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Mathematical modelling of lipid transbilayer movement in the human erythrocyte plasma membrane

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Abstract. A model is presented to simulate transverse lipid movement in the human erythrocyte membrane. The model is based on a system of differential equations describing the time-dependence of phospholipid redistribution and the steady state distribution between the inner and outer membrane monolayer. It takes into account several mechanisms of translocation: (i) ATP-dependent transport via the aminophospholipid translocase; (ii) protein-mediated facilitated and (iii) carrier independent transbilayer diffusion. A reasonable modelling of the known lipid asymmetry could only be achieved by introducing mechanism (iii). We have called this pathway the compensatory flux, which is proportional to the gradient of phospholipids between both membrane leaflets. Using realistic model parameters, the model allows the calculation of the transbilayer motion and distribution of endogenous phospholipids of the human erythrocyte membrane for several biologically relevant conditions. Moreover, the model can also be applied to experiments usually performed to assess phospholipid redistribution in biological membranes. Thus, it is possible to simulate transbilayer motion of exogenously added phospholipid analogues in erythrocyte membranes. Those experiments have been carried out here in parallel using spin labeled lipid analogues. The general application of this model to other membrane systems is outlined.

Key words: Phospholipid asymmetry – Aminophospholipid translocase – Transbilayer diffusion – Lipid flip-flop (erythrocyte membrane)

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Abbreviations: PBS, phosphate buffered saline, DFP, diisopropyl fluorophosphate; ESR, electron spin resonance; RBC, red blood cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine, PS, phosphatidylserine; SM, sphingomyelin; (0,2)PC, 1-palmitoyl-2(4-doxylpentanoyl)-PC; (0.2)PE, 1-palmitoyl-2(4-doxylpentanoyl)-PE, (0,2)PS, 1-palmitoyl-2(4-doxylpentanoyl)-PS

Introduction

Phospholipids can cross biological membranes by different mechanisms: which are (*i*) simple diffusion, (*ii*) facilitated diffusion and (*iii*) active transport (Zachowski and Devaux 1990). Simple diffusion refers to the spontaneous transmembrane diffusion occuring in the bulk lipid phase, while facilitated diffusion describes the accelerated passive transbilayer motion mediated by membrane proteins. The existence of an active transverse lipid transport mechanism was first suggested by Devaux and colleagues (Seigneuret and Devaux 1984) who incorporated spin-labeled phospholipids into the outer leaflet of human erythrocyte membranes: in the presence of cytosolic ATP PS- and PE-analogues transferred rapidly to the inner leaflet while the flip of PC-analogues was slow and ATP independent. ATP depletion resulted in a slow flip of PS and PE similar to that of PC (Seigneuret and Devaux 1984). There is considerable evidence that the ATP dependent lipid transport is mediated by a membrane protein, the so called aminophospholipid translocase, although this has not been identified so far (see Devaux 1991). The characteristics of this transport (lipid specifity, inhibition, dependence on substrate and ATP concentration) has been investigated in detail (Morrot et al. 1989; Zachowski et al. 1986). In the following years, such active aminophospholipid transport was found in other mammalian plasma membranes by several independent methods (for a review see Devaux 1991). The generalization has been made that the active protein mediated translocation of aminophospholipids is typical for eucaryotic plasma membranes. Moreover, in some subcellular membranes of eucaryotic cells active aminophospholipid transport has been found as well (Zachowski et al. 1989).

Although the physiological significance of the aminophospholipid translocase remains to be elucidated, it has been suggested that this membrane protein might be responsible for the well known phospholipid asymmetry in erythrocyte membranes. Besides experimental support (Calvez et al. 1988), this hypothesis has been strengthened by a simple diffusion model (Herrmann and Müller 1986)

based on experimental data with spin-labeled phospholipid analogues. The main outcome of this study was that phospholipid asymmetry can be explained solely by different rate constants of inward and outward translocation of aminophospholipids (Herrmann and Müller 1986). This was contradicted by Williamson et al. (1987) using a modified diffusion model. They concluded that the efficiency of the translocase was the same for the inward and outward motion of the aminophospholipids and underlined the specific aminophospholipid-spectrin interaction for maintaining the pronounced lipid asymmetry in erythrocyte membranes. However, these diffusion models are comparatively simple, neglecting the enzymatic character of the active lipid translocation. For that reason they cannot describe the complex behaviour of lipid translocation.

The purpose of this investigation is to develop a more elaborate model for describing transverse lipid movement in the human erythrocyte membrane. The main features of the model presented are carrier mediated mechanisms for the active as well as facilitated passive translocation of lipids and a carrier independent transbilayer diffusion. For several biologically relevant conditions the transbilayer motion and distribution of endogenous phospholipids of the human erythrocyte membrane is simulated. Although the model does not consider any interaction between phospholipids and spectrin it describes in a satisfying manner the transverse distribution and mobility of phospholipids in the human erythrocyte membrane. This supports the view that the active translocation of aminophospholipids PS and PE might be responsible for the asymmetric distribution of phospholipids. Moreover, the model allows one to describe the dynamics of transbilayer redistribution of exogenously added phospholipid analogues in erythrocyte membranes, which we have assessed experimentally using spin labeled analogues.

Since the model takes into account different mechanisms of transbilayer lipid motion it is not confined to the erythrocyte membrane but may be also applicable to other cellular membranes.

Materials and methods

The mathematical model

Experimental basis of the model. In the following the current knowledge of transverse motion of phospholipids, mainly obtained from investigations of the human erythrocyte membrane, is summarized.

(*i*) Aminophospholipids PS and PE are rapidly translocated from the outer to the inner monolayer of the cell membrane in the presence of cytosolic ATP, while outward translocation to the external layer is slow (Seigneuret and Devaux 1984; Bitbol and Devaux 1988).
(*ii*) ATP dependent translocation of PS and PE is mediated by the same membrane protein, the aminophospholipid translocase. Both lipids compete for the same binding site (Zachowski et al. 1986). The stoichiomety between aminophospholipid translocation and ATP consumption is almost one on the molar level (Beleznay et al. 1993).
(*iii*) The number of copies of the aminophospholipid translocase in a single erythrocyte is of the order of 100 to 400 (Morrot et al. 1990).
(*iv*) Transverse motion of PC is ATP independent and slow with similar rates in both directions. It does not compete for the binding sites of the translocase (Zachowski et al. 1986).
(*v*) In the absence of ATP, or after inhibition of the translocation, passive diffusion of PS and PE is similar to that of PC (Seigneuret and Devaux 1984).
(*vi*) Passive transverse diffusion of PC, PE, and PS is significantly slower in pure lipid membranes composed of erythrocyte lipids than in intact erythrocyte membranes (Zachowski et al. 1985). This suggests that in erythrocyte membranes sites exist supporting passive transverse diffusion. This facilitated diffusion is unspecific with respect to phospholipids and direction of movement. Candidates for those sites are integral membrane proteins such as glycophorin or band 3 protein (Gerritsen et al. 1980). About 10^6 copies of those proteins per erythrocyte exist (Goodman and Shiffer 1983).
(*vii*) Passive diffusion of SM between both layers in the erythrocyte membrane is considerably slower compared to that of PC, even in liposomes of erythrocyte lipids (Zachowski et al. 1985). It has been suggested that SM-SM interaction via hydrogen bonds could be responsible for such a slow transmembrane motion (Boegheim et al. 1983).
(*viii*) Phospholipid transverse distribution in intact human erythrocytes can be viewed as a steady-state with a continuous exchange of lipids in both directions (Herrmann and Müller 1986; Bitbol and Devaux 1988).

