ATP/Mg\textsuperscript{2+}-dependent Cardiac Transport System for Glutathione S-Conjugates

A STUDY USING RAT HEART SARCOLEMA VESICLES\textsuperscript{*}

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In the present study, the transport of glutathione S-conjugate across rat heart sarclemma has directly been proved to be an ATP-dependent process. Incubation of sarclemma vesicles with S-(2,4-dinitrophenyl)glutathione (DNP-SG) in the presence of ATP resulted in a substantial uptake of DNP-SG into the vesicles; Mg\textsuperscript{2+} was required for ATP-stimulated transport. The rate of glutathione S-conjugate uptake was saturated with respect to ATP and DNP-SG concentrations with apparent \( K_a \) values of 30 \( \mu \text{M} \) for ATP and 20 \( \mu \text{M} \) for DNP-SG. However, other nucleoside triphosphates, viz., GTP, UTP, CTP, and TTP, did not stimulate the transport effectively. The ATP-stimulated DNP-SG uptake was not affected by ouabain, EGTA, or by valinomycin-induced \( K^+ \)-diffusion potential, suggesting that \( Na^+ \), \( K^+ \), and \( Ca^{2+} \)-ATPase activities as well as the membrane potential are not involved in the transport mechanism. ATP could not be replaced by ADP, AMP, or by ATP analogues, adenosine 5'- (\( \beta,\gamma \)-methylene)triphosphate and adenosine 5'- (\( \beta,\gamma \)-iminoo)triphosphate. From these observations, it is proposed that hydrolysis of \( \gamma \)-phosphate of ATP is essential for the transport mechanism.

The transport of DNP-SG by the sarclemma vesicles, on the other hand, was inhibited by several different types of glutathione S-conjugates including 4-hydroxynonenal glutathione S-conjugate and leukotriene C\textsubscript{4}, and not by GSH. The transport system is suggested to have high affinities toward glutathione S-conjugates carrying a long aliphatic carbon chain (\( n \geq 6 \)) and may play an important role in elimination of naturally occurring glutathione S-conjugates, such as leukotriene C\textsubscript{4}.

Glutathione and its related enzymes play physiologically important roles in various metabolic reactions involving protein synthesis, transport, and detoxication (1-6). Glutathione disulfide (GSSG) and glutathione S-conjugates, generated through the reactions of glutathione peroxidase and glutathione S-transferase, are eliminated from the cells, which is one of the important steps in interorgan glutathione turnover and in mercapturic acid formation in the kidney (1, 4, 5). A sufficient capacity for the transport is required not only for the interorgan metabolism but also for the maintenance of low levels of the intracellular concentrations of these compounds, since GSSG inhibits protein synthesis (6) and some glutathione S-conjugates inhibit glutathione reductase and glutathione S-transferase (7-9).

Previous studies using the isolated perfused rat heart showed the existence of a common export system for the elimination of GSSG and glutathione S-conjugates from cardiac cells (9, 10). The transport system is closely linked to the cystolic free ATP/ADP ratio (11), and energy-dependent active transport of GSSG and glutathione S-conjugates across sarcomella is suggested.

To date, however, it has not been elucidated whether ATP is required for the transport in the heart, and the present work has been undertaken to answer this question using sarclemma membrane vesicles prepared from rat hearts. Direct evidence is here presented for an ATP-dependent transport of glutathione S-conjugate, S-(2,4-dinitrophenyl) glutathione (DNP-SG),\textsuperscript{1} across heart sarclemma. Based on the properties of the ATP-dependent transport system, the potential physiologic role of the transport system is discussed in relation to the metabolism of endogenously generated glutathione S-conjugates.

