Transport of somatostatin and substance P by human P-glycoprotein

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Abstract P-glycoprotein is an efflux pump for a broad spectrum of hydrophobic agents. We found that bioactive peptides including somatostatin and substance P inhibit ATP-dependent vincristine binding to P-glycoprotein-overexpressing K562/ADM membrane vesicles. Some of these bioactive peptides including somatostatin stimulate basal ATPase activity of P-glycoprotein; in contrast, other peptides including substance P inhibit it. The K562/ADM membrane vesicles showed an ATP-dependent, osmotically sensitive uptake of somatostatin and substance P, which was inhibited by valspodar, an inhibitor of P-glycoprotein. These findings suggested that certain bioactive peptides such as somatostatin and substance P directly interact with human P-glycoprotein as endogenous substrates for P-glycoprotein-mediated transport.

Keywords: P-glycoprotein; Bioactive peptide; Adenosine 5′-triphosphatase; Transport

1. Introduction

P-glycoprotein, a 170–180-kDa membrane glycoprotein, is a member of the adenosine 5′-triphosphate (ATP)-binding cassette transporter family. It has been well documented that overexpression of P-glycoprotein mediates one type of multidrug resistance (MDR) in tumor cells [1,2]. This protein is composed of two homologous subunits which contain 6 transmembrane segments and an ATP-binding cassette domain that catalyzes ATP hydrolysis [3–6]. Utilizing the energy produced by the hydrolysis of ATP, P-glycoprotein actively pumps out a broad spectrum of uncharged and positively charged amphipathic agents, such as anthracyclines, epipodophyllotoxins, steroids and short chain phospholipid analogues. P-glycoprotein is present not only in tumor cells but also in various non-tumorous epithelial tissues including adrenals, liver, kidney, intestine, placenta, pregnant uterus and capillary endothelial cells of brain and testis [7]. The tissue distribution pattern of P-glycoprotein and its known function strongly suggest the important physiological roles of P-glycoprotein in the disposition of xenobiotics and drug metabolites by a urinary excretion mechanism in the kidney, a biliary excretion mechanism in the liver absorption barrier, as well as a blood–brain barrier mechanism that limits accumulation of drugs in the brain [8].

In addition to the xenobiotics, various endogenous substrates for P-glycoprotein-mediated transport have been investigated in normal tissues. One of the physiological substrates for P-glycoprotein is steroid hormone in adrenal cortex, gravid uterus and placenta. Several steroids, such as aldosterone, corticosterone and cortisol are transported by P-glycoprotein [9]. Orlowski et al. [10] showed that progesterone and desoxycorticosterone stimulated P-glycoprotein-ATPase activity. The observation that P-glycoprotein in the adrenal gland has ATPase activity like those in the MDR cells [11] suggests another physiological role of P-glycoprotein in secretion of steroid hormones from normal tissues.

Gastrointestine functions as an important organ for endocrinology by secretion of peptide hormones, e.g., somatostatin and substance P that are known as bioactive peptides with diverse physiological functions. Within the gastrointestinal tract, somatostatin works as a potent inhibitor of fluid and electrolyte secretion, primarily by activation of somatostatin receptor [12]. Substance P is involved in the physiological control of several digestive functions, including motility, fluid and electrolyte secretion, blood flow and tissue homeostasis [13,14]. Since P-glycoprotein is found in high concentrations on the epithelial cells in gastrointestinal tracts [15], P-glycoprotein may interact with those peptide hormones as endogenous substrates. In the present study, we report that certain bioactive peptides such as somatostatin and substance P are the substrates for transport by human P-glycoprotein.

2. Materials and methods

2.1. Materials and tumor cells

Bombesin, neuromedin B, neuromedin C, gastrin releasing peptide (GRP, human), substance P, luteinizing hormone-releasing hormone (LH-RH, human), dynorphin A (human), mastoparan, α-mating factor and Leu–Pro–Leu–Arg–Phe-NH_2 were purchased from Peptide Institute Inc. (Osaka, Japan). Somatostatin, creatine phosphate, creatine kinase, ATP and vinblastine were purchased from Sigma (St. Louis, MO). All peptides were dissolved in water and stored at −20 °C. Precast peptide-polyacrylamide gel electrophoresis (peptide-PAGE) gels were from TEFCO (Tokyo, Japan). Anti-somatostatin mouse monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Anti-substance P rat monoclonal

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Abbreviations: ATP, adenosine 5′-triphosphate; MDR, multidrug resistance; GRP, gastrin releasing peptide; LH-RH, luteinizing hormone-releasing hormone

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antibody was obtained from Accurate Chemical & Scientific Corp (Westbury, New York). Horseradish peroxidase-conjugated anti-mouse and anti-rat IgG were purchased from Amersham Pharmacia Biotech (Uppsala, Sweeden). ImmobilonTM Transfer Membranes (0.45 μm pore size) were obtained from Millipore (Tokyo, Japan). [prolyl^{2,4}-3,4(*n*)-^{3}H]Substance P ([^{3}H]substance P, specific activity, 39 Ci/mmol), (3-[^{125}I]iodotyrosyl^{11})-Try^{11}-somatostatin-14 ([^{125}I]somatostatin, specific activity, 2000 Ci/mmol) and [G-^{3}H]vincristine sulfate ([^{3}H]vincristine, specific activity, 6.9 Ci/mmol) were purchased from Amersham (Tokyo, Japan). The purity of the ligand was confirmed by using HPLC or TLC, [^{3}H]substance P, [^{125}I]somatostatin and [^{3}H]vincristine were 98.6%, >90% and 97.6% pure, respectively. Valspodar (PSC 833) was supplied by Novartis Pharma. Ltd. (Basel, Switzerland) and was prepared as 10 mM stock solutions in 100% DMSO.

