The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage

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Platelet aggregation is initiated by the release of mediators as adenosine diphosphate (ADP) stored in platelet granules. Possible candidates for transport proteins mediating accumulation of these mediators in granules include multidrug resistance protein 4 (MRP4, ABCC4), a transport pump for cyclic nucleotides and nucleotide analogs. We investigated the expression of MRP4 in human platelets by immunoblotting, detecting a strong signal at 170 kDa. Immunofluorescence microscopy using 2 MRP4-specific antibodies revealed staining mainly in intracellular structures, which largely colocalized with the accumulation of mepacrine as marker for delta-granules and to a lower extent at the plasma membrane. Furthermore, an altered distribution of MRP4 was observed in platelets from a patient with Hermansky-Pudlak syndrome with defective delta-granules. Adenosine triphosphate (ATP)-dependent cyclic guanosine monophosphate (cGMP) transport codistributed with MRP4 detection in subcellular fractions, with highest activities in the dense granule and plasma membrane fractions. This transport was inhibited by dipyramidole, indomethacin, and MK571 with median inhibitory concentration (IC50) values of 12, 22, and 43 μM, and by ibuprofen. Transport studies with [3H]ADP indicated the presence of an orthovanadate-sensitive ADP transporting system, inhibited by dipyramidole, MK571, and cyclic nucleotides. The results indicate a function of MRP4 in platelet mediator storage and inhibition of MRP4 may represent a novel mechanism for inhibition of platelet function by some anti-inflammatory drugs. (Blood. 2004; 104:3603-3610)

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Introduction

The critical role played by platelets in hemostasis and thrombosis is related to their function as exocytotic cells that secrete effector molecules at the side of vascular injury. Platelets contain at least 3 types of intracelluar granules, in which these mediators are stored and concentrated, known as alpha, dense, and lysosomal granules. While alpha granules contain mainly polypeptides, as fibrinogen, von Willebrand factor, growth factors, and protease inhibitors, dense granules contain small molecules, specifically adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, and calcium. Humans with defective dense granule exocytosis suffer from delta storage pool disease associated with a moderate bleeding tendency. The most severe delta storage pool disease is observed in Hermansky-Pudlak syndrome (HPS), a rare autosomal recessive disorder in which ocucolitocanuran albiniun, bleeding, and lysosomal ceroid storage result from defects of melanosomes, platelet-dense granules, and lysosomes. Little is known, however, about transport proteins mediating accumulation of the effector molecules in granules or their transport across the plasma membrane. Possible candidates include the multidrug resistance protein 4 (MRP4/ABCC4) and MRP5 (ABCC5). These belong to the C-branch of the human ATP-binding cassette (ABC) transporter superfamily, which consists of 12 members, 9 of which comprise the group of multidrug resistance proteins (MRP1-9; ABCC1-6 and ABCC10-12). MRPs are integral membrane glycoproteins that mediate the primary active unidirectional export of organic anions from cells. Conjugates of lipophilic compounds with glutathione, glucuronate, and sulfate are preferred substrates of MRP1-3, while cyclic purine nucleotides and nucleotide analogs have been identified as substrates for MRP4 and MRP5. MRP5 mRNA has been detected in many tissues, and the MRP5 protein could be localized in erythrocytes, smooth muscle cells of the genitourinary tract, and in human heart cardiomyocytes, vascular endothelial, and smooth muscle cells. MRP4 mRNA was detected in prostate, liver, testis, ovary, brain, kidney, and adrenal gland. Studies in membrane vesicles containing recombinant MRP4 indicate that it represents a transporter with a relatively broad substrate spectrum, including cyclic nucleotides as well as bile acids in cotransport with reduced glutathione, and prostaglandins. It also confers resistance to antiviral nucleoside analogs and cytotoxic thiopurine nucleosides, probably by the cellular export of the intracellularly stored respective nucleotide.

Proceeding from the initial finding that MRP4 is highly expressed in human platelets, we investigated the subcellular localization and function of MRP4 in these blood cells. The results indicate a function of MRP4 in platelet-dense granule...
storage and shed light on a novel molecular component of the action of dipyramido and some other nonsteroidal anti-inflammatory drugs (NSAIDs).