MATERIALS AND METHODS

Biochemicals and Enzymes—GSH, GSSG, ATP, ADP, AMP, GTP, creatine phosphate, creatine kinase, and phenylmethylsulfonyl fluoride (Boehringer Mannheim), 1-chloro-2,4-dinitro-benzene, \( S-(\beta-nitrobenzyl) \)glutathione, \( S-(\beta,\gamma\text{-chloro}) \)glutathione, \( S-(p\text{-chlorophenacyl}) \)glutathione, \( S-(\beta,\gamma\text{-methylene}) \)triphosphate and \( S-(\beta,\gamma\text{-iminoo}) \)triphosphate, adenosine 5'- (\( \beta,\gamma \)-iminoo)triphosphate, adenosine 5'- (\( \beta,\gamma \)-methylene)triphosphate, TTP, UTP, CTP, ouabain, valinomycin, and neuraminidase (Sigma), N-acetylglycaminic acid (Nacalai Tesque Ltd., Kyoto), and leukotrienes C\textsubscript{5}, D\textsubscript{5}, and E\textsubscript{5} (Salford Ultrafine Chemicals and Research Ltd., Manchester) were obtained from the commercial sources indicated. 4-Hydroxynonenal was kindly provided by Prof. H. Esterbauer (Universität Graz, Austria). Glutathione S-conjugate of 4-hydroxynenal was prepared as described previously (9). Anthglutin was a generous gift from Dr. Komai (Sankyo Co. Ltd., Tokyo).

Preparation of \( 3\text{H}\)-Labeled \( S\)-Dinitrophenyl Glutathione (DNP-SG) — Dithiothreitol was first removed from \( [2\text{-}^3\text{H}] \)glycine-labeled GSH sample (Du Pont-New England Nuclear) by extraction with ethyl acetate under acidic condition (10 ml HCl, pH 2.2) according to Ref. 12. \( 3\text{H}\)-Labeled DNP-SG was enzymatically synthesized with the radiolabeled GSH and 1-chloro-2,4-dinitrobenzene (CDNB) using basic isoxymes of glutathione S-transferase prepared from rat liver

The abbreviations used are: DNP-SG, \( (S-(2,4\text{-dinitrophenyl}) \)glutathione; AMP-PCP, adenosine 5'- (\( \beta,\gamma \)-methylene)triphosphate; AMP-PNP, adenosine 5'- (\( \beta,\gamma \)-iminoo)triphosphate; 4-hydroxynonenal, trans-4-hydroxynen-2enal; HPLC, high performance liquid chromatography; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; CDNB, 1-chloro-2,4-dinitrobenzene.

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\textsuperscript{1} The abbreviations used are: DNP-SG, \( (S-(2,4\text{-dinitrophenyl}) \)glutathione; AMP-PCP, adenosine 5'- (\( \beta,\gamma \)-methylene)triphosphate; AMP-PNP, adenosine 5'- (\( \beta,\gamma \)-iminoo)triphosphate; 4-hydroxynonenal, trans-4-hydroxynen-2enal; HPLC, high performance liquid chromatography; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; CDNB, 1-chloro-2,4-dinitrobenzene.
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(9). The incubation medium contained 0.25 \(\mu\)mol of \(^{14}C\)-labeled GSH, 10 nM Tris/HCl buffer (pH 7.4), and 20 \(\mu\)g of the glutathione S-transferase in a final volume of 1 ml. After 2 h of incubation at 37°C, the incubation medium was diluted with 1 ml of ice cold water and then applied onto a QAE-Sephadex column (1 ml bed volume and pre-equilibrated with 0.1 M Tris/HCl (pH 7.4)). The column was washed with 2 ml of 0.1 M Tris/HCl (pH 7.4) to remove the excess of CDNB and glutathione S-transferase and followed by an additional washing with 3 ml of water. The retained DNP-SG was eluted with 0.7 N formic acid. The radioactive yellowish fraction was collected, lyophilized, and stored at −20°C until used. In two preparatory experiments, the product recovery was 89 and 92% as estimated from the initial radioactivity of the \([^{14}C]GSH\) sample.

In high performance liquid chromatography on a reversed phase column (COSMOSIL 5C18, 4.6 x 250 mm, Nacalai Tesque Ltd., Kyoto) using 50% aqueous acetonitrile as solvent, the conjugate gave a sharp single peak for the absorbance at 240 nm as well as for the radioactivity, and the elution time of the peak (3.2 min) was identical with that observed with DNP-SG prepared from nonradiolabeled GSH and CDNB in the same way. Spectrum of the radioactive product was the same as that of DNP-SG, showing an absorbance peak at 340 nm. Concentration of \([^{14}C]DNP-SG\) was estimated using \(c_{\text{DNP}} = 9.6 \text{ nmol}^{-1} \times X^{-1}\).

Preparation of Sarcolemma Vesicles from Rat Heart—Male Wistar rats were anesthetized with pentobarbital (25 mg/kg body weight). Hearts were dissected out and immediately immersed in ice-cold Krebs-Henseleit bicarbonate buffer solution equilibrated with 95% O\(_2\), 5% CO\(_2\). In order to remove blood, the hearts were perfused with the Krebs-Henseleit solution at 37°C for 2 min as described in Ref. 10.

