720/FTY720-P in platelets. However, to exclude the possibility that the FTY720-P release was caused by membrane damage, we assayed for leakage of the cytosolic protein GAPDH. Almost no GAPDH was detected in the medium of any platelet cultures (Fig. 1B), suggesting that platelets were not so damaged as to allow leakage of cellular constituents, or at least proteins.

Since it has been reported that some sphingosine kinase type 1 (SPHK1) is secreted from cells (Ancellin et al., 2002), we also tested the ability of plasma itself to produce FTY720-P. However, we could not detect any FTY720-P after incubating FTY720 with plasma, utilizing either endogenous ATP (Fig. 1A) or exogenously added ATP (2 mM; data not shown) as the phosphate donor.

Endothelial cells are also exposed to plasma, so we next investigated whether such cells could produce FTY720-P. For this purpose, we used HUVECs, a commonly studied

endothelial cell type. However, almost no FTY720-P was detected in HUVECs or in the incubation medium (Fig. 1C). These results imply that endothelial cells do not contribute to the production of plasma FTY720-P, although the possibility that other endothelial cells are involved *in vivo* cannot be excluded.

We next measured the *in vitro* kinase activity of cells and plasma towards FTY720. Platelets lysates did exhibit a significant kinase activity toward the agent, whereas only background levels were detected with lysates from erythrocytes, plasma, and HUVECs (Fig. 2A). Platelet *in vitro* kinase activities correlate well with the amounts of FTY720-P observed in Fig. 1.

To date, two sphingosine kinases, SPHK1 and SPHK2, have been reported (Kohama et al., 1998; Liu et al., 2000). Of these, only SPHK2 can efficiently phosphorylate FTY720 (Billich et al., 2003; Kharel et al., 2005). SPHK2 activity is inhibited by high concentrations of Triton X-100 (Billich et al., 2003; Liu et al., 2000), so measuring sphingosine kinase activity in the presence of low (0.005%) or high (0.5%) Triton X-100 concentrations enables us to discriminate between the activities of SPHK1 and SPHK2. Platelets exhibited high sphingosine kinase activity (69 pmol/min/mg) in low Triton X-100 concentrations, yet in the presence of 0.5% Triton X-100 displayed an ~25% decrease in activity (52 pmol/min/mg) (Fig. 2B). These results suggest that both SPHK1 and SPHK2 exist in platelets, and are responsible for approximately 3/4 and 1/4 of the resident kinase activity, respectively.

3.2 FTY720-P release is enhanced by platelet activation

Most S1P release from platelets is known to be dependent on stimuli that activate platelets such as thrombin and protein kinase C activation (Yatomi et al., 2000; Yatomi et al.,

1997b). Therefore, we tested whether FTY720-P release could be stimulated by platelet activation. A significant amount of FTY720-P was released in the absence of any stimuli, but the release was enhanced ~1.9 fold by treatment with thrombin (Fig. 3). The protein kinase C inhibitor staurosporine inhibits stimuli-dependent S1P release (Yatomi et al., 1997b), and, likewise, inhibited the thrombin-dependent increase in FTY720-P release (Fig. 3). However, staurosporine had no effect on the stimulus-independent FTY720-P release (Fig. 3).

3.2 Two FTY720-P transporters exist in platelets

Two transporters for S1P have been proposed to exist and function in S1P release from platelets, an ATP-dependent transporter stimulated by thrombin and an ATP-independent transporter (Kobayashi et al., 2006). To examine whether S1P and FTY720-P are released via one or more common transporter, we added FTY720, sphingosine, or both to platelets, with or without activation, and measured their released phosphorylated derivatives. In platelets incubated with 0.5 μ M FTY720 without platelet activation (i.e. in the presence of staurosporine), a significant amount of FTY720-P, but only a small amount of S1P (generated endogenously), was detected in the medium (Fig. 4A). In contrast, treatment with thrombin stimulated considerable S1P release (5.9 fold) and FTY720-P release (1.7 fold) into the medium (Fig. 4A). However, the total amounts of S1P and FTY720-P in the medium (Fig. 4A) and platelet (Fig. 4B) fractions were nearly equal in activated and unstimulated platelets, indicating that platelet activation does not enhance S1P or FTY720-P production.

