Interaction of a Substance P Agonist and of Substance P Antagonists with Lipid Membranes. A Thermodynamic Analysis†

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ABSTRACT: The molecular characteristics of the neuropeptide substance P (SP), its agonist [Sar^9,Met(O$_2$)^{11}]SP, and three of its antagonists [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP, [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP, and [D-Pro^2,D-Trp^{7,9}]SP were investigated at the air/water interface and when bound to lipid monolayers and bilayers. Measurement of the Gibbs adsorption isotherm showed that the surface areas of SP and its agonist (240 ± 5 Å^2 at biologically relevant concentrations) were distinctly larger than those of the antagonists (138 ± 5 Å^2) [Seelig, A. (1990) *Biochim. Biophys. Acta 1030*, 111–118]. The surface activity of the peptides increased in the order [Sar^9,Met(O$_2$)^{11}]SP < SP < [D-Pro^2,D-Trp^{7,9}]SP < [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP = [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP and correlated with the respective binding affinities to lipid membranes. The agonist did not insert into neutral and negatively charged bilayers or into densely packed lipid monolayers (at surface pressures > 31 mN/m). In contrast, the three antagonists gave rise to a strong binding both to neutral and to charged lipid monolayers and bilayers. The degree of binding was evaluated from the area increase of lipid monolayers upon peptide insertion, and the binding isotherms were analyzed in terms of the Gouy–Chapman theory. At the monolayer–bilayer equivalence pressure of ~32 mN/m, the binding can be described by a surface partition equilibrium with binding constants of (4.5 ± 0.1) × 10^3 M^{-1} for [D-Pro^2,D-Trp^{7,9}]SP and (1.3 ± 0.1) × 10^4 M^{-1} for both [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP and [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP for pure palmitoyloleoylphosphatidylcholine (POPC) membranes. For mixed POPC/palmitoyloleoylphosphatidylglycerol (POPG) or pure POPG bilayers the Gouy–Chapman analysis yields rather similar binding constants with small variations depending on the membrane composition. A comparison of the intrinsic binding constants demonstrates that the biological activity of SP and its analogues is *inversely* proportional to their hydrophobic lipid affinity.

Substance P (SP)[1] (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$) is a neuropeptide which is widely distributed in both the central and the peripheral nervous systems (Aronin et al., 1983). Its best investigated role is that of a potential neurotransmitter or neuromodulator (Nicoll et al., 1980) which conveys responses of noxious stimuli from sensory neurons to the central nervous system. SP is stored in proteolipid vesicles at the terminals of presynaptic neurons, from where it appears to be released by positively charged ions (Saria et al., 1980). After crossing the synaptic cleft, SP binds specifically to a protein receptor embedded in the postsynaptic membrane, initiating the transmission process. On its way to the postsynaptic receptor, SP encounters at least three types of lipidic environments: the storage vesicles, the presynaptic membranes, and the postsynaptic membranes, respectively. Membrane lipids may hence be of considerable importance for the action mechanism of SP, either by simply increasing its concentration at the membrane surface or by inducing the pharmacologically active conformation of SP after penetration into the lipid membrane (Schwyzer et al., 1986; Schwyzer, 1987).

Using the monolayer technique, we have previously shown that the binding of SP to lipid membranes is mainly due to electrostatic interactions (Seelig & Macdonald, 1989), in agreement with previous observations that the SP binding to negatively charged lipids decreases with increasing ionic strength (Lembeck et al., 1977; Schäfer et al., 1984). Hydrophobic interactions play only a minor role, and the binding constant of SP to neutral lipid membranes or monolayers (at the bilayer–monolayer equivalence pressure of 32 mN/m) was found to be 1–2 M^{-1} (Seelig & Macdonald, 1989; Seelig, 1990a). Indeed, SP does not insert into electrically neutral lipid bilayers (Wu et al., 1982; Rolka et al., 1986; Williams & Weaver, 1990), nor does it partition into an organic solution containing phosphatidylcholine or noncharged phosphatidylserine (Lembeck et al., 1978).[2]

If the membrane carries a negative surface charge, the SP concentration at the membrane surface strongly increases (Seelig & Macdonald, 1989). As a result, SP is driven into the membrane according to the law of mass action despite the weak hydrophobic interactions. Even though the intrinsic binding constant remains at 1–2 M^{-1}, the apparent binding constant which includes the electrostatic interactions may increase to 10^3–10^4 M^{-1} under physiological conditions (Schäfer et al., 1984; Seelig & Macdonald, 1989).

On the basis of the binding studies of SP alone, it is difficult to decide whether penetration of SP into lipid membranes is a necessary prerequisite for receptor binding or not. In the present study, we have therefore investigated the lipid binding properties of an agonist (Regoli et al., 1988) and of three of the most potent peptidic antagonists, ANT I, ANT II (Folkers

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SP, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$); ANT I, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP; ANT II, [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP; ANT III, [D-Pro^2,D-Trp^{7,9}]SP.

[2] In contrast to these observations, substantial binding of SP to neutral lipids has been reported for black lipid membranes containing residual amounts of solvent (Sargent et al., 1989).

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et al., 1984), and ANT III (Björkroth et al., 1982), of SP:

SP	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu MetNH_2
Agonist	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Sar	Leu Met$(O_2)NH_2$
ANT I	D-Arg	D-Pro	Lys	Pro	Gln	Gln	D-Trp	Phe	D-Trp	Leu LeuNH_2
ANT II	D-Arg	Pro	Lys	Pro	Gln	Gln	D-Trp	Phe	D-Trp	Leu LeuNH_2
ANT III	Arg	D-Pro	Lys	Pro	Gln	Gln	D-Trp	Phe	D-Trp	Leu MetNH_2

The binding of these peptides to neutral and negatively charged lipids was elucidated by the monolayer insertion technique since lipid monolayers constitute a simple model system to mimic bilayer membranes at different experimental conditions (e.g., lateral surface pressure, lipid composition). If the measurements are made at constant lateral pressure, the monolayer expansion due to peptide penetration allows the evaluation of the binding isotherm (Tamm, 1986; Seelig, 1987). Studies on erythrocyte membranes (Demel et al., 1975) have revealed a monolayer–bilayer equivalence pressure of about 32 mN/m. In addition, insertion studies with a charged local anesthetic and with a charged peptide hormone have demonstrated that the monolayer binding isotherms at a pressure of 32 mN/m are identical to the bilayer binding isotherms (Seelig, 1987; Beschiaschvili & Seelig, 1990a).

From the binding isotherms the apparent binding constants (overall binding constants obtained via Scatchard-type analysis at a low degree of binding) and the intrinsic binding constant (obtained after correction for electrostatic effects by means of the Gouy–Chapman theory) have been derived. The thermodynamic analysis was paralleled by spectroscopic measurements on peptide binding to small unilamellar vesicles in order to obtain independent evidence for peptide insertion. The present investigations were made under physiological conditions and yield information on the role of lipids in the functioning of SP and its analogues.

