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Uptake of Reconstituted Na,K-ATPase Vesicles by Isolated Lymphocytes Measured by FACS, Confocal Microscopy and Spectrofluorometry

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ABSTRACT

Na,K-ATPase (EC 3.6.1.37, Na,K-ATPase) is a fundamental vital membrane transport and receptor system which, after biosynthesis, is exported to the plasma membrane in inside-out vesicles. Na,K-ATPase can be extracted form the natural membrane and inserted into artificially formed phosphatidylcholine vesicles (liposomes). The ultrastructure of the reconstituted vesicles has been fully described. In the present work, the Na,K-ATPase-vesicles were labeled with fluorescent tracers either in their water or membrane phase, incubated with freshly isolated human lymphocytes, and the resulting cellular fluorescence measured with fluorescence activated cell sorting (FACS), confocal microscopy and spectrofluorometry. The FACS data show that all lymphocytes take up Na,K-ATPase-vesicles in a dose- and temperature-dependent fashion. Three-dimensional analysis of the fluorescence by confocal microscopy reveals that the fluorescence is contained within the cells. Quantitative determination by spectrofluorometry indicates that depending on the

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vesicle/cell ratio, a single lymphocyte takes up 650 to 36,500 vesicles within 30 min at 37°C together with up to about 200,000 renal Na,K-ATPase molecules.

Index Entries: Reconstituted Na,K-ATPase-vesicles; lymphocytes; uptake; FACS; confocal microscopy; spectrofluorometry; quantitative analysis.

INTRODUCTION

The cytoplasm of eukaryotic cells is rich in vesicles of 20–100 nm diameter. They serve as shuttles for neurotransmitters of hormones or they deliver or retrieve from the plasma membrane the integral proteins *(1,2)*. The Na,K-ATPase (Na,K-ATPase) or sodium pump is a fundamental membrane system that is responsible for the electrolyte balance of the organism, organs, cells, and organelles (*3*); it expels Na from the cell and replaces it by K by an electrogenic process (3 Na out vs 2 K in) coupled to hydrolysis of 1 ATP molecule. The process is modulated by an inhibitory extracellular receptor for cardioactive steroids such as ouabain and their presumed endogenous analogs *(4)*. The resulting transmembrane ion gradient and membrane potential is underlying cell excitability, impulse conduction *(5)*, and muscle contraction *(6)* in nerve and muscle. In epithelia, the polarized localization of the Na,K-ATPase leads to transepithelial Na transport and is the motor of Na reabsorption, e.g., in the kidney.

Vesicles similar to the ones found within cells can be formed artificially and integral membrane proteins, e.g., Na,K-ATPase, inserted into their membranes *(7)*. Their ultrastructure has revealed resemblance in size and aspect *(8,9)* to natural intracellular vesicles as both types are reaching apparently the same minimal energy state because of their like chemical composition *(10)*. The artificially formed Na,K-ATPase vesicles carry 50% of the reconstituted Na,K-ATPase in inside-out orientation and are, therefore, partially analogous to the natural intracellular inside-out vesicles delivering the Na,K-ATPase to the plasma membrane. Therefore, it should be possible to let them enter into the cell where they are predicted to join the normal intracellular vesicle traffic.

Lymphocytes take up externally added phospholipid vesicles or liposomes *(11–13)*. Liposome uptake involves binding to the cell surface followed by endocytosis *(14)*. Recently it was shown that they are able also to take up nanospheres without apparent toxicity

(15). However, it has not been examined whether isolated human lymphocytes take up vesicles containing foreign membrane proteins.

In the present work Na,K-ATPase-vesicles are labeled by fluorescent markers of the membrane or of the water phase and their uptake by isolated human lymphocytes is demonstrated by three different techniques. FACS analysis shows vesicle uptake by all cells; confocal microscopy visualizes the vesicle entry on a single cell level and spectrofluorometry reveals that the cells are able to take up about three times their own amount of membrane surface and about 10 times their own amount of Na,K-ATPase protein within 30 min at 37°C.

METHODS

Materials, Isolation of Na,K-ATPase, and Biochemical Measurements

Phosphate buffered saline (PBS), RPMI-1640 medium, and Hank's balanced salt solution (HBSS) were from Tecnomara (Zürich, CH). Phosphatidylcholine (grade II) was from Lipid products (Nutfield, UK); CF was purchased from Fluka (Buchs, CH). N-(5-fluoresceinthiocarbamoyl)1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethyl ammonium salt (Fluo-PE) was from Molecular Probes (Eugene, OR). LeucoSep tubes (Esquire Chemie AG, Zürich, CH) were used for the isolation of peripheral blood mononuclear cells. Na,K-ATPase was purified from the outer medulla of rabbit kidneys to a specific activity of 1200 to 1800 μmol Pi/mg protein/h by treatment with SDS and centrifugation across a sucrose gradient *(16)*. The Na,K-ATPase activity was measured by the linked-enzyme assay *(17)* and the protein was determined as described by Smith et al. *(18)*. The Na,K-ATPase was separated into α and β subunits by PAGE, which together make up about 90% if the protein as estimated by Coomassie blue staining and laser densitometry *(17)*.

