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Binding of substance P to monolayers and vesicles made of phosphatidylcholine and/or phosphatidylserine

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Analyses of interactions between substance P (SP) and phospholipids were performed by combined surface pressure and surface potential measurements in monolayers and by ^{13}C-NMR experiments on liposomes. This study was carried out using synthetic SP molecules: [1-^{13}C-Gly^{9}]SP and [1-^{13}C-Gly^{2}]SP. Injection of SP into the aqueous subphase led to an expansion of phosphatidylcholine (PtdCho) or phosphatidylserine (PtdSer) monolayer surface area. An apparent association constant of SP for PtdSer was estimated to be around 10^{6}–10^{7} M^{-1}. The surface potential $\Delta V/n$ varied linearly with the molecular area whereas the variation of surface pressure was biphasic, suggesting that at least two binding states contributed to the monolayer expansion. These two states Si (SP is inserted into the bilayer) and Ss (SP is stuck on the surface) were observed on vesicular membranes by ^{13}C-NMR. The kinetic of interconversion between these two states can be estimated by NMR, the Ss state being the stablest one. No perpendicular insertion of SP into these vesicular preparations seemed to occur, as previously postulated. However, SP might form aggregates in contact with these model systems, leading to a loss of permeability of the lipid vesicles.

Substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$) belongs to the tachykinins, a family of peptides sharing a common C-terminal amino acids sequence (Phe-X-Gly-Leu-Met-NH$_2$ [1]). The peripheral actions of tachykinins include hypotension, vasodilatation, salivation and contraction of various smooth muscles. Three different receptors NK-1, NK-2 and NK-3 have been identified on the basis of the different rank order of potencies of the tachykinins [2,3] and on binding data [4–8]. Structure-affinity or activity relationships of constrained analogues have allowed to confirm that SP was the endogenous ligand of NK-1 receptor [9] and that NK-1, NK-2, NK-3 receptors should probably bind three different secondary structures of tachykinins [10,11]. The binding selectivity should come from the different conformational requirement of the three receptors but also from the localization of the binding site pocket in the membrane, as proposed by Schwyzer [12]. Schwyzer et al. suggested that binding to the NK-1 receptor required hydrophobic membrane association of the C-terminal part of SP, whereas the hydrophilic N-terminal region remained in contact with the aqueous phase.

As the study of SP–cell membrane interactions was not presently possible, many groups have focused their researches on model systems simulating the membrane environment, i.e. hydrophobic solvents [13,14], micelles [15–17], vesicles [18,19] and monolayers [20,21]. The structural informations gained in these model media were more or less depending on the used physical methods.

In an isotropic medium (methanol), ^{1}H-NMR studies enabled us to determine the backbone conformation of SP. The core of SP, from Pro^{4} to Phe^{8}, was folded in an α-helical structure, the N-terminal part Arg-Pro-Lys being flexible, Leu^{10} adopted a C$_7$ conformation and the C-terminal carboxamide interacted with

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Abbreviations: SP, substance P; PEG, poly(ethylene glycol); 2-lysoPtdGro, lysophosphatidylglycerol; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; 2-lysoPtdCho, lysophosphatidylcholine; PtdGro, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; HMPA, hexamethylphosphoramide; THF, tetrahydrofuran; CD, circular dichroism.

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the primary amides from both glutamines stabilizing a U-turn conformation for the C-terminal hexapeptide [13]. Circular dichroism (CD) [14], infrared and Raman spectroscopy [16,19] measurements indicated that in the presence of methanol or trifluoroethanol, SP assumed a partially α-helical conformation in addition of other structure types like β-turns and β-sheet. But these techniques did not allow to localize these different foldings in the peptidic sequence. When SP was covalently linked to poly(ethylene glycol) (PEG), addition of trifluoroethanol did not induce any helical structure, interestingly. SP linked to PEG was shown to be physiologically inactive [22]. Structure-affinity or activity relationships have shown that only the 4–8 helical part was relevant to the bioaffine conformation whereas an helical structure in the 9–11 part was not required for high binding to NK-1 receptor [7]. However, no partial helical structure was detected in a polar isotropic medium (water) at concentrations around 5 mM [16,17]. Raman spectra of SP in water indicated that, at concentration close to 30 mM, SP formed a set of structures which all were distinct from completely unfolded peptide and from the aggregated β-sheet form observed in saline solution [19].

The addition of micelles or lipid vesicles in SP solution completely changed all the spectroscopic properties (NMR, IR, Raman and CD), indicating that strong interactions between the amphiphilic molecules and SP should occur. The formation of a partial α-helical structure was also observed in micellar and vesicular media [14,15,19]. The percentage of α-helix structure decreased with the charge of the detergents (2-lysoPtdGro > SDS > 2-lysoPtdCho) [15] and with the length of their skeleton [17]. The formation of a partial α-helix in SP was much more pronounced in the presence of detergent micelles than in the presence of negatively charged lipid vesicles [19]. ^{13}C-NMR analysis carried out in the presence of paramagnetic ions indicated that the association mode of SP was depending on the size of the micelles, i.e. SP crossed the SDS micelle whereas it weakly penetrated the lipid phase of 2-lysoPtdCho [23]. Moreover, preliminary ^{13}C-NMR results suggested the presence of different types of association of SP with lipid vesicles, in slow exchange on the NMR time scale [23]. Thus, these different complexation modes due to more or less penetration of SP into the lipidic phase could induce a more or less pronounced α-helical structure relevant to the bioactive conformation.