Materials and Methods

Materials. The acetate salts of the three substance P (SP) antagonists [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]SP, [D-Arg[1],D-Trp[7,9],Leu[11]]SP, and [D-Pro[2],-D-Trp[7,9]]SP and a SP agonist [Sar[9],Met(O_2)[11]]SP from Bachem (Bubendorf, Switzerland) were kindly provided by Merck (Darmstadt, Germany) and had a purity of better than 98%. The peptide concentrations were determined by weight, taking the acetate ions into account. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL).

Monolayer Measurements. The monolayer apparatus consisted of a round Teflon trough designed by Fromherz (1975) with a total area of 362 cm^2 divided into eight compartments (Type RML 2-T, Mayer Feintechnik, Göttingen, FRG). The surface pressure was measured by the Wilhelmy method using plates cut from filter paper (Whatman, No. 1). Lipid monolayers of pure POPC, pure POPG, and mixtures of the two were spread on an aqueous subphase (0.154 M NaCl, 10 mM Tris-HCl, pH 7.4). The lateral pressure was electronically adjusted to the desired value and was kept constant throughout the insertion experiments. Peptides (5 mg/mL) were dissolved in distilled water yielding clear solutions. Small amounts of the peptide stock solution, freshly prepared before use, were then injected into the buffer subphase. The insertion of the peptides into lipid monolayers was evaluated from the increase in area of the monolayer as described earlier [cf. Seelig (1987) and Seelig and Macdonald (1989)]. Measurements were performed at room temperature, 21 ± 1 °C.

Determination of the Surface Potential, ψ_0, by Means of the Gouy–Chapman Theory. The surface potential, ψ_0, for lipid membranes interacting with positively charged amphiphiles was calculated as described earlier [Seelig et al., 1988; Seelig & Macdonald, 1989; for reviews of the applicability of the Gouy–Chapman theory, see McLaughlin (1977, 1989)]. Na^+ binding to negatively charged headgroups was taken into account, as described by Beschiaschvili and Seelig (1990a). Briefly, the charge density, σ, is given as the ratio of total charge, Q_T, and total membrane area, A_T, according to (Seelig et al., 1988)

$$\sigma = Q_T/A_T = (e_0/A_L)\frac{X^0_{PG}(1 - X_{Na}) + z_pX_b}{1 + (A_P/A_L)X_b} \quad (1)$$

where e_0 is the elementary charge, X^0_{PG} is the mole fraction of phosphatidylglycerol, and X_b is the mole fraction of bound peptide per total lipid. In the present work, X_b was measured via the monolayer expansion. A_L and A_P are the surface areas of the lipid and the protein molecules, respectively, and were determined as described below. The unknown parameters in eq 1 are z_p, the effective charge of the peptide, and X_{Na}, the mole fraction of Na^+ bound to POPG. The latter is a function of the surface potential ψ_0 and can be calculated from the known Na^+ binding constant of $K_{Na} = 0.6\ M^{-1}$ (McLaughlin, 1989; Beschiaschvili & Seelig, 1990a).

A second independent relation between σ and ψ_0 is given by the Gouy–Chapman equation

$$\sigma = [2000\epsilon_r\epsilon_0 RT\sum_i C_{i,eq}(e^{-z_iF_0\psi_0/RT} - 1)]^{1/2} \quad (2)$$

where $\epsilon_r = 78$ is the dielectric constant for water (at 25 °C), ϵ_0 is permitivity of the free space, R is the gas constant, F_0 is the Faraday constant, $C_{i,eq}$ is the concentration of the ith electrolyte in the bulk aqueous phase (moles per liter), and z_i is the signed charge of the ith species. By combining the binding eq 1 with the Gouy–Chapman eq 2, a self-consistent solution for ψ_0 can be found for each experimental value of X_b.

Circular Dichroism and Fluorescence Spectroscopy. Circular dichroism (CD) measurements were performed with a Cary 61 instrument which was calibrated with α-1-camphorsulfonic acid. The optical length of the cuvette was 2 mm. Fluorescence spectra were recorded with a Jasco FP-777 spectrofluorometer (excitation at 282 nm, emission in the range 300–500 nm). The optical density in the exciting beam was always chosen to be smaller than 0.1. CD spectra of petpides in the presence of lipid vesicles were corrected for lipid absorption. Small unilamellar lipid vesicles (SUV) were prepared by sonification of lipid dispersions (2×10^{-3} M) under nitrogen for about 35 min (at 10 °C) until an almost clear solution was obtained.

Results

The Gibbs Adsorption Isotherm: Surface Activities and Surface Areas of the Peptides at the Air/Water Interface. SP and its analogues are amphiphilic molecules due to the segregation of charged amino acids at the N-terminus and uncharged amino acids at the C-terminal part of the molecule. They have the tendency to accumulate at the air/water interface and to lower the surface tension of water. This is reflected in the change of the surface pressure, π, which is defined as the difference between the surface tension of pure water, γ_0, and the surface tension of the surfactant solution, γ:

$$\pi = \gamma_0 - \gamma \quad (3)$$

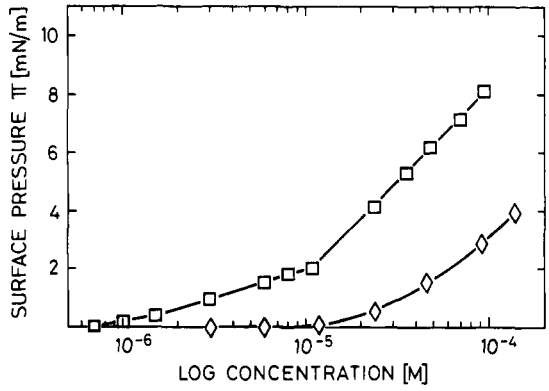

FIGURE 1: Surface pressure π as a function of the logarithm of the concentration of SP (□) and its agonist (◇) at pH 7.4 (154 mM NaCl).

In Figure 1, the surface pressure, π, of the agonist is plotted as a function of the logarithm of its concentration at pH 7.4 (154 mM NaCl). For comparison, measurements of SP under identical conditions (Seelig, 1990b) are included. The agonist is seen to be distinctly less surface active than SP in the whole concentration range. The lower surface activity of the agonist as compared to that of SP results from the increased hydrophilicity of its C-terminus due to the oxidation of Met[11]. Also taking into account the antagonists (Seelig, 1990b), the surface activities of SP and its four analogues increase in the order agonist < SP < ANT III < ANT II ≤ ANT I.