Insertion of Na,K-ATPase into Vesicles

Vesicles are formed by making use of the fact that phosphatidylcholine (PC), dissolved in a 1% cholate solution, forms closed vesicles upon detergent removal by dialysis *(8)*. Membrane fragments containing the purified Na,K-ATPase are treated with 1% cholate to disrupt the continuity of the lipid bilayer and single Na,K-ATPase molecules obtained in the supernatant of a 100,000*g* centrifugation ("soluble" Na,K-ATPase). When the "soluble" Na,K-

ATPase is added to the PC solution and the cholate removed by dialysis, Na,K-ATPase spontaneously inserts into the lipid bilayer and is seen by freeze fracture electron microscopy at regular distances *(8,9)* in the vesicles indicating noninteraction between the Na,K-ATPase molecules. The number of reconstituted Na,K-ATPase molecules is directly proportional to the protein/lipid ratio used for reconstitution and statistical analysis of the intramembrane particle distribution on concave and convex fracture faces has revealed random orientation *(9)*.

A lipid-cholate stock solution was prepared as follows: 60 mg PC in 600 µL chloroform-methanol was dried under a slow, steady stream of ultrapure N_2 in a 50-mL round-bottomed flask fixed to a Büchi minirotary apparatus in a water bath at 25°C for about 30 min in the dark until a thin film was formed to which 3 mL dried ether was added; this process was repeated. The third film was formed first without N_2 blow to avoid solvent capping and was then extensively dried by N_2 for 90 min at 25°C, followed by 2 min at 30°C. Then, 3 mL of a solution containing 50 m*M* NaCl, 50 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 30 m*M* L-histidine, and 1% Na-cholate, pH 7.2, (solution A) was added to the lipid film and the flask rotated until a clear solution was obtained from which 100 µL aliquots were put under nitrogen and stored at –70°C. Na,K-ATPase (600 µg protein) was pelleted at 100,000*g* for 15 min to in a Beckman airfuge at 0°C, resuspended in 100 µL of solution A and again centrifuged for 15 min at 100,000*g*; the Na,K-ATPase activity and protein content of the supernatant containing the "soluble" Na,K-ATPase were determined and the remaining 80 µL Na,K-ATPase solution added to 80 µL of PC solution; the Na,K-ATPase activity after PC addition was determined, the PC-Na,K-ATPase solution added to EDTA treated sterile dialysis tubing, and the cholate removed during 15 h at 0°C in 100 mL cholate-free solution A (solution B).

Fluorescent Labeling of the Vesicles

The vesicles were labeled in two manners: in the water phase by carboxyfluorescein (CF) according to published procedures *(19,20)* or in the membrane phase by Fluorescein-phosphatidylethanolamine (Fluo-PE). For CF-labeling 200 m*M* CF was added prior to dialysis. The pH was adjusted to 7.2 by 1 *M* NaOH, which brought 330 m*M* additional Na ions to the solution. The dialysis temperature was raised to 20°C to prevent crystalization; to eliminate the exter-

nal CF after dialysis, the liposomes were centrifuged four times for 60 min at 100,000*g* in a Beckman airfuge at 0°C and resuspended in 200 µL solution B. The tightness of the liposomes was controled by incubating them in the presence and absence of detergent and determining the entrapped and released CF by spectrofluorometry taking advantage of the CF selfquenching *(20,21)*. The entrapped fluorescence measured after detergent-lysis was found to be directly proportional to the liposome concentration in confirmation that there was no contaminating external dye. The quenching of the washed CF-liposomes only slightly diminished within 30 d at 5°C indicating negligible leak of CF. No CF-release was seen when the temperatures was increased from 0°C to 22°C or from 0°C to 37°C for 30 min. Thus, the CF-liposome stability was appropriate for incubation with cells. The ultrastructure of the CF-vesicles showed that the high CF and Na concentration induced multilayering, yielding a 330 nm average diameter *(10)*. Therefore, a membrane label was used: (i) to avoid multilayering; and (ii) to be able to follow the uptake of the vesicle membranes. For this purpose, 20% PC were replaced by Fluo-PE to form the lipid-cholate stock solution described above. Fluo-PE did not alter the ultrastructure of the vesicles *(10)*. Fluorescent vesicles without Na,K-ATPase protein were also prepared.