Basic assumptions of the mathematical model. Essentially, this is a carrier-mediated transport model. Two different carriers, *T* and *P*, are assumed. Active translocation of PS and PE from the outside to the inside at the expense of metabolic energy is achieved by the *T*-carrier, while the *P*-carrier mediates an unspecific passive transport of PS, PE and PC in both directions. Additionally, an unspecific transmembrane lipid flux is assumed for each lipid species in order to compensate unbalanced lipid carrier fluxes. This is what we call compensatory flux and for a given lipid species it is driven by the difference of total lipid content between both monolayers, weighted by the relative abundance of that species in the leaflet with the higher total lipid content. The schemes for both carrier systems are shown in Fig. 1 a and b.

The following assumptions for the binding of phospholipids to the carriers are made: *a*) rapid competitive binding of PS and PE to the *T*-carrier and of PS, PE and PC to the *P*-carrier, respectively, at both sides of the membrane; *b*) irreversible translocation of both, PS and PE by the *T*-carrier from the outer leaflet to the inner leaflet with consumption of ATP; *c*) reversible translocation of PS, PE and PC by the *P*-carrier and *d*) reversible switching of the unloaded forms of *T*- and *P*-carriers between both membrane layers.

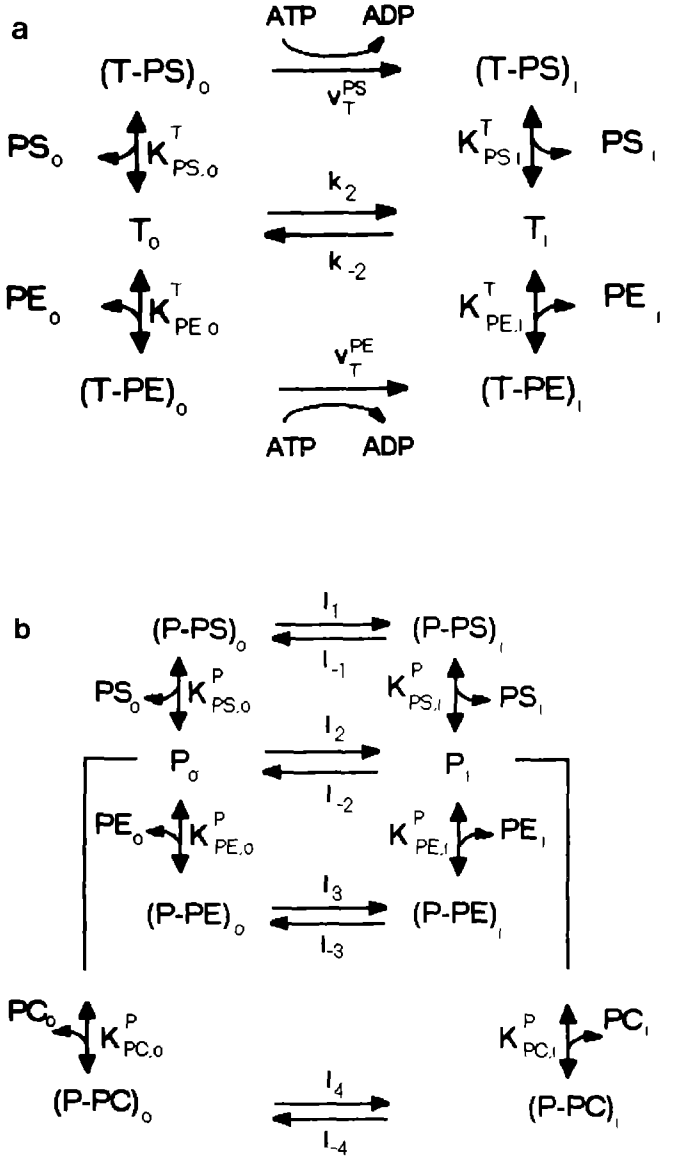

Fig. 1 a, b. Scheme of binding reactions considered for mathematical modelling of lipid translocation in the human erythrocyte membrane. **a** active carrier T, **b** passive carrier P. Indices o and i refer to the outer and inner membrane leaflet, respectively. $k_{\pm 2}$ and $l_{\pm 1}$, $l_{\pm 2}$, $l_{\pm 3}$, $l_{\pm 4}$ are the corresponding kinetic constants, $K^{P/T}_{PS,\,i/o}$ denote the equilibrium constants and $v_T^{PS/PE}$ denote the active translocation flux of the reactions where ATP consumption is involved

Translocation of other lipids is not considered in the model. It has been shown that the transverse mobility of SM is extremely slow (see above). Hence the transmembrane distribution of SM is regarded as time independent. Since the mechanism and kinetics of cholesterol flip-flop is still a matter of discussion, we have not included this lipid species in the model. However, owing to its relevance for membrane structure and dynamics we are well aware that future prospects of modelling have to include this species (see discussion).

Model equations. Time dependencies of concentrations of the phospholipids PS, PE and PC in the inner membrane monolayer can be described by the following system of differential equations

$$\frac{d[\mathrm{PS}]_i}{dt} = v_T^{\mathrm{PS}} + v_P^{\mathrm{PS}} + v_C^{\mathrm{PS}} \tag{1a}$$

$$\frac{d[\mathrm{PE}]_i}{dt} = v_T^{\mathrm{PE}} + v_P^{\mathrm{PE}} + v_C^{\mathrm{PE}} \tag{1b}$$

$$\frac{d[\mathrm{PC}]_i}{dt} = v_P^{\mathrm{PC}} + v_C^{\mathrm{PC}} \tag{1c}$$

v_T^{PS}, v_T^{PE} and v_P^{PS}, v_P^{PE}, v_P^{PC} denote the fluxes of phospholipids mediated by the active T-carrier and the passive P-carrier, respectively, while v_C^{PS}, v_C^{PE}, v_C^{PC} denote the unspecific compensatory fluxes of phospholipids.

