^{23}Na- and ^{1}H-NMR Studies of the Action of Chlorpromazine and Imipramine on Nigericin-Mediated Na^+ Transport across Phosphatidylcholine Vesicular Membranes

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In order to elucidate the action of chlorpromazine (CPZ) and imipramine (IMP) on nigericin-mediated Na^+ transport across phosphatidylcholine vesicular membranes, ^{23}Na nuclear magnetic resonance was applied to the exchange system of Na^+ ions present at the same concentration inside and outside unilamellar vesicles. CPZ and IMP added to the vesicles in micromolar concentrations produced an equal increase in the carrier-transport rate. The kinetic analysis, together with ^{1}H-NMR observations of the reduction in membrane fluidity produced by the drugs and on the direct interaction between drugs and nigericin, allowed us to conclude that the drug-induced promotion of transport occurred not from the formation step of the Na^+–nigericin complex nor from its diffusion step, but from its dissociation step. The formation of an adduct between drug and nigericin could be the cause of the drug effect and this proceeded much more efficiently at a membrane–water interface (stability constant K_b; 3×10^5 M^{-1}) than in methanol (K_b; 5×10^2 M^{-1}). The reason for the difference is also discussed.

Keywords ^{23}Na-NMR; ion transport; membrane; nigericin; chlorpromazine; imipramine

Some NMR methods,[1] combined with the use of unilamellar vesicular membranes, have recently been attracting increasing attention for the transmembrane transport of metal cations. One of these, the chemical exchange NMR method,[2] deals with a dynamic line-broadening induced by the chemical exchange of cations present inside and outside the vesicles under ionic equilibrium conditions and is applicable to rapid transport. We first applied[3] the chemical exchange NMR method to study the effects of chlorpromazine (CPZ) and imipramine (IMP) on Na^+ transport mediated by melittin channels. In the present study, we have extended this work to the nigericin transport system.

The major tranquilizer, CPZ, and antidepressant, IMP, are amphiphilic compounds consisting of a hydrophobic tricyclic ring and a polar alkylamine group. They produce various pharmacological and physiological effects[4] including actions on neuro-transmission and enzymatic processes. For a better understanding of these drug actions, studies[5-8] using simple model systems have been widely applied to drug–membrane interactions. However, the actions of these drugs on transmembrane ion-transport in model systems have been left unexplored.

Nigericin, one of the most highly characterized ion-carriers, is a polyether with both hydrofuran and hydropyran rings and is classified as an anionic carrier owing to its ionizable carboxylic acid group at one terminal.[9] It entraps a metal cation in the cavity formed by the head-to-tail hydrogen bond between the carboxyl group at one end and the hydroxyl group at the other.[10] Carrier-mediated transport has been treated[11] well in terms of a simple mobile carrier model, as presented by Painter and Pressman,[12] where the process is divided into three distinct phases. In the first phase, the carrier is initially held at a water–membrane interface and then binds a metal cation at this interface. In the second phase of transport, the neutral complex diffuses to the opposite interface. In the final phase, the complex releases the metal cation into the aqueous medium and the carrier is again trapped on the surface. To return to the original state at the first surface, a reversal of the sequence must occur, since the anionic carrier alone cannot pass through the hydrophobic core of bilayer membranes. This cycle is thus repeated without moving a net charge from one aqueous medium to the other, so that anionic carrier transport is a suitable system to be investigated by NMR chemical exchange phenomena. Riddell and his co-workers[11] have pointed out that the kinetics of Na^+ transport is first-order depending on the concentration of nigericin added to the vesicular membranes and the rate-determinig step is the dissociation of the Na^+–carrier complex. Transport in the form of a 1 : 1 complex has also been reported[13] from electrochemical measurements on planar black membranes.

With the aim of obtaining a molecular-based understanding of the effects of tricyclic tranquilizers on Na^+ transport, their action on the well-defined nigericin transport system was investigated by means of ^{23}Na- and ^{1}H-NMR. We report that CPZ and IMP exhibited a strong action on the carrier-mediated transport and the effect arises from a specific step of the three-step transport mechanism.