But, to demonstrate that proteins and polypeptides really penetrate into lipidic bilayers with natural or model membranes still remains a very complicated task. A simpler approach to this problem has been to use lipidic monolayers at the air/water interface. Surface pressure and surface potential measurements have proved to be very useful in demonstrating the interactions of proteins [28] and polypeptides [29] with phospholipids and their degree of penetration within the lipid layer. In this respect, the monolayer technique has confirmed a spontaneous insertion of SP into the molecules of PtdGro [20].

To get more insight into the structure of the molecular complexes which can be formed between SP and phospholipids and on the physical parameters which can affect these interactions, an investigation was performed which combined surface pressure and surface potential measurements in monolayers, with NMR experiments on liposomes. These studies were carried out using synthetic SP molecules which were ^{13}C-labeled either in position two or nine, i.e. [^{13}C-Gly^2]SP or [^{13}C-Gly^9]SP. [Gly^2]SP is an analogue of SP which is a strong inhibitor of specific ^{125}I-BHSP binding to rat brain synaptosomes (IC$_{50}$ values being 0.8 nM and 0.6 nM, respectively). The myotropic potencies of [Gly^2]SP and SP on the guinea-pig ileum are also similar. Furthermore, the conformational behaviour of [Gly^2]SP in methanolic solution was similar to that SP (unpublished results). In methanolic solution we have never evidenced *cis/trans* isomerisation for Pro^4 and Pro^2 residues. The presented data provided evidence that (1) SP should partly penetrate PtdSer into monolayers and bilayers made of PtdSer, but (2) this penetration was strongly dependent on the lipid molecular packing.

Materials and Methods

Materials

Egg yolk L-α-phosphatidylcholine (PtdCho) type III E and brain L-α-phosphatidyl-L-serine (PtdSer) were purchased from Sigma. Potassium [^{13}C]carbonate (99% enriched) was a gift from the Commissariat à l'Energie Atomique (CEA-France). Nitromethane, butyllithium, hexamethylphosphoramide (HMPA), tetrahydrofuran (THF), bromoethane and di-*tert*-butyl dicarbamate were purchased from Aldrich. HMPA and THF were, respectively, dried on calcium hydride and on ketyl. The water used was glass distilled. Dysprosium chloride and praseodymium chloride hexahydrate were purchased from Ventrom.

Synthesis of [1-^{13}C]Gly. The [1-^{13}C]Gly was prepared starting from potassium [^{13}C]carbonate.

Synthesis of [1-^{13}C]diethyl carbonate. A mixture of potassium carbonate ($K_2{}^{13}CO_3$) (10 mmol) previously dried in vacuo at 300°C, HMPA (2 ml), ethyl bromide (20 mmol) and calcium hydride (100 mg) was sonicated for 30 min. After the addition of Bu_3 SnCl (1 mol), the mixture was sonicated in a sealed tube at 60°C for 5 h. Gas chromatography analysis of the crude reaction mixture indicated the formation of diethyl carbonate (average yield 90%). The crude product was filtered under a stream of argon and used without further purification for the next step.

Synthesis of [1-^{13}C]ethyl nitroacetate. Doubly metalated nitromethane was C-alkylated by diethyl carbonate according to the procedure of Lehr et al. [30]. The experimental conditions were slightly modified in order to trap all the diethyl carbonate formed.

Under a stream of argon, two equivalents of *n*-butyllithium (52 mmol) (1.6 M in *n*-hexane) were slowly added to a mixture of nitromethane (26 mmol) in a THF/HMPT solution (5:1) at −90°C. This solution was stirred for 45 min at −70°C. At this temperature, the crude preparation of diethyl carbonate (10 mmol) was slowly added. After stirring for 7 h at −78°C, a mixture of acetic acid (12 ml) and THF (8 ml) was added. Then, at room temperature, the mixture was poured into 100 ml of water. The aqueous layer was extracted (twice) with 150 ml of diethyl ether. The combined organic phases were washed (five times) with 100 ml of water, dried and concentrated. The resulting residue was purified by column chromatography on silica gel using diethyl ether/hexane (1:1, v/v) as eluent, 1.17 g of ethyl nitroacetate were obtained (90% yield based on the diethyl carbonate).

Synthesis of [1-^{13}C]ethyl glycinate. [1-^{13}C]ethyl nitroacetate (9 mmol) in methanol (50 ml) was hydrogenated for 1 h in the presence of 2 ml of Raney-Nickel T-1 catalyst, at room temperature, in a Parr low-pressure hydrogenator. After filtration, the solution was concentrated to give the ethyl ester of glycine in 90% yield.

Synthesis of [1-^{13}C]N-Boc-glycine. The *N*-Boc-glycine was conveniently obtained by hydrolysis of the ethyl ester function followed by treatment of the resulting glycine with di-*tert*-butyl dicarbonate.

Solid-phase peptide synthesis of [1-^{13}C-Gly^9]SP and [1-^{13}C-Gly^2]SP. Peptide syntheses were carried out manually. The side chains of Arg and Lys were protected, respectively, by the tosyl (Tos) and benzyloxycarbonyl (Cbz) groups. Starting from a methylbenzhydrylamine resin (1 g, 0.29 mmol/g) all the amino acids were coupled by the dicyclohexylcarbodiimide 1-hydroxybenzotriazole method in DMF/dichloromethane (1:5, v/v), except for *N*-α-Boc-Gln which was coupled as its *p*-nitrophenyl ester in DMF. The coupling efficiency was monitored with the Kaiser test.