On the basis of the carrier mechanisms shown in Fig. 1 and with the assumptions given in the previous section all transmembrane fluxes can be expressed by the inner concentrations of PS, PE and PC. For that, the various fluxes are expressed in a first step as functions of the concentrations of the loaded carrier forms $[T-\mathrm{PL}]_i$, $[T-\mathrm{PL}]_o$ and $[P-\mathrm{PL}]_i$, $[P-\mathrm{PL}]_o$ where the indices i and o refer to the inner and outer membrane leaflet, respectively, and the notation PL stands for the phospholipids PS, PE or PC.

The ATP-dependent translocations are described by the following saturation functions:

$$v_T^{\mathrm{PS}} = \frac{k_1\,[\mathrm{ATP}]\,[T-\mathrm{PS}]_o}{K_m^1+[\mathrm{ATP}]};\quad v_T^{\mathrm{PE}} = \frac{k_3\,[\mathrm{ATP}]\,[T-\mathrm{PE}]_o}{K_m^3+[\mathrm{ATP}]} \tag{2}$$

where k_1, k_3 denote effective rate constants and K_m^1, K_m^3 the Michaelis-Menten constants for ATP of the irreversible translocations of PS and PE, respectively, mediated by the T-carrier.

The fluxes mediated by the passive P-carrier are written as:

$$v_P^{\mathrm{PS}} = l_1\,[P-\mathrm{PS}]_o - l_{-1}\,[P-\mathrm{PS}]_i \tag{3a}$$

$$v_P^{\mathrm{PE}} = l_3\,[P-\mathrm{PE}]_o - l_{-3}\,[P-\mathrm{PE}]_i \tag{3b}$$

$$v_P^{\mathrm{PC}} = l_4\,[P-\mathrm{PC}]_o - l_{-4}\,[P-\mathrm{PC}]_i \tag{3c}$$

where l_j and l_{-j} are the corresponding rate constants of inward and outward translocation.

The concentrations $[T-\mathrm{PL}]_{i/o}$, $[P-\mathrm{PL}]_{i/o}$ can be calculated as functions of the inner lipid concentration using the following set of equations:

a) Binding of lipidspecies to the T-carrier and P-carrier:

$$K^T_{\mathrm{PL},\,i} = \frac{[\mathrm{PL}]\,[T]_i}{[T-\mathrm{PL}]_i},\quad K^T_{\mathrm{PL},\,o} = \frac{[\mathrm{PL}]\,[T]_o}{[T-\mathrm{PL}]_o} \tag{4a,b}$$

(for PL = PS, PE) and

$$K^P_{\mathrm{PL},\,i} = \frac{[\mathrm{PL}]\,[P]_i}{[P-\mathrm{PL}]_i},\quad K^P_{\mathrm{PL},\,o} = \frac{[\mathrm{PL}]\,[P]_o}{[P-\mathrm{PL}]_o} \tag{5a,b}$$

(for PL = PS, PE, PC)

where $K^T_{\mathrm{PL},\,i/o}$ and $K^P_{\mathrm{PL},\,i/o}$ denote dissociation constants and $[T]_{i/o}$ and $[P]_{i/o}$ the concentrations of unloaded forms of the carriers with respect to the corresponding membrane monolayer.

b) Conservation of the total amount of the T-carrier and P-carrier:

$$[T]_{tot}=[T]_i+[T]_o+\sum_{PL=PS,\,PE}[T-PL]_i \qquad (6a)$$

$$+\sum_{PL=PS,\,PE}[T-PL]_o$$

$$[P]_{tot}=[P]_i+[P]_o+\sum_{PL=PS,\,PE,\,PC}[P-PL]_i \qquad (6b)$$

$$+\sum_{PL=PS,\,PE,\,PC}[P-PL]_o$$

c) Steady-state condition for different forms of each carrier:

$$v_T^0+v_T^{PS}+v_T^{PE}=0 \qquad (7a)$$

$$v_P^0+v_P^{PS}+v_P^{PE}+v_P^{PC}=0 \qquad (7b)$$

where $v_T^0=k_2\,[T]_o-k_{-2}\,[T]_i$ and $v_P^0=l_2\,[P]_0-l_{-2}\,[P]_i$ are the translocation fluxes of the unloaded carriers.

d) Conservation of the total lipid content:

$$[PL]_{tot}=[PL]_i+[PL]_o \quad PL=PS,\,PE,\,PC. \qquad (8)$$

Equation (8) implies that the concentrations of lipids bound to the carriers are assumed to be negligibly small compared to the concentrations of free lipids in the bulk phase of the monolayer (see Table 1).

The compensatory flux v_C is unspecific. The contributions v_C^{PL} (PL = PS, PE, PC) to this flux are determined:

a) by the difference between the total lipid concentrations in the inner and outer membrane monolayer

$$[L]_{i/o}=[PS]_{i/o}+[PE]_{i/o}+[PC]_{i/o}+[SM]_{i/o} \qquad (9)$$

including also sphingomyelin.

b) by the relative abundance of lipids within each membrane leaflet.

The compensatory fluxes are unidirectional and are directed towards the membrane layer with the lower total lipid content. For the condition $[L]_i>[L]_o$ they are written as

$$v_C^{PL}=-k_C\,([L]_i-[L]_o)\,\frac{[PL]_i}{[PS]_i+[PE]_i+[PC]_i} \qquad (10a)$$

and for $[L]_i<[L]_o$

$$v_C^{PL}=-k_C\,([L]_i-[L]_o)\,\frac{[PL]_o}{[PS]_o+[PE]_o+[PC]_o}. \qquad (10b)$$

At given values of $[PS]_t$, $[PE]_t$ and $[PC]_t$ Eqs. (1)–(10) represent a complete set of equations for the calculation of time-dependent and steady state distributions of lipids between the inner and outer monolayer. Calculations were performed numerically using a Runge-Kutta integration routine for solving the first order differential equations (1 a)–(1 c).

Model parameters. The model contains a rather high number of parameters. These are the intracellular concentration of ATP, the Michaelis Menten constant of ATP for active translocation of PS and PE, the total concentrations of the carriers and lipids, the dissociation constants of carrier-lipid complexes, the rate constants of translocations mediated by the two carriers and the rate constant of the unspecific compensatory lipid fluxes (see Table 1). It is generally assumed that the binding reactions between carriers and lipids occur in a three dimensional phase. Therefore, concentrations of lipids and carriers were recalculated in units [mmol/l] assuming a mean red blood cell volume of 107 μm^3 (Canham and Burton 1968). 2×10^8 lipids per erythrocyte (Shiga et al. 1979) converts to a total lipid concentration of 3 mmol/l. For the active carrier a value of 350 copies per cell is taken (see above). 7×10^5 copies of the passive *P*-carrier per cell are assumed. This value is of the same order as the total number of the integral membrane proteins band 3 and glycophorin in the erythrocyte membrane (Goodman and Shiffer 1983). A choice of other parameter values is discussed thoroughly in the Results section.