Experimental

Materials Egg yolk L-α-phosphatidylcholine (PC) was obtained as a chloroform solution from Sigma Chemical Co. and used without further purification. Ion-carrier, nigericin sodium salt, and detergent, *n*-octyl-β,D-glucopyranoside, were from Sigma Chemical Co. The hydrochlorides of CPZ ($CPZH^+Cl^-$) and IMP ($IMPH^+Cl^-$) from Sigma Chemical Co. were purified by recrystallization from benzene–ethanol. Sodium triphosphate (Na_5PPPi), choline chloride, and cholesterol were purchased from Tokyo Kasei Co. D_2O (purity 99.96%) and CD_3OD (purity 99.5%) were obtained from Aldrich Co. Deionized water (18 MΩ/cm) was used for every step of the vesicle preparation. To obtain a deuterated phosphate buffer, appropriate amounts of Na_2DPO_4 and NaD_2PO_4 were dissolved in D_2O. The respective phosphates were prepared from solutions of phosphoric acid-d_3 and anhydrous sodium carbonate in D_2O kept at pD 9.0 and 4.5.

Vesicle Preparation Large unilamellar vesicles (LUV) of, typically, 400 nm diameter were prepared according to the dialytic detergent-removal procedure described in the literature.[11] Briefly, this is as follows: 15 μmol PC and 225 μmol *n*-octyl-β,D-glucopyranoside were dissolved in 1.5 cm^3 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. The solution was dialyzed at 20 °C against 2 dm^3 of buffer over a period of 12 h. Removal of the detergent was completed by repeating the dialysis three times. Further dialysis was carried out twice with 10 mM sodium phosphate buffer containing 50 mM NaCl, 10 mM Na_5PPPi and 20 mM choline chloride. The last ingredient was added to balance the total ionic concentration on the inner and outer sides of the LUVs. All dialyses were performed in the dark under a constant stream of nitrogen, using media presaturated with nitrogen for 12 h. Thus, the resultant LUV suspension had the same concentration of Na^+, 100 mM plus that from the 10 mM sodium phosphate buffer (116 mM in total), inside and outside 10 mM PC LUVs. The internal anions were chloride and phosphate and the external anions were chloride, phosphate, and triphosphate. LUV samples containing cholesterol (cholesterol : PC = 2 : 10 mM/mM) were prepared in a similar manner as described above.

Small unilamellar vesicles (SUV) were prepared in the usual way[14]: 50 mM PC dispersions in 10 mM perdeuterated sodium phosphate buffer (pD 7.2) were irradiated on a bath-type sonicator (Branson B-220) under an Ar atmosphere.

NMR Measurements 1.2 cm^3 of the LUV suspension was transferred to a special NMR tube which was inserted into a 10 mm-o.d. tube containing D_2O to lock the external magnetic field. ^{23}Na-NMR measurements were carried out at 35 °C using a JEOL GX-270 FT spectrometer operating at 71.32 MHz. Typically, 8192 data points were taken over a 1 kHz spectral width and 1000 transients were collected at a pulse interval of 0.5 s with 90° pulse. To separate[11] intra- and extra-vesicular Na^+ lines, a small aliquot of $DyCl_3$ solution was added to form 2 mM $Dy(PPPi)_2^{-7}$ outside the vesicles, yielding a *ca.* 7 ppm shift difference. Nigericin sodium salt and the hydrochlorides of CPZ and IMP were used as standard solutions in methanol[1,11] and small aliquots (microliter amounts) of the solutions were added to the LUV sample. Using methanol in these amounts did not affect the Na^+ resonances. Under slow exchange conditions, the rate of Na^+ efflux (v) across a vesicular membrane can be determined from the broadening, $\Delta\nu_{in}$, of the inner Na^+ line. The latter is related[2] to the lifetime τ_{in} of a given inner Na^+ ion by $v = 1/\tau_{in} = \pi \cdot \Delta\nu_{in}$.

Spin-lattice relaxation times (T_1) of PC protons in SUVs were measured by an inversion-recovery technique[15] with a 180°–τ–90° pulse-sequence using a JEOL PFT-100 spectrometer operating at 100 MHz. All measurements were performed at 25 °C with accumulation of 9 transients, repeated at intervals of 5.0 s. Proton chemical shifts under an equilibrium of nigericin and drug in CD_3OD were observed at 35 °C using a JEOL GX-270FT spectrometer operating at 270 MHz.

