Sodium Ion Uptake into Isolated Plasma Membrane Vesicles: Indirect Effects of Other Ions

Jason R. Lane, Christopher G. Wigham, and Stuart A. Hodson
Laboratory of Ocular Biophysics, Department of Optometry and Vision Sciences, Cardiff University, Cardiff CF1 3XF, United Kingdom

ABSTRACT Vesicles derived from plasma membrane of corneal endothelium were agitated to their minimum size distribution. When isotonic salt solutions surrounding the vesicles were changed there were alterations to the vesicle size distribution: the modal point of the logarithmic distribution did not change but the log variance did, indicating that substantial fission and fusion of vesicles occurred depending upon the nature of the surrounding solute. Orientation and total membrane area was conserved in the transformed population of vesicles. Although the ions added to the external isotonic salt solutions in the present series of experiments have no direct effect upon sodium membrane transporters in these membranes, kinetics of sodium accumulation into the vesicles were affected in a way that correlated with changes to the vesicle size distribution. Early-saturating ($<$1 min) intravesicular concentrations of sodium corresponded with apparently stable populations. Late-saturating ($>$1 min) intravesicular concentrations of sodium corresponded with significant vesicle distribution shifts and included a few seconds of delay. During the linear accumulation phase, both populations showed similar magnitudes of sodium transport. The significance of these data is discussed.

INTRODUCTION

Deconvoluting the specific action of an exchanger-type membrane traffic protein in situ in a cell presents difficulties. One of the prime difficulties arises from the inability to control the ionic gradients across the exchanger-type membrane proteins (in particular, within the cell), in contrast to the ease with which channel-type membrane traffic proteins may be studied in excised patches. Except in special cases such as red cell ghosts and squid axons, it is usually difficult to control the ionic medium inside the cell. Isolating and purifying the plasma membrane and then forming sealed vesicles from the membrane sheets can, however, permit effective control of the ionic gradients across exchanger-type membrane proteins (Hopfer, 1989; Berteloot and Semenza, 1990).

A powerful technique to study exchanger-type activity is to systematically alter ionic gradients across the vesicle membrane and measure the resultant unidirectional ion fluxes (usually with radioisotopes) and, by utilizing Hill plots from flux data derived from candidate co- or anti-transported ions, it is possible in principle to deconvolute multiple activities and to work out the stoichiometries of total exchange processes (Turner, 1990; Boyer and Meier, 1990). Such studies can be augmented with procedures to control transvesicle electrical potentials (with the aid of ionophores), which are particularly informative when the exchange process is electrogenic. Transport inhibition studies with drugs provide additional useful insights into the nature of the exchanger processes, but their use in defining novel traffic protein activity is limited.

One way of noting uptake of ions into vesicles is to measure the whole process at suitable time intervals and to work out the rate constant (or constants) from an analysis of the uptake kinetics. In the current study, a simple relationship between ion uptake into the vesicle over time was not obtained. Added complexities in the uptake process were particularly noticeable when the vesicles were prepared in one defined medium and exposed to a second medium, which is the usual use of vesicles and one of the conditions used to develop Hill plots and estimate stoichiometric ratios for ion coupling studies.

MATERIALS AND METHODS

Preparation of corneal endothelial cell plasma membrane vesicles

Ion uptake into membrane vesicles was based on our earlier published methodology (Wigham et al., 1994; Lane et al., 1997). Bovine eyes were obtained from a local abattoir 2–4 h postmortem. The eyes were used immediately or refrigerated at 4°C for use within 24 h.

