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# Reversible binding of substance P to artificial lipid membranes studied by capacitance minimization techniques

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Interaction of substance P with electrically neutral, planar lipid bilayers prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and with anionic bilayers prepared from mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and brain phosphatidylserine was measured using the capacitance minimization method for monitoring the membrane surface potential caused by the positive charges and electric dipole moment of adsorbed peptide. Substance P bound to the electrically neutral bilayers from 9 mM KCl (buffered to pH 5.5 with 2.0 mM 2-(*N*-morpholino)ethanesulfonate) with a maximal binding density of about $1 \times 10^{-2}$ molecules per $nm^2$ and a dissociation constant of about $2 \times 10^{-4}$ M. Measurement of the surface potential at different ionic strengths (shielding of surface charges) allowed distinction between the fixed-charge surface potential and a dipole potential. Ascribing this dipole potential to membrane-bound substance P would imply an effective dipole moment normal to the bilayer surface of about 20 Debye per molecule. Magnitude and polarity are consistent with an $\alpha$-helical domain at the C-terminal end of substance P which is oriented normal to the surface of the membrane, and inserted so as to be inaccessible to the aqueous phase. Consistent measurements were obtained with anionic membranes at low substance P concentrations ($10^{-7}$–$10^{-6}$ M; pH 7.2). They indicated electrostatic accumulation of the triply charged peptide on the surface of the membrane followed by hydrophobic interaction with the same parameters as for neutral membranes. The results agree with the membrane structure of substance P determined with infrared attenuated total reflection spectroscopy, circular dichroism measurements, and thermodynamic estimations.

## 1. Introduction

According to the theory of membrane compartments [1], knowledge of the behavior of regulatory peptides in contact with lipid membranes is important for understanding the molecular mechanisms of receptor subtype selection [2,3] and the catalytic function of membranes in this respect [4].

Recently, we have studied membrane interactions of substance P, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$, an important neuropeptide reacting selectively with members of the neurokinin receptor family [5]. Using changes in accessible surface areas [6] as a basis for thermodynamic considerations, substance P was predicted to interact with lipid bilayer membranes by inserting its C-terminal message segment, residues 5–11, into the hydrophobic compartment as a perpendicularly oriented $\alpha$-helical domain, the N-terminal address segment, residues 1–4, remaining in the aqueous compartment [7]. This membrane structure of substance P is supported by circular dichroism (CD) measurements in the presence of anionic lipid vesicles [8] and by infrared and in-

Dedicated to Haruaki Yajima (Kyoto) on the occasion of his retirement.

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Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; PS, brain phosphatidylserine; $\sigma$, surface density (number/$nm^2$) of a species bound to the membrane; subscripts e, d, m, elementary charges, dipoles, molecules, respectively.

frared attenuated total reflection spectroscopy in organic solvents and on flat lipid bilayers [9]. Although our spectroscopic data are compatible with those of other authors using different systems (references cited in refs. 7–9), confirmation of our conclusions by an independent method was lacking. Furthermore, an experimental determination of the Gibbs free energy of association of substance P with an aqueous-hydrophobic interface is required to resolve an uncertainty in the calculation of the theoretical value. This uncertainty arises from the possibility of using different assumptions for numbers and energies of hydrogen bonds broken upon transfer of the C-terminal amide nitrogen and the methionine sulfur from the aqueous to the hydrophobic phase [3,7].

Few experimental studies of the interaction of substance P with lipid membranes have been reported [10–12], and the results have been inconsistent. Because of the interesting results obtained with other peptides such as adrenocorticotropin-(1–24)-tetracosapeptide [13], melittin [14], and dynorphin A-(1–13)-tridecapeptide [15], we have applied the method of membrane capacitance minimization [16–19] to investigate the interaction of substance P with electrically neutral POPC and anionic PS/DOPC bilayers. This method uses the membrane capacitance-voltage characteristics to monitor changes in the Gouy-Chapman and dipole potentials induced by the adsorption of substances bearing an electric charge and/or an electric dipole moment. The results obtained as a function of peptide concentration and ionic strength were interpreted in terms of preferred conformation, orientation, and electrostatic and hydrophobic accumulation of substance P on such lipid membranes. The study yielded results consistent with the predicted model for the membrane structure of substance P [7] and with data obtained via a revised method for estimating the hydrophobic association constant [3].

## 2. Experimental procedures and theory

### 2.1. Materials

Substance P (Bachem, Bubendorf, Switzerland) and lipids (Avanti Polar Lipids, Birmingham, AL) were commercial products. Solvents and chemicals were the highest quality available from Fluka (Buchs, Switzerland).