Results

Figure 1 shows ^{23}Na-NMR spectra in the LUV sample: extra-vesicularly existing $Dy(PPPi)_2^{-7}$ shifted the outer Na^+ line up-field and separated the inner and outer Na^+ lines. The line-width of inner Na^+ was taken as the standard in the case of absence of nigericin and drug (Fig. 1A). Addition of nigericin sodium salt at 50 μM broadened the inner Na^+ line by 10.1 Hz, initiating Na^+ exchange mediated by the ionophore (Fig. 1B). Subsequent additions of CPZ at 10 to 80 μM caused a further broadening of the inner line, indicating that CPZ could act as a promotor of the chemical exchange (Fig. 1C to F). An enhanced exchange was also apparent from the closer approach of the two lines as spectrum Fig. 1F was approached. CPZ, itself, does not transport the ions but acts only as an enhancer of nigericin transport, because the addition of CPZ to LUVs without nigericin induced no exchange of Na^+ ions (Fig. 2). Besides, the vesicular collapsing during the successive additions of CPZ was negligible since the inner peak-intensity, relative to the outer one, remained at 0.07—0.08. The rate of Na^+ efflux (v), determined from

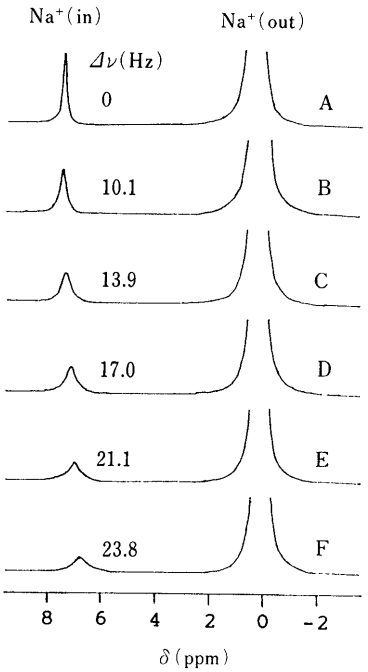

Fig. 1. NMR Spectra of Na^+ Ions Present Inside and Outside PC LUVs

[Nigericin] in μM and [CPZ] in μM: A 0, 0; B 50, 0; C 50, 10; D 50, 20; E 50, 40; F 50, 80, respectively. $\Delta\nu$: line-width broadening of inner Na^+.

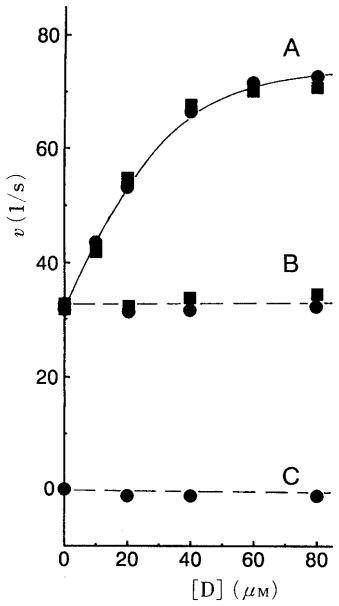

Fig. 2. Plots of Na^+ Efflux Rate v *vs.* Drug Concentration [D]

A, 50 μM nigericin in pure PC LUVs; B, 50 μM nigericin in cholesterol: PC (1 : 5) LUVs; C, no nigericin in pure PC LUVs. A solid line in A shows the curve of best fit determined by the kinetic analysis as described in the text. ●, CPZ; ■, IMP.

the relationship $v = \pi \cdot \Delta\nu_{in}$, is plotted against the concentration [D] of CPZ in Fig. 2. When the transport experiment was performed using IMP, instead of CPZ, a very similar transport enhancement was observed (Fig. 2). The slope of the v *vs.* [D] plots was reduced with an increase in [D]. The v values in the absence and presence of drug at 40 μM are listed in Table I. It was found that both drugs at this concentration induce almost two-fold faster transport.

TABLE I. Nigericin-Mediated Na^+ Efflux Rate v across LUV Membrane, with and without Drug[a]

Drug	Pure PC LUV	Chl[b]–PC (1 : 5) LUV
None	31.7	32.7
CPZ	66.6	32.4
IMP	67.8	34.6

a) v in s^{-1} ($\pm 2\,s^{-1}$ error) determined at 50 μM nigericin and at 40 μM drug. *b*) Chl; cholesterol.