From the slopes of the π/log C plots in Figure 1, the peptide areas at the air/water interface can be evaluated by applying the Gibbs adsorption equation [cf. Seelig (1990b)]. The agonist has a surface area of 240 ± 5 Å^2 at concentrations below 3×10^{-5} M. With increasing concentration, its area decreases and reaches a limiting value of 140 ± 3 Å^2 at concentrations larger than 10^{-4} M. The surface areas of the agonist correspond well to those obtained for SP, which are 240 ± 5 Å^2 and 142 ± 3 Å^2, respectively, under identical experimental conditions (pH 7.4, 154 mM NaCl). On the other hand, the surface areas of all three antagonists were 138 ± 5 Å^2 and were independent of concentration (Seelig, 1990b).

Insertion of Peptides into Lipid Monolayers at Different Lateral Pressures and Measurement of the Cutoff Pressure. The peptides were injected into the aqueous subphase (154 mM NaCl, 10 mM Tris-HCl, pH 7.4) of lipid monolayers composed of POPC, POPG, or mixtures of the two. The monolayers had an initial area, A, and were kept at a constant surface pressure during the insertion experiment by means of an electronic feedback system. The intercalation of the peptides between the ordered lipids produced an area expansion, ΔA. A stable equilibrium was reached after about 10 min. In Figure 2, the relative area increase $\Delta A/A$ due to the intercalation of the agonist and of ANT I is plotted as a function of the lateral pressure for pure POPC and POPG monolayers, respectively. Each data point corresponds to a fresh monolayer. ANT I in combination with negatively charged POPG exhibits the largest penetration power (cf. the two straight lines in the right half of Figure 2). If POPG is replaced by POPC, ANT I is considerably less efficient (see open symbols in Figure 2). Finally, the agonist exhibits only a very weak penetration power (cf. the two straight lines at the left lower corner of Figure 2). A quantitative measure of the penetration power is the so-called cutoff pressure of the different peptides (Seelig, 1987). It can be determined by extrapolating the $(\Delta A/A) - \pi$ isotherms toward the π-axis. Inspection of Figure 2 demonstrates that the slope of the straight lines increases in absolute value with increasing peptide concentration, i.e.,

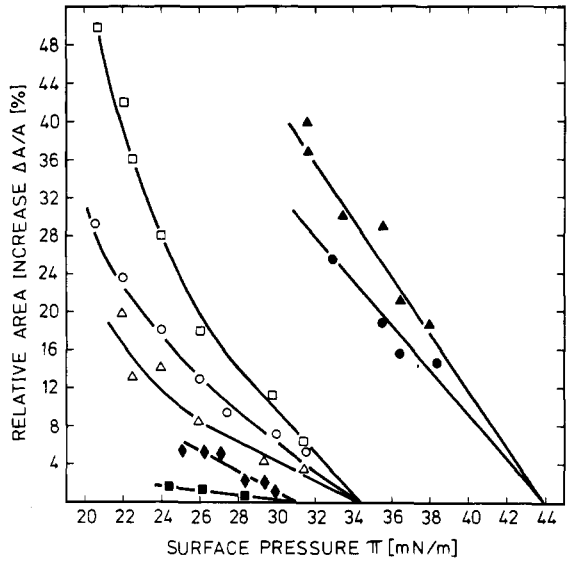

FIGURE 2: Relative area increase, $\Delta A/A$, of POPC monolayers (open symbols) and POPG monolayers (closed symbols) due to insertion of the agonist [2.3 μM (■), 4.7 μM (◆)] and ANT I [2.7 μM (△), 5.3 μM (○), 10.7 μM (□), 1.3 μM (●), 2.7 μM (▲)] as a function of the surface pressure. For every surface pressure, a new monolayer was prepared (pH 7.4, 10 mM Tris-HCl, 154 mM NaCl).

the higher the peptide concentration the larger the relative area increase. Nevertheless, for a given monolayer and peptide the corresponding $(\Delta A/A) - \pi$ isotherms intersect the π-axis at a common limiting pressure. Beyond this pressure, the peptide can no longer penetrate into the lipid film, not even at large peptide concentrations. For the agonist, this cutoff pressure is below 20 mN/m for insertion into a neutral POPC monolayer and 31 ± 0.5 mN/m for a charged POPG monolayer. For ANT I, the cutoff pressure increases to 34.5 ± 0.5 mN/m for a POPC monolayer and to 44 ± 0.5 mN/m for a POPG monolayer.

Binding Isotherms of the Antagonists. Several independent lines of evidence have demonstrated that a monolayer pressure of about 32 mN/m produces a lipid packing density which mimics closely the situation in lipid bilayers (Demel et al., 1975; Blume, 1979; Schindler, 1980; Schindler et al., 1984; Seelig, 1987; Beschiaschvili & Seelig, 1990b). As discussed above, only the antagonists, but not the agonist, will penetrate into the monolayer at this monolayer–bilayer equivalence pressure. The measurements of the peptide binding isotherm under biologically relevant conditions were thus limited to the antagonists. The relative area increase $\Delta A/A$ as a function of the concentration of ANT I and ANT II was measured for three lipid monolayers of different lipid composition, namely 100% POPC (Figure 3A), 75% POPC/25% POPG (Figure 3B), and 100% POPG (Figure 3C). All measurements were made at pH 7.4 (154 mM NaCl).

The expansion of the monolayer area, ΔA, is due to the incorporation of n_p peptide molecules with an area A_p. Since the pure lipid film (initial area A) contains n_L lipid molecules of area A_L, the extent of peptide binding, $X_b = n_p/n_L$, can be calculated according to (Seelig, 1987)

$$X_b = n_P/n_L = (\Delta A/A)(A_L/A_P) \quad (4)$$

The surface area of POPC and POPG can be estimated from monolayer measurements as A_L = 68 Å^2 at 32 mN/m (Evans et al., 1987). A peptide surface area of A_p = 110 Å^2 was evaluated for ANT I and ANT II under conditions where charge repulsion is minimal (Seelig, 1990b; Seelig & Dölz, 1991).[3] The exact value of A_p is not too critical in the fol-

