brief communication

^{23}Na and ^{1}H NMR studies on melittin channels activated by tricyclic tranquilizers

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ABSTRACT A dynamic ^{23}Na nuclear magnetic resonance (NMR) technique was applied to the exchange system of Na^+ ions present inside and outside large unilamellar vesicles at an equivalent concentration. Addition of melittin to phosphatidylcholine vesicles did not induce any detectable Na^+ transport across the membrane but subsequent addition of a trace of chlorpromazine or imipramine did induce Na^+ transport. Because the formation of a drug-melittin adduct in a solution was detected by ^{1}H NMR, the activation of melittin channels was assumed to originate from the direct interaction of the drug and melittin.

INTRODUCTION

Tricyclic tranquilizers, chlorpromazine (CPZ) and imipramine (IMP), are amphiphilic compounds that possess a hydrophobic tricyclic ring and a polar ammonium group. They have various pharmacological and physiological effects that include, in addition to a major action on neuro-receptors (Segawa et al., 1979; Seeman, 1980), hemolysis (Sheetz and Singer, 1974) and shape transformation in erythrocyte membranes (Fujii et al., 1979), anesthetic action (Seeman, 1972), and inhibition of phospholipase A_2 activity (Vanderhoek and Feinstein, 1979) and phagocytosis (Elferink, 1979). Although it is believed that some effects are protein based and others are membrane based, the molecular mechanisms remain enigmatic because of the complexity of the respective processes in biological systems. One approach would be to utilize simplified model systems. In the course of studies along this line, on the effects of the drugs on ionophore-mediated Na^+ transport across vesicular membranes as determined by the dynamic nuclear magnetic resonance (NMR) technique developed by Riddell and Hayer (1985), we have found that the drugs induce a dramatic action on the Na^+ transport using melittin as an ionophore. Melittin, the major protein component of bee venom, is an amphiphilic polypeptide consisting of 26 amino acid residues (Habermann and Jentsch, 1967) and has a lytic action on membranes (Sessa et al., 1969). At low concentrations, it is embedded in membranes in the tetrameric form (Tosteson and Tosteson, 1981; Vogel and Jähnig, 1986) and/or monomeric form (Stanislawski and Ruterjans, 1987; Altenbach and Hubbell, 1988) and functions as a voltage-dependent gated ion channel (Tosteson and Tosteson, 1984). We report here that CPZ and IMP activate melittin channels in phosphatidyl-choline (PC) vesicular membranes, probably through the direct interaction of drug and melittin.

METHODS

Large unilamellar vesicles (LUV) under conditions of ionic equilibrium were prepared by the dialytic detergent removal procedure of Riddell et al. (1988). In a typical preparation, 15 μm of egg yolk PC and 225 μm of *n*-octyl-β-glucopyranoside were dissolved in 1.5 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. The solution was dialyzed at 20°C against 2 liters of the buffer over a period of 12 h, followed by three repetitions. 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 both sides of the membrane. The LUV suspension was transferred into the inner part (1.2 ml in volume) of a double-walled NMR tube, of which the outer part contained D_2O to lock the magnetic field. ^{23}Na NMR measurements were carried out at 35°C on a JEOL (Japan Electron Optics Laboratory, Akishima, Tokyo, Japan) GX-270FT spectrometer driven at 71.32 MHz. For the separation of intra- and extravesicular Na^+ lines, a small aliquot of $DyCl_3$ solution was added to form 2 mM $Dy(PPPi)_2^{-7}$ outside the vesicles, yielding a 6–7 ppm shift difference between them. Melittin and hydrochlorides of CPZ and IMP were added by injection of small aliquots of the respective stock solutions to the vesicle solution inside the NMR tube. In a slow exchange regime, Na^+ transport rate v across the vesicular membrane can be determined from the linewidth broadening $\Delta\nu$ of inner Na^+ ions, which is related to the lifetime of the ions (τ) by the relationship $v = \pi \cdot \Delta\nu = 1/\tau$ (Riddell and Hayer, 1985). To observe the complex formation equilibrium between a drug and melittin, ^{1}H NMR was measured at 25°C on the same spectrometer operating at 270.05 MHz.