### 2.2. Lipid membranes

Planar lipid bilayer membranes covering a hole in a septum between two electrolyte compartments were formed according to the technique of Montal and Mueller [20]. The aqueous phase initially surrounding the membranes was 9.0 mM KCl buffered to pH 5.5 (neutral bilayers) with 2 mM 2-(*N*-morpholino)ethanesulfonate (Mes) or 7.2 (anionic bilayers) with 2 mM 3-(*N*-morpholino)propanesulfonate (Mops). The total ionic strength of each buffer was 10 mM. A mixture of 0.5 wt.% POPC dissolved in hexane containing 2.0 vol.% hexadecane was used to form the electrically neutral lipid bilayers. A mixture of 0.05 wt.% PS and 0.45 wt.% DOPC in the same solvents was used in the case of the anionic bilayers. This solvent combination results in some residual solvent being left in the lipid core region, thereby making the bilayer more fluid and more compressible than a solvent-free bilayer [18]. This in turn renders measurement of the capacitance minimization potential both technically easier and less susceptible to artefacts caused by non-electrostrictive phenomena. The presence of small amounts of the solvent has been shown not to affect the measured surface potential [18].

### 2.3. Electrical measurements

Four electrical parameters characterizing the lipid bilayer were monitored continuously: membrane capacitance ($C_m$), compressibility in an electric field ($\alpha_m$), membrane resistance ($R_m$), and the capacitance minimization potential ($V_{Cmin}$, see section 2.4). Measurements were carried out under the control of a microcomputer [14,18]. Each recorded point (e.g. fig. 2) is the average of 100 or 200 measurements made over a period of a few seconds. The recorded $V_{Cmin}$ values were stored and an average value could be calculated at any time and read off numerically. Typical values of the four parameters were: $C_m = 100$–$200$ pF, $\alpha_m \approx 2\ V^{-2}$ (i.e., bilayer capacitance increased by

2% for a transmembrane potential of 100 mV), and $R_m \approx 10^{10}$–$10^{11}$ Ω.

### 2.4. Surface potentials: capacitance minimization technique

Surface potentials of the lipid bilayers were measured using the capacitance minimization potential [16–19]. The technique is based on the compression of artificial lipid bilayers by a transmembrane electric field. The minimal capacitance (maximal bilayer thickness) is found when the potential difference across the bilayer is zero. As this transmembrane potential depends on both the intrinsic surface potentials of the bilayer itself (fixed charge and dipole potentials) and an externally applied potential, a change in the surface potentials can be compensated by an equal but oppositely directed external field. The externally applied voltage needed to achieve minimal capacitance is designated the 'capacitance minimization potential' ($V_{C\min}$) and is given directly by

$$V_{C\min} = -(\Delta V_{gc} + \Delta V_d), \quad (1)$$

where $\Delta V_{gc}$ is the difference in fixed-charge surface potentials (Gouy-Chapman potentials) between the two bilayer surfaces, and $\Delta V_d$ the corresponding difference in surface dipole potentials.

The Gouy-Chapman potential for a 1:1 electrolyte, adapted to convenient units, is described by eq. 2,

$$\Delta V_{gc}\ (\mathrm{mV}) = 50.8 \cdot \ln\left[s + (s^2 + 1)^{0.5}\right], \quad (2)$$

where $s = 1.36\sigma_e/c$; $c$ denotes the concentration of the 1:1 electrolyte (in mol $l^{-1}$), and $\sigma_e$ the surface charge density (in elementary charges per $nm^2$) which is related to the surface density (number/$nm^2$) of molecules of an adsorbed (bound) substance ($\sigma_m$) by $\sigma_m = \sigma_e/z$, where $z$ is the net charge of the substance. From eq. 2, one observes that the surface potential resulting from a given charge density depends on the ionic strength of the solution: higher ionic strengths are said to shield the surface charge, resulting in a lower surface potential. Curves for two different ionic strengths are shown in fig. 1.

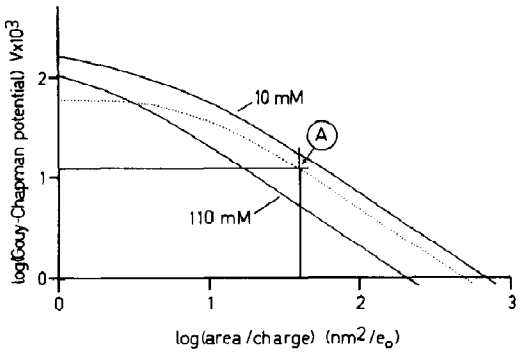



Fig. 1. Relationship between the Gouy-Chapman fixed-charge surface potential and the membrane surface area per unit charge ($nm^2/e_0$). For a given value of the membrane charge density, the Gouy-Chapman fixed-charge surface potential will be reduced when the ionic strength is increased. Concentrations refer to a 1:1 salt. The dotted curve represents the difference between the curves at 10 mM and at 110 mM. The change in $V_{C\min}$ in a shielding experiment can be matched to this curve and the surface charge density read off directly: point A corresponds to the shielding experiment described in fig. 3 ($\Delta V_{C\min} = 12$ mV, $\sigma_e^{-1} = 40\ nm^2/e_0$).

