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Reversal of Na^+-Dependent Glycine Transport in Sheep Reticulocyte Membrane Vesicles

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Abstract

Inside-out membrane vesicles have been prepared from sheep reticulocytes. With these vesicles, Na^+-dependent glycine uptake and net accumulation have been demonstrated to occur in reverse, i.e., from extravesicular (normal cytoplasmic) to intravesicular (normal extravesicular) surface. Uptake and accumulation are inhibited by energization of the sodium pump by ATP whereby the Na^+ electrochemical gradient is dissipated. Glycine-dependent Na^+ uptake was also observed, providing evidence that Na^+-dependent glycine influx into these vesicles, equivalent to normal efflux, is characterized by Na^+–glycine co-transport.

Key Words: Reticulocytes; vesicles; transport.

Introduction

Christensen *et al.* (1952) showed that there was a reduction in amino acid accumulation in duck red blood cells when medium containing NaCl was replaced by KCl. Since then, there have been numerous investigations concerning the nature, behavior, and mechanisms underlying Na^+-dependent solute transport systems. Among the solutes transported by Na^+-dependent routes are amino acids, sugars, vitamins, and several amines. It is now clear that Na^+ stimulates solute uptake by affecting the kinetic parameters, i.e., decreasing K_m and/or increasing V_{max}. This stimulation is usually reflected by Na^+–solute co-transport and, for neutral solutes, this is frequently an electrogenic process which responds to the transmembrane potential (for a recent review see Johnstone, 1979).

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The question of symmetry (or asymmetry) of Na^+-dependent solute transport systems has been addressed in several studies including those using lysed and restored pigeon red cells (Vidaver and Shepherd, 1968), intact Ehrlich ascites tumor cells (Christensen and Handlogten, 1968; Oxender and Christensen, 1963; Schafer and Heinz, 1971; Morville *et al.*, 1973; Johnstone, 1975), and rabbit ileum (Hajjar *et al.*, 1970). In the former studies, Vidaver and Shepherd showed that although the system operates in a reversible mode whereby Na^+-driven net glycine flow appears to occur in either direction, the system is kinetically asymmetric.

One of the difficulties inherent in experiments with cells is that it cannot be ascertained that the behavior, particularly efflux kinetics, is not complicated by the intracellular environment. Even in lysed and restored cells, the interior milieu is not completely replaced. Although membrane vesicles offer the advantage of eliminating this problem, until now preparations of distinct sidedness, particularly inside-out, which exhibit organic solute transport, have not been achieved (for a review see Lever, 1980).

In this paper we describe the use of reticulocyte membrane vesicles of either inside-out or right-side-out orientation to study Na^+-dependent glycine transport. Since reticulocytes possess relatively active amino acid transport activities (Benderoff *et al.*, 1978a, b), these vesicles, particularly those which are inside-out, have unique advantages for examining the sidedness of amino acid transport in mammalian cells.

Materials and Methods

Reticulocytes were produced in sheep by phlebotomy as follows: the sheep were initially bled (1 liter per day for 3 consecutive days) and then kept anemic by removing 0.6–0.7 liters twice a week thereafter. Iron stores were maintained by weekly intramuscular injections of iron dextran (250 mg Imferon, Fisons). Blood was collected from the jugular vein into heparinized bottles.

The cells were then centrifuged, washed three times with saline, and reticulocyte-enriched fractions collected by differential centrifugation according to the following modification of the procedure of Murphy (1973): Washed red cells (hematocrit 75–85%) are centrifuged in 50-ml tubes at 5000 rpm for 1 hr at 4°C in a Sorval RC-2B centrifuge using the HB-4 swinging bucket rotor. The top 20–25% of the cells is removed and centrifuged as above in 15-ml tubes. The first and second topmost (~10%) fractions are removed and washed with saline, and the layer of white blood cells is removed. The number of reticulocytes is counted after staining with new methylene blue (Cartwright, 1968).