Isolation of Lymphocytes and Incubation with Fluorescent Vesicles

Human blood was obtained from healthy voluntary blood donors. Peripheral blood mononuclear cells were isolated by centrifugation on a Ficoll gradient *(22)* in LeucoSep tubes, washed three times in HBSS, and kept in RPMI-1640 medium in the absence of serum. This procedure commonly yielded 90–95% lymphocytes and 5–10% monocytes as measured by flow cytometry. Cells were counted in a haemacytometer and viability was assessed before and after vesicle addition by Trypan blue exclusion. Increasing concentrations (0.5 to 200 µL) of vesicles were pelleted by centrifugation at 100,000*g* in the Beckman Airfuge at 0°C, 0.5 to 2 million peripheral blood mononuclear cells were added in 50 to 200 µL RPMI-1640 or PBS, and the suspension was incubated for 5 min to 2 h at 37°C; 1 mL PBS was added, the cells pelleted for 10 min at 400*g* at 20°C in a Sorvall H-2080 swing out rotor in a Sorvall Centrifuge RC-3B to remove the external liposomes and resuspended in 1 mL PBS. After incubation, the suspension was centrifuged at 400*g* for 10 min at

room temperature to remove the external liposomes and the pellet suspended in 1 mL PBS. Aliquots of the cell suspension were used:

1. For analysis of cellular fluorescence by flow cytometry.
2. For quantification of the intracellular Fluo-PE content by spectrofluorometry.
3. For measuring cell viability by Trypan blue exclusion.

Fluorescent Techniques for Measuring Vesicle Uptake

Cell pellets were suspended in 1 mL of PBS and analyzed in a Becton Dickinson FACScan (San Jose, CA). The lymphocyte and monocyte populations were differentiated by appropriate gating, excluding clusters and cell debris; 10,000 events from each cell population were analyzed. Mean cellular fluorescence and standard deviation were calculated for each sample and expressed in arbitrary fluorescence units, using the program Lysis II (Becton Dickinson). To determine the number of liposomes incorporated per cell, 100 µL cell suspension as well as the supernatants recovered after the 400*g* centrifugation step were added to 3 mL PBS in a quartz cuvet and the fluorescence measured in a Zeiss PMQ II (λ_{ex} 436 nm, λ_{em} 520 nm); 10 µL Triton X-100 (10%) was added after the first reading to release the Fluo-PE taken up by the cell. The quantity of Fluo-PE was determined by comparison with standard curves established with pure Fluo-PE. The number of vesicles was calculated on the basis of their size and composition and amounted to 3.58×10^4 60 nm vesicles (Na,K-ATPase-free) or 1.22×10^4 100 nm vesicles (with Na,K-ATPase) per mg Fluo-PE. For confocal microscopy, the cells were suspended in 10 µL fixing solution, mounted as described by Lenette *(21)*, put on a slide, and covered. Specimens were observed with a Zeiss confocal laser scan fluorescence inverted microscope (LSM 410, Carl Zeiss, Oberkochen, Germany) equipped with two different lasers: a Helium-Neon (He-Ne) laser (excitation wavelength at 543 nm) and an Argon laser (excitation wavelength at 488 nm). The argon laser was used to excite fluorescein and the emission spectra was detected with a photomultiplier preceded with a 510–525 nm narrow-band barrier filters; resulting from the He-Ne laser, the transmission image of the specimen was obtained on a second detector. Both lasers were used separately to define for each of them the value of the optical attenuating filters. Specimens were observed through an oil plan-neofluar ×63/1.4 objective with a zoom factor increasing the magnification 4 or 5 times. Optical sections were taken

at 1 μm intervals. The best level of section in the vertical axis was chosen through this optical sectioning. Images of 512 x 512 pixels were stored on an erasable optical disk (Sony Corporation, Tokyo, Japan) and then photographed on a Kodak Ektachrome Panther color slide film (100 Aza, Eastman Kodak, Rochester, NY) with a freeze-frame digital camera (Focus graphics, Geneva, CH).

RESULTS AND DISCUSSION

Vesicles Enter Lymphocytes in a Dose-Dependent Fashion

FACS is a convenient technique to assess the uptake of fluorescent particles by cells *(15)*. By appropriate window setting of the flow cytometer, the lymphocytes can be seen separately in three-dimensional (Fig. 1A) or two-dimensional (Fig. 1B,C) representation. When lymphocytes had been incubated with increasing concentrations of CF-vesicles (Fig. 1D) or Fluo-PE-vesicles (Fig. 1E,F) the lymphocyte peak shifts progressively to the right on the Log scale as the fluorescence associated with the cells increased by 1 to 3 orders of magnitude. No cells remained at the initial fluorescence level indicating that all the vesicles enter into all lymphocytes. Also, there is no appearance of cell debris or of additional peaks when cells have taken up vesicles, reflecting that their morphology and viability are preserved. In agreement with this observation, the Trypan blue analysis of the cells after incubation with fluorescent vesicles was 98.3% for the control preparation incubated without liposomes, 93.6, 85.4, 93.8, and 82.7% for cells incubated with increasing concentrations of Fluo-vesicles (Fig. 1E) and 86.4% for the control preparations and 83.8, 83.6, and 71.6% for the cells incubated with increasing concentrations of Fluo-vesicles shown in Fig. 1F. The data document that all isolated human lymphocytes take up fluorescent vesicles in a dose-dependent fashion without apparent toxicity.