The surface dipole potential, $V_d$, caused by adsorbed peptide molecules is given by eq. 3,

$$V_d\ (\mathrm{mV}) = 3.77 \times 10^4 \times \sigma_d(\mu_{mol}/\epsilon_a), \quad (3)$$

where $\sigma_d$ denotes the surface density (number/$nm^2$) of dipoles of dipole moment $\mu_{mol}$ perpendicular to the membrane surface, in a medium of dielectric constant $\epsilon_a$. If one only considers the dipole contribution of the adsorbed molecules, then $\sigma_d = \sigma_e/z = \sigma_m$, where $z$ is taken as +3 for substance P, and $\epsilon_a$ may be taken to be about 10 in the adsorption region (geometric average of values for the aqueous and lipid phases).

The magnitude and extent of a dipole field in the aqueous solution will be small, due to both the $1/\epsilon_a$ dependence of eq. 3 and direct shielding by counterions. For this reason, only dipolar components within the hydrophobic region of the bilayer will have an appreciable effect on the transmembrane potential. As the dipole potential of such sequestered molecular dipoles will not be influenced by ionic strength, in contrast to the Gouy-Chapman potential, the two components can be distinguished experimentally (shielding experiments).

The capacitance minimization technique, although not well known, has been used successfully in the determination of surface potentials in many systems, including the shielding of charged membranes by small cations [17,18,21], titration of membranes formed from phosphatidylethanolamine [22], and the binding of charged amphiphilic substances to neutral bilayers [13,14]. Given a resolution of about 1 mV in the measurement of $V_{C\min}$, the minimal surface charge density that can be detected in 10 mM electrolyte is about $1.4 \times 10^{-3}$ elementary charges/nm$^2$ (fig. 1, upper curve).

### 2.5. Analysis of $V_{C\min}$ measurements

After addition of substances (e.g., KCl, substance P) to one side of the bilayer, the aqueous phases were stirred for 15 s. No measurements were made during the period of stirring. Shifts of $V_{C\min}$ after changing the ionic strength were usually stable after the first stirring period (e.g., fig. 2A) and were measured immediately after cessation of stirring. Following changes in the bulk concentration of substance P, a new, stable level of $V_{C\min}$ took many minutes to establish itself (fig. 2B), and often several additional stirring periods were required before the final level was reached. A similar time course was reported in ref. 12 for the binding of substance P to surface monolayers. The shielding effect is thus rapid relative to possible variations in $V_{C\min}$ due to adsorption/desorption phenomena induced by the changed ionic strength (see section 2.6), and therefore can be determined unambiguously.

A quantitative analysis of $V_{C\min}$ measurements typically proceeds as follows. Starting from a symmetric situation (symmetric bilayer, identical aqueous phases on each side) for which $V_{C\min} = 0$, the substance to be tested is added to one side of the membrane. Following equilibration, the change in $V_{C\min}$ $[\Delta V_{C\min}(1) = -(\Delta V_{gc}(1) + \Delta V_d(1))]$ is noted. The ionic strength on the same side of the bilayer is then increased, and the immediate change in $V_{C\min}$ $[\Delta V_{C\min}(2) = -\Delta V_{gc}(2)$, $\Delta V_d(2)$ being zero as noted above] is recorded. Knowing the initial and final ionic strengths, the surface charge density, $\sigma_e$, can be read directly off difference curves such as that given in fig. 1. From the charge density, $V_{gc}(1)$ can be calculated, yielding in turn $\Delta V_d(1) = -\Delta V_{gc}(1) - \Delta V_{C\min}(1)$. Using the relationship $\sigma_e = \sigma_m z$, the measurements thus yield the surface densities of adsorbed molecules, $\sigma_m$, and of dipoles, $\sigma_d(\mu_{mol}/\epsilon_a)$, eq. 3 (see also section 4).