Inside-out vesicles (IOV[3]) were prepared from the cells according to the method of Steck *et al.* (1970) with slight modifications as follows: Cells were lysed by 1/50 dilution with 5 mM ice-cold PO_4 (Tris form), pH 8.3, stirred 2 min, and centrifuged at 17,000 rpm for 25 min in a Sorval RC-2Bcentrifuge (S-34 rotor). The white pellet was washed twice with lysing solution and the white membrane pellet allowed to vesiculate overnight at 0°C in 25 volumes 0.5 mM PO_4 (Tris form), pH 9.0. The membranes were centrifuged as before, sheared by passage 5–7 times through a 27-gauge needle, and then washed twice with 10 mM MOPS (Tris form), pH 7.4, containing 0.1 mM $MgCl_2$. Vesicles were stored in this buffer for up to 4 days at 0°C. Right-side out vesicles (ROV) were prepared similarly except 0.1 mM $MgSO_4$ was included in the vesiculation medium.

The sidedness of the vesicles was determined by measuring glyceraldehyde phosphate dehydrogenase (GAPD, cytoplasmic surface marker) (Beutler, 1975) and acetylcholinesterase (AchE, extracellular surface marker) (Steck, 1974), in the absence and presence of 0.2% Triton X-100.

The vesicles were equilibrated with the desired solutes either overnight or for 2 days at 0°C and then incubated at 37°C for varying lengths of time as indicated. Assays of [^{14}C]glycine or ^{22}Na transport were carried out by the Millipore filtration technique described by Blostein (1979). Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. ATP was obtained from Boehringer Mannheim, and choline chloride from Syntex. ^{22}Na (carrier-free) and [^{14}C]glycine were obtained from New England Nuclear.

Results

Na$^+$-Dependent Glycine Uptake into Inside-Out Vesicles

In order to test whether Na$^+$-dependent glycine transport in sheep reticulocyte vesicles can occur in the reverse mode whereby uptake is equivalent to normal efflux, we first ascertained that the vesicles are indeed inside-out. This was carried out by specific enzymic assays as described by Beutler (1975) and Steck (1974). Thus, measurements of AChE and GAPD in the absence and presence of Triton X-100 (0.2%) showed that the fraction of vesicles that were inside-out was $\geq$80% (see legends to Figs. 1–3 and Table I).

[3]Abbreviations: IOV, inside-out vesicles; ROV, right-side out vesicles; MOPS, morpholinopropanesulfonic acid; AChE, acetylcholinesterase; GAPD, glyceraldehyde phosphate dehydrogenase.

Table I. Na^+-Coupled Glycine Transport and Sodium Pump Activities of Inside-Out and Right-Side-Out Vesicles[a]

Property tested	Activity[b] (nmol/mg/min)	
	IOV	ROV
Na^+-dependent glycine influx	(9–4) 1.24	(25–3) 0.91 ± 0.05
Glycine-dependent Na^+ influx	(12–8) 2.01 ± 0.39	(25–3) 2.70 ± 0.32
ATP-dependent Na^+ influx	(17–3) 10.9 ± 0.9	(17–3) not detected

[a] Na^+-dependent [^{14}C]glycine influx was measured as described in Fig. 1. For ROV (25–3) two sets of triplicate tubes were incubated for 3 min at 37°C with 36 mM KCl substituting for 36 mM NaCl in the control tubes. The value for IOV (9–4) is calculated from the 2-min measurements in Fig. 1, after subtraction of the control values (Na^+ omitted). ATP-dependent ^{22}Na influx was measured as described in Fig. 3a; triplicate assays without (control) and with 0.5 mM ATP were carried out for 3 min at 37°C. Glycine-dependent ^{22}Na influx was measured under the same conditions used for Na^+-dependent [^{14}C]glycine influx except that ^{22}NaCl was included in the assay medium. Replicate assays without (control) and with 1 mM glycine were carried out for 8 min (IOV) or 3 min (ROV) at 37°C. IOV were not detected in ROV preparations and ROV were not detected in IOV preparations except IOV (9–4) in which 11% of the sealed vesicles were ROV. Values shown are differences (controls subtracted) ± S.E.M. of triplicate assays.