To isolate corneal endothelial cells, a small incision was made in the sclera ~3 mm from the limbus. This incision was extended around the globe and the cornea with the scleral rim removed from the rest of the eyeball. The cornea was washed to remove traces of iris pigment and unwanted tissue in 20 ml of 250 mM sorbitol. The endothelial cells were scraped off and transferred into 2 ml of 250 mM sorbitol. The cell suspension was carefully transferred to a 1-ml Potter-Elvehjem homogenizer and homogenized with four strokes of the plunger. This suspension was then centrifuged using self-forming Percoll gradients under isopycnic centrifugation (Hodson and Hodson, 1988) for 30 min at 4°C at 29,000 rpm (80,000 $\times$ g_{max}; 60,000 $\times$ g_{ave}). After centrifugation three distinct bands were visible in the Percoll gradient, previously identified as band 1, the nuclear enriched fraction; band 2, the plasma membrane enriched fraction; and band 3, the mitochondrial enriched fraction (Hodson and Hodson, 1988). The plasma membrane fraction was removed and transferred into a clean centrifuge tube. The plasma membrane fraction was centrifuged at 35,000 rpm (118,000 $\times$ g_{max}; 90,000 $\times$ g_{ave}) for 1 h to sediment out the

Received for publication 28 May 1998 and in final form 30 October 1998.
Address reprint requests to Dr. Stuart A. Hodson, Laboratory of Ocular Biophysics, Department of Optometry and Vision Sciences, Cardiff University, P.O. Box 905, Cardiff CF1 3XF, UK. Tel.: +44 1222 874374; Fax: +44 1222 874859; E-mail: hodsonsa@cardiff.ac.uk.

0006-3495/99/03/1452/05 $2.00

membranes from the Percoll. After removal of Percoll the final pellet containing the membranes was resuspended in a buffered sorbitol solution with a final composition of (in mM) 250 sorbitol, 0.2 $CaSO_4$, 10 $MgSO_4$, 10 Hepes, and 10 Trizma base at pH 7.5. This solution becomes the intravesicular composition at the start of every experiment carried out in this series. Plasma membrane suspensions were then passed rapidly through a 19G hypodermic needle four times.

Na^+ transport in the presence of inward KCl or $LiNO_3$ gradients

The goal of these experiments was to investigate the effects of the exposure of the corneal endothelial plasma membrane vesicles to a change of bathing solution from buffered sorbitol to an isoosmotic salt solutions while examining the kinetics of Na^+ uptake into the vesicles. It had earlier been established (Wigham et al., 1994; Lane et al., 1997) that none of the ions added to the bathing media co-transported with sodium or had any other direct effect upon sodium transporters present in the vesicle membranes. For purposes of illustration we report the results obtained with external KCl or $LiNO_3$ gradients, although combinations of potassium, lithium, choline and chloride, bromide, and nitrate were also investigated.

$^{22}Na^+$ uptake was measured using a rapid filtration technique (Renner et al., 1989; Soleimani and Aronson, 1989; Lynch and McGivan, 1987). To initiate the transport reaction, 10 μl of vesicle suspension and 10 μl of radiolabeled substrate (bathing solution) were mixed in a waterbath. The final composition of the bathing solution was (in mM) 0.2 $CaSO_4$, 10 $MgSO_4$, 0.2 $^{22}Na^+$ acetate (0.0115 Mbq), 125 KCl or $LiNO_3$, 10 Hepes, and 10 Trizma base at pH 7.5. This bathing solution defines the extravesicular composition at the start of the experiment. The reaction was terminated after the appropriate time interval by the addition of an ice-cold "stop" solution containing (in mM) 250 sorbitol, 0.2 $CaSO_4$, 10 $MgSO_4$, 10 Hepes, and 10 Trizma base at pH 7.5. The diluted suspension was quickly filtered through a pre-wetted membrane filter (HAWP 02500, 0.45 m pore size) attached to a Millipore vacuum pump assembly under light suction. The filters were then washed with 3 × 3 ml of ice-cold stop solution, removed, and placed in 10 ml of scintillation fluid (Ecoscint). The activity of the solution was measured in a liquid scintillation counter. The activities were used to calculate uptake per milligram protein. Vesicle protein was measured using the Coomassie blue protein assay reagent, using bovine serum albumin as standard.

All data were corrected for nonspecific trapping of ^{22}Na by the membranes and filters by subtracting the uptake observed at "zero time." Zero time uptake was determined in the presence of radioactive substrate when the vesicle and stop solutions were added simultaneously.