Doxorubicin-resistant human mylogenous leukemia K562 (K562/ADM) was established as previously described [16]. Cells were grown on 100-mm plastic tissue culture plates (Corning Glass Works, Corning, NY) containing RPMI-1640 medium (Sigma, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic- and antimycotic-solution (GIBCO BRL, Rockville, MD) in an atmosphere containing 5% CO_2 at 37 °C. The K562/ADM cells were maintained in the presence of 0.5 μg/ml doxorubicin. Human epidermoid carcinoma KB3-1 cell line and its transfectant with human wild-type MDR1 cDNA (GSV-2) were generous gifts from I.B. Roninson (Molecular Genetics, University of Illinois, Chicago). The cells were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Rockville, MD) supplemented with 10% heat-inactivated FBS and antibiotic- and antimycotic-solution.

2.2. Preparation of plasma membrane vesicles

A plasma membrane-enriched fraction of the cells was prepared as described previously [17,18]. Briefly, cell suspensions of either K562/ADM or K562 cells were centrifuged and resuspended in homogenate buffer (10 mM Tris–HCl, pH 8.0, 75 mM sucrose, 25 mM $MgCl_2$, 1.5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 150 mM KCl, and 5 mM dithiothreitol (DTT)). The pellets were disrupted by sonication for 30 s (Branson Sonifier model 450), and unbroken cells and nuclei were removed by centrifugation at $1000 \times g$ for 5 min. The supernatants were laid onto a discontinuous sucrose gradient consisting of 16%, 31% and 45% sucrose. Centrifugation was carried out using a SW41Ti rotor (Beckman) for 18 h at $76\,900 \times g$ at 4 °C. The opaque band at the 16/31% interface was collected and diluted with STM buffer (10 mM Tris–HCl, pH 7.5, 250 mM sucrose, 1.5 mM $MgCl_2$, 20 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The fraction was sedimented and the pellets were resuspended in STM buffer. The protein concentration was determined by the Lowry method [19] with bovine serum albumin as standard.

2.3. Preparation of P-glycoprotein-MRK-16–Protein A complexes

P-glycoprotein was immunoprecipitated with anti-P-glycoprotein monoclonal antibody MRK-16 as described previously [4]. The plasma membrane-enriched fractions from the K562/ADM cells were dissolved in solubilizing buffer composed of 50 mM Tris–HCl, pH 7.5, 150 mM NH_4Cl, 2 mM $MgCl_2$ and 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS) and sujected to immunoprecipitation. The membrane lysate (0.75 mg protein) was incubated with 20 μg of MRK-16 for 2 h at 4 °C and then 100 μl of Protein A–Sepharose CL4B suspension (20% by volume in solubilizing buffer) was added to 1 ml of the membrane lysate and left for 1 h. The precipitates were washed five times with the reaction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NH_4Cl, 2 mM $MgCl_2$ and 0.1% CHAPS).

2.4. Substance P and somatostatin transport by P-glycoprotein

Uptake of [^{3}H]substance P or [^{125}I]somatostatin into membrane vesicles was measured by the rapid filtration technique. Briefly, plasma membrane vesicles (approximately 50 μg of protein) prepared from K562, K562/ADM, KB3-1 or GSV-2 cells were added to a 200 μl of the transport assay solution containing 250 mM sucrose, 10 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 5 mM ATP and the radio-labeled peptide at the indicated concentration, which was pre-warmed at 37 °C. After incubation for the indicated time at 37 °C, the vesicles were rapidly filtered through nitrocellulose filters (0.45 μm pore, Schleicher and Schuell, Dassel, FRG) and immediately washed with 5 ml of ice-cold buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.0, and 1 mg/ml albumin). The filters were dried and radioactivity was quantified by liquid-scintillation counting. Non-specific binding of the tested peptides to the filter was determined in the absence of membrane vesicles and non-specific uptake into the vesicles was determined in the absence of ATP.

Initial transport of [^{3}H]substance P or [^{125}I]somatostatin was calculated by incubation with various substrate concentrations; the data were then fitted to Michealis–Menten type equation

$$V = \frac{V_{\max} \cdot [S]}{(K_m + S)}$$

in which V_{max} is the maximal initial transport rate and K_m is the substrate concentration at which the transport rate reaches the half-maximal value.