[b] Values shown are from different IOV and ROV preparations identified by the numbers in parentheses; these varied with respect to both percentage of reticulocytes in the original cell suspension and percentage of total membranes which were sealed vesicles.

Figure 1 depicts a typical time course of [^{14}C]glycine uptake into Na^+-free (choline chloride-loaded) vesicles incubated in media without and with 35 mM NaCl. As shown, glycine influx is stimulated by extravesicular Na^+ (normally cytoplasmic Na^+). Addition of ATP decreased Na^+-stimulated uptake. This decrease could be either a direct action of ATP on the Na^+-dependent glycine transport system, or indirect whereby ATP, by virtue of its direct access to the cytoplasmic surface of IOV, energizes the sodium pump. Therefore, we tested the effect of pump inhibition on Na^+-dependent glycine uptake with ATP present. Strophanthidin, rather than ouabain, was used because it is sufficiently lipophilic to permeate the vesicles and bind to the extracellular cardiac glycoside binding site. As shown in Fig. 2, Na^+-dependent glycine uptake was enchanced markedly with strophanthidin added to inhibit the Na^+-pump. This suggests that ATP inhibition is via activation of Na^+-pump activity which, in turn, dissipates the Na chemical gradient (see Fig. 3) and may generate a membrane potential (inside positive) (Hoffman *et al.*, 1979).

In another experiment, ATP-stimulated Na^+-pump activity in reticulocyte vesicles was demonstrated under conditions in which concurrent measurements of ATP-dependent ^{22}Na uptake and unidirectional [^{14}C]glycine efflux from [^{14}C]glycine-loaded vesicles were carried out. As shown, ATP-stimulation of ^{22}Na uptake (Fig. 3A) and [^{14}C]glycine efflux (Fig. 3B) were observed. These results support the notion that a sodium-pump-mediated

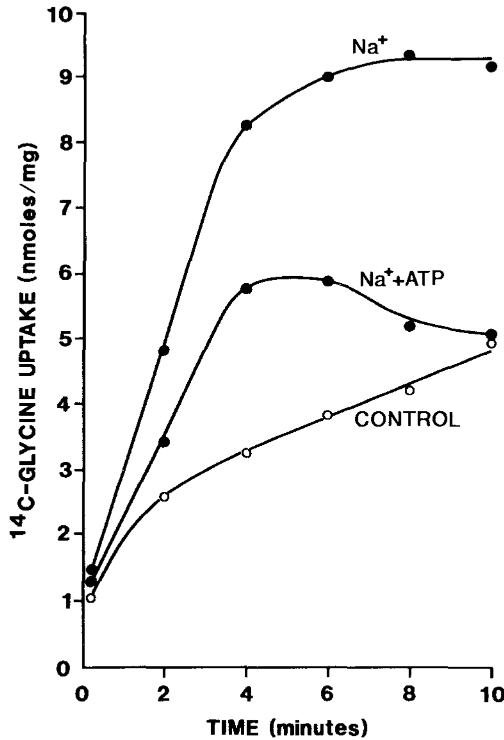

Fig. 1. Effects of Na^+ and ATP on glycine uptake. Vesicles (approx. 0.5 mg protein/ml) derived from reticulocytes (70%) were equilibrated overnight at 0°C, then for 23 min at 37°C with 38 mM choline chloride, 1 mM $MgCl_2$, 2 mM KCl, and 5 mM MOPS (Tris from), pH 7.4. They were then concentrated by centrifugation (10,000 rpm for 10 min at 4°C) to 1.1 mg protein/ml. The reaction was started by adding 660 μl prewarmed (37°C) isoosmotic medium containing 1.1 mM [^{14}C]glycine (1.93 × 10^3 cpm/nmole), 38 mM choline chloride or NaCl as indicated, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM MOPS (Tris form), pH 7.4, without or with 0.55 mM ATP, to 60 μl prewarmed (37°C) vesicles. Final concentrations were 34.5 mM NaCl, 0.5 mM ATP, and 1 mM [^{14}C]glycine. The reaction was terminated at the indicated times and the vesicles (100 μl aliquots) were filtered and washed as described in Materials and Methods using ice-cold solutions of identical ionic composition as the final reaction. Of the total membranes, 54% were inside-out and 6%, right-side-out.