Viability of the vesicle population was determined by Na^+- versus K^+-dependent L-alanine accumulation (Van Amelsvoort, 1978; Sanchis et al., 1994; Lane et al., 1997). Orientation of the vesicles was determined by ouabain inhibition studies. Vesicles were stabilized in the appropriate ionic solution and then were rapidly mixed to added ouabain in ionic solution to achieve a final concentration of 0.1 mM ouabain. The goal of this experiment was to distinguish between outside-out vesicles, where ouabain inactivation of Na^+-K^+-ATPase activity would be rapid, and inside-out vesicles, where ouabain inactivation, requiring penetration into the vesicles, would be slower. After exposures varying from 0 s to 1 h, the vesicles were rapidly washed as above on filters to remove all extraneous ouabain, and then the vesicles were solubilized in 2 ml ice-cold Triton X-100 (2.5%) and assessed for residual Na^+-K^+-ATPase activity in the spectrophotometer using the regenerating system of Horgan et al. (1972) where ATP hydrolyzed by the enzyme is regenerated by coupling to the oxidation of NADH, whose rate of loss was continuously monitored by absorbance at 340 nm. Nonspecific ATPase activity was estimated by the midterm addition of 0.1 mM ouabain to the cuvette in order to inhibit residual Na^+-K^+-ATPase activity.

Determination of vesicle size by photon correlation spectroscopy (PCS)

PCS (Hopfer, 1989) was used to determine the size distribution of the corneal endothelial plasma membrane vesicles produced during the present study using a Coulter N4M particle sizer. Size distributions were carried out at a measurement angle of 90° at 25°C, using an experiment time of 60 s.

Size measurements of vesicles exposed only to sorbitol solutions were taken fresh from the centrifuge precipitate and after every pass (1, 2, 3, or 4) through the hypodermic needle and finally after ultrasonication.

Vesicles prepared with internal 250 mM sorbitol were incubated in 125 mM $LiNO_3$ or 125 mM KCl or 250 mM sorbitol solutions. All solutions were buffered at pH 7.4 with 50 mM Hepes and the particle size distributions were obtained to determine the effect on the vesicle configuration of these solutions.

Reagents and materials

Percoll was obtained from Pharmacia LKB, Uppsala, Sweden; membrane filters from Millipore, UK; ^{22}Na acetate from Du Pont-NEN, Wilmington, DE; Ecoscint from National Diagnostics, U.S.A. All other reagents were obtained from Sigma, Poole, UK.

RESULTS

In all our procedures reported here the viability of the vesicles in either KCl or $LiNO_3$ solutions remained constant because there was noted a consistent and higher uptake of L-alanine in the presence of Na^+ compared to K^+ inward gradient in both preparations, including those exposed to different salt solutions (Lane et al., 1997). The orientation of the vesicles was further assessed by monitoring the fraction of the vesicles oriented outside-out by the availability of the total Na^+-K^+-ATPase to rapid or slow inhibition by added ouabain. The results are shown for vesicles immersed in KCl or $LiNO_3$ solutions (Fig. 1). Both suspensions of vesicles showed that a high and equal fraction of the total Na^+-K^+-ATPase activity was rapidly inhibited by ouabain within a few seconds of exposure, indicating that ~88–90% of the vesicles possessed their usual orientation of outside-out. The remaining 10% of the activity was only slowly inhibited with a half-time of inhibition >30 min, indicating that a small fraction of the vesicles was orientated inside-out.

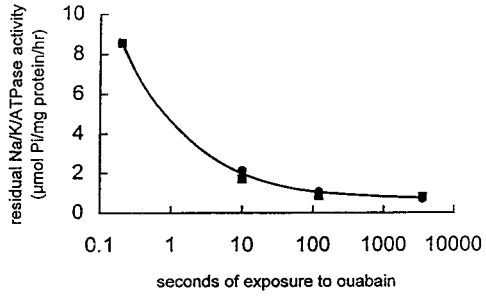

FIGURE 1 Residual total activity of membrane vesicular Na^+-K^+-ATPase after ouabain inhibition for various times (*ordinate*) in vesicles preexposed to buffered 125 mM KCl solutions (*circles*) or buffered 125 mM $LiNO_3$ (*squares*).