In each experiment, sarcolemma vesicles were prepared from 6 g of heart ventricles according to Kuwayama and Kanazawa (13). They were finally suspended in a small volume (500 \(\mu\)l) of 0.25 M sucrose containing 10 mM Tris/HCl (pH 7.4), frozen in liquid nitrogen, and stored at −70°C until used.

Measurement of Marker Enzyme Activities—The activities of Na\(^+\), K\(^+\)-ATPase, Ca\(^{2+}\)-ATPase, 5'-nucleotidase, and succinate dehydrogenase were measured according to Refs. 14 and 15. \(\gamma\)-Glutamyltransferase activity was measured according to Ref. 16 using \(\gamma\)-glutamyl-3-carboxy-4-nitroanilide and glycylglycine. Protein concentration was determined according to Lowry et al. (17).

Determination of Sialic Acid of Sarcolemma Vesicles—Specific measurement of exposed sialic acid of sarcolemma vesicles (right-side-out) was made by adding neuraminidase from Clostridium perfringens (100 \(\mu\)g of membrane protein) to the vesicles suspended in 0.16 M Tris acetate buffer (pH 5.2). The total amount of sialic acid was determined after treatment with neuraminidase in the presence of 0.1% (v/v) Triton X-100 (18). Liberated sialic acid was determined according to the thiobarbituric acid method (19).

Sarcolemma Vesicles—Sarcolemma vesicles without ATP were resuspended in 0.25 M sucrose and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C overnight. The sample was pelleted by centrifugation, washed with water three times, and postfixed in 1% osmium tetroxide at 4°C for 1 h. The specimens were then stained en bloc with 0.5% uranyl acetate in 0.1 M NaCl, dehydrated in graded ethanol solutions, and embedded in Epon 812. Ultrathin sections were prepared through several levels of the pellets with a glass knife on a Reichert Ultracut microtome and examined with a Hitachi H-7000 electron microscope.

Measurement of DNP-SG Uptake by Sarcolemma Vesicles—Frozen stored membrane vesicles were thawed quickly at 37°C and stored in ice until used. Unless otherwise stated, the standard incubation medium contained sarcolemma vesicles (150 \(\mu\)g of protein), 100 \(\mu\)M \([^{14}C]DNP-SG\), 0.25 mM sucrose, 10 mM Tris/HCl (pH 7.4), 10 mM MgCl\(_2\), 1 mM ATP, 10 mM creatinine phosphate, and 100 \(\mu\)M of creatine kinase in a final volume of 110 \(\mu\)l. The reaction was started by adding the vesicles to the incubation medium. The incubation was carried out at 37°C and, at appropriate times, aliquots of 20 \(\mu\l\) were taken from the incubation medium and immediately diluted to 1 ml with ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, and 10 mM Tris/HCl (pH 7.4)). \([^{14}C]DNP-SG\) incorporated into the vesicles was measured by a rapid filtration technique. The diluted sample was applied to a Millipore filter (GVWP, 0.22-\(\mu\)m pore size) under light suction. The filters were washed with 4 ml of the ice-cold stop solution and then dissolved in scintillation fluid (Clear-sol I, Nacalai Tesque Ltd., Kyoto). Radioactivity on the filter was measured in a liquid scintillation counter.

HPLC analysis of ATP, ADP, and AMP—Aliquots of 10 \(\mu\l\) were withdrawn from the incubation medium at appropriate times and mixed with 90 \(\mu\)l of 0.5 M perchloric acid containing 1 mM EDTA. The samples were kept in ice, neutralized by KHC\(_2\)O, and centrifuged. The supernatant was used for HPLC analysis. HPLC analysis was carried out according to Ref. 20 using a Shimadzu HPLC system LC-6A equipped with a reversed phase column (COSMOSIL 5C18, Naclai Tesque Ltd., 4.6 x 250 mm). Adenine nucleotides were detected photometrically at 259 nm with a Shimadzu absorbance detector, and the identification was made using authentic ATP, ADP, and AMP.