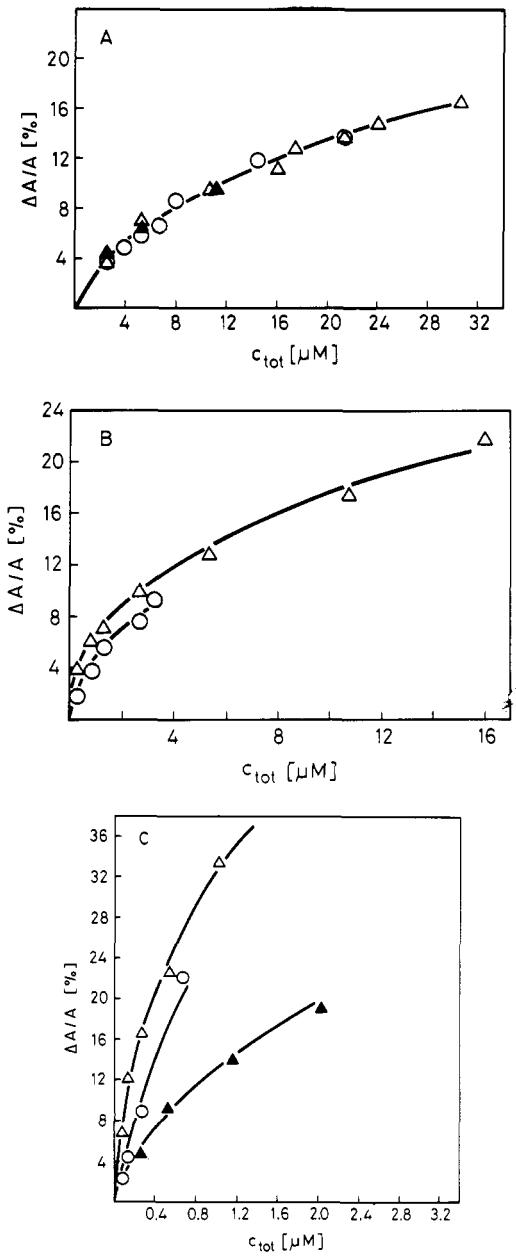

FIGURE 3: Relative area increase, $\Delta A/A$, of various lipid monolayers held at 32 mN/m, as a function of the total peptide concentration, C_{tot}, in the subphase: (○) ANT I at pH 7.4, (△) ANT II at pH 7.4, (▲) ANT II at pH 8.5. All measurements made in 154 mM NaCl. The monolayers contain (A) 100% POPC, (B) 25% POPG/75% POPC, and (C) 100% POPG.

lowing evaluations since an incorrect estimate of the peptide area by, e.g., 10% would lead to a 10% error in the binding constants which is within the accuracy of the measurements. With known surface areas A_L and A_P, the area expansion curves shown in Figure 3 can directly be transformed into true binding isotherms. C_{eq} was assumed to be equal to the total peptide concentration, C_{tot}, as long as the amount of bound peptide was smaller than 1% of C_{tot}. If the concentration of bound peptide, C_b, was larger than this values (e.g., for charged monolayers) a correction was made according to

$$C_{eq} = C_{tot} - C_b \quad (5)$$

[3] Under these conditions, the surface area is about 20% smaller than the experimental value at pH 7.4. The difference is due to electrostatic repulsion between peptides at this pH. Upon insertion into the lipid bilayer, the positive charge of the peptide is either compensated by negatively charged POPG or is at least diluted by zwitterionic POPC, leading to the smaller surface area.

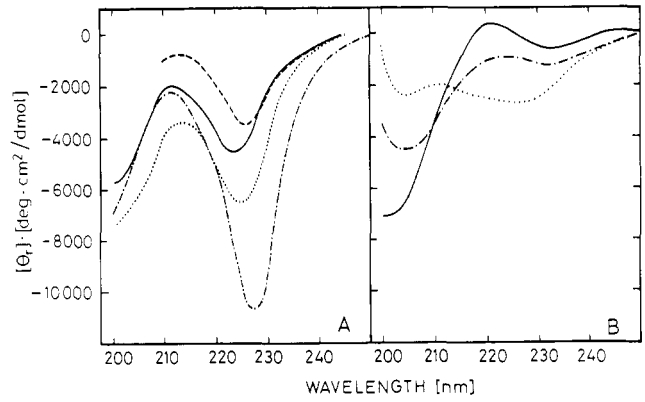

FIGURE 4: CD spectra of (A) ANT I (2×10^{-5} M) and (B) agonist (6×10^{-5} M) in solution and mixed with lipid vesicles: (—) Pure buffer (pH 7.4, 154 mM NaCl), (– –) in the same buffer containing 5 M guanidinium chloride, (···) in the presence of 30 mM SDS, (·–·) in the presence of lipid vesicles (2×10^{-3} M), composed of 100% POPG in (A) and 25% POPG/75% POPC in (B), respectively.

For electrically neutral POPC monolayers (Figure 3A), the binding isotherms of ANT I and ANT II are virtually identical. For negatively charged membranes, the insertion of ANT II is slightly stronger than that of ANT I (Figure 3B). With increasing POPG content, the difference in the insertion potential is enhanced (Figure 3C). Since ANT I and ANT II have the same amino acid sequence, the difference in their binding to negatively charged lipids can only be due to electrostatic effects. An apparent charge difference between the two diastereomers has been shown earlier (Seelig, 1990b). To further investigate the influence of the apparent peptide charge, the binding of ANT II to charged and uncharged lipid monolayers was also measured at pH 8.5 (154 mM NaCl) (cf. Figure 3A,C). As expected, the binding isotherms of ANT II at pH 7.4 and pH 8.5 are identical for neutral membranes (Figure 3A), at least at peptide concentrations up to 12 μM. In contrast, distinctly different binding isotherms are obtained for the two pH values if the membrane is composed of 100% POPG (Figure 3C). At the higher pH value, ANT II penetration is decreased due to a partial charge reduction (cf. below).

Peptide Insertion into SDS Micelles and Lipid Bilayers As Monitored by Circular Dichroism and Fluorescence Spectroscopy. Circular dichroism and fluorescence measurements were performed to obtain independent information on peptide/lipid interactions. For both types of measurements, a high lipid-to-protein ratio was chosen in order to ensure a maximum peptide binding to membranes.

For the agonist and the antagonists, CD spectra have not been reported before; in contrast, extensive literature exists on CD spectra of the parent compound SP (Mehlis et al., 1980; Wu et al., 1982; Wu & Yang, 1983; Rolka et al., 1986; Woolley & Deber, 1987; Williams & Weaver, 1980). The interpretation of the spectra of SP and its analogues is not trivial since both the peptide bonds and the phenylalanine and tryptophan side chains contribute to the absorption in the region around 220 nm.