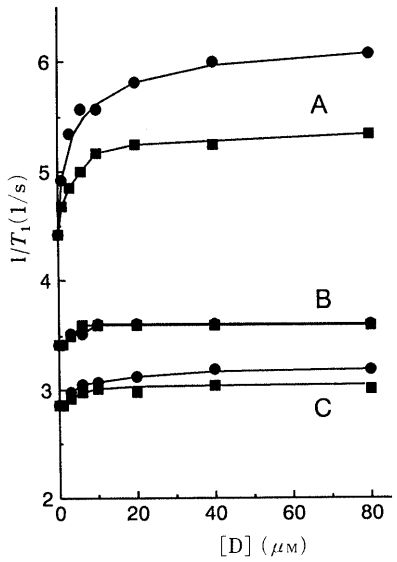

Fig. 3. Effects of Drug on $1/T_1$ in PC SUVs

A, N-methyl protons; B, chain-methylene protons; C, C-methyl protons. ●, CPZ; ■, IMP.

In order to investigate the drug effects in molecular terms, additional experiments were carried out. First, introduction of cholesterol into the PC vesicular membranes (cholesterol : PC = 1 : 5 mol/mol) did not produce any detectable change in the Na^+ efflux rate ($32\,s^{-1}$) in pure PC LUVs, irrespective of the presence or absence of drug (Fig. 2 and Table I). Second, the effect of drug on PC membrane fluidity was investigated by measuring the proton spin-lattice relaxation times (T_1) of PC SUVs in perdeuterated phosphate buffer. The relaxation rates ($1/T_1$) were determined for three kinds of protons yielding prominent peaks in the NMR spectrum; N-methyl protons at the polar end of the PC molecule, most[16] of the methylene protons in the hydrophobic acyl chains and C-methyl protons at the chain ends. As shown in Fig. 3, the addition of drug, up to 80 μM, increased the $1/T_1$ values of the respective protons: *e.g.*, the $1/T_1$ increments induced by 40 μM drug were 35.7 (N-methyl), 5.5 (methylene), and 11.5% (C-methyl) with CPZ and 19.8 (N-methyl), 5.3 (methylene), and 6.4% (C-methyl) with IMP. Considering[17] that $1/T_1$ is proportional to the motional correlation time τ in a fast-motional region, where PC molecules exist in a liquid crystalline phase, the results indicate that both drugs rigidify the membrane at the positions of the respective protons, especially at the N-methyl position. The effect of CPZ is twice as strong as that of IMP at positions where there are N-methyl and C-methyl groups.

Finally, a direct interaction between the drug and

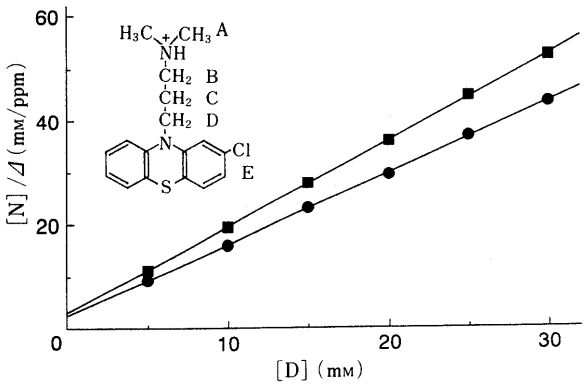

Fig. 4. Plots of $[N]/\Delta$ *vs.* [D] in CD_3OD

Correlation coefficient for a straight line; 0.9998 for CPZ (●) and 0.9999 for IMP (■). The plots are made for the proton "B" which is shown in the insert.

TABLE II. Parameters K_b and Δ_i of Drug–Nigericin Adducts in CD_3OD[a]

		CPZ–nigericin	IMP–nigericin
		K_b (M^{-1})	
		5.4×10^2	5.5×10^2
Δ_i (ppm)	A	0.57	0.50
	B	0.73	0.61
	C	0.21	0.18
	D	0.11	0.074
	E[b]	0.037	0.027

a) A to E denotes the drug proton and K_b and Δ_i have errors of $\pm 5\%$. *b*) The shift in E refers to a specific peak in the multiplet pattern.