RESULTS

Characterization of Sarcolemma Membrane Vesicles—The sarcolemma vesicles (0.92 ± 0.09 mg of protein (n = 6)) from 6-g heart ventricles exhibited high specific activities of 5'-nucleotidase (155 ± 29.7 \(\mu\)mol h\(^{-1}\) × mg of protein\(^{-1}\), n = 6) and Na\(^+\), K\(^+\)-ATPase (29.4 ± 8.0 \(\mu\)mol h\(^{-1}\) × mg of protein\(^{-1}\), n = 6), which are specific marker enzymes for the plasma membrane. Based on the activity of Na\(^+\), K\(^+\)-ATPase, the purification factor for the sarcolemma was estimated to be 29-fold with respect to the 1900 × g supernatant fraction.

Sarcoplasmic reticulum (SR) ATPase activity in sarcolemma vesicles was 2.9 ± 0.5 \(\mu\)mol h\(^{-1}\) × mg of protein\(^{-1}\) (n = 6). Succinate dehydrogenase activity detected in the sarcolemma vesicle preparation was 3.7 ± 1.0 \(\mu\)mol h\(^{-1}\) (n = 6), 0.56% of the activity observed in the 1900 × g supernatant, indicating a minimal contamination with mitochondrial fragments.

Electron micrograph of the isolated sarcolemma membrane vesicles revealed a variable-sized (0.2 to 1 \(\mu\m\) in diameter) population of membrane vesicles. The orientation of the sarcolemma vesicles was assessed from the accessibility of the membrane-associated sialic acid to neuraminidase. The amounts of sialic acid liberated by the neuraminidase treatment in the absence and presence of 0.1% (v/v) Triton X-100 were 79 ± 6 and 142 ± 11 nmol × mg of protein\(^{-1}\) (n = 4), respectively. From these results, 46% of the total population of the vesicles was estimated to be inside-out.

ATP-stimulated DNP-SG Uptake by Sarcolemma Vesicles—The activity of Mg\(^{2+}\)-dependent ATP phosphohydroladase (Mg\(^{2+}\)-ATPase) in sarcolemma vesicles was 203 ± 10 \(\mu\)mol h\(^{-1}\) × mg of protein\(^{-1}\) (n = 6), 7-fold higher than Na\(^+\), K\(^+\)-ATPase activity, and this is considered to be the direct contribution to ecto-ATPases localized at the outer surface of the plasma membrane (21). Because of the activity, externally added ATP is hydrolyzed by the vesicles very rapidly. As shown in Fig. 1A, when sarcolemma vesicles were incubated with 1 mM ATP, more than 90% of ATP was hydrolyzed within 10 min. To avoid rapid decreases in ATP concentration for the measurement of DNP-SG uptake, creatine phosphate (10 mM) and creatine kinase (100 \(\mu\)g/ml) were added to the incubation medium (standard medium) to regenerate ATP during prolonged incubation periods. Fig. 1B shows time courses of ATP, ADP, and AMP concentrations in the medium when sarcolemma vesicles (150 \(\mu\)g of protein) were incubated with 1 mM ATP in the presence of the ATP-regenerating system. ATP concentration was maintained constant (~1 mM) for about 20 min, and ADP and AMP concentrations remained at low levels. This steady state was followed by a rapid decrease of ATP concentration; ATP was hydrolyzed to ADP and successively to AMP.

Fig. 2 demonstrates the time course of uptake of \(^{14}C\)-labeled DNP-SG by sarcolemma vesicles. When the vesicles were incubated with 100 \(\mu\)M DNP-SG in the standard medium containing 1 mM ATP, 10 mM creatine phosphate, and creatine kinase (100 \(\mu\)g/ml), an increase in DNP-SG uptake into the vesicles was observed, and the uptake rate was almost constant at least up to 15 min. In contrast, the incubation of sarcolemma vesicles without ATP resulted in a decrease in the uptake rate. These results demonstrate that the uptake of...
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DNP-SG by sarcolemma vesicles is stimulated by ATP.

Table I summarizes the effect of ATP, creatinine phosphate/creatine kinase, and Mg$^{2+}$ on the DNP-SG uptake. After 10 min of incubation under the standard conditions (1 mM ATP, 10 mM creatine phosphate, and creatine kinase (100 μg/ml)), the amount of DNP-SG taken up by the vesicles was 0.3 nmol per 1 mg of protein, whereas in the case of incubation without creatine phosphate/creatine kinase, the value was 0.1 nmol per 1 mg of protein, one-third of that mentioned above, which is attributed to the rapid disappearance of ATP from the system as shown in Fig 1A. It is worth noting that removal of either ATP or Mg$^{2+}$ from the incubation medium suppressed the uptake to the background level (Table I). Therefore, Mg$^{2+}$ is required for the ATP-stimulated DNP-SG uptake.