When sphingosine was added to platelets, both intracellular and released S1P levels were increased (Fig. 4A and B). Nevertheless, the pattern of S1P release was indistinguishable from that observed in the absence of exogenous sphingosine, i.e. a slight

release in unstimulated platelets and a much greater release following thrombin treatment (Fig. 4A). When FTY720 and sphingosine were simultaneously added in the presence of staurosporine, the levels of released FTY720-P and S1P were nearly unchanged compared to those observed with each single treatment (Fig. 4A). In either case, FTY720-P was released efficiently, while S1P was not, indicating that the putative stimulus-independent transporter prefers FTY720-P over S1P as a substrate. However, the observed increase in FTY720-P release following thrombin stimulation of the platelets was significantly diminished in the presence of sphingosine (Fig. 4A). This reduction may be caused by a decrease in total FTY720-P levels, which fell from 37 pmol in the absence of exogenous sphingosine to 25 pmol upon sphingosine treatment (Fig. 4A and B). Competition for sphingosine kinase between FTY720 and sphingosine is one possible mechanism behind this reduction.

The involvement of an ATP-binding cassette (ABC) transporter in stimulus-dependent S1P release from platelets has been suggested, since ATP is required, and the ABC transporter inhibitor glyburide/glibenclamide inhibited the release (Kobayashi et al., 2006). The ABC transporter ABCC1/MRP1 reportedly functions in the release of S1P from mast cells (Mitra et al., 2006). To characterize the FTY720-P transporter in platelets, we tested the effects of the ABC transporter inhibitors glyburide, verapamil (an ABCB1/MDR inhibitor), and MK571 (an ABCC1 inhibitor), on FTY720-P release. No inhibitor affected the FTY720-P release in the absence of thrombin. However, glyburide, but not verapamil or MK571, inhibited the thrombin-induced increase in FTY720-P release (Fig. 5A). Consistent with the previous report (Kobayashi et al., 2006), glyburide also inhibited the thrombin-dependent release of endogenously generated S1P (Fig. 5B). These results suggest that an ABC transporter other than ABCB1 or ABCC1 is involved in the stimulus-dependent release of

both FTY720-P and S1P, but not in the stimulus-independent release.

4. Discussion

FTY720 is a prodrug, and its conversion to FTY720-P is necessary for its immunomodulating effects. Conversion occurs not only at the time of administration but also constitutively during its circulation, since FTY720 exhibits a cycle of phosphorylation/dephosphorylation (Brinkmann et al., 2002; Kharel et al., 2005). However, it has been unclear where FTY720-P is produced. We have demonstrated here that platelets have the ability to produce FTY720-P and supply it to the plasma.

FTY720-P is released from platelets both in a stimulus-dependent and a stimulus-independent manner, as is S1P (Fig. 3). However, the release of the two differs in that most S1P is released in a stimulus-dependent manner, whereas FTY720-P release is stimulus-independent. Such a stimulus-dependent release may be mediated by an ABC transporter, since glyburide decreased the FTY720-P release (Fig. 5A). Since neither verapamil nor MK571 inhibited stimulus-dependent FTY720-P release (Fig. 5A), an ABC transporter other than ABCB1 and ABCC1 may be involved.

Knowledge regarding a stimulus-independent transporter is limited, whereas the involvement of a Ca^{2+} -dependent transporter in the stimulus-independent S1P release has been reported (Kobayashi et al., 2006). However, further studies are needed to identify and characterize both transporters.

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Figure legends

Fig. 1. Platelets produce and release FTY720-P. (A) Platelets (3×10^7 cells), erythrocytes (1×10^8 cells), and plasma (2.5 mg protein) prepared from rats were treated with 3 μ M (300 pmol) FTY720 in 100 μ l total volume, then incubated at 37°C for 1 h. Platelets and erythrocytes were separated from the medium by centrifugation. Total lipids were prepared from both medium and cells, and FTY720 and FTY720-P were quantified by HPLC. Values are relative to the sum of the amounts of FTY720 and FTY720-P, and represent the mean $\pm$ SD from three independent experiments. (B) Platelets (3×10^7 in 100 μ l) were treated with nothing (-), 1 μ l ethanol, or 1 μ l of 300 μ M FTY720 in ethanol, and incubated at 37°C for 1 h. Proteins were prepared from the medium of each sample, as well as from untreated platelets, and separated by SDS-PAGE, followed by immunoblotting with anti-GAPDH antibodies. (C) HUVECs ($\sim 1 \times 10^6$ cells) were treated with 3 μ M FTY720 at 37°C for 1 h. Total lipids were prepared from both medium and cells, and FTY720 and FTY720-P were quantified by HPLC. Values are relative to the sum of the amounts of FTY720 and FTY720-P, and represent the mean $\pm$ SD from three independent experiments.