Temperature Dependence of Vesicle Entry

Recently, we discovered that the 200 m*M* CF used to label the vesicles in their water phase, in conjunction with the high Na concentration used to neutralize the acid CF, provoked multilayering of the vesicles and augmented their diameter from the 60 nm of Na,K-ATPase-free vesicles to 330 nm *(10)*. We exploited the fact to have small single-walled and large, multilayered vesicles to see whether there was a difference in the temperature dependence of their uptake

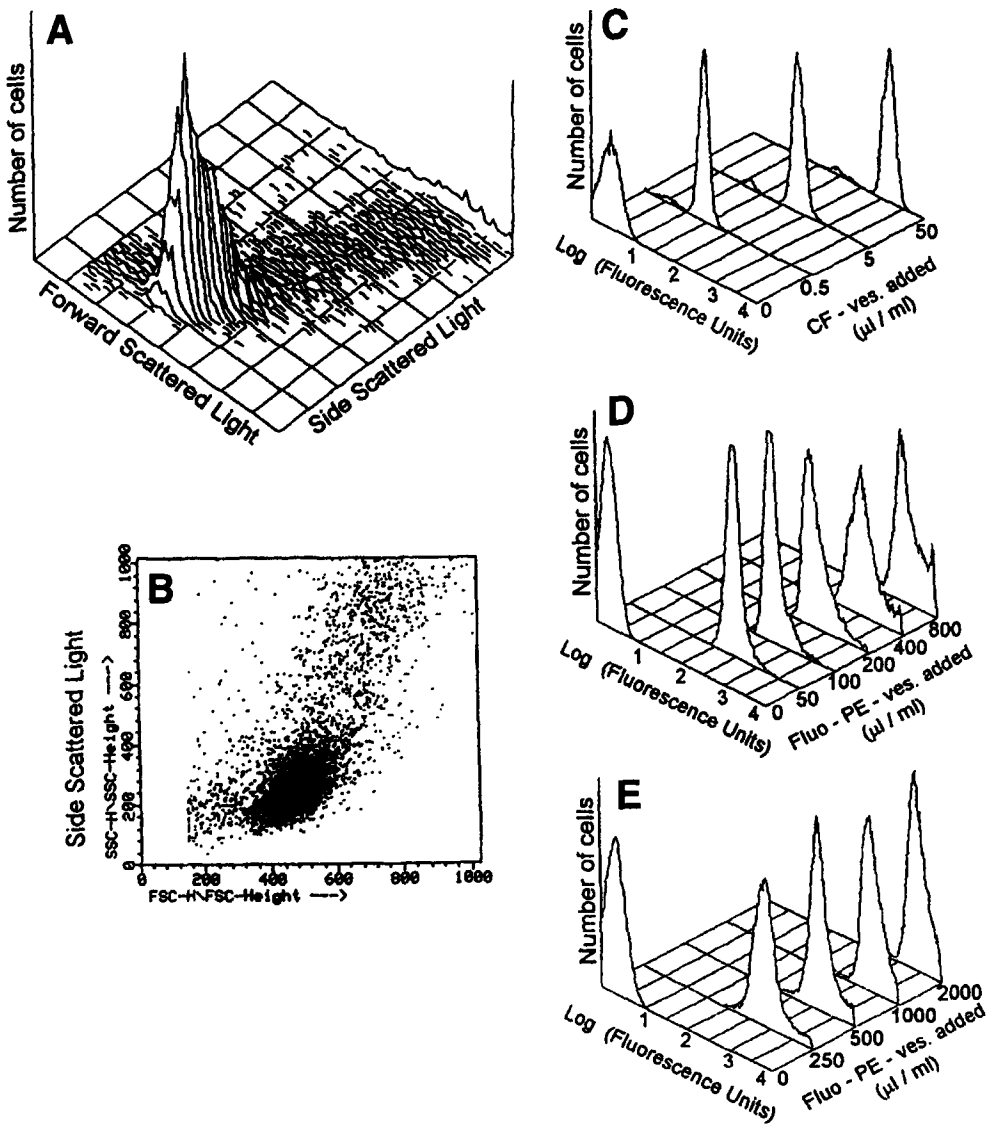

Fig. 1. Increase of cellular fluorescence with increasing vesicle/cell ratio. Freshly isolated human lymphocytes were incubated with increasing amounts of fluorescent vesicle for 30 min at 37°C. The vesicle quantity added is indicated in the figure in µL of suspension that had been pelleted and added to 1 mL cell suspension in the conditions described in *Methods;* 10^6 cells in 1 mL of PBS were added to the flow cytometer. The lymphocytes were sorted by specific window settings. Three-dimensional **(A)** or two-dimensional **(B,C)** representations of the 10^4 events (cells) analyzed by scattered light shows a relatively homogenous lymphocytes population. **(D)** FACS analysis of lymphocytes incubated with increasing con-

because this physical parameter is informative as to the possible mechanisms involved. The uptake of the two types of vesicles by cells incubated at 4, 20, or 37°C was very close (Fig. 2A) as seen clearly by the similar shifts of the fluorescence of CF-vesicles (Fig. 2B) and Fluo-vesicles (Fig. 2C) by a temperature increase form 4 to 37°C. The Q_{10} of the uptake was 1.46 ± 0.7 (S.E.M., n = 4). The temperature coefficient (Q_{10}) of a reaction is the ratio of the velocity measured at a given temperature (°C) to the velocity measured at 10 degrees below *(24)*. The Q_{10} of enzymatic reactions is generally around 2, i.e., the velocity doubles with a 10°C increase of the temperature.