Patients, materials, and methods

Materials

[8−3H] Cyclic guanosine monophosphate (cGMP, 326 GBq/mmol) and [2,8-3H] 3′,5′ cyclic adenosine monophosphate (cAMP, 480 GBq/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany) and [2,8-3H]ADP (1.1 TBq/mmol), from American Radiolabeled Chemicals (St Louis, MO). Unlabeled nucleotides, dipryridamole, serotonin, mepacrine (quinacrine), ibuprofen, indomethacin, and sodium orthovanadate were from Sigma (Munich, Germany). MK571 (3-[3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl]−[3-dimethylamino-3-oxopropyl)-thio]-methyl)thio) propanoic acid) was from Alexis (San Diego, CA).

Antibodies

The antibodies corresponding to the 15 amino-terminal and carboxy-terminal amino acids of the human MRFP sequence (MLPVYQEVKPNPLQD and propanoic acid) was from Alexis (San Diego, CA).

Preparation of platelet membrane vesicles. The washed platelets were pelleted and resuspended in homogenization buffer (100 mM KCl, 2 mM MgSO4, 12 mM Na2 citrate, 10 mM tris-glucose, 25 mM HEPES [N-2-hydroxylethylpiperazine-N’-2-ethanesulfonic acid], 5 mM ATP, 0.35% BSA, pH 7.0, 340 millionsmolar) supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.3 μM leupeptin, and 1 μM aprotinin). The platelets were lysed by repeated cycles of freezing and thawing (4 times) and centrifuged at 100 000g (30 minutes, 4°C) to obtain pellets of crude membranes or subjected to subcellular fractionation on a linear 30% to 60% sucrose density gradient. 

Patients

The patient is a member of a large kindred with the Hermansky-Pudlak syndrome, extensively studied as described before. He is an albino with white skin, numerous freckles, marked nystagmus, severely impaired vision, and a bleeding tendency characterized with easy bruising, gingival bleeding, and bleeding after tooth extraction. He has a prolonged bleeding time and a severe deficiency of granule-bound ATP and ADP in the platelets. A mutation in the HPS4 gene has been found in this patient.

Isolation of human platelets and subfractions

Preparation of human platelets for membrane studies. Platelets were obtained from a healthy donor, who did not take any medication during the studies, platelet-rich plasma (PRP) was isolated from citrated blood by differential centrifugation (180 g, 20 minutes).

Washing of platelets. PRP was centrifuged (7 minutes, at 650g) after addition of 111 μL/mL azide-citrate-dextrose-anticoagulant (ACD-A) and 5 μL/mL apyrase PRP. The supernatant was discarded and the platelets were washed with buffer containing 5 U/mL apyrase (Sigma, Taufkirchen, Germany) and 2 U/mL hirudin (Pharmion, Hamburg, Germany). Finally the platelets were resuspended in Tyrode buffer (150 mM NaCl, 12 mM NaHCO3, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5.5 mM tr-glucose, and 1 mL/g bovine serum albumin (BSA), pH 7.4).

Immunoblot analysis

Membrane fractions were loaded onto a 7.5% sodium dodecyl sulfate–polyacrylamid gel after incubation in sample buffer at 37°C for 30 minutes. Immunoblotting was performed using a tank blotting system (Bio-Rad, Hercules, CA) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NY). Primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 and 1% BSA to the following final concentrations: AMF and SNG sera, 1:1000; MLP, 1:500; and P-selectin, 1:500; and anti-GP Ib, 1:50. Secondary horseradish peroxidase–conjugated goat anti–rabbit and goat anti–mouse IgG antibodies (Bio-Rad) or horse anti–goat IgG antibody (Vector Laboratories, Burlingame, CA) was used at 1:2000 or 1:1000 dilutions.