The sarcolemma vesicles showed 0.9 ± 0.1 nmol × min$^{-1}$ × mg of protein$^{-1}$ (n = 4) of γ-glutamyltransferase activity measured with 5 mM L-γ-glutamyl-3-carboxy-4-nitranilide and 100 mM glycylglycine at 37 °C, pH 8.0. To check the decomposition of DNP-SG by γ-glutamyltransferase, DNP-SG was incubated with sarcolemma vesicles for 30 min in the standard medium for DNP-SG uptake measurement, the detectable reaction product was, however, not observed on HPLC analysis. A known inhibitor of γ-glutamyltransferase, anthglutin (22, 23), did not affect the uptake of DNP-SG into the vesicles. It is concluded, therefore, that the accumulation of radioactivity in the vesicles reflects directly the uptake of [3H]DNP-SG by the vesicles, not the decomposed product of DNP-SG through the γ-glutamyltransferase reaction.

**Effect of Osmolarity on DNP-SG Uptake**—To determine whether the uptake of DNP-SG by the vesicles reflects transmembrane movement of the compounds rather than binding at the membrane surface, the effect of medium osmolarity on the amount of the uptake was studied. An increase in osmolarity of the medium would be expected to lower the intravesicular volume without inhibiting the nonspecific binding of DNP-SG to the vesicle surface. When sarcolemma vesicles were incubated with 100 μM DNP-SG in the presence of 1 mM ATP and 30 mM creatine phosphate and creatine kinase for 1 h, the DNP-SG uptake reached the equilibrium amounts corresponding to different osmolarities. As shown in Fig. 3, the amount of DNP-SG taken up by vesicles was markedly

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**TABLE I**

Effect of ATP, creatine phosphate/creatine kinase, and Mg$^{2+}$ on DNP-SG uptake by sarcolemma vesicles

| Condition       | DNP-SG uptake (pmol/mg protein/10 min) |
|-----------------|---------------------------------------|
| Standard*       | 294 ± 25                               |
| Minus           | 95 ± 7                                 |
| ATP             | 21 ± 1                                 |
| M$^{2+}$        | 22 ± 2                                 |

*The standard incubation mixture contained 100 μM [3H]DNP-SG, 250 mM sucrose, 10 mM Tris/HC1, 10 mM MgCl$_2$, 1 mM ATP, 10 mM creatine phosphate, and creatine kinase (100 μg/ml).
affected neither by ouabain nor by EGTA, indicating that the uptake
showed a linear relationship against DNP-SG concentration (Fig. 2). DNP-SG uptake in the absence of ATP was not affected by the treatment (valinomycin/KCl), as shown in Table II, GSH had no inhibitory effect, whereas 100 mM GSSG inhibited DNP-SG transport by 18%.

Effect of GSH, GSSG, Glutathione S-conjugates, and Leukotrienes on DNP-SG Uptake—Inhibition of DNP-SG uptake by different types of glutathione-related compounds was examined. As shown in Table III, GSH had no inhibitory effect, whereas 100 mM GSSG inhibited DNP-SG transport by 18%.

Effect of ATP concentration on ATP-stimulated DNP-SG uptake by sarcolemma vesicles. The vesicles (150 µg of protein) were incubated with 100 µM [3H]DNP-SG in the presence of ATP (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM) in 110 µl of the incubation medium containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, 10 mM MgCl2, 10 mM creatine phosphate, and creatine kinase (100 µg/ml). After 10 min of incubation, DNP-SG incorporated into the vesicles was determined. Data are presented as mean ± S.E., n = 3.

Effect of various nucleotides on DNP-SG uptake by sarcolemma vesicles

Sarcolemma vesicles (150 µg of protein) were incubated at 37°C for 10 min with 100 µM [3H]DNP-SG in 110 µl of the incubation medium containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, and 4 mM MgCl2 in the presence of the indicated nucleotide (4 mM).