2.5. Binding assay

The ATP-dependent binding of [^{3}H]vincristine to the membranes of K562/ADM cells was examined in the presence or absence of bioactive peptides. Binding of [^{3}H]vincristine to membranes was measured by the filtration method as described previously [20]. Isolated plasma membranes containing 100 μg of protein was incubated at 25 °C with 250 nCi of [^{3}H]vincristine in 10 mM Tris–HCl (pH 7.4), 250 mM sucrose, 5 mM $MgCl_2$, and 3 mM ATP (buffer A) in a total volume of 100 μl. After 5 min, the reaction was stopped by adding 4 ml of ice-cold buffer A. The plasma membranes were collected by filtration on a membrane filter (Millipore MF-membrane, pore size 0.22 μm) pretreated with 3% bovine serum albumin solution. The samples were then washed with another 4 ml of ice-cold buffer A. By this method, approximately 60% of the membrane proteins were recovered on the filter. The filters were dried and radioactivity on each filter was measured.

2.6. P-glycoprotein ATPase assay

The P-glycoprotein-MRK-16–Protein A complexes were subjected to the ATPase assay. The ATPase activity was estimated by a coupled enzyme assay as described previously [21,22]. The immunocomplexes were dissolved in buffer A (10 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl and 1 mM DTT) supplemented with 5 mM Mg^{2+}/ATP, 0.1 mg/ml pyruvate kinase, 1 mM phosphoenolpyruvate, 0.1 mg/ml lactate dehydrogenase and 1 mM β-nicotinamide adenine dinucleotide (NADH). The absorbance at 340 nm was monitored and the rate of NADH degradation was determined by linear regression. Statistical difference from the basal ATPase activity was analyzed by ANOVA (Bonferroni/Dunn test; StatView ver 4.02).

2.7. Protease protection experiments

Protease protection experiments were performed as described previously [23] with minor modifications. Briefly, either K562/ADM or K562 plasma membrane vesicles (approximately 50 μg of protein) were added to a 200 μl pre-warmed (37 °C) transport assay buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.0 and 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 5 mM ATP) containing 250 μM somatostatin or 400 μM substance P (4 μg or 5.4 μg of total protein per transport reaction, respectively). After incubation for 1 min at 37 °C, the vesicles were washed with PBS (-). The samples were treated for 2 h at 4 °C with 250 units of trypsin in the presence or absence of Triton X-100 (1% w/v) in 10 mM Tris–HCl (pH 8.0). The reaction was terminated by adding a protease inhibitor mixture followed by elution of the proteins using 2 × sample buffer (0.2 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 40% glycerol, 125 mM DTT and 0.04% Coomassie blue G-250) for peptide–PAGE. The peptide separated by 16% peptide-PAGE was transferred electrophoretically to Immobilon sheets and the immunoblot analysis was carried out as described previously [24]. Blots were probed with 100-fold diluted anti-somatostatin mouse monoclonal antibody or anti-substance P rat monoclonal antibody for 1 h followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rat IgG diluted 2000-fold. All bands were visualized by SuperSignal West Femto Trial Kit (Pierce, Rockford, IL) using a Luminescent Image analyzer LAS-1000 (Fujifilm, Tokyo, Japan) and an Image Reader LAS-1000 Pro (version 2.11 for Windows, Fujifilm, Tokyo, Japan).

3. Results

3.1. Inhibition of vincristine binding to K562/ADM membrane vesicles by bioactive peptides

We examined the inhibitory activity of bioactive peptides on the ATP-dependent binding of vincristine to K562/ADM membrane vesicles to elucidate whether these peptides compete for the vincristine binding sites of P-glycoprotein. Table 1 shows that substance P and somatostatin inhibited the vincristine binding to K562/ADM membrane in the presence of ATP with the IC_{50} value of 41 ± 4.1 and 21 ± 1.2 μM, respectively (Table 1, means ± S.E. of three experiments).

We also examined the effects of some peptide hormones and a toxic peptide on vincristine binding. Nine bioactive peptides (bombesin, neuromedin B, neuromedin C, GRP, LH-RH, dynorphin A, mastoparan, α-mating factor and Leu–Pro–Leu–Arg–Phe-NH_2) effectively inhibited the binding of vincristine to K562/ADM membrane vesicles. The IC_{50} values were 3.6–83 μM in the range for nine peptides tested. GRP inhibited the vincristine binding most efficiently and showed the lowest IC_{50} values.

3.2. Effects of physiological bioactive peptides on basal P-glycoprotein ATPase activity

To examine the direct interaction of bioactive peptides with P-glycoprotein, we studied the effect of the peptides on the basal ATPase activity of P-glycoprotein. P-glycoprotein was immunoprecipitated with MRK-16 and the P-glycoprotein-MRK-16–Protein A–Sepharose complexes were subjected to a coupled enzyme ATPase assay. The immunocomplexes demonstrated an ATPase activity of 102 ± 11 nmol/min/mg P-glycoprotein (means ± S.E. of three experiments). To validate the ATPase assay for P-glycoprotein used in this study, we examined the effect of the presence of vinblastine which is known as a substrate for P-glycoprotein-mediated transport [25] and a stimulator of P-glycoprotein-ATPase activity [6]. As demonstrated in Fig. 1A, vinblastine at 40 μM stimulated ATPase of the immunocomplexes by 233%.