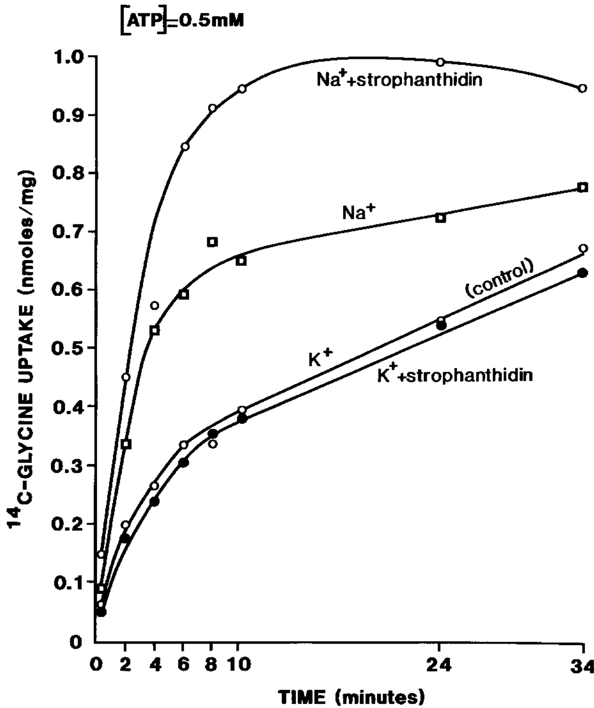

Fig. 2. Effect of strophanthidin on glycine uptake in the presence of Na^+ and ATP. Vesicles derived from reticuloytes (71%) were equilibrated overnight at 0°C, then 15 min at 37°C with 40 mM KCl, 1 mM $MgCl_2$, and 5 mM MOPS (Tris form), pH 7.4, and then concentrated to 1.23 mg protein/ml as described in Fig. 1. Assays were initiated by adding 600 μl isoosmotic Na^+ or K^+ medium to 150-μl vesicles and terminated by removing 80-μl aliquots at the indicated intervals as described in Fig. 1. Final concentrations were 0.1 mM [^{14}C]glycine (1.42 × 10^4 cpm/nmole), 32 mM NaCl or KCl, and 0.5 mM ATP with either 0.02 mM strophanthidin added as a 0.5% ethanolic solution or 0.5% ethanol (control). Of the total membranes, 57% were inside-out and 17%, right-side-out.

increase in intravesicular Na^+ (and/or change in membrane potential) stimulates Na^+-dependent amino acid efflux from these inverted membrane vesicles.

"Reverse" Accumulation of Glycine

Although the foregoing kinetic experiments show that Na^+-dependent glycine transport can operate in reverse, the question remained as to whether the system can perform net accumulation in reverse. Reverse accumulation

was evidenced in the following experiment. Vesicles (86% inside-out) were equilibrated with KCl and 0.1 mM [^{14}C]glycine and then diluted with medium containing [^{14}C]glycine of the same concentration and specific activity. As shown in Fig. 4, transfer to the K$^+$ medium did not result in further glycine uptake whereas imposition of the Na$^+$ gradient effected a substantial (twofold) accumulation of glycine. This was followed by a subsequent loss, to the level observed in the control. As shown, with 0.5 mM ATP present the peak accumulation or "overshoot" is decreased and the final amount of glycine inside the vesicles is somewhat less than in the control. Similar results were obtained with preparations lacking any detectable right-side-out vesicles (ROV).