The CD spectra of ANT I were measured in 6 M guanidinium chloride, in buffer, in SDS micelles, and in small unilamellar vesicles containing 25% POPG/75% POPC (Figure 4A). The dominant feature of the spectra is a negative CD band centered at ~225 nm which contains contributions from the aromatic ring systems (Holladay et al., 1977). The intensity of this band increases distinctly when the ANT I peptide is bound to SDS micelles or lipid vesicles and is paralleled by a similar intensity increase in the fluorescence

emission spectrum of ANT I. In aqueous solution, ANT I exhibits an unusual emission maximum at 337 nm (independent of concentration in the range of $3.8 \times 10^{-8} \leq c \leq 10^{-6}$ M) which is distinctly blue-shifted compared to that of a single tryptophan in solution ($\lambda_{max} \simeq 357$ nm). The molecular origin of this effect is unknown at present. One may speculate that the alignment of the hydrophobic ring systems D-Trp^7-Phe^8-D-Trp^9 causes a particularly hydrophobic environment. Addition of POPG vesicles to ANT I solutions induces a further blue shift to 331 nm and a more than 2-fold intensity increase of the emission maximum. Such spectral changes are often observed for tryptophan residues inserting into a more hydrophobic environment (Cowgill, 1967; Mollay & Kreil, 1973; Voges et al., 1987; Killian et al., 1990; Beschiaschvili & Seelig, 1990b; McKnight et al., 1991). However, on the basis of the unusual fluorescence emission spectrum of ANT I in aqueous solution, an alternative explanation is also possible, namely, that the same peptide interactions that make the Trp emission spectrum abnormal in the absence of lipid are further stabilized in the presence of lipid. Whatever the molecular origin of the increase in the CD and fluorescence intensity may be, the data nevertheless provide direct evidence for an interaction of ANT I with the lipid membrane.

No such interactions are expected for the agonist, at least not for densely packed planar bilayers. Inspection of Figure 2 reveals that the cutoff pressure of the agonist is close to 31 mN/m for *pure* POPG monolayers, which is not quite sufficient to match the monolayer–bilayer equivalence pressure of planar membranes. However, if highly curved unilamellar POPG vesicles are produced by sonication, the lipid packing density is reduced and the monolayer–bilayer equivalence pressure decreases to about 25 mN/m (Schindler, 1980). Since this value is below the cutoff pressure of the agonist, we expect an interaction of the peptide with *sonified* pure POPG vesicles. This is borne out experimentally. Figure 4B compares the CD spectra of the agonist measured in buffer, with unilamellar POPG vesicles, and with SDS micelles. The spectra are strikingly similar to those reported for the parent compound SP, under similar conditions [cf. Williams and Weaver (1990); Figure 2A). The CD spectrum of SP bound to POPG vesicles was interpreted, together with Raman data, as indicating a mixture of secondary structures with a significant contribution of type I and type III β-turns. By analogy, we conclude that the agonist also adopts some folded conformation which would be consistent with the large surface area requirement of the agonist (and of SP) as discussed above.

The results of the CD spectra support both the concept of the cutoff pressure and that of the bilayer–monolayer equivalence pressure. Knowledge of the two parameters thus allows predictions on the binding or nonbinding of a particular peptide to a specific membrane system.

It should be emphasized that the binding of SP and its agonist to sonified pure POPG vesicles is an exceptional situation which has only little relevance for the biological membrane. By having selected a lipid species for which the two peptides exhibit a rather high cutoff pressure and by reducing at the same time the lipid packing density via sonification, the agonist and SP were artificially forced into the membrane. A similar situation is observed for SDS micelles which exhibit an even lower molecular packing density than the sonified vesicles.

Discussion

A simple model to describe SP binding to lipid bilayers is to assume a Langmuir adsorption isotherm (Schäfter et al., 1984; Appu Rao et al., 1990). In this model, electrostatic interactions are not considered but are included in the apparent binding constant, K_{app}. The model further stipulates that a defined number of lipids constitute a SP binding site. Since SP and its analogues carry a positive charge of $z = +3$ and since the membrane systems are, in part, negatively charged, this model is only a crude approximation of the physical reality. As an alternative approach, we prefer to analyze the data by combining the Gouy–Chapman theory with a simple partition equilibrium, in accordance with the more recent literature on peptide binding (Seelig & Macdonald, 1989; Schwarz & Beschiaschvili, 1989; Kuchinka & Seelig, 1989; Beschiaschivili & Seelig, 1990b; Kim et al., 1991).

In the following, the detailed analysis will be presented only for the antagonists at the bilayer–monolayer equivalence pressure of 32 mN/m.

Analysis of Binding Isotherms Taking into Account Electrostatic Interactions. If a positively charged peptide inserts into a neutral POPC monolayer, the positive surface charge density, σ, increases and gives rise to a positive surface potential, ψ. As a consequence, molecules of like charge are repelled from the membrane surface and the peptide concentration at the membrane surface, C_M, becomes smaller than that in bulk solution, C_{eq}. If, however, the membrane contains negatively charged POPG, a negative surface potential arises and peptides are attracted toward the membrane surface leading to $C_M > C_{eq}$. The relationship between the membrane active concentration, C_M, and the bulk concentration, C_{eq}, is given by the Boltzmann equation

$$C_M = C_{eq} e^{-\psi_0 z_p F_0 / RT} \tag{6}$$

The surface potential ψ_0 can be approximated by means of the Gouy–Chapman theory (see Material and Methods). The effective charge of the peptides is denoted as z_p. The surface concentration, C_M, can then be entered into different chemical models of peptide binding. At the low degree of binding encountered in the present studies, the most realistic model is to assume a simple surface partition equilibrium of the form

$$X_b = K_p C_M \tag{7}$$

where K_p is the partition equilibrium constant. Indeed, the combination of a partition equilibrium with the Gouy–Chapman theory has been successful in explaining the binding of charged hydrophobic ions and drugs to lipid bilayers [for reviews, see McLaughlin (1977, 1989); cf. also Seelig et al. (1988), Gabev et al. (1989), and Bäuerle and Seelig (1991)].

For small molecules, the charge sensed at the membrane surface is identical to the total charge. In contrast, highly charged peptides typically exhibit a smaller z_p than predicted by the number of charged groups. (Seelig & Macdonald, 1989; Schwarz & Beschiaschvili, 1989; Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990a; Mosior & McLaughlin, 1991, 1992). Different reasons such as large charge separations (Carnie & McLaughlin, 1983), discrete charge effects (Stankowski, 1991), or clustering of like charges (Seelig, 1990b) could lead to a charge reduction. The quantitative analysis of binding isotherms of charged peptides thus requires the determination of two independent parametes, i.e., K_p and z_p. In agreement with previous authors, it was found that the two parameters are not strongly dependent on each other and can be evaluated within rather narrow limits.