RESULTS AND DISCUSSION

Fig. 1 shows ^{23}Na NMR spectra observed on addition of melittin at 2 μM to 10 mM PC-LUV's prepared by the above procedure and after the subsequent addition of CPZ at concentrations from 10 to 80 nM. Melittin alone, even after 2 h of incubation, caused no broadening of the inner Na^+ line within the limit of an experimental error (0.5 Hz), implying that melittin did not mediate detectable Na^+ exchange across the vesicular membrane. The result seems consistent with the view that melittin behaves as voltage-dependent gated channels (Tosteson and Tosteson, 1984) being substantially kept closed under the present conditions of ionic equilibrium, although a very slow Na^+ transport was observed in the time-course measurements of NMR intensities under nonionic-equilibrium conditions (Veiro and Hunt, 1986). In fact, the ion carrier nigericin and the ion channel gramicidin A in the same concentration range as em-

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0006-3495/92/08/569/04 $2.00

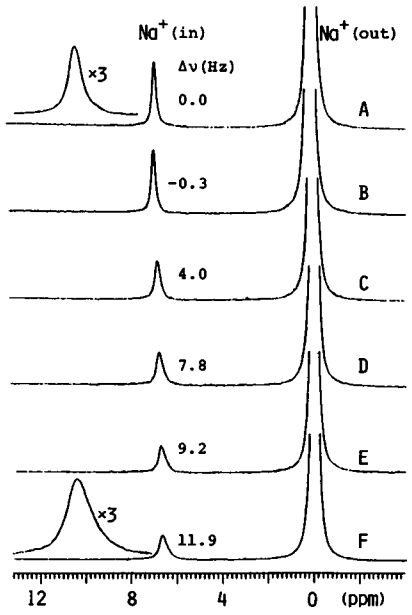

FIGURE 1 NMR spectra of Na^+ ions present inside and outside PC LUV's containing melittin at 0 (*A*) and 2.0 μM (*B–F*) and CPZ (used as $CPZH^+Cl^-$) at 0 (*A, B*), 10 (*C*), 20 (*D*), 40 (*E*), and 80 nM (*F*), together with the linewidth broadenings ($\Delta\nu$) of inner Na^+. LUV samples were prepared as described in the text and the spectra were recorded at 35°C with 8,192 data points taken over a 1-kHz spectral width and with 1,000 transients collected at a pulse interval of 0.5 s and pulse width of 90°.

ployed here, of which both act as mediators irrespective of transmembrane potential, were observed to induce exchange broadening in the present vesicles (Tanaka et al., manuscript in preparation), in accordance with previous reports (Riddell et al., 1988; Buster et al., 1988). As can be seen in Fig. 1, in the system with melittin, subsequent additions of CPZ induced a broadening of the inner Na^+ line, indicating the occurrence of exchange between the inner and outer Na^+ ions. During successive additions of CPZ, the integral ratio I_i/I_o of intra- and extravesicular Na^+ signals was held at 0.08, indicating no appreciable rupture of the vesicles. The drug-induced transport rate is plotted versus drug concentration in Fig. 2. Here, the rate (28.9 s^{-1}) at 40 nM was comparable to that induced by gramicidin (5 μM) under identical conditions (Tanaka et al., manuscript in preparation). A similar effect also could be detected for IMP but, in that case, the transport rate induced was reduced to almost a half (Fig. 2). Such effects were never observed in PC LUV's without melittin, showing that the tricyclic drugs themselves did not induce any permeability in the vesicular membrane.

The situation that a nanomolar range of drug concentration can cause a detectable Na^+ transport cooperatively with an ionophore seems specific to the combinations of these drugs and melittin. We have found (Tanaka et al., manuscript in preparation) that a micromolar range of CPZ or IMP is needed for any appreciable action on nigericin and gramicidin-mediated transports. A millimolar range of general anesthetics was used for investigation of their actions on melittin-mediated transport (Veiro and Hunt, 1986). To pursue the mechanism of the drug action, we performed additional experiments using LUV's consisting of 10 mM PC-phosphatidylserine (PS) mixture (4:1 mol/mol). Again, melittin alone induced no Na^+ transport and, indeed, the effect of CPZ observed in melittin-containing PC LUV's almost disappeared in the mixture (Fig. 2). The results may be explained by the preferential interaction of the cationic drug with anionic PS, which prevents a drug-melittin interaction. The role of the charged alkyl amine group of the drug was investigated by adding alkylammonium salts, $(CH_3)_4NCl$ and $(CH_3CH_2CH_2CH_2)_4NBr$ at 5 mM to pure PC and PC-PS (4:1 mol/mol) LUV's containing 2 μM melittin. In all cases, the Na^+ transport was not detected, suggesting that a whole drug molecule including its hydrophobic tricyclic portion is requisite for an effective action on melittin channels.