Experimental procedures

Materials. Diisopropyl fluorophosphate (DFP) was from Sigma (St. Louis, MI). Spin-labeled phospholipid analogues were a gift of P. F. Devaux and his group (Institut de Biologie Physico-Chimique, Paris). Fresh blood from healthy donors was obtained from the Blood Bank, Berlin-Lichtenberg.

Red blood cell preparation. After removal of buffy coat and plasma, red blood cells (RBC) were washed three times in PBS buffer (150 mM NaCl, 5.8 mM phosphate buffer, pH 7.4).

Spin-labeling. Labeling and back exchange procedures are essentially as described by Calvez et al. (1988). Before labeling, intact erythrocytes were incubated 5 min with 5 mM (final concentration) DFP, at 37 °C, to minimize the hydrolysis of the spin-labeled phospholipids (Colleau et al. 1991; Morrot et al. 1989). After the desired amount of the spin-labeled phospholipid analogues 1-palmitoyl-2-(4-doxylpentanoyl)-PS [(0, 2) PS], 1-palmitoyl-2-(4-doxylpentanoyl)-PE [(0, 2) PE] or 1-palmitoyl-2-(4-doxylpentanoyl)-PC [(0, 2) PC] from chloroform/methanol (1:1 v/v) were dryed and resuspended in PBS by vigorous vortexing, labeling of RBC was carried out at 33% hematocrit. The incorporation into the outer leaflet of erythrocyte membranes was completed within 1 min. The concentrations of spin-labeled phospholipids correspond to about 1% of endogenous erythrocyte membrane lipid.

Assay for transverse phospholipid motion and distribution. (A) Back-exchange-assay. The ESR determination of the transmembrane distribution of spin-labeled lipids was performed by the back exchange technique using bovine serum albumin (BSA) (Calvez et al. 1988; Morrot et al. 1989). At given times 120 µl aliquots were taken from the red blood cell suspension and mixed with 70 µl of 3.3% BSA (w/v). After 1 min the mixture was centrifuged for 1 min and the supernatant was kept. The amount of extractable probe was obtained by the intensity from the ESR spectrum of the supernatant after reoxidation by potassium ferrocyanide (10 mM).

(B) Reduction experiments. At 37 °C, the signal intensity of the paramagnetic moiety of labeled lipids is decreased on the inner layer owing to reducing properties of the erythrocyte cytoplasm (Seigneuret et al. 1984). Thus, signal loss of lipid analogues incorporated into the erythrocyte membrane provides a measure of inward motion.

Results

The model reference state

As the model reference state we consider the well known time independent asymmetric distribution of lipids within the human erythrocyte membrane. Mathematically, this reference state can be determined by numeric integration of the differential equations (1 a–c) over a long time period when model variables achieve time independent values. The initial distributions of PS, PE and PC at $t=0$ can be chosen arbitrarily. Figure 2 shows, for $t<3\,000$ min, transitions of $[PS]_i$, $[PE]_i$ and $[PC]_i$ towards the in vivo steady state characterized by an active translocase, and for $t>3\,000$ min, transitions towards the stationary state which is reached after inactivation of the translocase ($[ATP]=0$). In Tables 1 and 2 are listed the numerical values of the model parameters used for the integration and the values of the model variables (lipid concentrations and fluxes) within the reference state, respectively. The model parameters were chosen with the following constraints:

a) Concentration of free ATP is 1 mmol/l as measured in human erythrocytes.
b) The reference state of the model should correctly predict the stationary distribution of PS, PE and PC between the two membrane layers deduced from experiments.
c) Relations between model parameters were kept as simple as possible. For example, for the active carrier it was assumed that the rate constants for the translocation of PS and PE are identical ($k_1=k_3$). Furthermore, the intracellular concentration of ATP is considered to be half-saturating, i.e. $K_m^1=K_m^3=1$ mmol/l. The asymmetry of the active carrier was introduced by a comparitatively stronger binding of PS to the active carrier at the outer leaflet.
d) For the appropriate choice of kinetic constants k_1 and k_3, the predicted activity of the translocase was related to the rate of non-glycolytic consumption of ATP in human erythrocytes. Non-glycolytic consumption of ATP in erythrocytes is about 1.6 mmol/l/h. From that 0.4 mmol/l/h are utilized by the Na/K-ATPase (Maretzki et al. 1980;

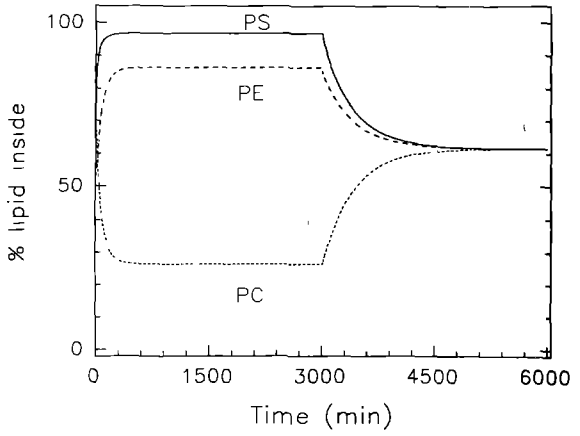

Fig. 2. Time dependence of transverse redistribution of different lipid species. The curves were obtained by numerical integration of the differential equations of the model with the initial values $[PL]_i/[PL]_{tot}=0\,50$ for PS and PE as well as $[PC]_i/[PC]_{tot}=0.77$. The second part of the time course ($t>3\,000$ min) shows the relaxation of the system after the active carrier T is switched off by setting the intracellular ATP-concentration to 0. The values of the model parameters are listed in Table 1