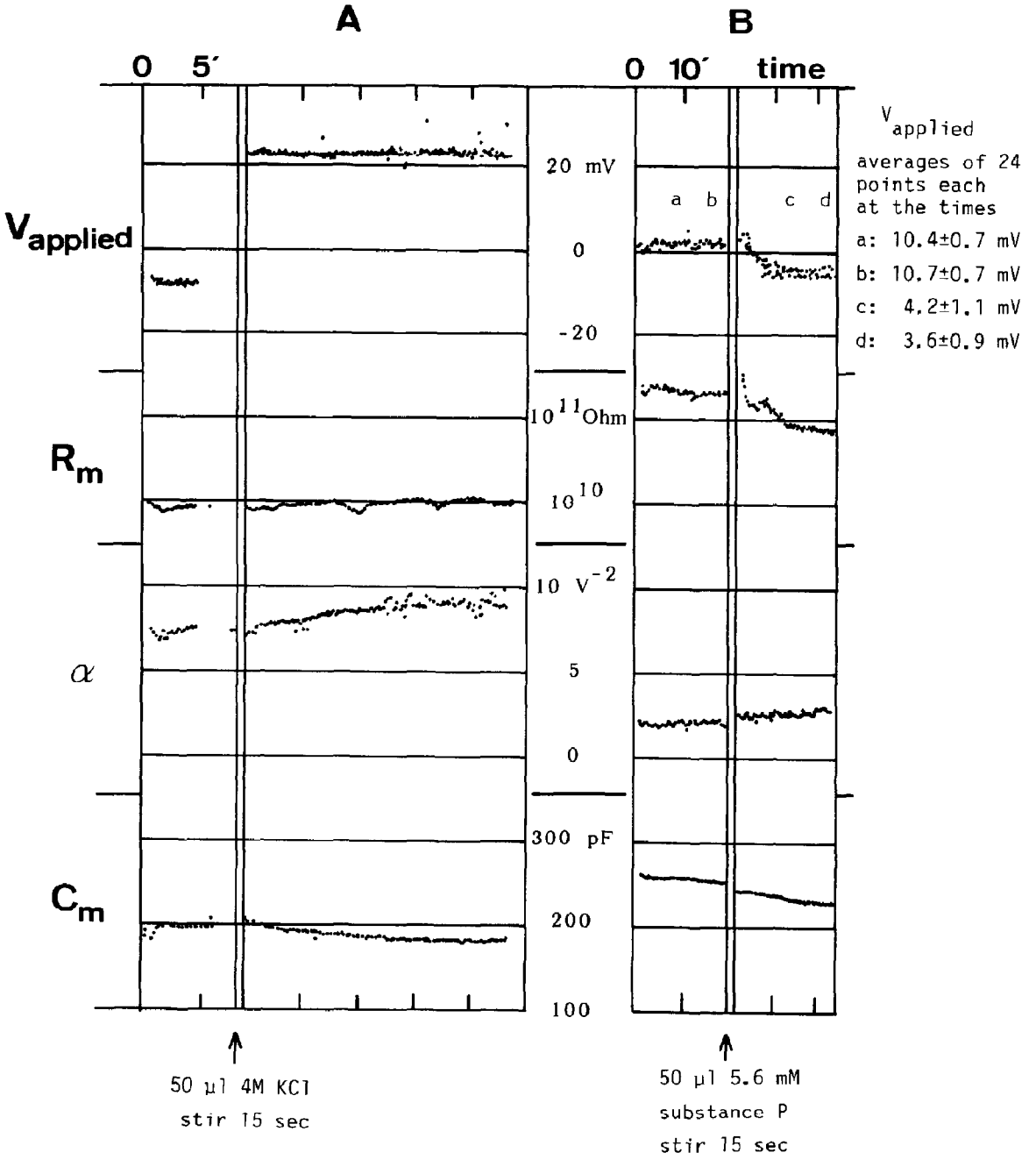
### 2.6. Analysis of binding data

The hydrophobic adsorption or binding of amphiphilic molecules to lipid bilayers has been studied by many authors. The Langmuir adsorption isotherm has been widely used [12,23–25]:

$$c_s/K_d^L = \sigma_m/(\sigma_{max}^L - \sigma_m), \qquad (4)$$

where $\sigma_{max}^L$ is the maximum binding concentration (molecules per unit area), $K_d^L$ the dissociation

Fig. 2. Representative experimental traces showing the continuous recording of membrane capacitance ($C_m$, in pF: bottom trace), compressibility ($\alpha$, in $V^{-2}$), resistance ($R_m$, in $\Omega$) and capacitance minimization potential ($V_{applied}$, in mV: top trace). The horizontal axis is time (5 min per major division). (A) PS/DOPC bilayer: initial ionic strength 10 mM. At the mark ($x$-axis), 50 $\mu$l of 4 M KCl was added to the rear chamber (volume 2 ml), yielding a total ionic strength of 110 mM. The change in $V_{C\min}$ ($-7.3 \pm 0.3$ to $22.3 \pm 0.3$ mV) was fully developed immediately after the 15 s stirring period. From the observed change of $V_{C\min}$ (29.6 mV) the total surface charge density can be determined (e.g., difference curve of fig. 1) to be $-46$ mV. A gap in the time axis was inserted in the recording for clarity to allow the altered conditions to be registered. The initial value of $-7$ mV is offset due to poorly matched measuring electrodes. The membrane had a high compressibility ($\alpha = 7$–$9$ V$^{-2}$), making the measurement of $V_{C\min}$ relatively easy, with a correspondingly low scatter. (B) POPC bilayer: initial ionic strength 10 mM. At the time indicated by the vertical line (about 36 min), 50 $\mu$l of 50 mM substance P was added to the front side of the membrane (volume of the compartment 2 ml), yielding a final concentration of 0.14 mM substance P. This trace, made with a different recorder from that in panel A, contains no gap in the time axis: the end of the 15 s stirring period is indicated by the vertical line across the whole trace. The change in $V_{C\min}$ resulting from the adsorption of substance P to the bilayer (0.7 to $-6.3$ mV) took about 9 min to develop in this case. The bilayer was less compressible than that in panel A ($\alpha = 2.5$ V$^{-2}$), with a corresponding increase in the scatter of the $V_{C\min}$ measurement. The slight decrease in $R_m$ after the addition of substance P is not significant: it is not found consistently, and shifts of this magnitude are often observed after stirring, even without the addition of substances.