The crude peptides obtained after cleavage from the resin by HF were purified as previously reported [31]. Thin-layer chromatography was carried out on precoated silica gel plates (Merck F-254) with the following solvent systems (v/v): eluent A, *n*-butanol/acetic acid/water (4:1:5); eluent B, *n*-butanol/acetic acid/pyridine/water (5:1:5:6).

[1-^{13}C-Gly^9]SP. TLC: R_f 0.13(A), 0.70(B). HPLC: (Lichrosorb RP-8), isocratic 1.5 ml/min 22%, CH_3CN in triethylammonium phosphate buffer (pH 3.0), R_t = 13.5 min (98% min. purity). $[\alpha]_D^{19}$ −82° (*c* 0.5, 10% acetic acid). amino acid analysis: Met, 0.99; Leu, 0.91; Gly, 0.97; Phe, 2.15; Gln, 2.3; Lys, 1.03; Arg, 1.1.

[1-^{13}C-Gly^2]SP. TLC: R_f 0.13(A), 0.70(B). HPLC: (Lichrosorb RP-8), isocratic 1.5 ml/min 19.8%, CH_3CN in triethylammonium phosphate buffer (pH 3.0), R_t = 13.6 min (98% min. purity). $[\alpha]_D^{19}$ −52° (*c* 0.5, 10% acetic acid). Amino acid analysis: Met, 0.94; Leu, 0.78; Gly, 1.8; Phe, 1.74; Gln, 1.84; Lys, 0.95; Arg, 0.94.

Preparation of the phospholipid vesicles: small unilamellar vesicles (SUV). Phospholipids alone or mixture of phospholipids were first dried under vacuum and then diluted in hexane and dried once again under vacuum overnight. The dry phospholipids were suspended in deionized water at pH 6.8 (no buffer) and sonicated using a Sonifier MSE, under argon. Sonication was carried out intermittently at 4°C and room temperature until no further change in turbidity occurred (30 min). The turbidity of the solution was measured in a 1 cm cell at 300 nm using a Beckman 34 spectrometer.

Methods

Electron microscopy. One drop of vesicles was placed on a formar-coated grid and after 30 s one drop of 2% uranyl acetate was added, 2 min later the solution was drained off with filter paper. The grid was dried under vacuum for 12 h The grids were examined in a Jeol JEM-100 CXII electron microscope. A uniform population of single bilayer vesicles having an average diameter of 300 Å was observed for egg phosphatidylcholines. Some small multilamellar vesicles were detected in the presence of brain phosphatidylserine.

NMR measurements. (a) Sample preparation. A 2 ml solution of small unilamellar vesicles (SUV) (10 mg/ml) made of a mixture of PtdCho and PtdSer (4:1, w/w) was introduced through a syringe, under argon, in a 10 mm NMR tube stopped with a rubber septum. Then, 0.2 ml of a solution of the peptide (0.5 to 1.5 μM) dissolved in water was added dropwise under vigorous vortexing, in order to avoid the precipitation of SP-SUV complex.

(b) Fourier transform ^{13}C-NMR. The spectra were obtained at 125.76 MHz on a Brüker AM 500 using a 10 mm VSP probe; 90° pulses (13 μs) and composite pulse decoupling (CPD) centered around 5 ppm in the ^{1}H spectrum were used for the acquisition of routine spectra. ^2H_2O was used as the lock and the methyl of choline served as a test for the homogeneity. Sample temperature (40°C) was controlled with the Bruker variable temperature unit. A correct signal/noise ratio was obtained after 3000 accumulations by using a 200 ppm spectral width (digital resolution = 0.6 Hz/pt) and a recycle time of 2 s.

Monolayer measurements. Surface pressure was measured by means of a platinum plate connected to a torsion balance of our fabrication. Surface potentials were measured by using two Americium electrodes. Details of the experimental set-up and the way to obtain compression isotherms are described elsewhere [32].

Lipids were spread as chloroform/methanol (5:1, v/v) solutions. Ultrapure water from a Milli-Q apparatus (Millipore) was used to prepare the subphases which consisted of 20 mM sodium phosphate at pH 5.8. These conditions of low ionic strength and slightly acidic pH were used in order to limit the polypeptide auto-association [27,34]. Through all experiments, reference surface potentials of aqueous subphases were around 20–30 mV. Film compressions were reproducible to within 1% ($\pm 5 \cdot 10^{-3}$ nm^2) whereas the reproducibility of surface potential determinations was estimated to be around ±5 mV. Experiments were carried out at 20°C.

For measuring the binding of substance P to phosphatidylserine in monolayers (Fig. 1), a small circular trough (40 ml volume) milled from Perpex was used. The lipid was spread at an initial surface pressure of 10 mN/m. Then, a 4 mM substance P solution in the 20 mM sodium phosphate buffer was stepwise added underneath the lipid film, in order to obtain the desired bulk concentration. In the low concentrations range used (< 1 μM), auto-association of substance P was very limited [34,27]. The subphase was gently stirred using a small bar magnet in order to facilitate the diffusion of substance P from the bulk towards the water/lipid interface. Equilibrium was reached within 10 min, in agreement with other reports [20].