Vesicle transport studies

Transport of cyclic nucleotides. The ATP-dependent transport of [3H]cGMP or [3H]cAMP into membrane vesicles was measured by rapid filtration through nitrocellulose filters essentially as described. Vesicles were incubated in the presence of 4 mM ATP, 10 mM MgCl2, 10 mM creatine phosphate, 100 μg/mL creatine kinase, and [3H]cGMP or [3H]cAMP in the concentrations indicated in incubation buffer containing 250 mM sucrose and 10 mM Tris/HCl (pH 7.4). The final incubation volume was 75 μL. For inhibition studies, compounds were added from a stock solution in an appropriate solvent (incubation buffer, dimethyl sulfoxide, or ethanol at a final concentration of the solvent below 0.5% vol/vol); identical concentrations of the solvent were used in control samples. Aliquots (20 μL) of the incubations were taken at the times indicated, diluted in 1 mL ice-cold incubation buffer and filtered immediately through nitrocellulose filters (0.2-μm pore size, presoaked in incubation buffer). Filters were rinsed with 5 mL incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5′-AMP. Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of 5′-AMP as a blank from those in the presence of ATP and are given in pmol [3H]cGMP or [3H]cAMP × mg protein−1 (1 pmol × mg protein−1 = 528 atomic disintegrations per minute [DPM] or 780 DPM, respectively).
Transport of [3H]ADP. ATP-dependent transport of [3H]ADP (1 μM) into membrane vesicles was measured by rapid filtration as described for cyclic nucleotides, except that vesicles were incubated in incubation buffer supplemented with 0.4 mM ATP and 10 mM MgCl₂ in the presence or absence of sodium orthovanadate (1 mM). For studying the effect of an increased osmolarity of the extravesicular medium, the vesicles were preincubated for 45 minutes at 4°C in buffer containing 1 M sucrose or in standard incubation buffer containing 250 mM sucrose.

Immunofluorescence microscopy

Coverslips cleaned in acetone (5 minutes) and washed with water were covered with 40 μL human collagen type I (1 mg/mL; Sigma, Munich, Germany), incubated (15 hours, 37°C), washed twice with 1 mL phosphate-buffered saline (PBS), blocked by BSA (2 mg/mL, one hour, 37°C), washed twice with 1 mL PBS, incubated with 20 μL platelet suspension (100 × 10⁹/L, 30 minutes, room temperature [RT]), washed with 1 mL PBS, pH 7.3, thrice, and fixed with formaldehyde in PBS (1%, 30 minutes, RT). After 3 washes with PBS, platelets were permeabilized with 1% saponin in PBS (30 minutes RT) and blocked by 20% human serum in PBS (15 minutes). Antibody staining was carried out using the primary antibodies at the following dilutions: SNG (or preimmune serum), 1:125; MLP (or preimmune serum), 1:50; anti-LAMP2, 1:10; and anti-P-selectin (CD62P), 1:5. The respective secondary antibodies, either conjugated to Alexa Fluor488 or Alexa Fluor568, were used at a dilution of 1:250 or 1:50, respectively. Fluorescence micrographs were taken with a confocal laser scanning microscope (Chromaphor Analysen Technik, Duisburg, Germany). Samples were observed with a Nikon inverted microscope and a 100 × oil-immersion objective. A CCD camera and VoxCell scan software from VisiTech International (Sunderland, United Kingdom) were used for analysis. For peptide competition experiments, the SNG serum was preincubated for 60 minutes at room temperature with 90 μM of the synthetic peptide used to generate this antibody. For staining of dense granules with mepacrine, platelets were incubated with 100 μM mepacrine and washed twice with calcium-free Tyrode buffer before they were placed on coverslips.

For comparison of normal platelets and Hermansky-Pudlak syndrome platelets, staining intensities were assessed by immunofluorescence microscopy using MetaMorph Imaging series 4.6 software (Visitron Systems, Puchheim, Germany).

Results

Detection of MRP4 in human platelets

Expression of MRP4 and MRP5 was analyzed by immunoblot analysis as shown in Figure 1A. The SNG antibody directed against the carboxyl terminus of human MRP4 detected a strong broad double-band at 170 to 180 kDa in crude membranes (100 000 pellets) from human platelets (Figure 1A, left panel). The double-band is probably due to different glycosylation, since both bands were shifted to one major band with a lower apparent molecular mass after treatment of the membrane proteins with peptide N-glycosidase F prior to immunoblot analysis. Both bands disappeared when the SNG antiserum was preincubated with the synthetic SNG peptide, while an additional band at about 130 kDa appeared when the SNG antiserum was preincubated with the synthetic peptide used to generate this antibody. For staining of dense granules with mepacrine, platelets were incubated with 100 μM mepacrine and washed twice with calcium-free Tyrode buffer before they were placed on coverslips.