Such energy-requiring processes are described by an equation derived from the Van't Hoff equation *(25)* stating that the reaction rate k is proportional to a transmission coefficient (probability that a molecule in a transition state will give the product of the reaction), and proportional to kT/h, which indicates the rate at which a molecule in the transition state is transformed into product (= Boltzmann constant x Absolute temperature/Planck constant). By contrast, if a purely physical process were implicated such as liposome adsorption to the cell surface, the cellular fluorescence would be inversely related to temperature. According to the Gibb's law, the difference of the adsorbed amount (ΔA) of a compound is a function of its concentration (ΔC). The concentration gradient within the adsorbed layer (a) = $-(1/RT) \times (\Delta A/\Delta C)$ where R is the gas constant and T is the absolute temperature. Thus, the high temperature dependence speaks against vesicle adsorption on the cell surface as a cause of the cell-associated fluorescence in line with the other analyses of data obtained by FACS and confocal microscopy presented herein.

Na,K-ATPase in the Vesicles Does Not Interfere with Their Cellular Uptake

Considering the rapid vesicle uptake by the isolated lymphocytes, we wished to see whether the presence of Na,K-ATPase in the vesicle membrane altered their uptake. No statistically significant differences in the cellular fluorescence measured by FACS was observed when the cells were incubated with CF-vesicles or Fluo-

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centrations of CF-vesicles added to 3.7×10^6 cells in 200 µL RPMI-1640. **(E,F)** Lymphocytes incubated with increasing concentrations of Fluo-PE-vesicles in the experimental conditions described in *Methods*.