Film compression experiments were carried out on preformed subphases containing substance P at a concentration of 1 μM. In this case, and before starting film compression, equilibrium completion (within 10 min) was assessed by the stability of the ΔV recording.

Results

Monolayer experiments

Fig. 1 shows the change in surface pressure as a function of SP concentrations in the subphase, in the absence (curve a) and in the presence (curve b) of a film of phosphatidylserine (PtdSer).

In the absence of lipid, substance P already affected the water surface tension for a concentration as low as $5 \cdot 10^{-9}$ M ($\pi \sim 2$ mN/m). Increasing substance P concentration in the subphase led to a substantial and sigmoidal-shaped pressure increase which reached its maximum value ($\pi = 16$ mN/m) for a SP concentration of 10^{-5} M, with an inflexion point for a SP concentration of $2 \cdot 10^{-7}$ M. These data indicated a marked amphiphilic character of SP, with a propensity to form a monomolecular film by itself at the air/water interface.

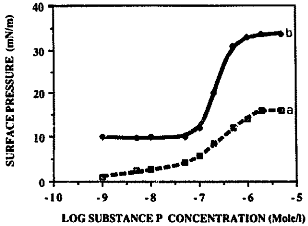

Fig. 1. Changes in surface pressure π versus substance P concentration in the subphase, at constant surface area. Curve (a) was obtained with Substance P alone. Curve (b) was recorded in the presence of a film of bovine brain phosphatidylserine spread, at an initial surface pressure of 10 mN/m.

In the presence of PtdSer (initial surface pressure: 10 mN/m), addition of SP into the subphase brought about a significant pressure increase for concentrations higher than 10^{-7} M. Then, a marked S-shaped pressure increase was observed, which reached its maximum value ($\Delta\pi = 24$ mN/m) for a SP concentration of $2 \cdot 10^{-6}$ M. It should be noted that curves (a) and (b) are practically homothetic. However, the fact that $\Delta\pi$ was larger in the presence than in the absence of lipids provided evidence for interactions between the polypeptide and the lipid molecules in the monolayer and suggested partial penetration of the polypeptide into the lipid film.

Data in Fig. 1 indicate that SP–PtdSer interactions reached nearly saturation for 1 μM SP concentration in the subphase. This concentration was used in the following film compression experiments.

As shown in Fig. 2, PtdCho and PtdSer exhibited similar compression curves in the absence of SP. In both cases, a film collapse was observed at a surface pressure of 42 mN/m corresponding to a limiting molecular area of 0.45 nm^2. In the presence of SP, a slight film expansion, around 0.1 nm^2, was found for PtdCho, at any surface pressure, indicating a slight interaction of polypeptide with lipid film. A large film expansion was observed for PtdSer up to a surface pressure of 30 mN/m. Above this pressure the compression isotherm tended to converge with that recorded in the absence of SP. A film collapse was still observed, at a slightly higher surface pressure of 45 mN/m but with the same limiting molecular area of 0.45 nm^2. Such observation strongly suggests that the interactions of SP with PtdSer molecules was depending on the lipid molecular packing. This is more clearly evidenced in Fig. 3 where the data are expressed in terms of relative area increase $\Delta A/A$, a parameter which was representative of the number of SP

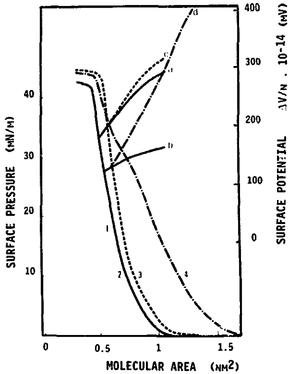

Fig. 2. Compression isotherms (1, 2, 3, 4) and surface potential data (a, b, c, d) for egg phosphatidylcholine (1, 3; a, c) and bovine brain phosphatidylserine (2, 4; b, d) in the absence (1, 2; a, b) and in the presence (3, 4; c, b) of substance P in the subphase. The subphase was 20 mM sodium phosphate, at pH 5.8. Subtance P concentrations was 1 μM. The curves shown are recorder traces. Symbols are just used for curve identification.

molecules which were in interaction with the lipid film [20]. More precisely, assuming that the conformation of SP did not change upon film compression and that of PtdSer was not significantly affected by SP interactions, then $\Delta A/A$ is directly proportional to the SP/PtdSer molar ratio. On these grounds, the curve of Fig. 3 indicates that the SP molecules which are able to penetrate into the lipid film at low and moderate surface pressure are squeezed out from the lipidic film for surface pressure above 35 mN/m.

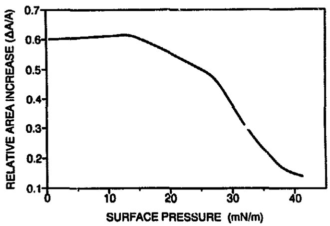

Fig. 3. Relative area increases $\Delta A/A$ versus surface pressure for a film of bovine brain phosphatidylserine in the presence of 1 μM substance P in the subphase. This curve was obtained from compression isotherms (1) and (4) in Fig. 2.

These experiments were completed by surface potential measurements which gave interesting information on the permanent dipole moment and net electric charge of any molecule present at the air/water interface [35]. Data are given in terms of $\Delta V/n$, which takes into account changes in surface lipid density resulting from film compression [36] (Fig. 2). Relatively high surface potential values were recorded for PtdCho, which is a zwitterionic and therefore neutral molecule over a wide pH range [37].