Table II

| Nucleotides | DNP-SG uptake (pmol/mg protein/10 min) |
|------------|---------------------------------------|
| None       | 23                                    |
| ATP        | 206                                   |
| AMP-PNP    | 21                                    |
| AMP-PCP    | 23                                    |
| ADP        | 22                                    |
| AMP        | 19                                    |
| CTP        | 32                                    |
| GTP        | 46                                    |
| TTP        | 35                                    |
| UTP        | 33                                    |

ATP was most effective for DNP-SG uptake among the nucleotides studied (Table II). ATP analogues, i.e. AMP-PNP and AMP-PCP, as well as ADP and AMP, were not effective, suggesting that hydrolysis of γ-phosphate is essential for the uptake. Other nucleotide triphosphates, i.e. CTP, GTP, TTP, and UTP, were less effective as compared to ATP.

Effect of EGTA, Ouabain, and Vanadate on ATP-stimulated DNP-SG Uptake—The ATP-stimulated DNP-SG uptake was affected neither by ouabain nor by EGTA, indicating that the transport is independent of Na+,K+- and Ca2+-ATPase activities. On the other hand, the uptake was inhibited by vanadate (VO43-) (Fig. 5); half-maximal inhibition was observed at 30 µM, and 1 mM vanadate inhibited the uptake almost completely.

Effect of Valinomycin-induced K+ Diffusion Potential on DNP-SG Uptake—Involvement of the membrane potential as a driving force for the uptake of DNP-SG by the vesicles was examined. For this purpose, sarcolemma vesicles (150 µg of protein) were incubated for 30 s in the presence of valinomycin (0.1 mg/ml) and 100 mM KCl under the conditions described in Fig. 2. DNP-SG uptake in the absence of ATP was significantly enhanced by the valinomycin-induced K+ diffusion potential (inside-vesicle-positive) from 0.95 ± 0.06 pmol x mg of protein−1 (control) to 1.84 ± 0.09 pmol x mg of protein−1 (valinomycin/KCl), n = 6 (p < 0.01). However, the ATP-dependent uptake was not affected by the treatment and showed 10.5 ± 0.9 and 11.4 ± 1.1 pmol x mg of protein−1 (n = 6) for the incubation in the absence and presence of valinomycin/KCl, respectively. ATP-dependent DNP-SG uptake is considered to be independent of the membrane potential.

Dose-dependent Uptake of DNP-SG by Sarcolemma Vesicles—As shown in Fig. 6, the ATP-dependent uptake exhibited a saturation kinetics with respect to DNP-SG concentration (apparent Km, 20 µM), while the uptake without ATP showed a linear relationship against DNP-SG concentration.

Effect of GSH, GSSG, Glutathione S-conjugates, and Leukotrienes on DNP-SG Uptake—Inhibition of DNP-SG uptake by different types of glutathione-related compounds was examined. As shown in Table III, GSH had no inhibitory effect, whereas 100 µM GSSG inhibited DNP-SG transport by 18%.
Fig. 5. Effect of vanadate on ATP-stimulated DNP-SG uptake by sarcolemma vesicles. The vesicles were incubated with 100 μM [3H]-DNP-SG in the presence of sodium vanadate (Na3PO4) under the conditions as described in Fig. 2. The concentrations of sodium vanadate are indicated in the figure. After 10 min of incubation, radioactivity incorporated into the vesicles was determined as described under "Materials and Methods." The compounds (except leukotrienes) indicated in the table were present in the incubation medium at a concentration of 100 μM. The concentration of leukotrienes C4, D4, and E4 in the medium was 20 μM. Data are presented as mean values of triplicate measurements.

The extent of inhibition increased with the increase in the length of the aliphatic carbon chain. Glutathione S-conjugate of 4-hydroxynonenal (100 μM), which is derived from the reaction of a lipid peroxidation product with GSH, inhibited DNP-SG uptake into the vesicle by 60%. It is noteworthy that leukotriene C4 inhibited the uptake even at a lower concentration (20 μM), whereas leukotrienes D4 and E4, which lack γ-glutamyl moiety, were less effective. Creatine kinase was not inhibited by leukotrienes at this concentration.

DISCUSSION

DNP-SG Transport across Sarcolemma Is an ATP-dependent Process—A common transport system closely linked to the cytosolic energy state for the elimination of GSSG and glutathione S-conjugates in heart had been reported (9-11). In the present study using sarcolemma vesicles prepared from rat heart, it has been demonstrated that the transport of glutathione S-conjugate, i.e. DNP-SG, across sarcolemma is an ATP-dependent process. The ATP-dependent transport of DNP-SG is considered to be a primary active transport process rather than a secondary active transport mediated by the membrane potential or ion gradient across the membrane, as exemplified by the noninhibitory effect on the DNP-SG uptake through the inhibition of Na+-K+-ATPase by ouabain or Ca2+-ATPase by EGTA. Nonetheless, the ATP-dependent DNP-SG uptake was little affected by valinomycin-induced K+-diffusion potential, being different from the transport of DNP-SG across rat liver canalicular membrane reported by Inoue et al. (24).