A
B
0 5′
0 10′ time
$V_{applied}$
$R_m$
$\alpha$
$C_m$
20 mV
0
-20
$10^{11}$Ohm
$10^{10}$
10 $V^{-2}$
5
0
300 pF
200
100
a b
c d
$V_{applied}$
averages of 24 points each at the times
a: 10.4±0.7 mV
b: 10.7±0.7 mV
c: 4.2±1.1 mV
d: 3.6±0.9 mV
50 µl 4M KCl
stir 15 sec
50 µl 5.6 mM
substance P
stir 15 sec

constant, and $c_s$ the concentration of substance next to the bilayer surface. This equation is based on the assumption that the adsorption sites are spatially fixed. For adsorption to a fluid membrane, the Volmer isotherm, which assumes the adsorption sites not to be localized in space, may be more appropriate (e.g., see ref. 26):

$$c_s/K_d^V = \left[\sigma_m/\left(\sigma_{max}^V - \sigma_m\right)\right] \cdot \exp\left[\sigma_m/\left(\sigma_{max}^V - \sigma_m\right)\right] \quad (5)$$

where $K_d^V$ and $\sigma_{max}^V$ are analogous to $K_d^L$ and $\sigma_{max}^L$. For $\sigma \ll \sigma_{max}^V$, eq. 5 reduces to a form which is formally equivalent to eq. 4, but with $K_d^L = K_d^V/2$ and $\sigma_{max}^L = \sigma_{max}^V/2$. The data on the binding of substance P to the neutral membranes were fitted by a nonlinear least-squares fitting program [27] to both eqs. 4 and 5.

The relationship of $c_s$ to the free (bulk) concentration, $c_f$, is given by the Boltzmann equation,

$$c_s = c_f \exp(-zFV/RT), \quad (6)$$

where $z$ denotes the net charge of the molecule, $F$ the Faraday constant, $R$ the gas constant, $T$ the absolute temperature, and $V$ the effective electrical potential at the point of adsorption.

In many situations, $V_{gc}$, the Gouy-Chapman potential, is substituted for $V$ in eq. 6, leading to the so-called Gouy-Chapman-Stern equation for binding of charged substances. The Gouy-Chapman potential is calculated assuming a uniformly smeared surface charge density, and has been applied successfully in a great many situations involving averaged quantities and moderate charge densities.

At low bound concentrations, however, the discrete charge effect must be considered. Just as the surface potential decreases with increasing distance from the membrane surface, there is a lateral decay moving away from a bound charge along the membrane surface. A charged molecule approaching the surface will naturally be diverted to the areas of lower potential between previously bound charges, so that the effective potential at the point of binding will be less than the average surface potential. An analytical treatment of this effect is given in ref. 14. In analogy with those studies, and based on the relatively low surface density of the bound substance P molecules, we apply the same treatment in the present case. Detailed analysis shows that the effective potential is at most 5% of the averaged potential ($V_{gc}$), so that such surface depletion effects are minor.

For the binding of substance P to the anionic PS/DOPC bilayers, there is a net attractive force, and an enhancement of the binding through surface accumulation is both expected and observed.

It is important to note that the measured compression of the bilayer caused by an electric field represents an average over the whole surface, in contrast to the binding of a charged species, for which the local field at the binding site is relevant. Thus, the experimental $V_{C\min}$ value is a good measure of the idealized Gouy-Chapman potential. A more detailed discussion of these effects was given by Schoch and Sargent [14].

The dissociation constant ($K_d^L$ or $K_d^V$) depends implicitly on the chemical potential of the adsorbing species both on the membrane surface and in the aqueous phase, and may thus be expected to change with ionic strength. In such a case, the equilibrium bound concentration could also depend on the ionic strength, which should be revealed by slow changes in $V_{C\min}$ following the initial jump resulting from shielding. Such an effect was found for melittin [14], for which the ratio bound/free increased at higher salt concentration.