Lower $\Delta V/n$ values were obtained for PtdSer, owing to the presence of one negative charge on this lipid, in the conditions of pH and ionic strength used [37]. A slight ΔV increase was observed for PtdCho in the presence of SP, accounting for a slight adsorption of the cationic polypeptide onto the lipid film. This change in ΔV tended to be nil at high surface pressure. SP–PtdSer interactions brought about a very large and positive $\Delta\Delta V$ change at low surface pressure, but which also rapidly decreased down to be almost nil near the film collapse. Altogether, π and ΔV data clearly indicate that SP is capable to interact with PtdSer molecules, but to an extent strongly depending on the lipid molecular packing. All these data support the idea that SP is expelled from the lipid layer at high surface pressure.

Vesicles

The interactions of SP with lipids have been studied by ^{13}C-NMR with neutral and negatively charged vesicles made of, respectively, PtdCho and a mixture of PtdCho and PtdSer (4:1, w/w).

Characterization of vesicles. Sonication of pure PtdCho or of a mixture of PtdCho and PtdSer led to small unilamellar vesicles which gave a well resolved ^{13}C-NMR spectrum. Addition of Dy^{3+} or Pr^{3+} ion shift reagents allowed us to differentiate the inner and outer layers of the vesicle [38]. The ratio of the *N*-methyl carbon (Fig. 6a ^{1}) resonances for inner and outer layers was close to 2/1 but it significantly decreased when PtdCho/PtdSer mixtures were sonicated due to the presence of little amount of small multilamellar vesicles as detected by electron microscopy. However, this ratio remained constant during 24 h at 45°C, demonstrating the stability of the vesicles.

Addition of SP to vesicles of PtdCho or PtdCho/PtdSer. By electron microscopy, no structural modification of the vesicle could be noticed upon addition of SP.

Addition of [1-^{13}C-Gly^9]SP (0.3 μM) to vesicles of PtdCho or PtdCho/PtdSer (25 μM) produced no noticeable change in the ^{13}C-resonances of the phospholipids (Fig. 6b). However, some modifications in the resonances of the peptide were detected. With PtdCho

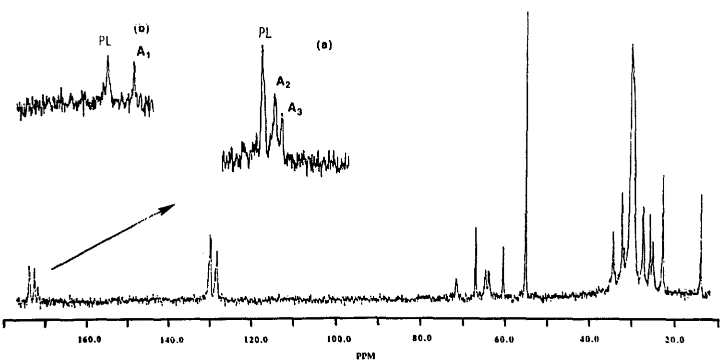

Fig. 4. NMR spectrum of SP (1.5 mM) PtdCho/PtdSer vesicles (10/2.5 mM) complex. (a) Enlargment of the carbonyl region of [1-^{13}C-Gly^9]SP. (b) Enlargment of the carbonyl region of [1-^{13}C-Gly^2]SP.

vesicles, the resonance of carbonyl Gly^9 was deshielded (0.2 ppm) in comparison to its chemical shift in aqueous solution. With PtdCho/PtdSer vesicles, the resonance of carbonyl Gly^9 was splitted in two major absorptions at 172.74 ppm (A$_2$) and 171.65 ppm (A$_3$) (Fig. 4a). The linewidths of these resonances $\nu_{1/2} = 35$ Hz (A$_2$) and $\nu_{1/2} = 25$ Hz (A$_3$) were larger than those observed in water ($\nu_{1/2} = 4$ Hz). In some experiments, two other transitory resonances of very weak intensity were observed either at 170.14 ppm or 173.2 ppm. But the half-lifetime of these states were too small to be analyzed.

The carbonyl resonance of [1-^{13}C-Gly^2]SP (A$_1$) was not splitted in the presence of PtdCho vesicles or PtdCho/PtdSer vesicles (Fig. 4b). It was broadened ($\nu_{1/2} = 20$ Hz) and shielded (0.3 ppm) in comparison to the parameters obtained in aqueous solution (171.31 ppm, $\nu_{1/2} = 3$ Hz).

Effect of SP concentration. The chemical shifts of A$_2$ and A$_3$ resonances at 172.74 ppm and 171.65 ppm varied with the concentration of SP. However, this shift was not accompanied by any modification of the linewidths. The A$_3$ resonance at 171.65 ppm was deshielded to 171.86 ppm.

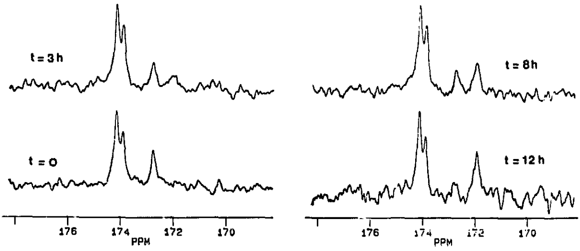

Fig. 5. NMR spectrum of SP (1.5 mM) with PtdCho (10 mM)/PtdSer (2.5 mM) vesicles. Evolution of the carbonyl region during the time. $t = 0$, A$_2$ resonance is the only one observed, $t = 12$ h, A$_3$ resonance is the only one remaining. The two upfield resonances correspond to the carbonyl resonance of the phospholipids outside and inside the bilayer [46].