Table 1. Model parameters used for the calculation of the reference state

$K^T_{PS,o}$	: 0.1 mmol/l
$K^T_{PS,i}=K^T_{PE,i}=K^T_{PE,o}$	: 0.5 mmol/l
$K^P_{PL,i}=K^P_{PL,o}$ (PL = PS, PE, PC)	: 0.05 mmol/l
$k_1=k_3$	: 1.21×10^5 min^{-1}
$k_2=k_{-2}$	: 2.42×10^5 min^{-1}
$l_j=l_{-j}$ $(j=1,\ldots,4)$	: 0.242 min^{-1}
k_c	: 0.1 min^{-1}
$K_m^1=K_m^3$	: 1.0 mmol/l
$[Lipid]_{tot}$	: 3.0 mmol/l[a]
$[T]_{tot}$	: 5.5×10^{-6} mmol/l[a]
$[P]_{tot}$	: 1.1×10^{-2} mmol/l[a]
[ATP]	: 1.0 mmol/l[b]
$[PS]_{tot}/[Lipid]_{tot}$	: 0.14[c]
$[PE]_{tot}/[Lipid]_{tot}$	: 0.28[c]
$[PC]_{tot}/[Lipid]_{tot}$	: 0.315[c]
$[SM]_{tot}/[Lipid]_{tot}$	: 0.265[c]
$[SM]_t/[SM]_{tot}$	: 0.18[c]

[a] These values were calculated using the values of 2×10^8 lipid molecules, 350 copies of T-carrier and 7×10^5 copies of P-carrier, respectively, per erythrocyte and the volume of the erythrocyte as 107 μm^3 (details are given in the section on model parameters).
[b] from Clark (1988)
[c] from Luly (1989)

Table 2. Values of model variables in the model reference state. Values in brackets refer to a quasi-stationary state when the active carrier is not operating, i.e. $[ATP]=0$

Lipid concentrations	
$[L]_i$	: 1.52 (1.4999) mmol/l
$[L]_o$	: 1.48 (1.5001) mmol/l
$[PS]_t/[PS]_{tot}$	: 0.97 (0.62)
$[PE]_t/[PE]_{tot}$	: 0.86 (0.62)
$[PC]_i/[PC]_{tot}$	: 0.26 (0.62)

Translocation fluxes ($\times10^{-4}$ mmol/l/min)

	PS	PE	PC
v_T	17.19 (0)	29.29 (0)	–
v_P: outward	− 3.79 (−2.45)	− 6.76 (−4.90)	− 2.31 (−5.51)
inward	0.20 (2.40)	1.75 (4.80)	10.62 (5.40)
total	− 3.59 (−0.05)	− 5.01 (−0.10)	8.31 (−0.11)
v_C	−13.60 (0.05)	−24.28 (0.10)	− 8.31 (0.11)

Brumen and Heinrich 1984). This gives about 1.2 mmol/l/h metabolic energy available for ATP-consuming processes including the active translocation of phospholipids. With a stoichiometry between lipid translocation and ATP consumption equal to unity one obtains, with the model parameters listed in Table 1, within the reference state an ATP consumption for the active carrier of 0.29 mmol/l/h, which is a reasonable value in comparison to the available metabolic energy.

e) Kinetic constants of the passive carrier were chosen to simulate fairly reasonably the time dependence of redistribution of endogenous lipids after the translocase is switched off. The characteristic half time is expected to be that of translocation of PC which is of the order of a few hours (Fig. 2) (Middelkoop et al. 1986).

f) The value of the parameter k_C of the compensatory flux was chosen in order to attain a total lipid content of nearly the same magnitude for both membrane layers (Table 2). In the model reference state the total concentration of lipids in the inner layer is slightly greater than that in the outer layer; consequently, the compensatory flux is directed outwards.

The dependence of the stationary lipid distribution on the intracellular ATP-concentration is shown in Fig. 3. In the close neighbourhood of the reference state (i.e. at [ATP] = 1 mmol/l) no dramatic changes could be predicted. Over the major range of ATP concentration the stationary distributions of PE and PC are more sensitive with respect to changes of the ATP-concentration than the distribution of PS. The latter responds sharply at very low concentrations of ATP. When the carrier is inactivated the equilibrium distributions of PS, PE and PC are equal, but they remain asymmetric between the two membrane layers (cf. Fig. 2). The residual asymmetry is due to the asymmetric distribution of SM, assumed to be fixed in the present model.

It is worth mentioning that for [ATP] = 0, i.e. $v_T^{PS} = v_T^{PE} = 0$, the total rates v_P as well as the rates of the compensatory fluxes v_C for PS, PE and PC are much smaller than the unidirectional fluxes mediated by the passive carrier but do not vanish. At first sight, non-vanishing values for the net rates v_P and v_C at [ATP] = 0 seem to be in contradiction to the principle of detailed balance which states that under equilibrium conditions any molecular process and the reverse of that process should take place at the same rate (Katchalsky and Curran 1967). However, within the present model, stationary states with [ATP] = 0 are not equilibrium states but quasi-steady states owing to the constraint of a fixed asymetric distribution of SM between the two membrane layers. True equilibrium states could only be reached within a much longer time scale, where all components of the membrane may relax.

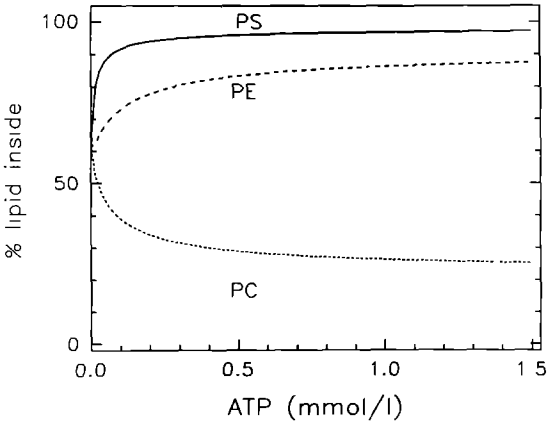

Fig. 3. Steady state distributions of phospholipids as a function of free ATP. The values of the model parameters are listed in Table 1

Simulation of experiments on redistribution of spin-labeled lipid analogues

Redistribution of incorporated spin-labeled lipid analogues. The rates of inward translocation of lipids are measured by incorporating labeled lipid analogues into the outer membrane leaflet and, subsequently by monitoring the time course of disappearance of labeled species from the outer leaflet (see Experimental Procedures). In order to simulate those experiments in the framework of the model a differential equation describing the transverse redistribution of lipid analogues is introduced to the system of (1). For example, the time dependence of the concentration of labeled PS on the inner layer is given by equation:

$$\frac{d[PS^*]_i}{dt} = v_+^{PS}\frac{[PS^*]_o}{[PS]_o} - v_-^{PS}\frac{[PS^*]_i}{[PS]_i} \tag{11}$$

[PS*] denotes the concentration of labeled PS, indices i and o refer to the inner and outer membrane layer, respectively, and v_+^{PS} and v_-^{PS} denote the total inward and the total outward translocation flux of PS, respectively. The redistribution of lipids can then be simulated by simultaneous numerical integration of Eqs. (1 a–c) and (11) (Note, that the experimental situation where more than one labeled species is added can also be simulated in a similar manner). Equations (1 a–c) consider the endogeneous as well as exogeneous lipids as one common species whereas (11) refers only to the labeled lipids. Thus, endogenous, as well as exogenous lipids, are treated as a common pool having the same rate of translocation. Therefore, it is easy to envisage that for stationary conditions where $d[PS^*]_i/dt = 0$ and both translocation fluxes are of the same magnitude, the distribution of endogenous and exogeneous lipids is equal:

$$\frac{\overline{[PS]}_i}{\overline{[PS]}_o} = \frac{\overline{[PS^*]}_i}{\overline{[PS^*]}_o}. \tag{12}$$

The bar denotes values in the stationary state. A new stationary state distribution is attained after incorporation of exogeneous lipids, however, the perturbed stationary distribution differs only slightly from the reference state under typical experimental conditions (i.e. the amount of lipid analogues corresponds to 1–2% of endogenous phospholipids).