Somatostatin stimulated the basal ATPase activity approximately 2-fold with a half maximum level (i.e., SC_{50}) of 22 ± 16 μM (means ± S.E. of three experiments) (Fig. 1A). Other peptides such as bombesin, neuromedin B, neuromedin C, somatostatin, LH-RH and Leu–Pro–Leu–Arg–Phe-NH_2 also

Table 1
Effects of bioactive peptides on the ATP-dependent binding of [^{3}H]vincristine to K562/ADM membrane vesicles

Peptide	Amino acid composition	IC_{50} (μM)
Somatostatin	A-G-C-K-N-**F**-**F**-W-K-T-**F**-T-S-C-NH_2	20.7 ± 1.2
Substance P	R-P-K-P-Q-Q-**F**-**F**-G-L-M-NH_2	41.0 ± 4.1
Bombesin	pE-Q-R-L-G-N-Q-**W**-A-V-G-H-L-M-NH_2	29.9 ± 4.0
Neuromedin B	G-N-L-**W**-A-T-G-H-**F**-M-NH_2	25.8 ± 2.5
Neuromedin C	G-N-H-**W**-A-V-G-H-L-M-NH_2	12.1 ± 1.3
GRP	V-P-L-P-A-G-G-G-T-V-L-T-K-M-**Y**-P-R-G-N-H-**W**-A-V-G-H-L-M-NH_2	3.6 ± 0.5
LH-RH	pE-H-W-S-**Y**-G-L-R-P-G-NH_2	83.0 ± 16.5
Dynorphin A	Y-G-G-**F**-L-R-R-I-R-P-K-L-K-**W**-D-N-Q	47.5 ± 6.2
Mastoparan	I-N-L-K-A-L-A-A-L-A-K-K-I-L-NH_2	10.7 ± 1.7
α-Mating Factor	**W**-H-**W**-L-Q-L-K-P-G-Q-P-M-**Y**	10.1 ± 0.5
Leu–Pro–Leu–Arg–Phe–NH_2	L-P-L-R-**F**-NH_2	32.0 ± 0.4

The IC_{50} value is the concentration of the peptide which inhibites [^{3}H]vincristine binding by 50%. Data are expressed as the means ± S.E. of triplicate experiments. Aromatic amino acids are depicted in bold letters. pE: pyroglutamic acid.

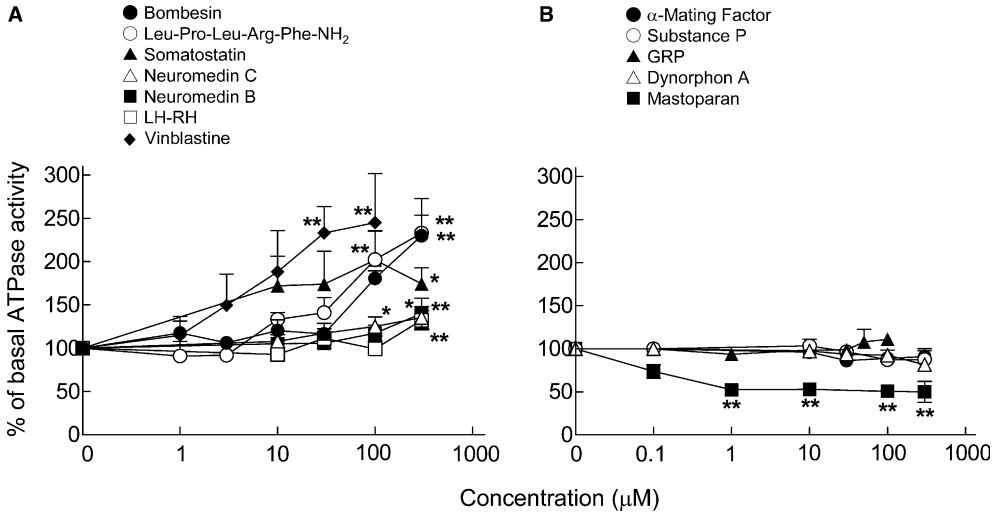

Fig. 1. Effects of physiological bioactive peptides on the basal P-glycoprotein ATPase activity. Panels A and B show ATPase stimulators and inhibitors, respectively. P-glycoprotein in the membrane fraction prepared from K562/ADM cells was immunoprecipitated with MRK-16. The P-glycoprotein-MRK-16–Protein A–Sepharose complexes were subjected to the ATPase assay. The ATPase activity in the presence or absence of bioactive peptides is expressed as a percentage of the basal ATPase activity of three independent membrane preparations (100% = 102 ± 11 nmol/min/mg P-glycoprotein). The concentrations of the bioactive peptides used were 1–300 μM. Each point with bars represents the mean value and S.E. of triplicate experiments. Significant difference from basal ATPase activity was determined by Bonferroni/Dunn test (two tail) (* $P < 0.05$, ** $P < 0.01$).