For comparison of normal platelets and Hermansky-Pudlak syndrome platelets, staining intensities were assessed by immunofluorescence microscopy using MetaMorph Imaging series 4.6 software (Visitron Systems, Puchheim, Germany).
mixed fraction containing lysosomes but also a high proportion of plasma membrane, as indicated by GPIb. Accumulation of the fluorescent mepacrine was used as an additional dense granule marker. Mepacrine has been shown to be rapidly and specifically concentrated in dense granules. Labeling appeared only in the densest fraction. However, note that here the intact platelets were loaded with mepacrine prior to homogenization, and, thus, transport into membrane vesicles, formed during the preparation, could not occur, in contrast to the cGMP transport measured in the isolated fractions.

Immunolocalization of MRP4 in human platelets

The subcellular localization of MRP4 in human platelets was further investigated using immunofluorescence microscopy (Figures 2-3). As shown in Figure 2, both anti-MRP4 antibodies SNG, directed against the carboxyl terminus, and MLP, directed against the amino terminus of MRP4, revealed a similar positive staining, mainly in intracellular structures and to a lower extent at the plasma membrane depending on the activation of the platelets indicated by the different shape of platelets (Figure 2A, C, E). Control staining with the preimmune sera or after preincubation with the recognition peptide indicates the specificity of the signal (Figure 2 B, D, F). To identify the intracellular structures, where MRP4 is localized, double-label experiments were performed using costaining with P-selectin as marker for α-granules and mepacrine, as well as the anti-LAMP2 antibody, as markers for dense granules (Figure 3). The overlay images in Figure 3 (C, F, I) indicate no colocalization with the α-granule marker but a partial colocalization with mepacrine-storing and LAMP2-positive structures, most likely to be dense granules (Figure 3D-I).

Altered localization of MRP4 in dense granule–deficient platelets (HPS)

Furthermore, the distribution of MRP4 in platelets from a patient with HPS was studied. It has been established before that the patient had a severe deficiency of granule-bound ATP (0.08 μmol/1011 platelets; controls, 2.02 ± 0.20) and granule-bound ADP (0.06 μmol/1011 platelets; controls, 1.74 ± 0.21). Immuno-fluorescence microscopy of permeabilized and nonpermeabilized platelets from this patient revealed surface expression of MRP4 with decreased intracellular staining compared with controls (Figure 4). By immunoblot analysis of whole platelet lysates, MRP4 was also found in HPS platelets, most likely due to the plasma membrane–expressed MRP4 (not shown).
MRP4 transport activity in vesicular fractions from platelets

ATP-dependent transport of [3H]cGMP and [3H]cAMP, established substrates of MRP4,14 was studied as index of MRP4 transport activity (Figure 5). ATP-dependent transport can proceed into the fraction of inside-out–oriented membrane vesicles or into granules representing preformed inside-out vesicles. [3H]cGMP transport was observed in crude membranes as well as in all membranous fractions of the density gradient analyzed. The relative transport activities correlated with the detection of MRP4 (Figure 1B). The absolute rates of net ATP-dependent [3H]cGMP transport amounted to 1.50 ± 0.29 pmol × mg protein⁻¹ × minute⁻¹ and 2.28 ± 0.13 pmol × mg protein⁻¹ × minute⁻¹ (mean values ± SD, n = 3) in the plasma membrane and dense granule fraction, respectively, at a cGMP concentration of 2 μM and 75 μg membrane protein/75 μL incubation volume. ATP-dependent [3H]cAMP transport could also be detected with a transport rate of 0.45 pmol × mg protein⁻¹ × minute⁻¹ at a substrate concentration of 4 μM (light membrane fraction; Figure 5, right panel). The [3H]cGMP transport was further characterized by inhibition studies with several compounds previously shown to affect recombinant MRP4,24,25 including dipyridamole and the leukotriene analog MK571 as well as the nonsteroidal anti-inflammatory drug indomethacin (Figure 6). The median inhibitory concentration (IC₅₀) values calculated from the concentration dependency curves for dipyridamole, indomethacin, and MK571 were 12, 22, and 43 μM, respectively. Dipyridamole also inhibited the ATP-dependent cAMP transport by 42% at 10 μM (Figure 5, right panel). The [3H]cGMP transport was further inhibited by ibuprofen to 64.5 ± 15.4% of the control at 50 μM. In concentrations up to 100 μM, no significant inhibition by serotonin was observed, suggesting that serotonin is not a high-affinity substrate for MRP4. Also mepacrine only slightly affected cGMP transport (69.7 ± 5.6% of control at 100 μM).