nigericin was found by following[3] the chemical shifts of the drug protons. The sample solution was prepared so that it contained nigericin sodium salt fixed at [N] = 1 mM and an excess of drug ([D] ≥ 5 mM) in CD_3OD. In the presence of nigericin, the chemical shifts moved significantly up-field (net shift Δ). Figure 4 shows the plots of $[N]/\Delta$ *vs.* [D] made for the "B" protons (see the formula inserted in Fig. 4). The plots could be fitted closely to a modified Hanna–Ashbaugh relation[3] for 1 : 1 complex formation (D + N = DN):

$$[N]/\Delta = (1/\Delta_i)[D] + 1/(K_b \cdot \Delta_i)$$

Intrinsic shifts in the complex Δ_i and its bonding constants K_b were determined by least-squares fitting and are listed in Table II, together with the Δ_is for the remaining drug protons which were estimated from the K_b and Δs of those protons at 5 mM drug using the equation. It is rather surprising that the K_bs (*ca.* $5 \times 10^2\,M^{-1}$) of CPZ and IMP were so similar. Δ_i decreased rapidly upon going from protons "A" and "B", positioned at the polar terminal, towards the ring protons "E". This suggests that the positively charged ammonium group is an interactive locus and a counterpart of the interaction is the negatively charged carboxyl group of nigericin, the origin of the Δs coming from an electrostatic shielding effect.[18]

Discussion

As described earlier, the transport system can be treated in terms of three distinct steps:

$$N^- + M^+ \xrightarrow{k_f} NM \quad (1)$$

$$NM \xrightarrow{k_{dif}} NM \quad (2)$$

$$NM \xrightarrow{k_d} N^- + M^+ \quad (3)$$

Step 1 represents complex formation between the nigericin anion (N^-) and the Na cation (M^+), occuring at a membrane surface, step 2 represents the diffusion of the neutral complex NM towards the other membrane surface, while step 3 represents the dissociation of the complex at the surface. The same process occurs in the opposite direction, and the cycle is repeated. Thus, the transport system is in complete equilibrium which, in turn, allows Eqs. 1 to 3 to be also written[12] as equilibrium equations. The drug can participate in step 1 by means of complex formation between the drug cation (D^+) and nigericin anion. Then, the process ($N^- + D^+ = ND$) should compete with that of N^-–M^+ complex formation and lead to a reduction in the formation rate of the latter. If step 1 were rate-determining, the presence of drug would thus decrease the overall transport rate. However, this disagrees with the dynamic line-broadening of Na^+ observed with the drugs. How do the drugs act in step 2? The fluidity of the medium in which a molecule diffuses is an essential factor in the diffusion process. As shown by the T_1 measurements on PC SUVs, the reduction in membrane fluidity at both its surface and interior, on addition of the drugs at up to 80 μM, allows us to predict that the diffusion step will be slowed by the drugs. Furthermore, a stronger effect exhibited by CPZ compared with IMP in terms of membrane stiffening leads us to anticipate a larger reduction in diffusion rate produced by CPZ than by IMP. This fails to explain the observed transport, promoted equally by both drugs. A question may arise about the difference in the vesicular size used (*ca.* 20 nm for SUV and 400 nm for LUV). Cholesterol can be used[11,19] to stiffen membranes in a liquid crystalline phase and so slow the diffusion process. When the transport experiment was performed using cholesterol–PC (1 : 5 mol/mol) LUVs, no appreciable rate change was found (Table I). Again, this indicates that the diffusion step is not rate-determining.

Therefore, only step 3 can be regarded as responsible for the drug effect. This deduction is also supported strongly by a previous finding[11] that the dissociation step is rate-determining. In this way the drug would interact with the N^-–M^+ complex and destabilize it, hence hastening the release of Na^+ ion. Such a catalytic drug-action is suggested by the finding of an association between nigericin sodium salt and drug in CD_3OD (Table II). Besides, a similar association constant for CPZ and IMP with nigericin supports well as equal promotion of transport by both drugs. The working mode of drug at a water–membrane interface is then expressed by the scheme:

$$NM + D^+ \overset{K_b}{\rightleftharpoons} NMD^+ \xrightarrow{k'_d} N^- + D^+ + M^+ \quad (4)$$