Figure 4 shows the time courses of appearance of labeled lipid analogues of PS, PE and PC in the inner layer

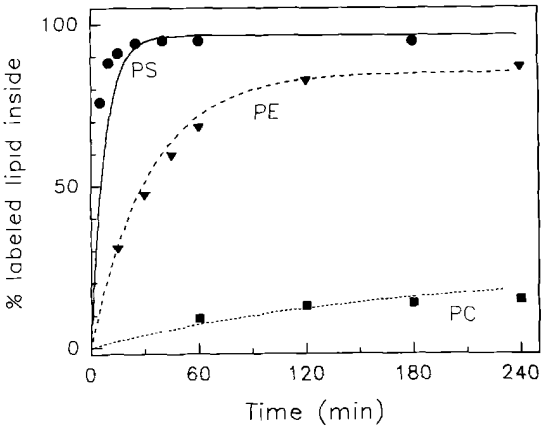

Fig. 4. The percentage of labeled lipids in the inner membrane leaflet as a function of time. Dots are experimental data obtained at 37 °C. Curves correspond to simulation by the present model. Values of the model parameters used are listed in Table 1

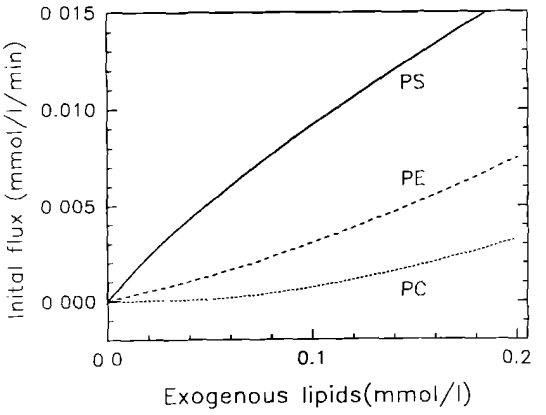

Fig. 5. Initial rate of reorientation of labeled lipids as a function of their amount added to outer erythrocyte membrane leaflet. Predictions of the model. Values of the model parameters used are given in Table 1

after incorporation into the outer layer. The dots are experimental data obtained at 37 °C while curves correspond to a model simulation. Both the initial rate of translocation and equilibrium distributions are in accordance with the previous experimental results in that PS and PE are rapidly translocated to the inner leaflet compared to the choline-containing lipid PC (Seigneuret and Devaux 1984; Calvez et al. 1988; Morrot et al. 1990). It has been shown that the behaviour of these analogues with respect to translocation kinetics as well as equilibrium distribution reflects that of endogenous phospholipids of the human erythrocyte membrane (Morrot et al. 1989).

The values of the model parameters are given in Table 1. Owing to the nonlinearity of the model, which does not provide for analytical solutions, the fitting of the model parameters has been performed by repeated numerical integration with a limited number of different sets of model parameters. Figure 4 shows that the model, besides a satisfying description of the reference state, also allows a good simulation of the redistribution of all three spin labeled lipid species. The characteristic half times of lipid redistribution estimated from the model simulation are: 6 min (PS), 23 min (PE) and 131 min (PC). It is interesting to compare these values with the characteristic half times deduced from the experimental data shown in Fig. 4: 2.0 min (PS), 26.5 min (PE) and 305 min (PC). The latter values are obtained by fitting the experimental data for each individual lipid species with the exponential equation:

$$[PL^*]_i(t) = [PL^*]_i|_{t\to\infty}(1-\exp(-kt)) \tag{13}$$

which in light of the present model has, of course, no mechanistic basis (Herrmann and Müller 1986).

Initial rate of redistribution. In order to characterize the ATP-dependent protein mediated translocation of aminophospholipids in the human erythrocyte membrane the initial rate of outside-inside translocation has been investigated at 4 °C (Zachowski et al. 1986). In these measurements labeled lipids were incorporated at various concentrations beween 0.2% and 6.0% of the endogenous phospholipid concentration. The observed saturation behaviour of the initial rate of inward translocation was described by Michaelis-Menten kinetics, estimating the maximum velocities and half-saturation constants K_m for phospholipids. The fact that the apparent K_m value for PS is almost 10 times smaller than that of PE was interpreted by the corresponding higher affinity of PS for the outer binding site on the active carrier.

To simulate those experiments the initial inward fluxes have to be calculated as a function of the amounts of lipids incorporated into the outer membrane leaflet. The expression can easily be obtained from (11) by considering only the term representing the inward flux:

$$\left.\frac{d[PL^*]_i}{dt}\right|_{t=0} = v_+^{PL}\left.\frac{[PL^*]_o}{\overline{[PL]}_o + [PL^*]_o}\right|_{t=0} \tag{14}$$

with PL = PS, PE, PC. $\overline{[PL]}_o$ is the stationary concentration of PL in the outer leaflet of the model reference state and $[PL^*]_o$ is the concentration of labeled lipid in the outer layer. By calculating the inward velocities v_+^{PL}, initial rates for redistribution of PS, PE and PC as functions of the amount of incorporated lipids were obtained (Fig. 5). It can be seen that the curves do not have a hyperbolic shape characteristic for the Michaelis-Menten equations, at least not in the concentration range of exogenous lipids considered here. One reason for this discrepancy might be that experimental observations were performed at low temperature while the model is adjusted to 37 °C. However, one has to be aware that the initial rate of redistribution is a rather complex function of added analogues. According to (14) the initial rate for the redistribution of a labeled lipid can be expressed as a product of two terms. The first one represents the inward velocity of the total lipid, and the second one the concentration ratio of added lipid to total lipid in the given membrane layer. Each term is a saturation function of the amount of lipid added. The inward flux is a saturation function due to characteristics of a carrier mediated transport system. The second term reveals a saturation

behaviour with respect to the concentration $[PL^*]_o$ with a half-saturation constant $K_m = [PL]_o$. Assuming the inward flux v_+^{PL} is saturated, the observed half-saturation constant has the meaning of the concentration of endogenous lipid in the outer layer of the model reference state. Experimentally determined K_m-values for phospholipids are indeed close to the values of concentration of PS and PE in the outer membrane leaflet (PS: ~16 μM, PE: ~165 μM) (Zachowski et al. 1986). It is interesting to note that the observed ratio of apparent K_m between PS and PE deduced from the model is the same as the ratio of their concentrations in the outer membrane leaflet, as one would actually expect from the above discussion.