The results of the analysis of ANT I and ANT II binding isotherms via the Gouy–Chapman theory are summarized in Table I. Input parameters are the experimentally determined binding X_b and the corresponding equilibrium concentration C_{eq}, from which we calculate the surface charge density and

Table I: Gouy-Chapman Analysis of the Insertion of ANT I and ANT II into POPC/POPG Monolayers at 32 mN/m (22 ± 1 °C, 0.154 M NaCl, 10 mM Tris-HCl, pH 7.4)

C_{eq} (μM)	X_b (mmol/mol)	σ (mC/m²)	ψ_0 (mV)	C_M (μM)	X_b/C_M (M^{-1})	K_p (M^{-1})
			100% POPC; ANT I; z = 1.3			
2.67	23.3	6.9	7.2	1.84	12603	(1.3 ± 0.1) × 10^4
4.00	30.2	8.8	9.2	2.49	12121	
5.34	35.8	10.4	10.9	3.06	11698	
6.68	40.2	11.6	12.1	3.60	11154	
8.02	52.6	14.8	15.5	3.63	14458	
14.69	73.5	20.1	20.7	5.08	14461	
21.44	84.0	22.7	23.2	6.54	12842	
			75% POPC/25% POPG; ANT I; z = 1.3			
0.26	11.1	−37.5	−36.7	1.69	6543	(5.1 ± 1.0) × 10^3
0.79	22.8	−34.5	−34.2	4.53	5037	
1.31	34.6	−31.5	−31.6	6.62	5228	
2.64	46.3	−28.7	−29.0	11.66	3969	
3.17	58.1	−25.9	−26.4	12.26	4737	
			100% POPG; ANT I; z = 1.3			
0.06	18.5	−86.5	−68.9	2.02	9145	(9.3 ● 0.9) × 10^3
0.11	29.7	−84.3	−67.8	3.68	8048	
0.23	66.7	−77.2	−64.0	6.16	10827	
0.47	98.9	−71.7	−60.8	10.64	9288	
			100% POPC; ANT II; z = 1.3			
2.67	23.5	6.9	7.4	1.82	12881	(1.3 ± 0.1) × 10^4
5.34	39.4	11.3	12.1	2.88	13671	
10.7	58.7	16.4	17.2	4.43	13249	
16.0	69.2	19.1	19.9	5.79	11933	
17.36	79.7	21.6	22.4	5.52	14423	
21.44	83.4	22.5	23.2	6.53	12770	
24.00	91.5	24.4	15.0	6.66	13730	
30.70	102.6	26.9	27.4	7.54	13594	
			75% POPC/25% POPG; ANT II; z = 2			
0.253	23.5	−31.6	−31.7	3.06	7673	(6.2 ● 0.5) × 10^3
0.779	37.1	−26.7	−27.2	6.63	5594	
1.303	43.3	−24.5	25.1	9.42	4592	
2.63	60.6	−18.4	−19.2	11.91	5084	
5.30	79.2	−11.9	−12.6	14.31	5528	
10.65	108.8	−1.7	1.9	12.37	8793	
15.90	134.8	7.1	7.4	8.86	15209	
			100% POPG; ANT II; z = 2			
0.045	42.6	−78.7	−64.8	7.33	5818	(6.4 ± 0.6) × 10^3
0.093	75.1	−70.1	−60.3	10.62	7073	
0.207	102.6	64.5	−56.4	17.55	5843	
0.451	141.5	−56.3	−52.1	25.10	5642	
0.947	205.8	−44.0	−42.0	25.83	7966	
1.356	216.3	−42.1	−40.5	32.80	6585	
			100% POPG; ANT II; z = 1, pH 8.5			
0.252	26.0	−85.9	−68.7	3.76	6898	(6.7 ± 0.5) × 10^3
0.510	57.0	−80.8	−66.0	6.85	8323	
1.12	86.8	−76.3	−63.5	13.70	6345	
2.02	117.8	−71.8	−61.0	22.30	5289	

the surface potential ψ_0 of the monolayer as well as the interfacial concentration, C_M, of the peptide. Of particular relevance is the ratio $X_b/C_M = K_p$ which according to our model should remain constant. This is indeed borne out by the experimental results (cf. penultimate column in Table I). The average value of the individual measurements is denoted K_p and is listed in the last column of Table I. In contrast, the ratio X_b/C_{eq} was found to vary up to 1 order of magnitude depending on the peptide and membrane system investigated. The variation of the partition constant K_p with the lipid composition is shown in Figure 5A (solid symbols). For ANT I and ANT II, K_p is found in the narrow range of 5×10^3 M^{-1} $< K_p < 1.3 \times 10^4$ M^{-1} (at 32 mN/m). For ANT III, which has a less hydrophobic C-terminus, the partition constant is $K_p = 4.5 \times 10^3$ M^{-1} (pure POPC monolayer, 32 mN/m) (data not shown). Also included in Figure 5 is the partition constant of SP (solid symbols; Figure 5B) determined previously (Seelig & Macdonald, 1989). It is obvious that SP binds 3 orders of magnitude less than its antagonists.

Within the framework of the surface partition model, the best fit of the binding data was obtained with an effective charge of $z_p = 1.3$ for ANT I and ANT II. In the presence of charged monolayers, the effective charge of ANT II increased to $z_p = 2$. This z_p value is closer to that of the parent compound SP ($z_p = 2.4$) (Seelig & Macdonald, 1989) in the presence of the same negatively charged membrane (Seelig & Macdonald, 1989). A larger effective charge for ANT II than for ANT I is consistent with the slightly higher pK values of ANT II as compared to those of ANT I (Seelig, 1990b). However, no simple correlation can be found between the pK values of a peptide in solution and its apparent peptide charge. Nevertheless, it can be concluded that the magnitude of the effective charge, z_p, is very sensitive to the individual steric arrangements of the charged amino acids at the membrane surface and the resulting different electrostatic interactions. At pH 8.5, the effective charge of ANT II is reduced to $z_p = 1$.

For the sake of comparison with the biochemical literature,

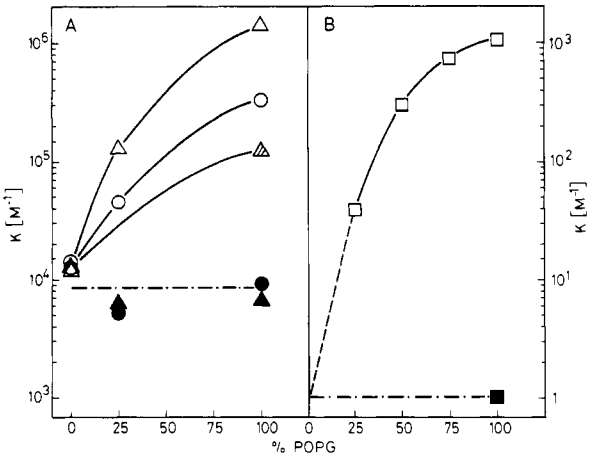

FIGURE 5: Intrinsic (K_p) and apparent binding (K_{app}) constants of the agonists (A) and of substance P (B) as a function of the POPG content of POPG/POPC monolayers at a constant surface pressure of 32 mN/m. Closed symbols: Intrinsic binding constants describing a surface partition equilibrium according to $X_b = K_p C_M$, where the interfacial concentration C_M is calculated with the Gouy–Chapman theory. K_p describes the binding isotherms (Figure 3) over the whole concentration range and is almost independent of the membrane composition. Open symbols: Apparent binding constant evaluated from a Scatchard plot at a *low* degree of binding X_b. The data facilitate the comparison with earlier analyses. However, $X_b/(1 - nX_b) = K_{app}C_{eq}$ is not a good model because (i) the Scatchard plot is nonlinear and K_{app} varies with X_b and (ii) K_{app} is furthermore distinctly influenced by membrane composition because it includes electrostatic effects (cf. text). Panel A: ANT I (circles) at pH 7.4; ANT II (triangles) at pH 7.4 and (hatched triangle) at pH 8.5. Panel B: SP (squares) at pH 7.4. K_{app} of SP/100% POPG was taken from Seelig and Macdonald (1989); K_{app} for 25%, 50%, and 75% POPG were from Alt and Seelig (unpublished results). All solutions contained 10 mM Tris buffer and 154 mM NaCl.