To obtain another support for drug-melittin interaction, experiments were performed to measure the chemical shifts of drug protons in a solution containing melittin at $[M]_t$ = 2.5 mM and the drug at concentrations above $[D]_t$ = 10 mM in CD_3OD. Fig. 3 shows the shifts (Δ) of CPZ protons, taking those in the absence of melittin as standards. The peculiar changes of Δ's with $[D]_t$ can be interpreted on the basis of the competition of two complex formation equilibria:

$$D + M = DM \quad \Delta = \Delta_{DM} \cdot K_{DM} \cdot [M]_t/(1 + K_{DM} \cdot [D]_t) \quad (1)$$

$$D + D = DD \quad \Delta = 2\Delta_{DD} \cdot K_{DD} \cdot [D]_t. \quad (2)$$

Here, Δ_{DM} and Δ_{DD} are the shifts Δ's in the drug-melittin adduct and in the drug dimer, respectively, and K_{DM} and

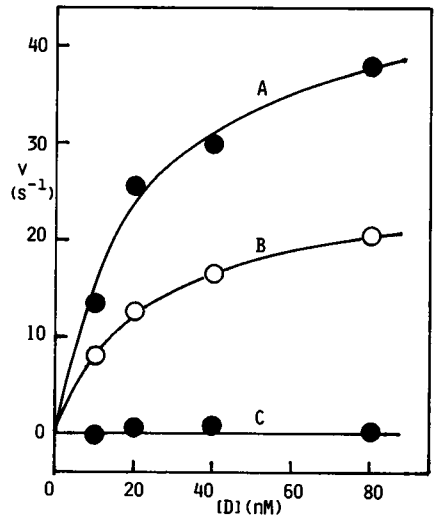

FIGURE 2 Plots of Na^+ transport rate v vs drug concentration $[D]$ in 2 μM melittin-containing LUV's at 35°C: *A;* 10 mM PC LUV with $CPZH^+Cl^-$, *B;* 10 mM PC LUV with $IMPH^+Cl^-$, and *C;* 10 mM PC-PS (4:1 mol/mol) LUV with $CPZH^+Cl^-$.

K_{DD} are the formation constants of the respective complexes. Eq. 1 was derived from $\Delta = \Delta_{DM}([D]_{DM}/[D]_t)$ and $K_{DM} = [DM]/([D] \cdot [M])$ and Eq. 2 from $\Delta = \Delta_{DD}([D]_{DD}/[D]_t)$ and $K_{DD} = [DD]/([D] \cdot [D])$ under conditions of $[D]_t \gg [M]_t$ and $[D]_t \gg [D]_{DD}$. As shown in Fig. 3, the Δ's of methyl (*A*) and methylene (*B*) attached to the alkylammonium *N* atom obey, especially in the lower range of $[D]_t$, Eq. 1 where the Δ is in a hyperbolic decay with respect to $[D]_t$. Upon going from protons *A* or *B* to ring protons (*E*), the contribution of *D-M* complexation to the corresponding Δ decreases rapidly while that of *D-D* dimerization increases slowly. The results suggest that the interactive locus of CPZ is the positively charged ammonium group, allowing us to infer that the counterpart of the interaction is the carboxyl group of Gln-26, the only negatively charged moiety in melittin. The *D-M* equilibrium was analyzed by applying the linear plot of $[M]_t/\Delta$ vs $[D]_t$ transformed from Eq. 1 to the data for protons *A* and *B* below $[D]_t$ = 100 mM. The K_{DM} and Δ_{DM} values are listed in Table 1, together with those for IMP determined by a similar procedure. The averaged K_{DM} values, 1.1×10^2 for CPZ and 2.4×10 M^{-1} for IMP, may be related to the stronger effect of CPZ than IMP on the melittin-mediated Na^+ transport, even