[3H]ADP transport in vesicular fractions from platelets

To assess whether ADP could be a substrate for MRP4-mediated ATP-dependent transport, the uptake of [3H]ADP (1 μM) into platelet membrane vesicles (crude membranes) was measured in the presence of 0.4 mM ATP during a time period of 2 minutes. As shown in Figure 7, a time-dependent increase of the vesicle-associated [3H]ADP was observed at a rate of 6.74 ± 1.9 pmol × mg protein⁻¹ × minute⁻¹ (mean value ± SD of 3 different experiments with triplicate determinations). When ATP was replaced by 5’-AMP or the nonhydrolyzable ATP analog AMPPNP, conventionally used in the control incubations to calculate the ATP-dependent component of transport, we observed about a 3- to 4-fold higher background binding of the [3H]ADP to the membranes, which was only slightly increasing with time (not shown). This lower unspecific binding of the [3H]ADP in the presence of ATP compared with 5’-AMP is probably due to the presence of unlabeled ADP, competing with and diluting the [3H]-labeled ADP. Source of this unlabeled ADP could be ADP contamination in the commercially available ATP and the formation of ADP through ATP hydrolysis. To demonstrate ATP dependency and ensure at the same time equal initial ADP concentrations, we measured transport in the presence of ATP with or without 1 mM orthovanadate, an inhibitor of ATP hydrolysis. We observed a time-dependent increase of the vesicle-associated radioactivity in the presence of ATP, despite the fact that the [3H]ADP was diluted by the simultaneous formation of unlabeled ADP. This indicates that there is an active incorporation countering the dilution effect. The

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**Figure 4.** Altered localization of MRP4 in platelets from HPS patients. Platelets adhered to collagen were stained by the anti-MRP4 antibody SNG and a tetramethylrhodamine isothiocyanate–labeled secondary antibody (1:200) as described in “Patients, materials, and methods.” Differences in fluorescence were enhanced using pseudocolor (< 100 magnification; MetaMorph Imaging series 4.6 software, Visi/ron Systems, Puchheim, Germany). Control platelets (A) show membrane staining and 1 to 3 strong staining clusters, most likely resembling dense granules, whereas HPS platelets mostly showed membrane staining only (B). Higher magnification of HPS platelet (C).

**Figure 5.** Transport of cyclic nucleotides in platelet membranes. Platelet membrane vesicles (100 μg protein) were incubated with [3H]cGMP (left panel) or [3H]cAMP (right panel) (4 μM) in the presence of 4 mM ATP (▴) or 4 mM 5’-AMP (●), and the vesicle-associated radioactivity was determined as described in “Patients, materials, and methods” (mean values ± SD, n = 3). The rate of net ATP-dependent transport (▵) was calculated by subtracting transport in the presence of 5’-AMP as a blank from transport in the presence of ATP. Right panel: ATP-dependent transport of cAMP in the presence (●) or absence ( ○) of 10 μM dipyridamole.