Fig. 2 shows the particle size distribution of the plasma membrane fraction after one pass through the end of a hypodermic needle. The vesicle size distribution was found to be heterogeneous, and no difference in the distribution was obtained after four passes through the needle. This suggests that the vesicles are formed into their minimum energy distribution after the first pass through the needle, or even earlier because similar distributions were found after the initial precipitation in the centrifuge. Ultrasonication caused no further changes to the distribution.

When the vesicles were incubated in buffered isotonic KCl there was no apparent change in the vesicle size distribution. However, when the vesicles were exposed to isotonic $LiNO_3$ there was a change in the particle size distribution with a tighter distribution resulting from a smaller log standard deviation (Fig. 3). Exposure to predominantly lithium-containing solutions resulted in the fission of larger vesicles, the fusion of smaller vesicles, and a resulting enhancement of the number of vesicles of modal size. At the same time there was no apparent loss of membrane area nor any significant change in the sidedness of the vesicles that remained predominantly outside-out (Fig. 1).

Fig. 4 represents $^{22}Na^+$ uptake in the presence of either a KCl or $LiNO_3$ inward gradient. Immediately after external sorbitol was substituted by KCl gradient, uptake of $^{22}Na^+$ was linear for 30 s, after which time the signal increased at 60 s and then significantly decreased after 2 min. When sorbitol was substituted by $LiNO_3$, $^{22}Na^+$ uptake showed an initial delay equivalent to a 7-s latent period followed by linear uptake for the next 50 s, after which the signal tended to saturate. During the interval of linear sodium accumulation after both KCl and $LiNO_3$ substitution, there was no significant difference between the rates of sodium uptake (13.3 ± 0.5, KCl exposure, and 13.7 ± 0.6, $LiNO_3$ exposure, in units of pmol Na^+/mg protein · s, respectively, mean ± SE, using five determinations in three preparations)

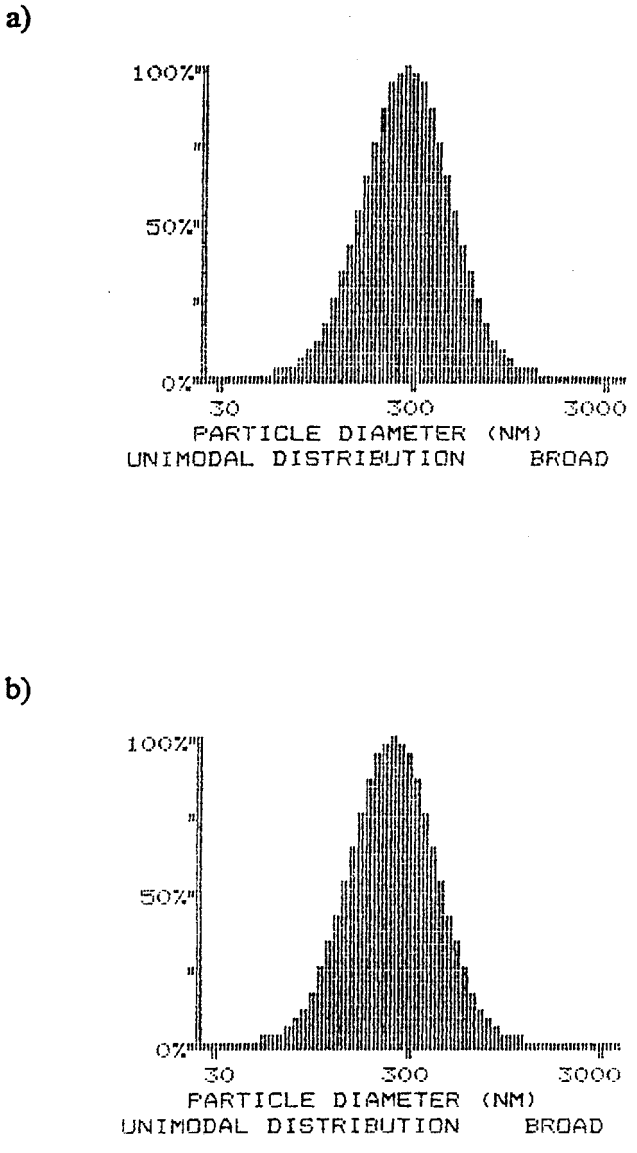

FIGURE 2 Size distribution (direct output reading) of corneal endothelial plasma membrane vesicles obtained by photon correlation spectroscopy after (*a*) one pass through the end of a hypodermic needle and (*b*) after four passes. The vesicles were incubated in buffered 250 mM sorbitol.