the experimental data of Table I (columns 1 and 2) were also plotted according to a conventional Scatchard analysis

$$X_b/C_{eq} = K_{app}(1 - nX_b) \quad (8)$$

even though saturation was not reached. All Scatchard plots were nonlinear (not shown). The apparent binding constants, K_{app}, were evaluated for *low* X_b values. These are summarized in Figure 5A for ANT I and ANT II and in Figure 5B for the parent compound SP [data from Seelig and Macdonald (1989)] (note the different scale for SP). As anticipated, the K_{app} coincides with K_p only for neutral membranes and low degrees of binding since electrostatic interactions are of minor importance under these conditions. On the other hand, K_{app} is 2–3 orders of magnitude larger than K_p for charged membranes. The binding of SP to charged lipids (Figure 5B) is distinctly weaker than that observed for the antagonists under similar conditions. The binding of SP to pure zwitterionic bilayers or zwitterionic monolayers at high pressure was too weak to be measured (Lembeck et al., 1978; Schäfer et al., 1984; Rolka et al., 1986; Seelig & Macdonald, 1989). However, the apparent binding constant for a zwitterionic lipid can be estimated by extrapolating the data of Figure 5B to 0% POPC. A value of $K_{app} \simeq 1$ M^{-1} is obtained which corresponds well with the intrinsic binding constants $K_p \simeq 1$ M^{-1} determined earlier (Seelig & Macdonald, 1989). Figure 5 also demonstrates that the *variation* of K_{app} with POPG (i.e., negative surface charge density) is most pronounced for SP and decreases in the order SP > ANT II > ANT I. This can be traced back to the fact that the effective peptide charge decreases in the same order, namely SP, $z = 2.4$ > ANT II, $z = 2$ > ANT I, $z_p = 1.3$ (cf. Table I).

Cutoff Pressure for Peptide Insertion. Peptide insertion into lipid monolayers depends strongly on the lateral packing density and the surface charge of the lipids. For SP (Seelig & Macdonald, 1989) and its analogues (present study), the cutoff pressures, i.e., the pressure above which peptide insertion is no longer possible, were found to be proportional to the surface activities of the respective compounds. The cutoff pressures increased in the following order: agonist (<20 mN/m for 100% POPC; ~31 mN/m for 100% POPG) < SP (<20 mN/m for 100% POPC; ~35 mM/m for 100% POPG) < ANT I (~34.5 mN/m for 100% POPC; ~44 mN/m for 100% POPG).

A comparison of the cutoff pressures in monolayers with the packing density in micelles (estimated lateral pressure ~10 mN/m), lipid vesicles (25–35 mN/m depending on vesicle size; Schindler 1980, and planar lipid bilayers (32–35 mN/m; Demel et al., 1975; Seelig, 1987) allows the following conclusions. (i) The agonist, which has the lowest surface activity and the lowest cutoff pressure, does not insert into planar POPG or POPC lipid bilayers. (ii) SP can penetrate to some extent into planar membranes provided they contain an appreciable amount of negatively charged lipids. (iii) The antagonists, finally, are expected to insert also into uncharged membranes since they exhibit the largest surface activities and the largest cutoff pressures. The cutoff pressures, which are easily accessible experimental parameters, thus confirm the conclusions on the basis of the more elaborate analysis of the binding isotherms.

Biological Relevance. The present investigation demonstrates that small modifications of the amino acid sequence of SP may lead to large differences in the surface activities and the thermodynamic binding parameters of these peptide molecules.

The agonist has a greater selectivity for the NK-1 receptor than SP (Regoli et al., 1988, 1989). It is, however, less surface active than SP, and it does not partition into well-packed biological membranes. Its intrinsic hydrophobic binding constant, K_P, is even smaller than that of SP [1–2 M^{-1} for monolayers at the monolayer–bilayer equivalence pressure (Seelig & Macdonald, 1989; Seelig, 1990a)]. The lipid binding constants of SP and its agonist are thus inversely proportional to their respective receptor binding constants. Even though the agonist does not insert into the lipid phase, the membrane might nevertheless have a functional role by accumulating, orienting, and preshaping the peptide molecules at the membrane surface in order to facilitate receptor binding. SP (Seelig, 1990b) and its agonist adopt similar conformations at the air/water interface as judged by their identical cross-sectional areas (240 ± 5 Å^2 at pH 7.4, 154 mM NaCl). The relatively large area requirements suggest a folded conformation at low concentrations. Comparable conformations have been proposed for SP in solution (Sandberg & Iversen, 1982; Chassaing et al., 1986). Membrane penetration and a concomitant α-helix formation (Schwyzer, 1987) seem not to be a prerequisite for the binding of these two neuropeptides to the NK-1 receptor.

The antagonists are much more surface active than SP, and they insert strongly into lipid membranes. Even though they have the same formal charge, their effective charge experienced by the membrane surface differs and depends on the isomerization of the N-terminus. This, in turn, could explain the different activities of ANT I and ANT II in different tissues (Post & Folkers, 1985).

The physiological potency of the antagonists increases with their tendency to partition into lipid membranes. As the affinity of the antagonists for the NK-1 receptor is relatively low, (10^7 M^{-1} or lower; Weinrich et al., 1988), it can be

concluded that the potency increase of the antagonists with increasing hydrophobicity of the C-terminus is an unspecific effect and results from an increasing tendency to partition into amphiphilic membrane sites of lipidic and peptidic nature. The strong tendency of the antagonists to insert into membranes might be responsible for different side effects, especially for their local anesthetic effect (Post et al., 1985; Post & Folkers, 1985). Even if the peptide is injected at low concentrations (micromolar), the amount of bound peptide per lipid, X_b, easily reaches a value of ~30 mmol/mol which is known to be the limiting membrane concentration for the onset of local anesthesia (Roth, 1979).

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REFERENCES

Appu Rao, A. G., Cann, J. R., Stewart, J. M., & Winzor, D. J. (1990) *Int. J. Pept. Protein Res. 35*, 258–262.

Aronin, N., Difiglia, M., & Leeman, S. (1983) in *Brain Peptides* (Krieger, D. T., Brownstein, M. J., & Martin, J. B., Eds.) pp 783–803, Wiley, New York.