**Figure 6.** Inhibition of cGMP transport into platelet membranes. Membrane vesicles were incubated with [3H]cGMP (2 μM) for 10 minutes at 37°C in the presence of dipyridamole (□), indomethacin (●), or MK571 (▲) at the concentrations indicated. Rates of ATP-dependent [3H]GMP transport were determined as described in “Patients, materials, and methods” and calculated as percent inhibition of control [3H]cGMP transport in the presence of the identical concentrations of the solvent used. Data represent mean values ± SD from 3 determinations.
vesicle-associated radioactivity was significantly reduced in the presence of ATP plus orthovanadate, despite the fact that inhibition of ADP formation should enhance binding of the labeled compound, thus indicating that the observed decrease represents inhibition of the active transport process. The dilution effect on the $[^3H]$ADP uptake by the vesicles in the presence and absence of orthovanadate reflects transmembrane movement rather than binding to the membrane surface, the influence of high osmolarity was studied. At a concentration of 1 M sucrose outside the vesicles, the rate of $[^3H]$ADP transport in the absence of orthovanadate was markedly reduced, indicating active transport (Figure 7B). In the presence of orthovanadate, however, 1 M sucrose only slightly affected $[^3H]$ADP association to the vesicles, indicating that the amount of radioactivity measured represents the proportion of $[^3H]$ADP binding to the vesicle membrane independent of transmembranal transport. Furthermore, the vanadate-sensitive ADP accumulation could also be detected in the dense granule fraction of the sucrose gradient and was inhibited by dipyridamole, MK571, and cGMP (Table 1).

**Discussion**

The release of platelet-dense body constituents such as ADP has a fundamental role in hemostasis. The content in platelet-dense bodies is probably established in megakaryocytes, with ADP concentrations exceeding 0.6 M and indicating an active transport. However, very little is known about this process. The presence of plasma membrane proteins such as GPIb in the dense granule membrane suggests that dense granules arise from both endoge-

![Table 1. Inhibition of $[^3H]$ADP transport into platelet dense vesicles](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, µM</th>
<th>$[^3H]$ADP transport, % of control</th>
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<tbody>
<tr>
<td>None (control)</td>
<td>NA</td>
<td>100</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>10</td>
<td>52.7 ± 5.2</td>
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<tr>
<td></td>
<td>100</td>
<td>8.8 ± 0.4</td>
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<tr>
<td>MK571</td>
<td>100</td>
<td>40.7 ± 3.9</td>
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<tr>
<td>cGMP</td>
<td>100</td>
<td>28.7 ± 2.3</td>
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Platelet-dense membrane vesicles (100 µg protein) were incubated with $[^3H]$ADP (1 µM) in the presence of 0.4 mM ATP or 0.4 mM ATP + 1 mM orthovanadate for 2 minutes, and the difference in the vesicle-associated radioactivity was calculated. The compounds listed were added in the given concentrations to incubations with and without orthovanadate. The difference is given as percent of control (mean values ± SD from 3 determinations). The control vanadate-sensitive $[^3H]$ADP transport at 2 minutes in these experiments was 3.0 ± 0.4 pmol/mg protein.$[^3H]$ADP transport in platelet membranes. (A-D) Platelet membrane vesicles (100 µg protein; crude membranes) were incubated with $[^3H]$ADP (1 µM) in the presence of 0.4 mM ATP (■) or 0.4 mM ATP + 1 mM orthovanadate (▲), and the vesicle-associated radioactivity was determined as described in “Patients, materials, and methods” (mean values ± SD, n = 3; 1 pmol/mg protein $^{-1}$ = 1724 DPM). (A-B) Vesicles were preincubated for 45 minutes at 4°C in standard incubation buffer containing 250 mM sucrose (A) or in buffer containing 1 M sucrose (B). (C-D) ADP transport in the absence (C) or presence (D) of 100 µM dipyridamole.