DISCUSSION

The vesicle size distributions measured in the current investigation are typical for membrane vesicles derived from bacterial and animal cells and used in other transport studies (Kaback, 1974; Quinlan and Hochstadt, 1976; Lever, 1977, 1980). Under the conditions used in these experiments, sodium enters the vesicles via a sodium hydrogen exchanger (NHE type I) and none of the ionic conditions to which the vesicles were exposed have any effect on the activity of NHE I (Wigham et al., 1994; Lane et al., 1997) other than the sodium concentration and pH, both of which were constant in all trials. Our solution mixing seemed adequate

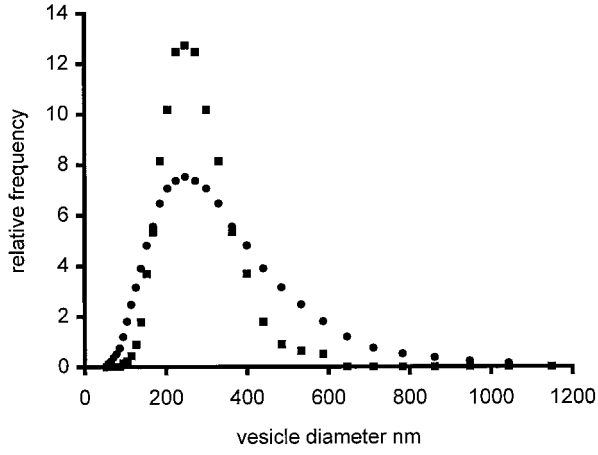

FIGURE 3 Size distribution of corneal endothelial plasma membrane vesicles obtained by photon correlation spectroscopy after initial preparation in buffered 250 mM sorbitol followed by exposure incubation in buffered 125 mM KCl (*circles*) or buffered 125 mM $LiNO_3$ (*squares*). The KCl exposed vesicles indicate an identical population distribution to those shown in Fig. 2.

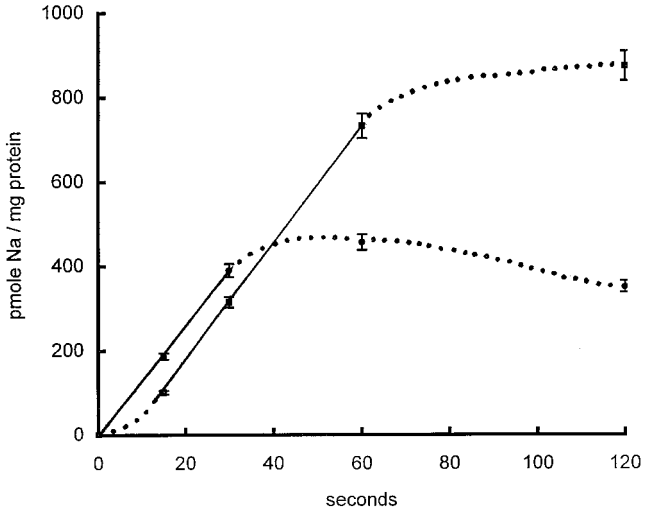

FIGURE 4 Accumulation rate of $^{22}Na^+$ into corneal endothelial plasma membrane vesicles immediately after substitution of external buffered 250 mM sorbitol for buffered 125 mM KCl (*circles*) or buffered 125 mM $LiNO_3$ (*squares*). The results represent the mean ± SE of values derived from means of five determinations from three separate experiments. Where the interpolation is less certain the points are joined by broken lines.

because there was <1 s delay before the transport process was in full action (Fig. 4) if the vesicles did not alter their size distribution.