When we assume fast equilibrium of the NMD^+ adduct formation, the Na^+ efflux rate v is given in the form of the competitive rate equation of 3 and 4:

$$v = k_d[NM] + k'_d[NMD]$$

Under the condition that steps 1 and 2 are rapid relative to steps 3 and 4, the equation can be rewritten in the following analytical form,[11,20] using the total concentrations of nigericin ([N]) and drug ([D]) and taking the rate constant k'_d and bonding constant K_b as variables:

$$v = k_d[N] + (k'_d/2)([N] + [D] + 1/K_b - \sqrt{([N] + [D] + 1/K_b)^2 - 4[N][D]})$$

Here, the first term $k_d[N]$, the rate in the absence of drug, is 31.7 s^{-1}. This equation was applied to the plots of v *vs.* [D] in PC LUVs. The optimum k'_d and K_b were examined by a curve-fitting procedure and were found to be within the narrow ranges of $1.0(\pm 0.1)\,s^{-1}/\mu M$ NMD and $3(\pm 1) \times 10^5\,M^{-1}$, respectively, for both CPZ and IMP. The calculated curve is seen to follow closely the plots of v *vs.* [D] (Fig. 2), indicating that the above kinetic treatment is reasonable. The fact that k'_d ($1.0\,s^{-1}/\mu M$ NMD) is larger than k_d ($0.63\,s^{-1}/\mu M$ NM) indicates the effectiveness of the catalytic step 4.

The apparent bonding constant K_b, as found in the membrane–water phase, is compared with that of *ca.* $5 \times 10^2\,M^{-1}$ in methanol, the former being nearly 600-fold greater than the latter. This is very interesting when compared with that[11] in the Na^+–nigericin complex where the K_b is much lower in membrane–water phase ($22\,M^{-1}$) than in methanol ($4.8 \times 10^4\,M^{-1}$). The reason for this needs to be discussed. Amphiphilic drugs have a high affinity[5] for lipid membranes so that those used in the present experiment will be almost completely taken up by the membranes. Incorporation of the drug into PC membranes is achieved in a state[6] where the ammonium group is located near the phosphate of the lipid polar head group and the tricyclic ring is near the α-methylenes of the fatty acyl chains. Water-insoluble nigericin is also assumed[11] to be completely taken up by the LUV membranes and to be arranged with its carboxyl group projecting into the polar aqueous region while the rest of the molecule remains in the interior of the membrane. A similar orientation of drug and carrier in a membrane surface region leads to an increased association probability for both molecules through fast two-dimensional diffusion[21] in vesicular membranes and an improved molecular interaction between them. Part of the increase in the membrane/water phase K_b must be attributed to an orientation effect as described above. The remainder may come from the hydrophobicity of the tricyclic ring of drug: it stabilizes the free drug molecule in methanol, unlike the case of the metal cation. A difference in the solvation energies[22] of the alkylammonium ion of the drug and the metal ion may also be an important factor.

The similar effect of CPZ and IMP on nigericin-mediated Na^+ transport is an intriguing observation. Generally, CPZ is know to be stronger than IMP in terms of its actions on membranes: *e.g.*, hemolysis[7] of erythrocytes, membrane ordering,[8] and membrane stiffening, as found in this study. Also in terms of ion transport, our ^{23}Na-NMR studies[3] with PC LUVs have shown 2.2-fold and 1.5-fold stronger effects due to CPZ, com-

pared with IMP, on melittin-mediated transport and gramicidin-mediated transport, respectively. For the former, the difference in drug effect was considered to be due to the difference in bonding strength of both drugs to amphiphilic polypeptide melittin. A difference in the tricyclic ring structures of CPZ and IMP induced a different drug-effect on melittin-mediated transport, but not on nigericin-mediated transport. This may mean that, in the complex of the drug with nigericin, the interaction of the hydrophobic portions is less important than the electrostatic one of the polar groups. Finally, a comment should be made on the finding that cholesterol introduced into the membrane eliminated almost entirely the drug-induced effect on nigericin-mediated Na^+ transport. This phenomenon may be interpreted in terms of the preferential interaction of cholesterol with the drug and/or nigericin and so in terms of a change in the drug-nigericin interaction. Further discussion of this is beyond the scope of this paper.

In conclusion, the cationic tricyclic drugs CPZ and IMP promoted nigericin-mediated Na^+ transport across PC LUV membranes equally. The observed drug-effect was understood on the basis of drug action on the dissociation step which determines the overall rate of transport. The action at a molecular level was attributed to an attack by the drug molecule on the Na^+–nigericin complex, which could be made much more easily at the membrane-water interface.

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