![Figure 8. Possible involvement of MRP4 in platelet transmitter storage and release. Platelets contain the ATP-dependent export pump MRP4 in the membrane of dense (β) granules and in the plasma membrane depending on platelet activation. MRP4 may be involved in the active transport of ADP into the dense granules and in the ADP release from the platelets. MTS indicates microtubular system; DTS, dense tubular system.](image)
and cGMP transport in comparison with that of the other marker proteins indicates an enrichment in dense granules besides the plasma membrane fraction. Furthermore, the inhibition profile of the observed transport (Figure 6) points at MRP4 as a major candidate for mediating this transport. The determined IC_{50} values of 12 μM, 22 μM, and 43 μM for dipiridamole, indomethacin, and MK571, respectively, were slightly higher but in the same order of magnitude than those that have been found to inhibit MRP4-mediated transport in other systems. In membranes from MRP4-containing SF9 cells, 5 μM indomethacin inhibited transport to about 50%.^{12} MRP4-mediated PMEA (9-(2-phosphonylmethoxyethyl)adenine) efflux from intact cells was inhibited by dipiridamole and MK571 with IC_{50} values of 2 μM and 10 μM, respectively.^{12} Amongst the MRPs, MRP4 has a unique broad substrate and inhibitor specificity. The leukotriene antagonist MK571 also effectively inhibits MRP1,^{13} which, however, does not transport cyclic nucleotides. MRP5, a cyclic nucleotide transporter like MRP4,^{12} is inhibited by dipiridamole but is not or only slightly affected by MK571.^{12,14} Thus, the combined inhibition by dipiridamole, MK571, and indomethacin strongly argues for a major contribution of MRP4. In addition, we detected MRP5 only at a low level in platelets (Figure 1A). However, we cannot fully exclude a contribution of other not-yet-identified proteins to the observed transport.

Dipiridamole and, in particular, dipiridamole in combination with low-dose aspirin are very effective in preventing recurrent stroke.^{39} However, the mechanisms underlying this dipiridamole effect have not been fully elucidated. Dipiridamole has been shown to enhance nitric oxide (NO)/cGMP-mediated effects in intact human platelets^{30} as well as to interfere with the ADP-dependent platelet activation.^{41} Both effects could be related to MRP4, assuming a possible effect of this transporter on the cytosolic cGMP concentration^{28} as well as a role in adenosine nucleotide transport. Similarly, inhibition of MRP4-mediated transmitter storage and release may represent a novel molecular mechanism for the action of indomethacin and ibuprofen, besides the known inhibition of cyclooxygenases (COX1 and COX2).

Beside ADP and ATP, serotonin is also a well-established compound of platelet-dense granules. Platelets exhibit a sodium- and chloride-coupled serotonin uptake transporter (5-hydroxytryptamine transporter (5-HTT); SLC6A4) in their plasma membrane.^{42} In addition, a pH gradient–dependent serotonin transporter system has been described in platelet-dense granules,^{43} however, the molecular identity of this transporter has not been elucidated so far. The weak inhibition of the MRP4-mediated cGMP transport by serotonin suggests that serotonin is not a high-affinity substrate for MRP4.

Since ADP is the major signaling molecule concentrated in platelet-dense granules, the question of whether MRP4 represents an ADP transporter is of central interest. The fact that MRP4 mediates the transport of cyclic nucleotides and nucleotide analogs and exhibits a remarkably broad substrate specificity among the MRP4s supports this speculation. However, ADP translocation by ABC transporter is experimentally difficult to demonstrate due to the complex coupling of substrate transport to hydrolysis of ATP at the 2 nucleotide binding domains resulting in ADP formation. However, we could observe a time-dependent increase of the vesicle-associated [^{3}H]ADP in the presence of ATP, which was significantly reduced by the addition of orthovanadate, which inhibits the ATP hydrolysis (Figure 7). This vanadate-sensitive uptake of [^{3}H]ADP was markedly reduced by an increase in osmolality of the extravesicular medium, which is expected to decrease the intravesicular volume without affecting the surface, suggesting that this process reflects transport into an osmotically sensitive space rather than binding to the membrane surface. Furthermore, this uptake was significantly affected by dipiridamole, MK571, and cGMP (Table 1). These data indicate the presence of an ATP-driven ADP transporting system in platelet membrane vesicles and granules and pinpoint MRP4 as a major candidate protein involved in this process.

The novel concept of the involvement of an ABC transporter in platelet transmitter storage and release (Figure 7) remains to be speculative but is strongly supported by our findings. This may have a major impact on understanding the role of dipiridamole and other NSAIDs in primary and secondary prevention of arterial occlusions. Further evidence for the role of MRP4 as a platelet ADP transporter may be provided by ADP transport assays with recombinant MRP4 or by the identification of mutations in the MRP4 gene affecting platelet function.

Acknowledgments

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References


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