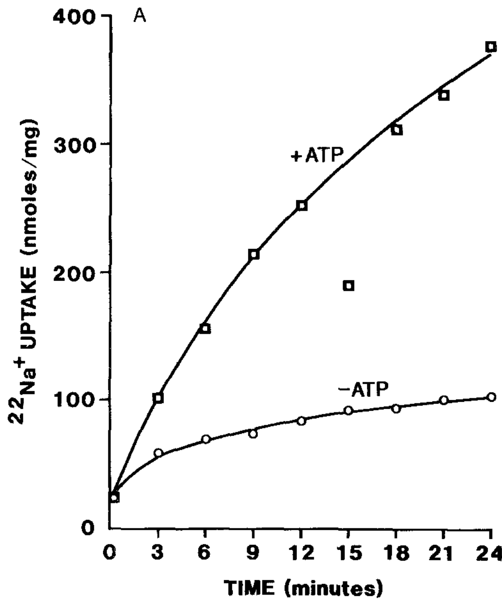

Fig. 3. ATP-dependent $^{22}Na^+$-uptake (A) and [^{14}C]glycine efflux (B). Vesicles derived from reticulocytes (55%) were equilibrated overnight at 0°C, then 2 hr at 37°C in 40 mM KCl, 5 mM MOPS (Tris form), [^{14}C]glycine (1.3×10^3 cpm/nmole, pH 7.4), and then concentrated to 3.85 mg protein/ml. The assays were initiated by adding 450 μl medium containing 5 mM MOPS (Tris form), 1 mM $MgCl_2$, 0.1 mM nonradioactive glycine, and 40 mM ^{22}NaCl (63 cpm/nmole) without or with ATP to 50-μl vesicles. The reaction was terminated (50-μl aliquots) as indicated in Fig. 1. Final concentrations were 36 mM NaCl, 1 mM ATP, and 0.1 mM glycine. Of the total membranes, 46% were inside-out and none were right-side-out. (A) ^{22}Na uptake; (B) [^{14}C]glycine efflux.

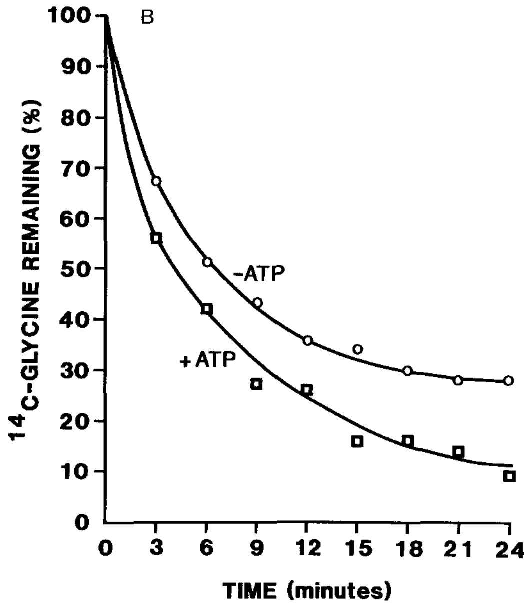

Fig. 3. Continued.

As noted above and shown in Fig. 3, ATP can stimulate glycine efflux indirectly via activation of the sodium pump; this effect of ATP and the fact that these vesicles are inside-out substantiate the conclusion that Na^+-dependent glycine accumulation is occurring "in reverse."

Evidence for Na^+–Glycine Co-Transport in Both Inside-Out and Right-Side-Out Vesicles

Table I summarizes data from transport experiments carried out with right-side-out as well as inside-out vesicles. As might be expected, Na^+-dependent uptake is observed in both types of vesicles, whereas extravesicular ATP increased ^{22}Na uptake in IOV but not in ROV. This is consistent with the idea that the ATP site for Na,K-ATPase is at the outer surface of IOV and at the inner surface of ROV. Because of the relatively large size of these vesicles (5–10 μl intravesicular space per milligram protein, not shown) initial rates of sodium as well as glycine uptake should be amenable to quantitative estimates (c.f. Fig. 3a). The results of experiments to test the effect of glycine on net Na^+ uptake (Table I) indicate that glycine enhances ^{22}Na uptake in IOV and ROV, consistent with the conclusion that Na^+-stimulated glycine transport in *both* directions involves Na^+–glycine co-transport.