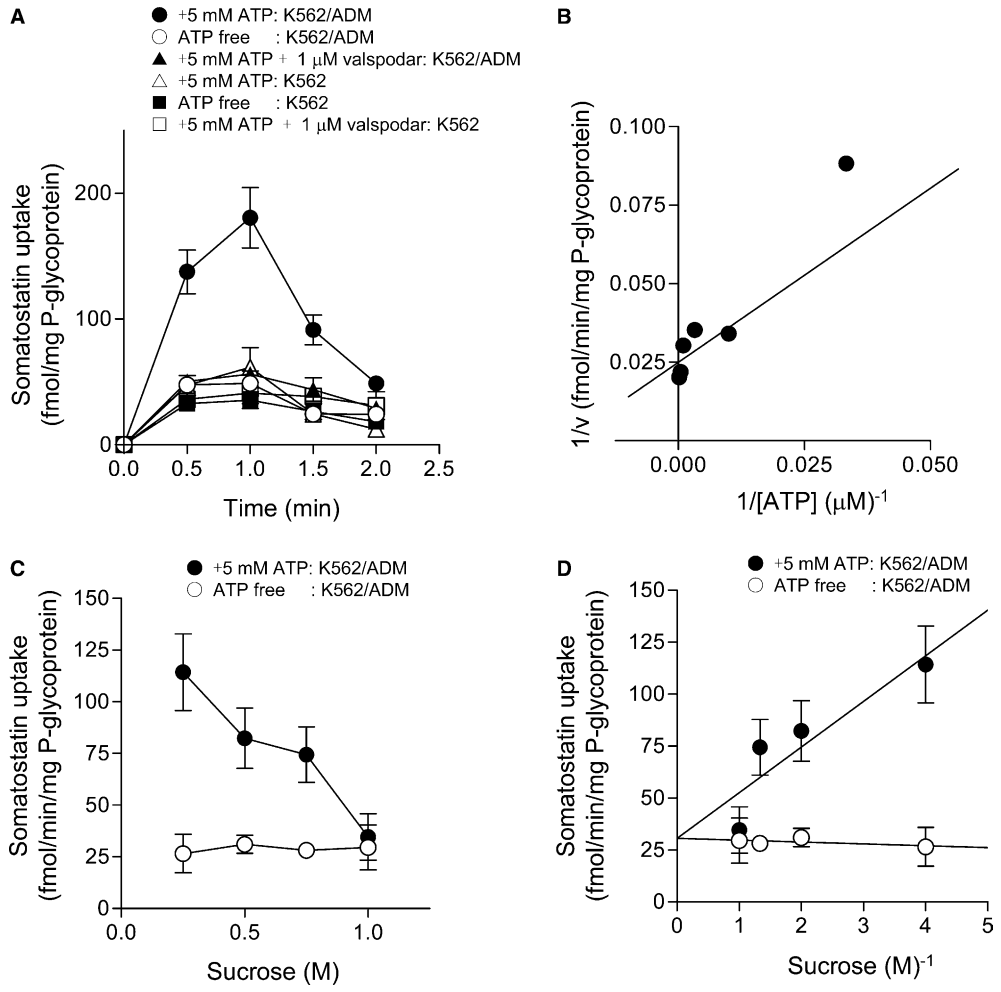

Fig. 2. Somatostatin transport by P-glycoprotein. (A) Time course of ATP-dependent uptake of somatostatin in membrane vesicles prepared from K562/ADM and K562 cells. The uptake experiment was performed with membrane vesicles of K562/ADM and K562 cells in the presence or absence of 5 mM ATP. Membrane vesicles were incubated with radio-labeled somatostatin at a final concentration of 400 pM for the indicated time. Uptake in the presence of 1 μM valspodar was measured. (B) Kinetic parameters of P-glycoprotein-mediated somatostatin uptake. Double reciprocal plot of the uptake of somatostatin in membrane vesicles from K562/ADM cells. Initial transport rates were measured by incubation for 0.5 min. Reciprocal values of the initial transport rates were plotted against 1/ATP concentrations. (C,D) Effect of medium osmolality on somatostatin uptake by membrane vesicles of K562/ADM. Transports of somatostatin was carried out in the transport buffer with different osmolarlty (250 mM to 1 M sucrose) in the presence of ATP. Each point and bar represent the mean value and S.E. of triplicate experiments.

stimulated basal ATPase with SC_{50} values of 22–255 μM. In contrast, substance P slightly inhibited the basal ATPase activity by 13% at 100 μM (Fig. 1B). Mastoparan inhibited the basal ATPase activity in a concentration-dependent manner (IC_{50} value was 1.3 ± 0.3 μM, means ± S.E. of three experiments) (Fig. 1B).

3.3. Somatostatin and substance P transport by P-glycoprotein

We carried out the P-glycoprotein-mediated transport assays of somatostatin and substance P with plasma membrane vesicles containing significant amounts of P-glycoprotein. Time courses of somatostatin and substance P uptake in membrane vesicles were shown in Fig. 2A and 3A, respectively. Vesicle-associated peptides included the peptides that were bound to and/or transported into vesicles. ATP increased the amounts of vesicle-associated somatostatin and substance P in K562/ADM, but not in K562 vesicles, suggesting that these peptides were transported into the vesicles by an energy-dependent, P-glycoprotein-mediated mechanism. The uptake of somatostatin was rapid, the maximal within 1 min being reached. On the other hand, substance P uptake was slow and increasing even after 2 min. In K562/ADM vesicles, ATP-dependent transport was inhibited by 1 μM valspodar, a known MDR reversal agent [26], which further supports the role of P-glycoprotein in the peptide transport. Initial rates of ATP-dependent uptake of both somatostatin and substance P were determined by incubating vesicles with various concentrations of ATP (Fig. 2B and 3B). The uptake values followed simple Michealis–Menten kinetics with apparent K_m and V_{max} values of 49 μM and 44 fmol/min/mg P-glycoprotein for somatostatin and 180 μM and 554 pmol/min/mg P-glycoprotein for substance P, respectively.