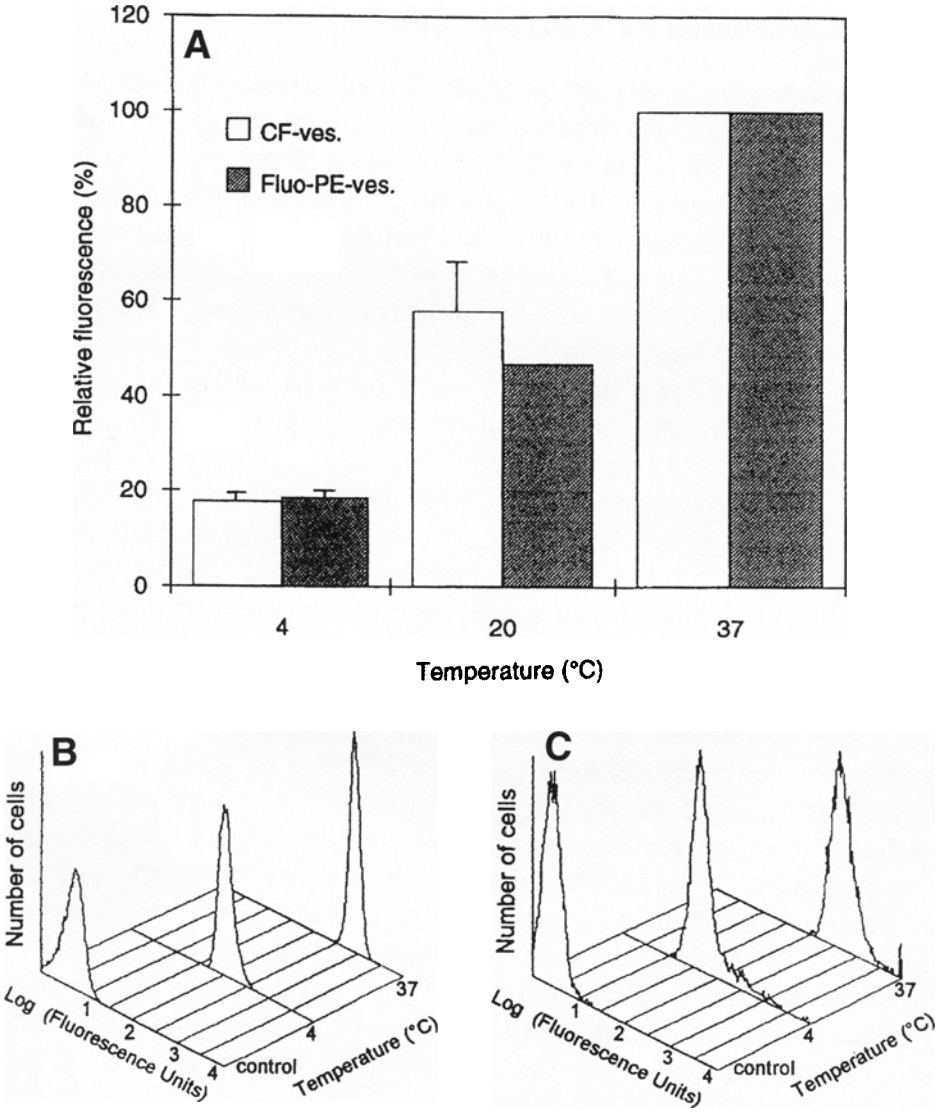

Fig. 2. Temperature-dependence of vesicle entry; 1.5×10^6 cells were incubated in 100 µL RPMI-1640 with 50 µL/mL CF-vesicles **(A,B)** or with 50 µL/mL Fluo-vesicles **(A,C)** in 80 µL PBS for 30 min at 37°C. Results are expressed in percentage of the maximum fluorescence obtained in the experiment. After the incubation, cells were washed once in PBS and analyzed by FACS. Results are expressed in percentage of the maximum fluorescence obtained in the experiment. Data represent mean of two or three measurements (± S.E.M.).

vesicles with or without reconstituted Na,K-ATPase (data now shown).

Confocal microscopy was used to visualize the fluorescent vesicles taken up with or without reconstituted Na,K-ATPase. This new technique analyzes the fluorescence by a laser beam combined with powerful software for virtual sections across the cells yielding finally a three-dimensional map of the intracellular fluorescence. As the same cell is seen in fluorescent or transmission mode the intracellular fluorescence can be attributed to cellular structures in the limits of the microscopic resolution.

When cells were incubated with Fluo-vesicles without Na,K-ATPase for 5 (Fig. 3A) or 30 (Fig. 3B) min, they became strongly fluorescent (left panels). By comparison with the transmission images (right panels), it is evident that the vesicles have entered all cells. The same result is seen with cells incubated for 5 (Fig. 3C) or 30 (Fig. 3D) min with vesicles reconstituted with Na,K-ATPase. Obviously, the presence of renal Na,K-ATPase in the vesicles does not prevent their uptake by all lymphocytes, in agreement with the data obtained by FACS.

That the fluorescence is not absorbed on the cell surface and is contained within the cells is shown in a typical scanned cell that has been incubated with Na,K-ATPase-vesicles for 20 min (Fig. 4) and was then analyzed in three dimensions by nine virtual 1 μm cross-sections. The first scan, on top of the cell, reveals no fluorescence; two small fluorescent spots became apparent on the next scanned plane; the next section, deeper within the cell, uncovers more fluorescence. The maximal fluorescence was seen in the four sections going through the middle of the cell, sparing apparently the nuclear region.

Quantification of the Uptake

To evaluate the importance of the Na,K-ATPase-vesicle entry into lymphocytes, it was important to quantify the process: how many vesicles enter and how much foreign material is imported with them expressed as a fraction of intrinsic? To answer this question, the size and composition of the vesicles as well as the size of the cell must be known and a method to quantify the internalized membranes is required. The ultrastructure of our vesicles with and without Na,K-ATPase is known *(8,9)* and from there their content in phospholipids and Na,K-ATPase molecules was calculated as shown in Table 1. When the number of phospholipids per vesicle is known, the internal fluorescent phospholipid can be extracted from the cell, its quantity determined by spectrofluorometry, and the vesicle num-