Discussion

In this study, we show that the procedure of preparing vesicles of uniform sidedness (inside-out or right-side-out) orientation, originally developed for mature human red cells, can be adapted to sheep reticulocytes. These vesicles have an advantage over intact cells, or even lysed and restored cells, in that there can be no solute compartmentalization, interference from other substrate amino acids, or nonhomogeneity with respect to the effective intracellular solute concentration. It is also clear that these vesicles reflect rather faithfully the transport properties of the intact cell (Table I) with respect to both glycine transport and sodium pump activity. Moreover, reticulocytes, unlike mature mammalian red cells, have amino acid transport activities

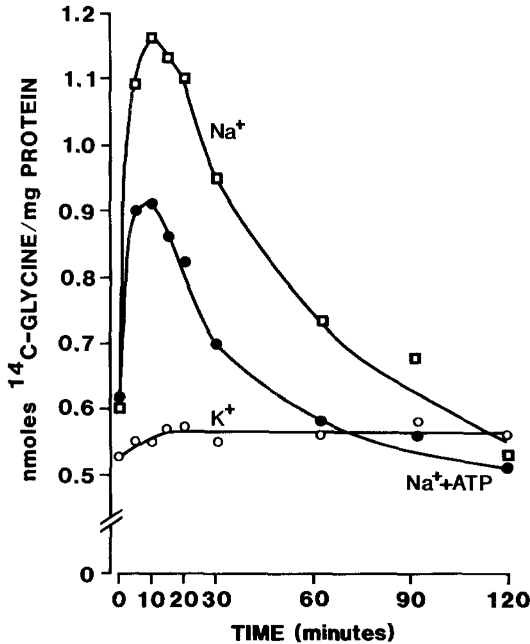

Fig. 4. Na^+-gradient-stimulated glycine accumulation. Vesicles derived from reticulocytes (58%) were equilibrated with 0.1 mM [^{14}C]glycine (2.5×10^4 cpm/nmole) as described in Fig. 3, concentrated to 6.56 mg protein/ml, and then assayed as described in Fig. 3 except that 0.1 mM [^{14}C]glycine of the same specific activity was included in the medium and an additional sample with KCl replacing NaCl in the medium was included as indicated. ATP (1 mM) was present as indicated. Of the total membranes, 50% were inside-out, and 8%, right-side-out.

including both Na^+-independent exchange and Na^+-dependent transport systems. Of the latter type, Na^+-stimulated glycine uptake is particularly active. In intact cells and, to a lesser extent, right-side-out "ghosts," Na^+-stimulated uptake of glycine results in substantial net accumulation (Benderoff *et al.* 1978a). Our results show that in mammalian red cells, as in avian cells (Vidaver, 1964a and 1964b), Na^+-dependent solute (glycine) transport system can not only operate kinetically in the reverse mode whereby *both* Na and glycine are co-transported from cytoplasmic to extracellular surface, but can also perform osmotic work in reverse. Assuming that the inhibition of glycine uptake by ATP is mediated by activation of the sodium pump, only inside-out vesicles should respond. The fact that net glycine accumulation in IOV, equivalent to normal net loss, is inhibited by ATP substantiates the conclusion that accumulation is occurring into the inside-out vesicles and not into the small fraction ($\leq 20\%$) of vesicles that are right-side-out. Moreover, these data indicate that the sodium pump and amino acid transport systems are not segregated in different vesicle populations.

Evidence for coupled flows of Na^+ and solute in *both* directions has been obtained in experiments with intact cells (Curran *et al.,* 1970; Koser and Christensen, 1971). However, adequate precision in the measurement of the coupling ratio has not been possible for fluxes occurring from the cytoplasmic to extracellular medium because of interfering substrate (see, for example, Koser and Christensen, 1971). Although we have not yet examined in detail the coupling ratio of Na influx:glycine influx, the experiments shown in Table I confirm bidirectional co-transport and indicate that IOV and ROV derived from sheep reticulocytes should provide an uncomplicated system for evaluating true coupling ratios.

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