Bäuerle, H. D., & Seelig, J. (1991) *Biochemistry 30*, 7203–7211.

Beschiaschvili, G., & Seelig, J. (1990a) *Biochemistry 29*, 10995–11000.

Beschiaschvili, G., & Seelig, J. (1990b) *Biochemistry 29*, 52–58.

Beschiaschvili, G., & Seelig, J. (1991) *Biochim. Biophys. Acta 1061*, 78–84.

Björkroth, U., Rosell, S., Xu, J.-C., & Folkers, K. (1982) *Acta Physiol. Scand. 116*, 167–173.

Blume, A. (1979) *Biochim. Biophys. Acta 557*, 32–44.

Carnie, S., & McLaughlin, S. (1983) *Biophys. J. 44*, 325–332.

Chassaing, G., Convert, O., & Lavielle, S. (1986) *Eur. J. Biochem. 154*, 77–85.

Cowgill, R. W. (1967) *Biochim. Biophys. Acta 316*, 196–203.

Demel, R. A., Geurts van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B., & Van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 97–107.

Evans, R. W., Williams, M. A., & Tinoco, J. (1987) *Biochem. J. 245*, 455–462.

Folkers, K., Hakanson, R., Hörig, J., Xu, J.-C., & Leander, S. (1984) *Br. J. Pharmacol. 83*, 449–456.

Fromherz, P. (1975) *Rev. Sci. Instrum. 46*, 1380–1385.

Gabev, E., Kasianowicz, J., Abbott, T., & McLaughlin, S. (1989) *Biochim. Biophys. Acta 979*, 105–112.

Holladay, L. A., Rivier, J., & Puett, D. (1977) *Biochemistry 16*, 4895–4900.

Killian, J. A., Keller, R. C. A., Struyvé, M., de Kroon A. I. P. M., Tommassen, J., & de Kruijff, B. (1990) *Biochemistry 29*, 8131–8137.

Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135–148.

Kuchinka, E., & Seelig, J. (1989) *Biochemistry 28*, 4216–4221.

Lembeck, F., Mayer, N., & Schindler, G. (1977) *Naunyn-Schmiedeberg's Arch. Pharmacol. 301*, 17–22.

Lembeck, F., Mayer, N., & Schindler, G. (1978) *Naunyn-Schmiedeberg's Arch. Pharmacol. 303*, 79–86.

Lembeck, F., Folkers, K., & Donner, J. (1981) *Biochem. Biophys. Res. Commun. 103*, 1318–1321.

McKnight, C. J., Rafalski, M., & Gierasch, L. M. (1991) *Biochemistry 30*, 6241–6246.

McLaughlin, S. A. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.

McLaughlin, S. A. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 113–134.

Mehlis, B., Rueger, M., Becker, M., Bienert, M., Niedrich, H., & Oehme, P. (1980) *Int. J. Pept. Protein Res. 15*, 20–28.

Mollay, C., & Kreil, G. (1973) *Biochim. Biophys. Acta 316*, 196–203.

Mosior, M., & McLaughlin, S. (1991) *Protein Kinase C: Current Concepts and Future Prospectives* (Epand, R., & Lester, D., Eds.) Ellis Horwood, Chichester, England.

Mosior, M., & McLaughlin, S. (1992) *Biochemistry* (in press).

Nicoll, R. A., Schenker, C., & Leeman, S. E. (1980) *Annu. Rev. Neurosci. 3*, 227–268.

Post, C., & Folkers, K. (1985) *Eur. J. Pharmacol. 113*, 335–343.

Post, C., Butterworth, J. F., Strichartz, G. R., Karlsson, J. A., & Persson, C. G. A. (1985) *Eur. J. Pharmacol. 117*, 347–354.

Regoli, K., Drapeau, G., Dion, S., & Couture, R. (1988) *Trends Pharmacol. Sci. 9*, 290–295.

Regoli, D., Dion, S., Rhaleb, N. E., Rouissi, N., Tousignant, C., Jukic, D., D'Orleans-Juste, P., & Drapeau, G. (1989) *Biopolymers 28*, 81–90.

Rolka, K., Erne, D., & Schwyzer, R. (1986) *Helv. Chim. Acta 69*, 1798–1806.

Roth, S. H. (1979) *Annu. Rev. Pharmacol. Toxicol. 19*, 159–178.

Sandberg, B. E. B., & Iversen, L. L. (1982) *J. Med. Chem. 25*, 1015–1017.

Sargent, D. F., Bean, J. W., & Schwyzer, R. (1989) *Biophys. Chem. 34*, 103–114.

Saria, A., Mayer, N., Lembeck, F., & Pabst, M. (1980) *Naunyn-Schmiedeberg's Arch. Pharmacol. 311*, 151–157.

Schäfer, H., Schmidt, W., Lachmann, U., & Bienert, M. (1984) *Pharmazie 39*, 765–769.

Schindler, H. (1980) *FEBS Lett. 122*, 77–79.

Schwarz, G., & Beschiaschvili, G. (1989) *Biochim. Biophys. Acta 979*, 82–90.

Schwyzer, R. (1987) *EMBO J. 6*, 2255–2259.

Schwyzer, R., Erne, D., & Rolka, K. (1986) *Helv. Chim. Acta 69*, 1789–1797.

Seelig, A. (1987) *Biochim. Biophys. Acta 899*, 196–204.

Seelig, A. (1990a) *Cell. Biol. Int. Rep. 14*, 369–380.

Seelig, A. (1990b) *Biochim. Biophys. Acta 1030*, 111–118.

Seelig, A., & Macdonald, P. M. (1989) *Biochemistry 28*, 2490–2496.

Seelig, A. & Dölz, R. (1991) *Ann. N.Y. Acad. Sci. 632*, 468–471.

Seelig, A., Allegrini, P. R., & Seelig, J. (1988) *Biochim. Biophys. Acta 939*, 267–276.

Stankowski, S. (1991) *Biophys. J. 60*, 341–351.

Tamm, L. K. (1986) *Biochemistry 25*, 7470–7476.

Voges, K. P., Jung, G. & Sawyer, W. H. (1987) *Biochim. Biophys. Acta 896*, 64–76.

Wienrich, M., Bartoszky, G. D., Greiner, H. E., Lues, J., & Wild, A. (1988) *Regul. Pept. 22*, 1988.

Williams, R. W., & Weaver, J. L. (1990) *J. Biol. Chem. 265*, 2505–2513.

Woolley, G. A., & Deber, C. M. (1987) *Biopolymers 26*, S109–S121.

Wu, C. S. C., & Yang, J. T. (1983) *Biochim. Biophys. Acta 746*, 72–80.

Wu, C. S. C., Hachimori, A., & Yang, J. T. (1982) *Biochemistry 21*, 4556–4562.