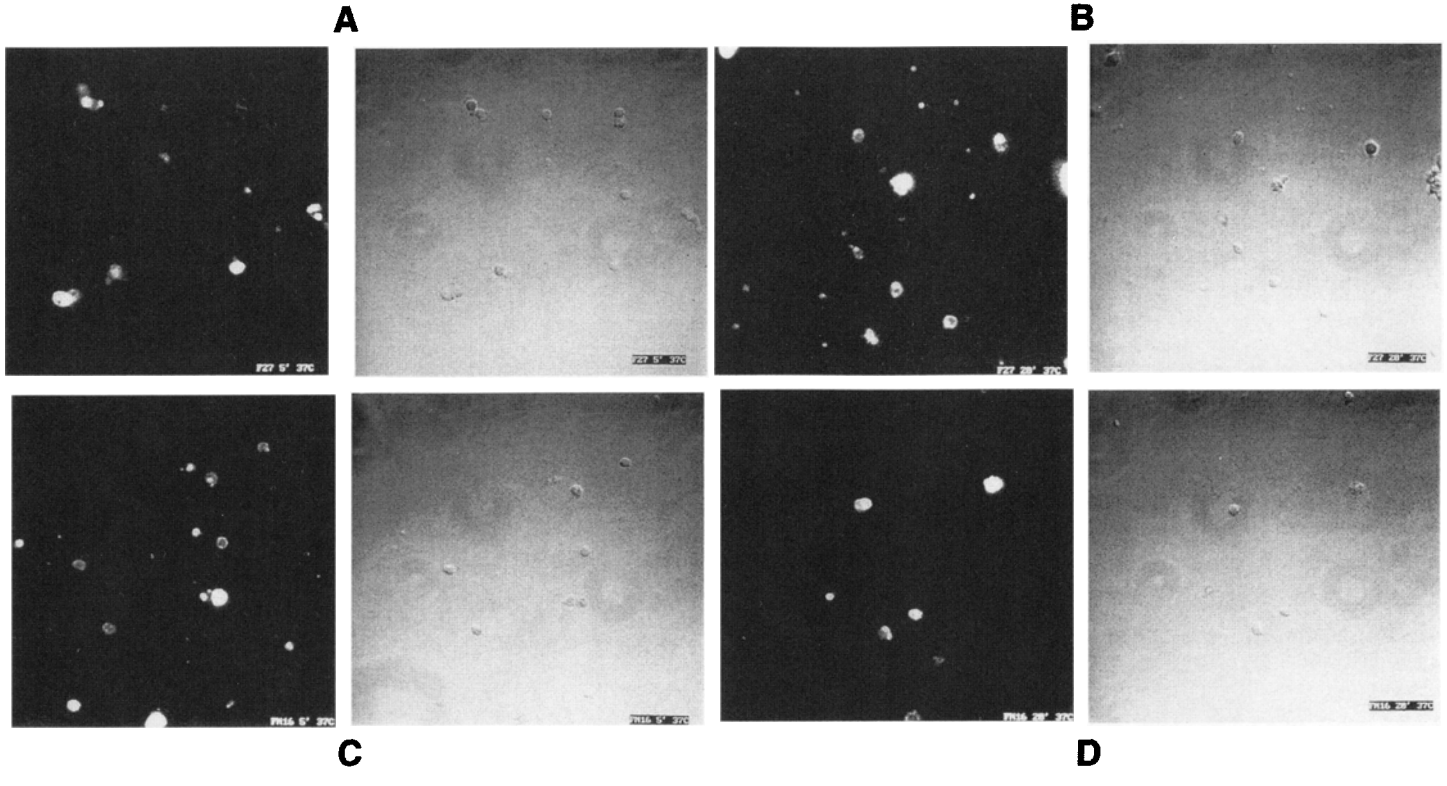

Fig. 3. Confocal micrographs of lymphocytes incubated with Fluo-vesicles without Na,K-ATPase show that all cells take up liposomes. Cells (1.76×10^{6}) in 95 µL RPMI-1640 have been incubated for 5 **(A,B)** or 30 **(C,D)** min at 37°C with 5 µL vesicles formed without (A,B) or with (C,D) Na,K-ATPase and analyzed by fluorescence /left pannels) and transmission (right pannels). After incubation, the suspension was diluted with 1 mL PBS and centrifuged for 10 min at 400*g* at 18°C. Cell pellets were resuspended in 1 mL polyvinyl alcohol (PVA), one drop of the suspension was put between slide and coverslip and the preparation examined by confocal microscopy as described in *Methods*.

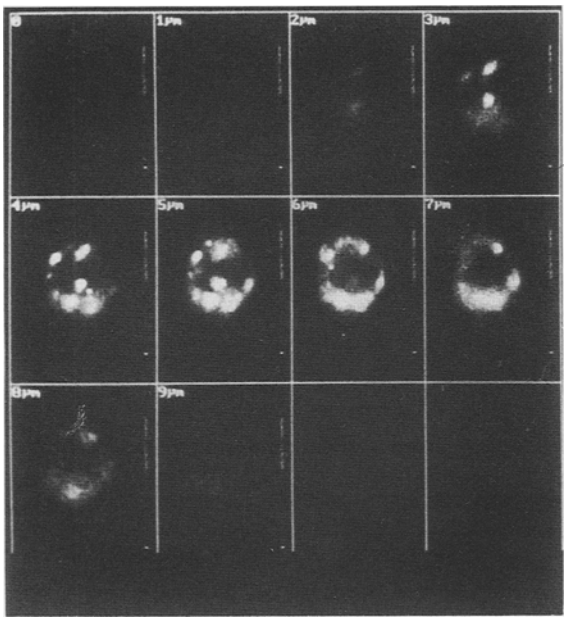

Fig. 4. Three-dimensional demonstration of intracellular fluorescence resulting from Na,K-ATPase-vesicle uptake. Virtual 1 μm z-sectioning of a typical cell that has been incubated with fluorescent vesicles for 20 min in the conditions described for Fig. 3.

ber taken up per cell calculated. However, the lymphocytes are contaminated by monocytes, which are phagocytic cells and take up more vesicles than lymphocytes. The average percentage of monocytes present in the preparation was determined by FACS in a series of three different preparations (± S.E.M.) to which 5 μL CF-liposomes had been added (to 2×10^6 cells in 100 μL RPMI-1640 at 37°C): 8.3 ± 0.1 %, 8.7 ± 0.6%, 9.1 ± 0.1%, 9.4 ± 0.3%, and 10.3 ± 0.3% after 5, 10, 30, 60, and 120 min of vesicle addition, respectively. An average value of 9% was chosen for the calculations.