Time dependence. The ratio of the resonances at 172.75 ppm and 171.65 ppm changed during the experiment (Fig. 5). At the beginning of the experiment, the resonance at 172.74 ppm was predominant. After eight hours, the resonance at 171.65 ppm was the only one observed. This change in the A_2/A_3 resonances ratio was similar when SP was added to vesicles which were first left for four hours at room temperature before adding SP. Furthermore the addition of NaCl (50 μM) speeded up this effect.

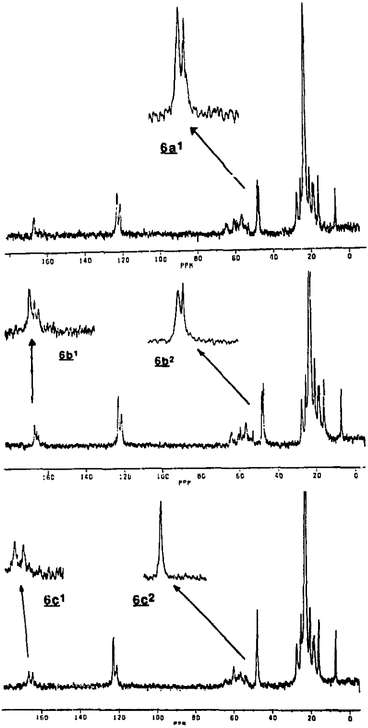

Fig. 6. An NMR spectrum of PtdCho/PtdSer vesicles (10/2.5 mM) and 0.5 mM $DysCl_3$. ($6a^1$) Enlargment of the *N*-methyl resonances, the *N*-methyl resonances of the outer face are deshielded. ($6b^1$) Enlargment of the carbonyl region showing the increase of the A_3 resonance. ($6b^2$) Enlargment of the *N*-methyl region indicating the ionic impermeability of the vesicles in the presence of SP. (6c) The same sample 12 h later. ($6c^1$) Enlargment of the carbonyl region indicating the absence of Si state. ($6c^2$) Enlargment of the *N*-methyl region showing the loss of permeability.

Salt effect. The addition of paramagnetic ions (Dy^{3+} or Pr^{3+}) to the SP/PtdCho-PtdSer mixture produced a deshielding and a broadening of the *N*-methyl carbon resonance of PtdCho. The amplitudes of these modifications were depending on the molar ratio SP/Dy^{3+}. Increasing Dy^{3+} concentration from 0.1 μM to 0.5 μM diminished the intensity of the A_2 resonance of [1-^{13}C-Gly^9]SP without any broadening of shift. At higher concentration of Dy^{3+} (10 mM), only a sharp resonance A_3 at 171.82 ppm was detected. The disturbance due to the presence of Dy^{3+} on the carbonyl carbon of Gly^9 was similar to that observed upon addition of NaCl (50 μM). Thus, the modifications observed were not due to the use of paramagnetic ions but should correspond to an equilibrium between different associated states of SP with the lipid vesicles.

Ionic permeability. In the presence of Dy^{3+} (0.1 μM) at low concentration of SP (0.5 μM), the resonances of the *N*-methyl carbons were splitted even after 24 h. When the concentration of SP was increased to 1 μM, the splitting faded over a period of 8 h (Fig. 6c). The resulting resonance was large and its chemical shift positioned between the initial resonances.

Discussion

Substance P is a cationic and amphiphilic polypeptide which has recently been shown to display surface activity [40]. The monolayer experiments reported above confirm these observations. In the conditions of pH and ionic strength used, they indicate that adsorption of SP at the air/water interface reached saturation for a polypeptide concentration of 1 μM in the subphase. These monolayer experiments also showed that for subphase concentration above 0.1 μM, SP interacted with neutral and acidic phospholipids with, presumably a modification of their molecular packing.

The present results clearly show that insertion of SP did occur at low ionic strength with uncharged monolayers while no insertion was observed at high ionic strength [20]. Since PtdCho is an electrically neutral lipid in the conditions of pH used, it is more than likely that hydrophobic forces were involved in the interactions of SP with this lipid and that the film expansion which was observed should be related to the insertion of hydrophobic residues of SP into the lipid matrix.

The extent of the monolayer expansion depends on the electric charge borne by the polar head of the phospholipids. Injection of SP underneath a film of PtdCho, induced a weak film expansion which was independent of the surface pressure. Changes in the surface potential were also observed.

Films expansions were larger with negatively charged monolayers (PtdCho/PtdSer mixtures), as compared to PtdCho, and reached a maximum with PtdSer alone. Large and positive changes in surface potential were also observed in the presence of PtdSer, indicating that, in addition to hydrophobic forces, electrostatic forces stabilized SP–PtdSer interactions. But, quite interestingly, with PtdSer, the film expansion was shown to strongly depend on the lipid molecular packing. Indeed, increasing the surface pressure resulted in a progressive decrease of $\Delta\Delta V$ and in a biphasic decrease of $\Delta A/A$, both parameters being nearly nil near film collapse.