## 3. Results

### 3.1. Binding to neutral membranes

The experiments with neutral membranes were performed at pH 5.5 to ensure protonation of the peptide amino group, $pK_a \approx 6.5$ [28]. Addition of substance P at concentrations higher than $10^{-5}$ M caused measurable changes in the surface potential of the neutral bilayers. Short-term shielding experiments were used to distinguish fixed-charge surface potentials from the dipole potentials induced by the bound molecules. Fig. 3 shows the concentration dependence of $V_{C\min}$ for a series of measurements on a single bilayer (lowest curve,

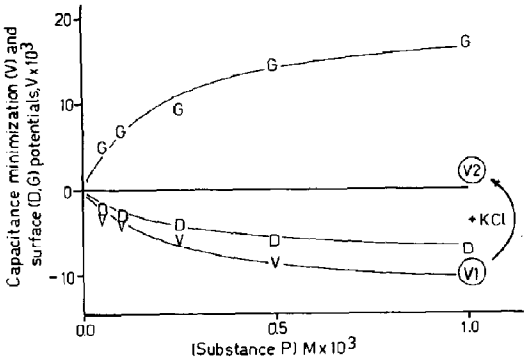



Fig. 3. Surface potentials induced on a neutral POPC bilayer by bound substance P. The points denoted by V indicate the measured $V_{C\min}$ values in 10 mM buffer. At the highest concentration of substance P ($10^{-3}$ M, point V1, $V_{C\min} = -10$ mV), KCl was added to bring the total ionic strength to 110 mM (point V2, $V_{C\min} = 2$ mV). From this jump of 12 mV (on raising the ionic strength from 10 to 110 mM), a surface charge density of $2.5 \times 10^{-2}$ charges/nm$^2$ and a Gouy-Chapman potential of 17 mV (in 10 mM buffer) are indicated (point A in fig. 1). Application of eq. 1 yields a dipole potential of $-7$ mV. The average ratio of $V_d$ to the surface density of bound molecules, $\sigma_m$, from three experiments on both neutral and anionic membranes ($800 \pm 100$ mV nm$^2$) was used to derive separate $V_{gc}$ (G) and $V_d$ (D) values for the rest of the curve. The potentials corresponding to the binding parameters fitted to the Langmuir isotherm (eq. 4) are indicated by the solid lines.

'V'). Only one shielding experiment was attempted per membrane, as exchanging the aqueous phase often led to mechanical rupture. In the experiment shown, $V_{C\min}$ reached a value of $-10$ mV at $10^{-3}$ M substance P in the 10 mM buffer (point V1). Upon raising the ionic strength to 110 mM by addition of KCl, $V_{C\min}$ changed to 2 mV (point V2). Application of eq. 2 indicated a fixed-charge surface potential of 17 mV and a surface charge density of $2.4 \times 10^{-2}$ charges/nm$^2$, corresponding to a surface density of bound substance P of $\sigma_m = 8 \times 10^{-3}$ molecules/nm$^2$.

As determined from eq. 1, there is a dipole potential of $-7$ mV. This potential represents a change induced by the adsorbed substance P and cannot be given a unique interpretation. It might represent solely the contribution of bound substance P. In this case, $\sigma_m$ is substituted for $\sigma_d$ in eq. 3, yielding $\mu_{mol} \approx 22$ Debye (assuming $\epsilon_a \approx 10$ in the interfacial region [14]). Alternatively, the measured dipole potential might reflect a different orientation of the dipole moment of the phospholipid molecules, induced by the bound substance P. The intrinsic dipole potential of a phospholipid monolayer is of the order of 440 mV [29]. The measured $V_d$ of 7 mV could represent a very minor alteration in the average conformation, or a larger change in only a few phospholipids, for example. Table 1 lists the values of surface charge densities and dipole potentials measured at three different concentrations of substance P both on neutral bilayers and on anionic bilayers (see section 3.2). Combining all determinations yields $V_d/\sigma_m = (8 \pm 1) \times 10^2$ mV nm$^2$ (mean ± S.D.), i.e.,

Table 1

Selected binding data and measured dipole potentials for substance P on neutral and anionic lipid bilayer membranes

| Lipid type | Substance P in solution (M) | | Substance P bound (molecules/nm$^2$) ($\sigma_m$) [b] | Dipole potential | |
|---|---|---|---|---|---|
| | Bulk ($c_f$) | Surface ($c_s$) [a] | | ($V_d$) (mV) [c] | ($V_d/\sigma_m$) (mV nm$^2$) |
| POPC | $1 \times 10^{-4}$ | $1 \times 10^{-4}$ | $3.3 \times 10^{-3}$ | −3 | 900 |
| | $8 \times 10^{-4}$ | $8 \times 10^{-4}$ | $8.0 \times 10^{-3}$ | −7 | 900 |
| | $1 \times 10^{-3}$ | $1 \times 10^{-3}$ | $8.3 \times 10^{-3}$ | −7 | 800 |
| PS/DOPC (1:9) | $2 \times 10^{-7}$ | $2.6 \times 10^{-5}$ | $3.4 \times 10^{-3}$ | −2.5 | 800 |
| | $5 \times 10^{-7}$ | $3.6 \times 10^{-5}$ | $6.6 \times 10^{-3}$ | −5 | 800 |
| | $1 \times 10^{-6}$ | $5.0 \times 10^{-5}$ | $8.1 \times 10^{-3}$ | −6 | 700 |

[a] Concentration adjacent to surface from eq. 6 using $V = 0$ for POPC, and $V = -46$ mV for PS/DOPC.
[b] Experimental uncertainty: $\pm 1 \times 10^{-3}$ molecules/nm$^2$.
[c] Experimental uncertainty: ±1–2 mV.