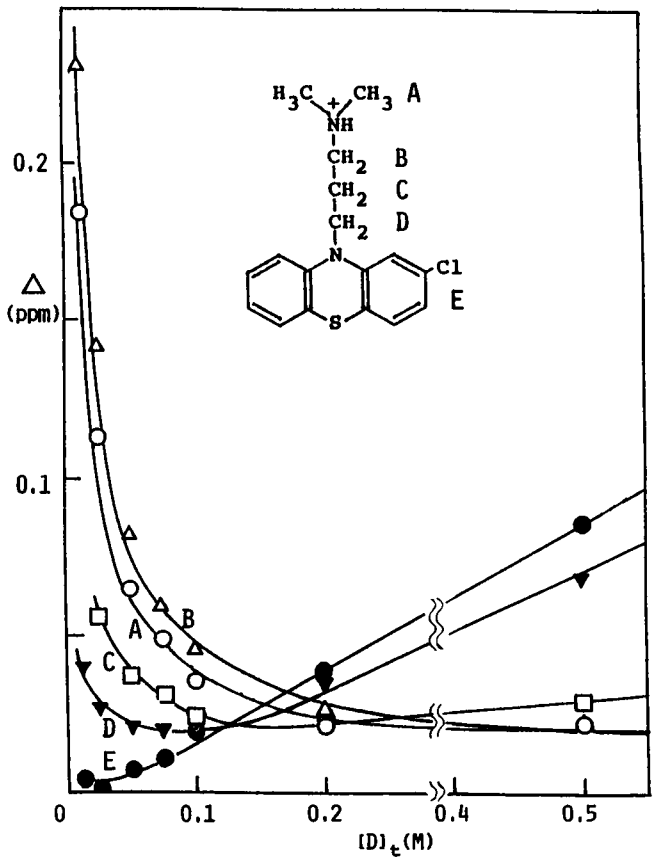

FIGURE 3 The shifts Δ's as a function of the total concentration $[D]_t$ of $CPZH^+Cl^-$ in a solution containing 2.5 mM melittin in CD_3OD. ^1H NMR spectra were recorded at 25°C and the shift of ring protons (*E*) refers to a specific peak in the multiplet pattern.

TABLE 1 The formation constants (K_{DM}) and the intrinsic shifts (Δ_{DM}) of drug protons *A* and *B* in the drug-melittin complexes

Drug	Proton	K_{DM}/M^{-1}	Δ_{DM}/ppm
CPZ	A	1.2×10^2	1.52
	B	1.0×10^2	2.00
IMP	A	2.0×10	2.86
	B	2.8×10	2.72

though the K_{DM} values could be altered for the membrane system. The large Δ_{DM} values of 1.5–2.8 ppm in the *D-M* adduct, a possible origin of which is the electrostatic shielding effect (Buckingham, 1960), suggest a close proximity between the drug protons and the carboxyl group of Gln-26.

Possible models of drug action on melittin channels may be drawn from the view of the drug-melittin adduct and the previously presented conformation of CPZ in membranes where the ammonium group is located near the phosphate of lipid polar head group and the tricyclic ring is near the α-methylenes of the fatty acyl chains (Kuroda and Kitamura, 1984). In the model of tetrameric-melittin channel with an internal pore, in which the hydrophilic random-coil segment of residues 21–26 is exposed at the membrane surface whereas the α-helical segments of the rest penetrate to the membrane interior (Vogel and Jähnig, 1986), a plausible model of drug action on the channel opening is an anchoring effect in which the drug molecule drags the aggregate deeper so as to span the membrane bilayer. An analogous model has been proposed for the effect of an applied electric field on alamethicin channels (Fox and Richards, 1982). In the monomeric model where a melittin molecule is located in the membrane to induce the wedge effect leading to the formation of disordered phase of lipids such as a pore (Terwilliger et al., 1982), the drug action may be related to the stabilization and/or extension of such a phase. It is noteworthy that, taking into account the concentrations of the drug and melittin employed here, the activation of only a part of the melittin channels incorporated into membranes was enough to induce the detectable Na^+ transport, though a definitive explanation on the drug action must await an elucidation of the working states of melittin channels.

Received for publication 7 October 1991 and in final form 18 March 1992.

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