It is worth emphasizing that these observations strongly differ from those reported for SP interacting with PtdGro [20]. In this case, lower film expansions were observed, larger SP concentrations in the subphase were required and $\Delta A/A$ exhibited a regular monophasic decrease with increasing surface pressure. This suggests that, not only electrostatic forces, and as mentionned above hydrophobic forces, are involved in the interaction of SP with lipids, but that the structure of the lipid polar headgroups also plays a role. The importance of polar headgroups in drug/lipid interactions was clearly put forward when comparing the binding properties of the anti cancer drug Celiptium with phosphatidylglycerol and cardiolipin [41,42].

The fact that $\Delta A/A$ decreased in a biphasic manner strongly suggested that at least two binding states were required to describe the binding of SP to PtdSer, respective contributions of which depend on the lipid molecular packing. State one, which existed at low and moderate surface pressure ($\pi < 35$ mN/m) and led to a film expansion, might correspond to a partial penetration of SP between the lipid molecules. State two, which prevailed at high surface pressure ($\pi > 35$ mN/m) might correspond to a simple adsorption of SP at the lipid/water interface.

In these conditions, it was difficult to determine a binding constant for SP to PtdSer at any surface pressure, in particular for pressures above 35 mN/m where, more than likely, state one and state two still coexist in the monolayer. This is why experiments of the type shown in Fig. 1 were restricted to the surface pressure range of 10–35 mN/m which, in Fig. 2, should correspond only to state one of binding of SP to PtdSer. From data in Fig. 1, one can roughly estimate an 'apparent' binding constant of 10^6 to 10^7 M^{-1} for this state one. In the case of acidic lipids, SP, which is a positively charged molecule, is expected to accumulate at the lipid/water interface, in response to the negative electrical potential ψ_0 which is generated by the negative charges borne by the lipid molecules. Correcting for this effect would lead to an 'intrinsic' binding constant of lower value. In any case, an apparent binding constant around 10^6–10^7 M^{-1} should account for a relatively high affinity of SP for PtdSer. This is in agreement with previous determinations which also yielded rather high binding constants of SP to phospholipid. These values, which were obtained by means of different approaches and for different phospholipid species, also correspond to a rather wide SP concentration range. Thus, several values have been estimated: 10^7 M^{-1} from the partition of a mixture of SP and PtdSer between buffers and chloroform/methanol solutions at pH 7.21 [39], or a value of $2.5 \cdot 10^5 < K_a < 3.7 \cdot 10^4$ M^{-1} for the association of radioactive analogues of SP [^{125}I-Tyr^8]SP or [^{3}H-Nle^{11}]SP to unilamellar vesicles of phosphatidylcholine and phosphatidic acid mixtures [43], 21 M^{-1} and $2.7 \cdot 10^7$ M^{-1} for the association of SP to anionic membranes with Gouy-Chapman surface potentials of 0 mV and −120 mV, respectively [44], and $3.3 \cdot 10^4$ M^{-1} for the binding of SP to a monolayer of PtdGro [20].

The lateral pressure of PtdCho/PtdSer vesicles has been estimated to be around 25–30 mN/m [46]. Accordingly and taking into account the monolayer experiments, the insertion of SP into the lipid bilayer was expected to be possible. Therefore, SP molecules which were selectively ^{13}C-labeled in position 2 or 9 were incubated with vesicles made of a mixture of PtdCho and PtdSer. ^{13}C-NMR spectra of these labeled SP molecules also enabled different association states of SP with the lipid bilayer to be characterized. Only one resonance A_1 was observed for [1-^{13}C-Gly^2]SP whereas two resonances A_2 and A_3 were detected for [1-^{13}C-Gly^9]SP. The vesicles are metastable states which anneal over many hours and substance P could trap or induce certain states. In these states, the chemical shifts of carbonyl Gly^9 of SP could have different values. This hypothesis can be dismissed since: (1) the same spectra were observed with freshly prepared vesicles or with vesicles left for 4 h at room temperature before the addition of SP; (2) no noticeable vesicle interaction was observed by electron microscopy; (3) the stability of the vesicles during the experiment was clearly established for low concentrations of SP and Dy^{3+} since the ratio of the A_2 and A_3 resonances changes with time whereas both in and out N-CH_3 choline resonances remained invariant. The A_1, A_2 and A_3 ^{13}C-carbonyl resonances were all deshielded as compared to those found for SP alone in the absence of lipids, indicating a strengthening of hydrogen bonds. A plausible interpretation was that the two carbonyl groups which were related to the A_1 and A_3 resonances would correspond to carboxamides located at the lipid/water interface and that the more deshielded A_2 resonance of [1-^{13}C-Gly^9]SP would account for the C-terminal part of SP inserted into the lipid membrane.