Translocation of spin-labeled lipid analogues observed by the spin-label reduction technique. For intact erythrocytes the spin-labeled phospholipid analogues have been shown to become rapidly reduced at 37 °C when they come in contact with the cytoplasm on the inner membrane leaflet (Seigneuret et al. 1984). Therefore, the outside-inside translocation of spin-labeled phospholipids can also be measured simply by the loss of the label signal intensity. The remaining signal corresponds to labeled lipids in the outer leaflet. The measured kinetics of signal reduction of spin labeled lipid analogues in membranes of intact human erythrocytes at 37 °C are shown in Fig. 6 (symbols). Consistent with the fast translocation of aminophospholipids the ESR signal of labeled analogues of PS and PE is almost abolished after 30 and 120 min, respectively, while the PC-associated intensity is only slowly reduced. Fitting the measured kinetics to a monoexponential function gives the rate constant k of intensity decrease as 0.046 min^{-1} for PS, 0.020 min^{-1} for PE and 0.005 min^{-1} for PC.

To simulate these measurements by the present model the following reaction scheme is proposed:

$$PL_o^* \xrightarrow{v_+^{PL}} PL_i^{\#} .$$

The spin-labeled lipid analogues PL* (PL = PS, PE, PC) are translocated to the inner membrane leaflet where – to a first approximation – instantaneous reduction is assumed to take place. $PL^{\#}$ refers to the reduced spin label. This leads to the following differential equation

$$\frac{d[PL^*]_o}{dt} = -v_+^{PL} \frac{[PL^*]_o}{[PL]_o + [PL^*]_o + [PL^{\#}]_o} . \quad (15)$$

The inward velocity v_+^{PL} has the meaning of the total inward flux. The reduced spin-labeled lipid analogue $PL^{\#}$ exhibits transverse motion in both directions. However, for calculation $[PL^{\#}]_o$ can be neglected because its concentration in the outer leaflet is small compared to $[PL]_o + [PL^*]_o$ in the experimental window of measuring. The spin-labeled analogue is considered as a part of a common pool of this lipid species and is therefore treated in the same way as was already introduced (see above). Under the assumption that the spin label is instantaneously reduced when it enters the inner monolayer mathematical simulation was performed by numerical integration of the basic system of differential equations (1) together with Eq. (15). Calculated curves are shown in Fig. 6. A reasonable coincidence between experimental and simulated data was obtained for PE and PC. However, a significant deviation between experiment and model was obtained for PS. This can be explained by the fact that the rate constant of internal reduction is in the order of that of inward translocation of PS. Indeed, the rate constant of intracellular spin label reduction is 0.036 min^{-1} (assuming a first order mechanism of reduction kinetics), which we have determined from independent experiments (data not shown).

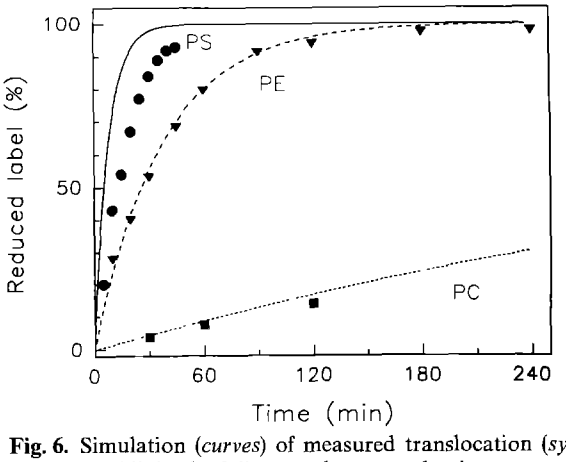

Fig. 6. Simulation (*curves*) of measured translocation (*symbols*) of spin-labeled lipids by following the spin reduction occurring on the inner leaflet of human erythrocyte membranes (for details see results)

Discussion

In the present study we have developed a model describing the translocation of phospholipids across the human erythrocyte membrane. This model is based on the current knowledge of components involved in transverse motion of phospholipids, the specifity of the translocation as well its regulation. An important feature of this model is the ATP-dependent translocation of aminophospholipids PS and PE mediated by the aminophospholipid translocase (Seigneuret and Devaux 1984). Other models have already been presented to simulate transverse lipid motion in erythrocyte membranes (Herrmann and Müller 1986; Williamson et al. 1987; Bitbol and Devaux 1988); however, their main disadvantage is that they do not allow one to differentiate between active and passive translocation of phospholipids.

In our model, transverse lipid motion is assumed to occur by different pathways: (*i*) active transport by the aminophospholipid translocase; passive motion via (*ii*) protein-mediated facilitated diffusion and via (*iii*) diffusion in the bulk lipid phase. While the existence of the translocase in the human erythrocyte membrane (and other mammalian plasma membranes) is acknowledged, candidates for those membrane proteins involved in the facilitated lipid diffusion in the red blood cell membrane are not identified and only poorly investigated. Although evidence for the existence of passive carriers (flipases) me-

diating the transverse motion of lipids has been given for subcellular membranes, such a carrier has never been reported for the human erythrocyte membrane. A typical example for such a flipase was recently described in membranes of the endoplasmic reticulum, showing that a protein in these membranes exists, which mediates a rapid, transverse motion of lipids independent of ATP (Herrmann et al. 1990). This flipase-mediated motion is (a) saturable, arguing for a limited number of binding sites and (b) unspecific with respect to lipid species and direction of transverse motion. Thus, more accurately, this protein should be named as a 'flip-flopase' as recently suggested by Devaux (1992). The molecular mechanism of this protein-mediated lipid passage is unknown. A reasonable assumption might be the existence of protein-structure induced defects at the protein-lipid boundary, facilitating the transverse lipid motion. In this respect, one may wonder whether most of integral membrane proteins – if not all – can function as a 'flip-flopase'. The effectiveness of those proteins in mediating an unspecific flip-flop may depend mainly on the ability to create appropriate defects in the protein-lipid boundary. For this reason we have made the assumption in our model that the protein-mediated facilitated lipid diffusion in human erythrocyte membranes deduced from experiments (Van Zoelen et al. 1978; Gerritsen et al. 1980; Zachowski et al. 1985) can be treated as a passive carrier mechanism (see The Mathematical Model). Moreover, with this assumption we can keep the generality of the model and allow it to be applied to other biological membranes.