To measure the amount of transported peptides in the vesicle-associated ones, we examined the peptide transport in various concentrations of sucrose. As shown in Fig. 2C and 3C, ATP-dependent somatostatin or substance P transport decreased when osmolality increased from 250 mM to 1 M. The values obtained from extrapolation to the infinite high

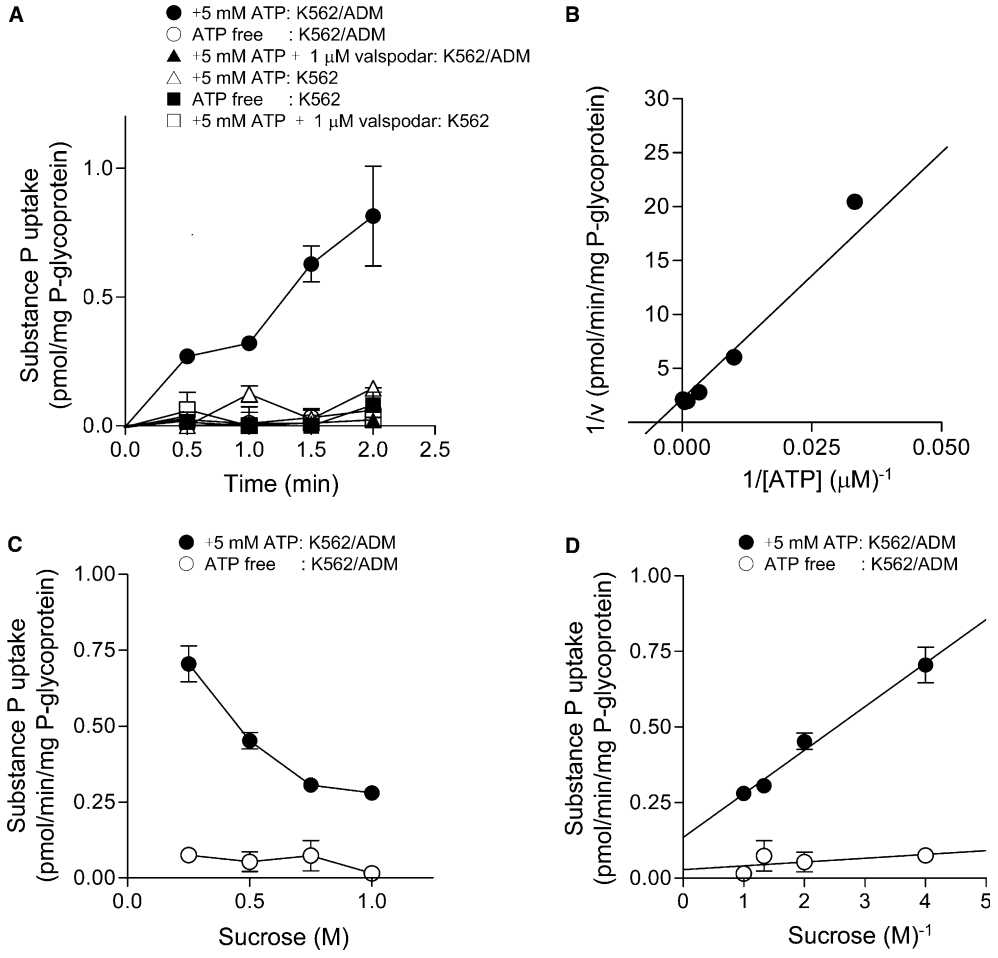

Fig. 3. Substance P transport by P-glycoprotein. (A) Time course of ATP-dependent uptake of substance P in membrane vesicles prepared from K562/ADM and K562 cells. Membrane vesicles were incubated with radio-labeled substance P at a final concentration of 15 nM for the indicated time. (B) Kinetic parameters of P-glycoprotein-mediated substance P uptake. Double reciprocal plot of the uptake of substance P in membrane vesicles from K562/ADM cells. (C,D) Effect of medium osmolality on substance P uptake by membrane vesicles of K562/ADM. Each point and bar represent the mean value and S.E. of triplicate experiments.