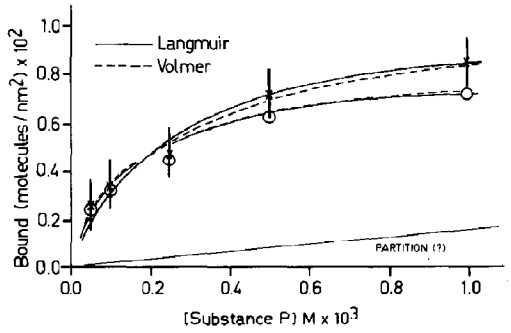



Fig. 4. Number of substance P molecules bound per unit area of POPC membrane surface vs. the free solution concentration of substance P (abscissa) in buffer of 10 mM ionic strength, at pH 5.5. (×) Binding derived from a direct analysis of the data of fig. 3. Error bars show the potential error resulting from the ±1 mV experimental uncertainty. The thick, continuous line indicates the fit using the Langmuir adsorption isotherm, eq. 5($K_d^L = 2.2\times10^{-4}$ M, $\sigma_{max}^L = 1.0\times10^{-2}$ molecules/nm$^2$), the dashed line using the Volmer isotherm, eq. 5 ($K_d^V = 4\times10^{-4}$ M, $\sigma_{max}^V = 1.7\times10^{-2}$ molecules/nm$^2$). The straight line at the bottom indicates the amount of substance P expected to bind based on the partitioning reaction reported by Seelig and MacDonald [12], assuming $K_a = 1$ M$^{-1}$ and an area/lipid molecule of 0.7 nm$^2$. Subtracting this curve from the upper curve yields the corrected values (○; error bars omitted for clarity). The best fits for the Langmuir and Volmer isotherms are shown by the thin, continuous and dashed lines ($K_d^{L'} = 1.5\times10^{-4}$ M, $\sigma_{max}^{L'} = 0.8\times10^{-2}$ molecules/nm$^2$, $K_d^{V'} = 1.5\times10^{-4}$ M, $\sigma_{max}^{V'} = 1.1\times10^{-2}$ molecules/nm$^2$). These $\sigma_{max}$ values correspond to 1 substance P molecule per 190 and 130 POPC molecules for the Langmuir and Volmer isotherms, respectively.

the ratio $V_d/\sigma_m$ is constant within experimental error.

This average value of $V_d/\sigma_m$ was used to derive $V_{gc}$ and $V_d$ for every datapoint in fig. 3. The results are designated in the plots as G and D, respectively. The surface density of bound molecules, $\sigma_m$, was calculated from $V_{gc}$. The binding curve to the data of fig. 3 is shown in fig. 4. A nonlinear least-squares fit of the binding data to eq. 4 yielded a maximal bound surface density of $\sigma_{max}^L = (1.0 \pm 0.1)\times10^{-2}$ molecules of substance P per nm$^2$ of surface, and a dissociation constant of $K_d^L = (2.2 \pm 0.3)\times10^{-4}$ M (continuous line). Fitting the data to eq. 5 yielded $\sigma_{max}^V = (1.7 \pm 0.3)\times10^{-2}$ molecules/nm$^2$ and $K_d^V = (4 \pm 2)\times10^{-4}$ M (dashed line).

### 3.2. Binding to anionic bilayers

The intrinsic surface charge density of the PS/DOPC bilayers was determined in a shielding experiment. The value obtained ($V_{gc} = -46$ mV, corresponding to $7.7\times10^{-2}$ negative charges/nm$^2$) was lower than one would expect for the lipid mixture by a factor of approx. 2. Lower surface charge densities than expected in phosphatidylcholine : PS mixtures (95 : 5) have been reported previously [30]. Part of the difference in our case may have been due to incomplete uptake of PS into the membrane-forming phase. If a drop of chloroform is added to the hexane/hexadecane mixture used to dissolve the lipids, an increased surface charge density is found.

The surface charge density attributable to bound substance P is the difference between the surface charge density before and after the addition of peptide. The results of three experiments at low substance P concentrations (less than 20% saturation) are given in table 1. The ratios $\sigma_m/c_f$ (bound/free) of substance P were much higher than for the neutral membranes. However, application of the Boltzmann equation (eq. 6) to calculate the concentration of substance P immediately adjacent to the bilayer, $c_s$, yielded $\sigma_m/c_s$ ratios close to the $\sigma_m/c_f$ ratios for the neutral bilayers.

Three independent determinations of the dipole potential with shielding experiments yielded values of $V_d/\sigma_m = (7.6 \pm 0.6)\times10^2$ mV nm$^2$. Given the overall experimental uncertainty, these results agree with those determined for neutral bilayers.