With regard to the vesicle uptake, the following monocyte/lymphocyte fluorescence ratios were obtained in triplicates (± S.E.M.) in six different preparations that incorporated, respectively, 36 ± 1.5, 28 ± 1, 27 ± 3, 17 ± 0.3, 23 ± 0.2, and 10 ± 0.3 times more fluorescence within 60 min at 37°C; the average of the six separate determinations was 23. On the basis of the 9% monocyte fraction and the 23-fold difference of uptake by lymphocytes and monocytes, the distribution of Fluo-PE-liposomes between monocytes and lympho-

Table 1

Parameters Used for Quantifying Vesicle Uptake

Average diameter	Lymphocyte		10 μm[a]
	Na,K-ATPase-vesicle total		100 nm[b]
	Inner compartment (–4 nm membrane)		92 nm
Phospholipid No.	per 100 nm vesicle	outer leaflet	44,882
		inner leaflet	37,988
		total	82,870
Surface	Lymphocyte		$3.14 \times 10^{-10} m^2$
	Na,K-ATPase-vesicle		$3.14 \times 10^{-14} m^2$
	PC headgroup		$0.7 \times 10^{-18} m^2$[c]
Volume	Lymphocyte		$5.24 \times 10^{-16} m^3$
	Na,K-ATPase-vesicles		$4.08 \times 10^{-22} m^3$

[a]The average cell size was estimated by optical and electronic microscopy and found compatible with published data *(28)*.

[b]The vesicle size was determined by freeze fracture *(8,9)* and transmission electronic microscopy *(10)*.

[c]The diameter of a phosphatidylcholine headgroup was found in the work of Mimms et al. *(29)*.

cytes was calculated considering that 9% of the cells (monocytes) take up 23 parts of the fluorescence and 91% of the cells (lymphocytes) take up one part, which is equivalent to 72% of vesicles taken up by monocytes and 28% by lymphocytes. When this correction ismade, the number of vesicles incorporated per lymphocytes at increasing vesicle/cell ratios amounts to the values listed in Table 2. By combining these data with the data of Table 1, the amount of internalized membrane and volume was also calculated, as well as the number of Na,K-ATPase molecules (Table 2).

In previous work, the vesicular Na,K-ATPase was radiolabeled by ^{110m}Ag. The cells were then incubated with the ^{110m}Ag-labeled Na,K-ATPase-vesicles, washed in presence of external dimercaptopropanesulfonic acid (DMPS) to remove all external silver and the ^{110m}Ag taken up by the cells was counted. According to previous determinations, there were four molecules Na,K-ATPase per liposome at the protein/lipid ratio used and, in conjunction with the value of 2 silver ions bound per Na,K-ATPase molecule, the number of liposomes incorporated was found to be 3114 ± 215 (S.E.M., n = 21) for an incubation period of 120 min at 35°C at a liposome/cell ratio of $3.1. \times 10^5$ *(26,27)*. Thus, two entirely different methods yield

Table 2

Foreign Material Brought to a Lymphocyte by Vesicle Uptake for 30 Min

Vesicles entered	Foreign membrane		Foreign volume		Foreign Na,K-ATPase	
Number	(m^2)	(% of intrinsic)	(m^3)	(% of intrinsic)	No.	% of intrinsic
1910[a]	2.158×10^{-11}	7	1.406×10^{-19}	0.03	11,460	38
4122[a]	4.658×10^{-11}	15	3.035×10^{-19}	0.06	24,732	82
8748[a]	9.885×10^{-11}	32	6.440×10^{-19}	0.12	52,488	175
16,087[a]	1.818×10^{-10}	60	1.184×10^{-18}	0.23	96,522	322
39,414[a]	4.454×10^{-10}	142	2.902×10^{-18}	0.55	236,484	788
18,098[b]	2.045×10^{-10}	65	1.332×10^{-18}	0.25	108,588	362
19,004[b]	2.147×10^{-10}	68	1.399×10^{-18}	0.27	114,024	380
41,526[b]	4.692×10^{-10}	149	3.057×10^{-18}	0.58	249,156	830
106,982[b]	1.208×10^{-9}	385	7.876×10^{-18}	1.50	641,892	2140

Two different Fluo-vesicle preparations (a) and (b) were pelleted and incubated with 1.4×10^6 cells for 30 min at 37°C, resulting in ratios of 0.27, 0.59, 1.58, 3.99, and 8.88×10^5 (a) and 0.6, 1.1, 1.8, and 4.7×10^6 vesicles (b) per cell. After the incubation, the cells were processed and the entrapped Fluo-PE measured by spectrofluorometry as described in *Methods* and calculated according to the data of Table 1. The number of 6 Na,K-ATPase molecules per vesicle was taken from previous determinations of freeze fractured preparation prepared in the same conditions. A number of 30,000 Na,K-ATPase molecules per lymphocyte had been determined by ^{3}H-ouabain *(30)*.

close values for the number of Na,K-ATPase-vesicles incorporated per cell.

It is impressive that a lymphocyte incubated in the presence of 4.7 million externally added Na,K-ATPase vesicles has taken up 3.85 times its own membrane surface, replaced 1.5% of its volume by foreign solution, and taken up more than 20 times more foreign Na,K-ATPase as compared to the intrinsic number (Table 2).

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