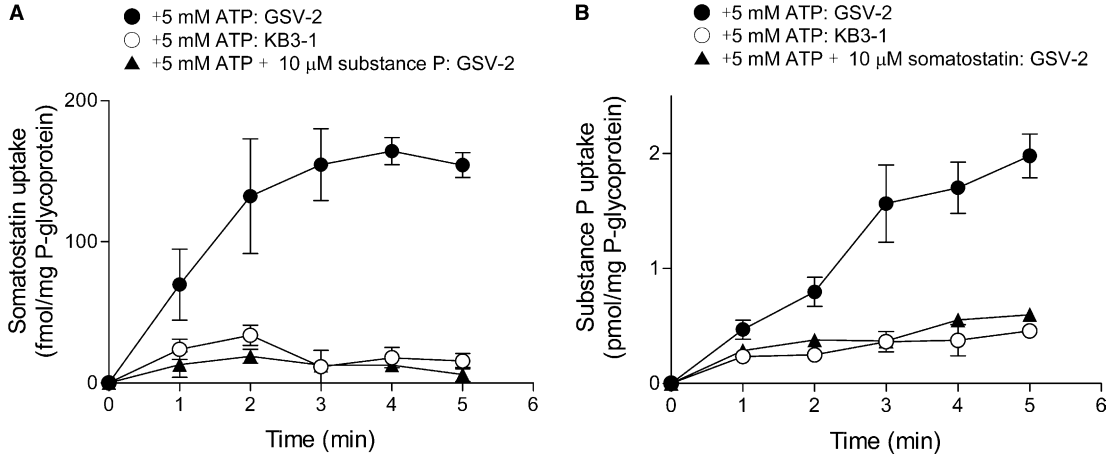

Fig. 4. Somatostatin and substance P uptake in the membrane vesicles from human MDR1 transfected KB3-1 cells (GSV-2 cells). Membrane vesicles from KB3-1 cells and GSV-2 cells were incubated with radio-labeled somatostatin at 400 pM or substance P at 15 nM for the indicated time. The competition of peptide uptake was performed in the presence of excess amount of non-labeled peptide (10 μM). Each point and bar represent the mean value and S.E. of triplicate experiments.

osmolality indicate that approximately 95% of somatostatin or 75% of substance P associated with vesicles represents specific transport into the intravesicular space (Fig. 2D and 3D). In contrast, the amount of the peptides associated with the vesicles showed no response to increased medium osmolality in the absence of ATP. These results indicated that somatostatin and substance P are transported by P-glycoprotein in an energy dependent manner.

To exclude the possible involvement of other transporters for bioactive peptide transport, an uptake experiment was also performed with membrane vesicles prepared from human MDR1-transfected KB3-1 cells (GSV-2 cells) or vector transfected KB3-1 cells. In the presence of ATP, the uptake of somatostatin and substance P increased time-dependently in GSV-2 cells, but not in KB3-1 cells (Fig. 4). In contrast, the peptide uptake in GSV-2 cells was not observed in the absence of ATP (data not shown). The ATP-dependent transport was inhibited by 10 μM non-labeled bioactive peptides. Therefore, the bioactive peptides competitively bound to the same binding site(s) of P-glycoprotein for somatostatin and substance P.

3.4. *Protease protection experiments of somatostatin or substance P transport*

To distinguish between simple binding and transport of the peptides to membrane vesicles, a transport of the peptides was analyzed by protease protection experiments. In the standard condition with an ATP-regenerating system, somatostatin and substance P were detectable in the fraction containing vesicles (Fig. 5A, lanes 1 and 2). When a protease solution was added, a major part of the peptides was not degraded (Fig. 5A, lane 3); however, the majority of peptides was proteolyzed by addition of detergent (Fig. 5A, lane 4). Furthermore, the vesicle associated peptides were not extracted with high salt concentrations (data not shown). The results demonstrated that a significant population of peptides was transported into the lumen of these vesicles. As compared with the standard condition with an ATP-regenerating system (Fig. 5A, lane 3), ATP depletion inhibited the peptide transport into the lumen (Fig. 5B, lane 3). In addition, an ATP-regenerating system combined with 1 μM valspoder also showed inhibition of the peptide transport (Fig. 5B and C, lane 3).

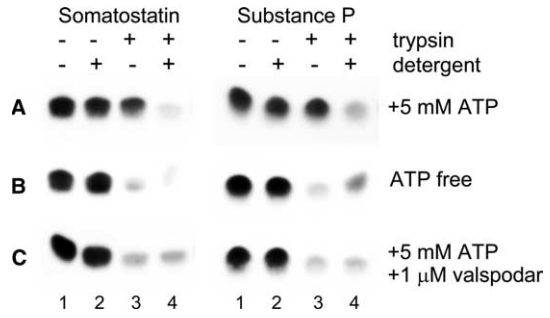

Fig. 5. Uptake of somatostatin and substance P across the plasma membrane of vesicles from K562/ADM. After the uptake of substance P, the vesicles were treated with trypsin in the presence or absence of detergent. Trypsin mediated proteolysis was terminated by adding protease inhibitors followed by the addition of Laemmli sample buffer. Proteins were separated on SDS gels and transferred to Immobilon sheets in order to detect peptides by immunoblot analysis. Lane 1, untreated sample; lane 2, membranes treated with detergent; lane 3, membranes treated with trypsin in the absence of detergent; lane 4, membranes treated with trypsin in the presence of detergent. (A) Incubation in the presence of an ATP-regenerating system. (B) Incubation in the absence of ATP. (C) Incubation in the presence of an ATP-regenerating system and 1 μM valspodar.