Fig. 2. Platelets exhibit high kinase activity towards FTY720. (A) Plasma (250 μ g) and total lysates (25 μ g) prepared from rat platelets, erythrocytes, and HUVECs were incubated with 20 μ M FTY720, 2 mM ATP, and 5.4 μ Ci [γ - 32 P]ATP at 37°C for 15 min. Lipids were extracted and separated by TLC. Radioactivities associated with S1P were quantified using a bioimaging analyzer BAS-2500. Values shown represent the mean $\pm$ SD from three independent experiments. (B) Total lysates (25 μ g protein) prepared from human platelets were incubated for 15 min at 37°C with 20 μ M sphingosine, 2 mM ATP, and 5.4 μ Ci

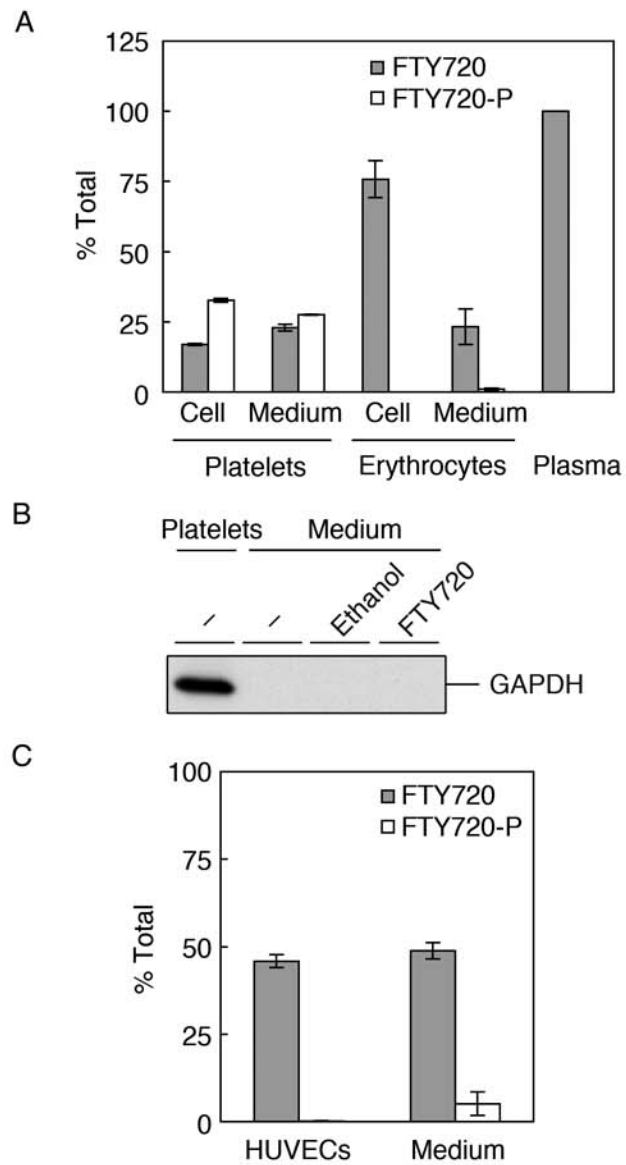
[γ - 32 P]ATP in buffer containing 0.005% (low) or 0.5% (high) Triton X-100. Lipids were extracted, separated by TLC, and quantified. Values shown represent the mean $\pm$ SD from three independent experiments.