When developing the model we expected from experimental investigations (Zachowski et al. 1985) that the contribution of the lipid flip-flop in the bulk lipid phase (pathway *iii*) would be rather slow and small (see also The Mathematical Model) in comparison to mechanisms (*i*) and (*ii*) and, thus, could be neglected. However, we had to realize that a third pathway is required to account for the almost equal number of lipid sites on both leaflets of the cell membrane. We have called this pathway the compensatory flux (see below) which is one of the important outcomes of this study. A stationary state in which nearly equal numbers of lipids in the monolayers are attained could only be modeled reasonably by introducing this passive compensatory flux for each lipid species. The total compensatory flux is unidirectional and depends on the difference between the total lipid sites for both halves of the membrane. With increasing differences for the amount of lipids in both layers, the passive diffusion becomes enhanced. For the model reference state the extent of this lipid flux is of the order of that mediated by the active carrier and it is larger than facilitated diffusion fluxes (Table 2). It is important to note that the necessity of the assumption of such a flux does not result from an inappropriate choice of parameter values. Without the compensatory flux the model could fit only if strong coupling between active and facilitated diffusion were assumed, so that unidirectional active translocation of aminophospholipids PS and PE could be counterbalanced by facilitated diffusion of PS, PE and PC. So far, there is no experimental evidence for such coupling. However, it is obvious that the compensatory flux depends on the activity of the translocase (Table 2). When the aminophospholipid translocase is active, the extent of the compensatory flux, in particular for PS and PE, somewhat exceeds or is even much higher than that of the inward and outward fluxes mediated via facilitated diffusion (pathway *ii*). On the other hand, when the translocase is assumed to be inhibited, the unidirectional compensatory fluxes are more than one order of magnitude slower compared to the fluxes of the facilitated diffusion pathway (see Table 2). This is in agreement with experimental data obtained in the absence of intracellular ATP (inhibition of the translocase) (Zachowski et al. 1985). Under these conditions the passive diffusion in erythrocyte membranes was significantly higher compared with the extent of transverse motion in liposomes prepared from endogenous erythrocyte lipids.

The experimental investigation of the passive PS and PE diffusion in red blood cell membranes in the presence of translocase activity is hampered by the very fast inward translocation of these aminophospholipids. While such a, under certain conditions rather high, passive (compensatory) motion (see Table 2) has never been assumed or concluded to occur in erythrocyte membranes, it is interesting to note that an enhanced outward translocation of phospholipids has been observed when the translocase is active (Bitbol and Devaux 1988; Connor et al. 1992). This has been ascribed to an active outward translocation mediated by the translocase (Seigneuret and Devaux 1984; Williamson et al. 1987). So far convincing evidence is lacking. An alternative explanation could be given in the framework of the model. Owing to the fast and effective ATP-dependent translocation of aminophospholipids from the outer to the inner leaflet both the facilitated diffusion as well as the compensatory flux are enhanced to account for an almost equal distribution of total lipid sites between both leaflets (see below). This might give also some hint for the biological significance of such a compensatory flux: Of course, it is easy to envisage that the stability of a bilayer membrane requires that the difference in the numbers of lipids between both monolayers does not exceed a certain value, since a membrane could only resist a certain maximal area difference between its two halves. Owing to the incompressibility of lipid bilayers, raising the difference in area between both layers may result in bending and, eventually, in local rupture of the bilayer (vesiculation). Similarly, increasing lateral pressure in one monolayer and bending of the bilayer may act as a driving force for translocation of phospholipids.

In the framework of our model the compensatory flux is limited to the phospholipids PC, PE and PS. We are well aware that experimental evidence is as yet lacking. However, the existence of this flux might also be discussed in the light of other membrane constituents; in particular we want to emphasize a possible involvement of cholesterol. The transverse distribution, as well as the kinetics of flip-flop of cholesterol, are still under discussion. It is important to mention that a very fast half time of cholesterol translocation of the order of seconds has been reported (Lange et al. 1981). Taking into account the fact that the molar ratio cholesterol/phospholipid is about 1

in the human erythrocyte membrane, cholesterol has to be considered as a candidate to be involved in the compensatory flux.

Another important outcome of this model is that lipid asymmetry can be explained solely by the common existense of specific and unspecific translocation pathways across the erythrocyte membrane, which differ also in their characteristic half time. It was not necessary to include some specific interactions between aminophospholipids and other membrane constituents on the inner leaflet to create the pronounced lipid asymmetry as found in erythrocyte membranes. This conclusion is consistent with recent independent experimental investigations on erythrocyte membranes showing that neither a specific interaction of lipids with the membrane skeleton – in particular with spectrin – exists nor could explain the asymmetric distribution of phospholipids (Morrot et al. 1986; Calvez et al. 1988).

In the present paper it is also predicted that the asymmetric distribution of PE is more sensitive to changes in intracellular ATP-concentration compared to PS. This is consistent with previous experimental results (Calvez et al. 1988). As expected, the asymmetric distribution does not depend linearly on intracellular ATP-concentration. Obviously, increasing the concentration of ATP to infinity would not result in an asymmetric distribution of PS where this lipid is exclusively localized on the inner leaflet (to 100%), owing to the saturation of the carrier with respect to ATP.

Usually, the translocation and final distribution of phospholipid species is determined by experiments when the distribution of endogenous phospholipids is in the steady state. A small amount of labeled lipids is incorporated into the outer leaflet leading only to a negligible disturbance. Under these conditions the kinetics of different translocation mechanisms can be assessed. This leads to the conclusion that PC is only slowly translocated across the membrane. However, from our model we can infer that under certain conditions translocation of PC may depend significantly on that of aminophospholipids via the aminophospholipid translocase which is mainly due to the limited lipid sites in each layer. Assuming an almost abolished asymmetric distribution of phospholipids the reestablishment of the activity of aminophospholipid translocase leads not only to a fast enrichment of aminophospholipids on the inner layer, but also to a rapid asymmetric distribution of PC (Fig. 2). Experimental validation of this prediction is warranted in future. However, this might be a rather difficult task, since so far there is no experimental approach reported which ensures a complete scrambling and equal transbilayer distribution of endogenous membrane lipids.

As already mentioned an attractive aspect of our model is that experimental approaches for assessing lipid distribution and transbilayer motion can be rather easily simulated. The comparison between experimental data and those obtained from the simulation suggests that the model allows reasonable description of translocation of phospholipids in the erythrocyte membrane. However, the predictive value of the model should not be overestimated since with the present status of knowledge many details of the translocation mechanism, as well as parameter values, are lacking and have to be established and verified from future experiments.

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