Our results indicate that an isoosmotic change in the medium surrounding isolated plasma membrane vesicles results in a relatively permanent redistribution of sizes in the population according to PCS measurements. In this respect, PCS is probably a more reliable method for estimating true vesicle size distributions than simple light scattering measurements. In the latter case, light scattering properties may result from changes other than size alone, for example, changes in the refractive index of the interior milieu of the vesicles resulting from (say) ion binding differences between KCl and $LiNO_3$. However, PCS measures the time-dependent fluctuations in the scattering intensity and computes the autocorrelation function ($G\Gamma$, where Γ represents the time interval of the sampling frequency) many hundreds of times to deduce the size distribution of the population from its diffusional mobility derived from the fluctuations in $G\Gamma$. In this way PCS may be used relatively independently of the light scattering properties of individual particles in the population and may be used to estimate size distributions with suspensions of micelles or with suspensions of vesicles. We believe that the different distributions of vesicles after exposure to different solutions shown in Fig. 3 represent true size changes in the vesicles. It was not possible to examine the time course of the changes quantitatively because our sampling time necessary to determine size distribution was 1 min. Qualitative turbidity studies indicated, however, that the redistribution was substantially complete within a few seconds of exposure to the new solution. There was no apparent change in the orientation of the vesicles while they underwent fusion (the smaller size loss) or fission (the larger size loss) seen in Fig. 3. Because the mean size did not alter it seems as if the main change was associated with a narrowing of the tolerance in the packing angles between neighboring phospholipids. How this could be effected by the presence of external ions is not clear, but one possibility could result from a change in the Bjerrum length (Elliott and Hodson, 1998) and associated change in the spatial distribution of the force of repulsion between neighboring charged headgroups. It was interesting to observe (Fig. 4) that fission and fusion did not result in rapid substrate accumulation which would be associated with the vesicles opening and closing, and thereby effecting large leak pathways. Permeability barriers were maintained during fission and fusion. On the contrary, NHE I activity was substantially reduced while the vesicles redistributed (Fig. 4) and this observation raises several interesting possibilities concerning membrane transporter activity and the nature of its dependence on physical stability of the surrounding membrane. Both types of vesicle distributions result in a linear phase of sodium accumulation (Fig. 4) of similar magnitudes, which represents the specific transport activity under these conditions. Other transport activities are simultaneously taking place across the vesicle membrane because these membranes also contain K^+ channels and nonselective anion channels (Rae, 1985). Although electrochemical equilibrium is never established via exchangers (a gradient is an obligatory requirement for activity) channels can result in electrochemical equilibrium. It may be for this reason that the statistically significant ($p < 0.05$) diminution in signal after 2 min compared to 1 min for the preparations exposed to KCl occurs if the larger vesicles in the population break up during filtration if there is no gradient (and consequently osmotic/hydrostatic pressure gradients) during the filtration process which would tend to occur after the vesicles had substantially equilibrated. In support of this suggestion, it is interesting to note that the magnitude of the peaks of the two distributions (Fig. 3) corresponds with the maximal signals in the two preparations (Fig. 4).

Besides raising a number of interesting possibilities concerning the relationship between vesicle size and ionic environment, and the activity of transport membrane proteins in destabilized membranes, our study in part justifies the empirical criterion of uptake in the first 10–20 s of exposure as reflecting transport activity used by other researchers when carrying out kinetic studies of this kind (Kinne et al., 1975; Turner et al., 1986; Grassl, 1996; Vayro and Simmons, 1996). Yet other researchers, using the rapid filtration technique, have reported that it is possible to manually obtain the first samples after a 5-s incubation time (Murer and Kinne, 1980; Sanchis et al., 1994) and by using a semiautomatic apparatus time points of 1–2 s have been reported (Kessler et al., 1978; Aronson et al., 1983). Shorter uptake studies should, we suggest, be used with caution if vesicle population size changes are occurring.

This work was supported by the Biosciences and Biotechnology Science Research Council, UK.

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