The transport of DNP-SG across sarcolemma may require hydrolysis of γ-phosphate of ATP, since ADP and AMP, as well as ATP analogues, i.e. AMP-PCP and AMP-PNP, had no effect on DNP-SG uptake (Table II). In addition, the inhibition of transport by vanadate with an IC50 of 30 μM (Fig. 5) suggests the involvement of a specific ATPase and/or phosphorylation of the carrier protein as active intermediate
in the transport mechanism. In this context, the transport system in heart is apparently similar to the system in human erythrocytes (25, 26) and also to a recently reported transport system in rat liver plasma membrane (27). However, the \( K_m \) value of the sarcolemmal DNP-SG uptake for ATP is 30 \( \mu \)M (Fig. 4), which is one order smaller in magnitude than the values reported for DNP-SG transport in human erythrocytes (0.76–1.0 mM, Refs. 25 and 26) as well as in rat liver plasma membrane vesicles (0.32 mM, Ref. 27). On the other hand, if compared with \( K_m \) values of other ATPases, the \( K_m \) value (30 \( \mu \)M) obtained for DNP-SG transport in sarcolemma vesicles is similar to those of Ca\(^{2+}\)-pumping ATPases (16–30 \( \mu \)M, Refs. 28 and 29) and of ATP-dependent multidrug transporter, Pglycoprotein, (38 \( \mu \)M, Ref. 30).

**Physiological Role of ATP-dependent Transport System for Glutathione S-Conjugate in Heart Sarcolemma**—The glutathione conjugate reaction catalyzed by glutathione S-transferase is one of the most important detoxication processes in the body. In some cases, however, biologically active glutathione S-conjugates are also formed in different classes of compounds, as has been emphasized by Igme (31) and Kaufmann (32). The elimination of glutathione S-conjugates from living cells must be playing a significant role in cellular defense mechanisms (33).

In the present study, ATP-dependent transport of DNP-SG across sarcolemma was shown to be inhibited by different types of glutathione S-conjugates (Table III), which implies a broad substrate specificity for this transport system. Inhibition of DNP-SG transport by GSSG was 18% (relatively low), but this supports the previous conclusion that GSSG and glutathione S-conjugates are transported by a common carrier in the heart (9, 10). It is important to note that DNP-SG transport is inhibited by glutathione S-conjugates which bear a long aliphatic carbon chain, including glutathione S-conjugate of 4-hydroxynonenal. As shown previously, 4-hydroxynonenal is rapidly converted to the corresponding glutathione S-conjugate and then eliminated from the heart (9). It is likely that the elimination of the glutathione S-conjugate is mediated by the ATP-dependent transport system studied here.

The inhibition of DNP-SG transport by leukotriene \( \text{C}_4 \) is remarkable (Table III). \( K_i \) for the inhibition has been estimated to be 1.5 \( \mu \)M (34), and a high affinity of the transport system for leukotriene \( \text{C}_4 \) is suggested. Leukotrienes are a family of biologically active conjugated trienes derived from arachidonic acid and mediate a variety of diseases involving inflammatory or hypersensitivity reactions. The peptide leukotrienes, such as leukotriene \( \text{C}_4 \), increase vascular permeability and contract smooth muscles (35, 36). Rat heart contains near-neutral isozymes (\( \text{Yb}_1\text{Yb}_2 \) and \( \text{Yb}_2\text{Yn}_1 \)) of glutathione S-transferase as its major forms (37, 38), and they exhibit leukotriene \( \text{C}_4 \) synthesis activity (38–40). In fact, leukotriene \( \text{C}_4 \) as well as leukotriene \( \text{C}_1 \)-like immunoreactive substances are formed (41, 42) and released from rat heart (43). It is, therefore, of interest and importance to know whether the ATP-dependent transport system is responsible for the translocation of leukotriene \( \text{C}_4 \), which must be an important step in the metabolism of cysteinyl leukotrienes, i.e. leukotrienes \( \text{D}_4 \) and \( \text{E}_4 \), formation.

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