4. Discussion

We investigated in the present study whether somatostatin, substance P and certain bioactive peptides are able to interact with P-glycoprotein. First, we showed that somatostatin, substance P and nine bioactive peptides inhibited the ATP-dependent vincristine binding efficiently with IC_{50} values of micromolar concentrations, suggesting that they interact with P-glycoprotein at a vincristine binding locus. Second, these bioactive peptides stimulated or inhibited the basal P-glycoprotein-ATPase activity at almost equal concentration range for the inhibition of vincristine binding. Furthermore, we also showed that somatostatin and substance P were transported into plasma membrane vesicles prepared from K562/ADM cells. The transports of somatostatin and substance P were dependent on ATP and osmotically sensitive, and were inhibited by treatment of the vesicles with valspodar, a known MDR reversal agent. These results clearly indicate that P-glycoprotein transports both somatostatin and substance P.

Several peptide substrates for P-glycoprotein have been reported to modulate its ATPase. Sarkadi et al. [27] showed that the various biologically active hydrophobic peptide derivatives, e.g., proteinase inhibitors, chemoattractants, ionophores, enkephalins and immunosuppressants, stimulated the ATPase activity of P-glycoprotein in the vanadate-induced trap method. In our study, the ATPase assay using P-glycoprotein-MRK-16–Protein A–Sepharose immunocomplexes, the ability of bioactive peptides to modulate P-glycoprotein-ATPase was divided into two groups. While five peptides had inhibitory effects (substance P, GRP, dynorphin A, mastoparan and α-mating factor), other peptides had stimulatory effects (somatostatin, bombesin, neuromedin B, neuromedin C, LH-RH and Leu–Pro–Leu–Arg–Phe-NH_2) on the basal P-glycoprotein-ATPase in a concentration-dependent manner (Fig. 1A and B). Interestingly, while both somatostatin and substance P were transported by P-glycoprotein in an ATP-dependent manner (Fig. 2A and 3A, 5A and B, lane 3), somatostatin stimulated the P-glycoprotein-ATPase and substance P inhibited it. Previously, we demonstrated that the basal ATPase activity was inhibited by cyclosporine A, which is known as a substrate for P-glycoprotein-mediated transport [18,21]. In addition, all peptides used in our study inhibited the ATP-dependent vincristine binding efficiently (Table 1). Therefore, the effects of the peptides on ATPase activity are likely to be independent of their direct interactions with the vincristine binding site(s) of P-glycoprotein or their transports by P-glycoprotein. Recently, how drug transport by P-glycoprotein is coupled to ATP hydrolysis was elucidated [28]. The increase or decrease rate of ATP hydrolysis depends on the distance between the LSGGQ sequence in one nucleotide-binding domain (NBD) and the Walker A sequence in the other NBD. Thus, it was speculated that the substrates that stimulate P-glycoprotein-ATPase may bring the LSGGQ and Walker A sites closer together so that ATP hydrolysis occurs at a faster rate, while an inhibitory substrate, mastoparan, may make both sites farther apart and/or reduce the rate of ATP hydrolysis.

In addition, Oude Elferink and Zadina [29] demonstrated that C-terminal amidation containing aromatic amino acids is a crucial characteristic for peptides to be substrates. In their report, membrane from MDR1-transfected cells transports the synthetic opioid peptide DAMGO (Tyr–D-Ala–Gly–*N*-Methyl–Phe–Gly-ol) and various other bioactive peptides including substance P are good inhibitors of DAMGO transport.

In consistance with their findings, all peptides used in our study (except dynorphin A and α-mating factor) have C-terminal amidation, suggesting that these bioactive peptides also have the characteristics for P-glycoprotein substrate. One remarkable difference in the chemical structure between the inhibitors and the stimulators of P-glycoprotein-ATPase is that the inhibitor such as mastoparan does not carry aromatic amino acids, whereas other peptides do (Table 1). Lipophilic peptides with aromatic amino acids may induce conformational changes of the drug-binding site in P-glycoprotein leading to separation of the two NBDs.

The present findings raise the question whether the transport of bioactive peptides by P-glycoprotein plays any significant physiological role. A signal sequence-dependent protein translocation system is a well known secretory pathway of peptides. Somatostatin-producing cells synthesized prosomatostatin with a signal peptide, which was proteolytically processed to mature somatostatin, and the mature hormone was secreted by the cell [30]. Also, preprotachykinin was processed into substance P by the same secretory pathway [31]. P-glycoprotein is expressed in hormone-secreting organs including the adenohypophysis, adrenal cortex, placental trophoblasts and pancreas [32,33], and supports our hypothesis that an alternative pathway different from the classical secretory pathways is present.

Furthermore, a role of P-glycoprotein in disposition of the opioid peptide DPDPE ([D-penicillamine^{2,5}]enkephalin) from the brain was demonstrated by enhanced sensitivity of Mdr1a(-/-) mice for this peptide [34]. Another interesting hypothesis for P-glycoprotein's physiological role is that P-glycoproteins expressed in the gastrointestinal tracts and kidney are involved in peptide excretion mechanisms which are similar to those in the blood–brain barrier. Although the main pathway of downregulation of these bioactive peptide activities is achieved by hydrolysis with peptidases [35], the disposition of bioactive peptides by P-glycoprotein into the luminal space of intestines may also contribute to the down regulation of their signaling functions. Nevertheless, P-glycoprotein-mediated transport might have a role in the elimination of bioactive peptides that contribute to keep homeostatic balance in normal organs.

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