At a molecular level, the exact conformation of SP in interaction with lipids is still unknown. Recently, and on the ground of thermodynamic calculations, Schwyzer et al. have proposed a model for the insertion of SP into lipid bilayers. In this model, nine residues of the C-terminal sequence should be transferred into the hydrophobic phase as an α-helical domain, oriented nearly perpendicularly to the membrane surface, the N-terminal residues remaining in the aqueous phase with their charges exposed to water [44] (Fig. 7a). In such a configuration and orientation, SP would have a calculated molecular dipole moment of 124 debye, oriented perpendicularly to the membrane surface, with its positive end pointing towards the aqueous phase and a molecular area estimated at around 240 Å^2 [20,40]. According to simple electrostatic theory [45], one SP molecule should then contribute negatively to the surface potential by a value $\Delta V_p = 12\pi \cdot \mu(\text{millidebye})/A(\text{Å}^2) = -19\,500$ mV. When interacting with phosphatidylserine, SP led to a film expansion. At a surface pressure of 20 mN/m, a ΔA increase of 35 Å^2 was observed which, according to the above model, would correspond to the interaction of 0.14 molecule of SP per PtdSer molecule. A negative contribution to the surface potential of approx. -2850 mV would result. Even though these calculations were approximative, they indicate that according to this model, SP would considerably decrease the surface potential of PtdSer monolayers. In fact, only moderate and positive increases in ΔV were observed at low surface pressure ($+130$ mV at $\pi = 10$ mN/m), suggesting that the model of Schwyzer (Fig. 7) should be discarded. By itself, SP contributed only poorly to the surface potential and the positive ΔV changes which result from PtdSer–SP interactions may be partially accounted for by neutralization of the negative charges of PtdSer molecules by the positive charges of SP molecules. Quite recently and on the grounds of Raman and CD spectroscopy, this model of interaction of SP with lipids was also rejected [19]. SP would contain less than 20% helical structure and would form a β-turn when associated with negatively charged lipid vesicles. Thus, the contribution of SP to the polarization potential ΔV_p should be weak because of a small dipole moment in this molecule. However, one cannot exclude that when adsorbed at the membrane surface, SP might adopt a partial helical structure with its dipole moment oriented parallel to the membrane surface and therefore with no contribution to the surface potential.

As mentioned above, the lateral pressure of PtdCho/PtdSer vesicles is not known accurately, which makes difficult a strict correlation between monolayer and bilayer data to be achieved. Nevertheless, the pressure range of 25–30 mN/m which is estimated for these vesicles [44] corresponds to the pressure range over which a transition between two states was observed in monolayers (Fig. 2). Therefore, and on account of our monolayer and bilayer data, we propose a new model of binding of SP with lipids, with two binding states, which are tentatively described as state Si (SP is inserted into the lipid layer) and state Ss (SP is stuck on the lipid surface) in Figs. 7b and 7c, respectively.

According to the value of the surface potential, the peptidic backbone of Ss state should be on the surface with some hydrophobic side-chains pointing inside the membrane. In the Si state, there should be a penetration of at least the three last amino acids (Gly-Leu-Met-NH$_2$) inside the membrane. More experiments are necessary in order to determine the exact degree of penetration of SP into a lipid layer. In particular, a precise knowledge of the stoichiometry of the binding of SP with neutral and acidic lipids as well as of the corresponding binding constants should be of great help in such studies.

Whatever the amplitude of the penetration of SP into the bilayers should be, these two modes of interactions Si and Ss were in equilibrium. Since the chemical shifts of the resonances corresponding to Si and Ss, i.e. A$_2$ and A$_3$, respectively, depend on the concentration of SP, each resonance may reflect a fast equilibrium between other states. The shielding of these two resonances with increasing concentrations of SP could be relevant to the aggregation of SP in the Si state inside the membrane and to the fast exchange between Ss state and free SP. According to kinetic data, Si was the major state at the initial time whereas Ss became the

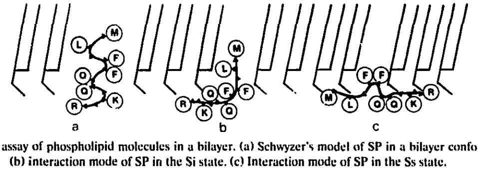

Fig. 7. Schematic view of an assay of phospholipid molecules in a bilayer. (a) Schwyzer's model of SP in a bilayer conformation and orientation. (b) interaction mode of SP in the Si state. (c) Interaction mode of SP in the Ss state.

major state after 12 h. Since the surface area of the vesicles cannot dramatically change, it was likely that the incorporation of SP (Si) into the vesicular membrane would increase the surface pressure, thus leading to a constrained complex (Si) which evolved towards a less constrained state (Ss). Even for a short time, only the Ss state was present at high ionic strength. This observation can be explained either by a speed up of the outflow of SP from the membrane or either by a speed down of the entry of SP into the membrane. The monolayer experiment agreed with the first explanation since a stable insertion was always obtained after 5–10 min regardless of the ionic strength. Interestingly, the change from Si to Ss was accompanied by the loss of ionic permeability at high concentration of SP: the addition of low concentration of paramagnetic ions allowed to distinguish the two ^{13}C resonances of methylcholine inside and outside the vesicle. At low concentration of SP the ratio of these two resonances was invariant during the experiment (24 h). At higher SP concentration, these two resonances coalesced after 6 hours, indicating that Dy^{3+} penetrated into the vesicle.

To conclude the physicochemical studies reported in this paper clearly indicate that different binding states are required to describe the association of SP with phospholipids and that their respective contribution depend onto the lipid molecular packing. This is an important observation which would be accounted for when considering the binding of SP with biological membranes. In this respect, whether the Si and Ss binding states we propose, are involved in the process of molecular recognition of SP to the NK-1 receptor, is an important question.

The surface pressure of synaptic membranes is still unknown. However, the surface pressure of some cell membranes are estimated to be around 32 mN/m [47]. At this pressure, Si state should not be stable. However, it can transiently exist and as the binding rate to the receptor is fast, it might be a useful step towards the binding to the NK-1 receptor.

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