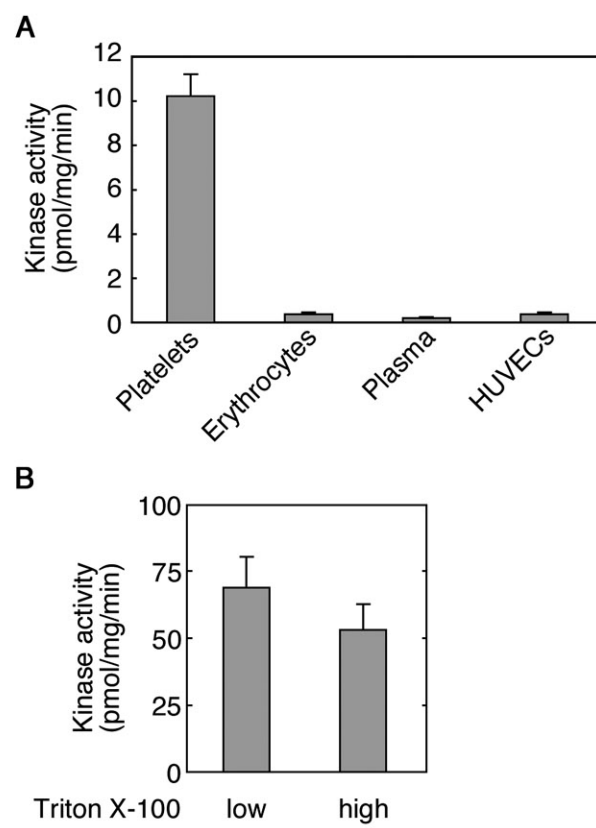
Fig. 3. Activation of platelets increases FTY720-P release. Rat platelets were pretreated for 5 min with 1 μ M staurosporine or its solvent dimethyl sulfoxide (DMSO), then loaded with 3 μ M FTY720 for 1 h, and incubated with 1 U/ml thrombin or buffer for 15 min, all at 37°C. Platelets were separated from medium by centrifugation. Total lipids were prepared from both medium and cells, and FTY720-P was quantified by HPLC. Values are relative to the total FTY720-P in cell and medium fractions, and represent the mean $\pm$ SD from three independent experiments. A statistically significant difference between values is indicated (*, $p < 0.002$; t-test).

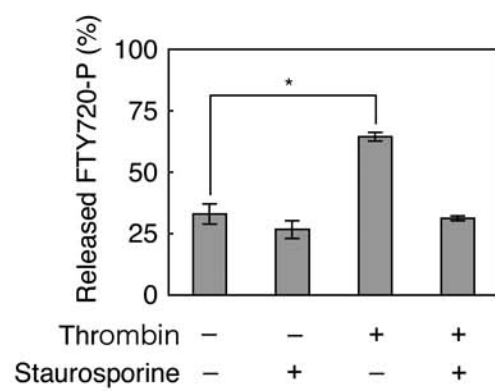
Fig. 4. FTY720-P release and S1P release from platelets are differentially dependent on two transporters. Rat platelets were pretreated for 5 min with 1 μ M staurosporine or its solvent DMSO, then loaded with 0.5 μ M (50 pmol) FTY720 and/or 0.5 μ M sphingosine for 1 h, and incubated with thrombin (1 U/ml) or buffer for 15 min, all at 37°C. Total lipids were prepared from medium (A) and platelets (B), and FTY720-P and S1P were quantified by HPLC. Values represent the mean $\pm$ SD of released S1P or FTY720-P from three independent experiments.

Fig. 5. The ABC transporter inhibitor glyburide inhibits the stimulus-dependent release of FTY720-P from platelets. Rat platelets were pretreated with 500 μ M glyburide, 15 μ M

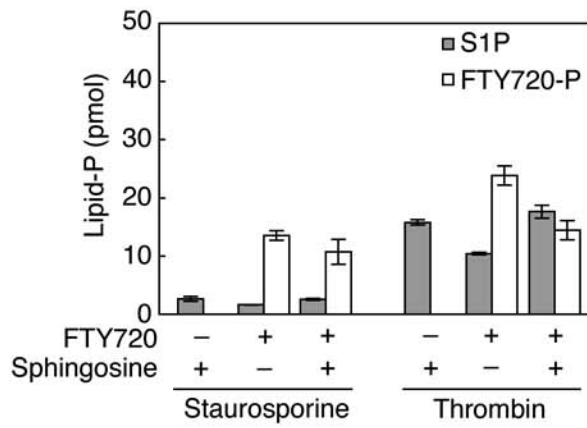
verapamil, or 15 μ M MK571 at 37°C for 5 min, then incubated with 3 μ M FTY720 at 37°C for 1 h. After further incubation with thrombin (1 U/ml) or buffer at 37°C for 15 min, total lipids were prepared from the medium and cells. FTY720-P (A) and S1P (B) were quantified by HPLC. Values shown are of the released FTY720-P or S1P relative to the total amount in cell and medium fractions and represent the mean $\pm$ SD from three independent experiments. Statistically significant differences are indicated (*, $p < 0.001$; t-test).







A Medium



B Platelets