## 4. Discussion

On the basis of our data (fig. 4) we cannot say whether the Langmuiror the Volmer isotherm is more appropriate. Measurements at higher substance P concentration would be required, but this is difficult due to self-association of substance P at aqueous concentrations much above 1 mM [31]. Depending upon which binding model one considers, our highest experimental value of bound substance P was about 83% (Langmuir) or about 50% (Volmer) of the saturation binding level. In the latter case, the potential errors in $K_d^V$ and $\sigma_{max}^V$

are considerable. This is reflected in the uncertainties given.

The dissociation constant ($K_d$) derived for the binding isotherms reflects the affinity of substance P for the lipid bilayer. Our measured value ($K_d^L = 2.2 \times 10^{-4}$ M or $K_d^V = 4 \times 10^{-4}$ M) agrees well with a theoretical estimate ($2.4 \times 10^{-4}$ M) given previously [3]. Lembeck et al. [10] measured the apparent $K_d$ ($10^{-7}$ M) for extraction of substance P into an organic phase by PS. A direct comparison of the two values is difficult as the experimental situation is very different – extraction with ion-pair formation into a bulk phase, compared with surface binding. A large Boltzmann factor will be involved, however, so the difference by a factor of about 1000 is entirely plausible. Schäfer et al. [11], in a study of the binding of substance P to liposomes of mixtures of phosphatidylcholine and phosphatidic acid, reported an apparent association constant of 0.037 $1/\mu$mol ($K_d = 2.7 \times 10^{-5}$ M) at a mole fraction of phosphatidic acid of 0.18 and an ionic strength of 0.01 M. This $K_d$ value is lower than ours by a factor of less than 10 in the case of neutral bilayers, in spite of the charged liposome surface. The results of Schäfer et al. for unilamellar liposomes were obtained using gel filtration to separate bound and free fractions. This non-equilibrium method may have resulted in an artefactual loss of apparent affinity.

In classical drug/receptor-binding studies the maximum bound concentration is generally equated with the number of 'binding sites'. In our situation, with no specific 'receptors' and a value of about 1 molecule of substance P per 100–150 lipid molecules, this interpretation is clearly not relevant. The limit to binding may be analogous to a limited 'solubility' in the bilayer, involving boundary lipids, etc. Schäfer et al. [11] determined similar values for the maximum molar binding capacities to unilamellar liposomes (1/750–1/120, depending on the conditions).

The shielding experiments were instrumental in determining the surface charge density resulting from the binding of substance P to neutral membranes. As mentioned above, in addition to shielding effects, a change in the ionic strength could result in differences in the surface potential through variation in the amount of bound substance P. From the relatively slow kinetics of binding of substance P to the bilayers, we were able to measure the shielding effect separately from any further adsorption or desorption phenomena. It is interesting to note, however, that no longer-term variations in $V_{C\,min}$ were observed following changes in ionic strength (data not shown). This suggests that there are no major shifts in the amount of substance P bound when the ionic strength is changed. The sensitivity of measurement is lower at the higher ionic strength, however, it appears as if the chemical potentials of both bound and free species are affected in a similar manner.

Interpretation of the results with the mixed PS/DOPC system is made more difficult by the heterogeneity of the system. As with the Boltzmann factor for the self-repulsion of bound, charged molecules, the attractive forces between a charged ligand and an oppositely charged membrane will be non-uniform due to the discrete nature of the charges. This situation may be compounded by potential heterogeneity of the lipid mixture, especially in the presence of multiply charged ligands. Complete segregation of phosphatidylcholine and phosphatidic acid mixtures has been observed in the presence of $Ca^{2+}$ [32]. Such segregation could lead to enhanced binding of substance P in domains of charged lipid, while the neutral domains would bind very little at the concentrations reported here for anionic bilayers. Given the exponential dependence of the Boltzmann factor on surface potential, a greater extent of total binding could be expected compared to a homogeneous distribution. Such an effect may be reflected in our observations, where the amount bound tends to be higher than that predicted assuming a homogeneous model. Schäfer et al. [11] also reported enhanced binding of substance P to clustered negative charges compared to single ones. The results with the charged bilayers must be interpreted with caution, but they give no indication of qualitatively different interactions with lipid membranes when the Boltzmann accumulation is accounted for.

The measured dipole potential cannot be given a definite interpretation. The linear dependence of the dipole potential on the number of bound

molecules of substance P ($V_d/\sigma_m$ constant) could be explained either by a dipole moment of the bound molecules themselves, or by a disturbance, proportional to the concentration of bound substance P, of the orientation of the membrane lipid molecules. If we assume the averaged contribution of the phospholipids to be unchanged by substance P, the dipole moment calculated for substance P itself would be about 20 Debye (eq. 3). While this compares very well with the value expected for the membrane-bound conformation and orientation indicated by previous studies [7–9], the possibility of other mechanisms resulting in a similar dipole moment cannot be ruled out.

Recently, Seelig and Macdonald [12] reported on the binding of substance P to neutral and anionic lipid monolayers, measuring the change of surface area at constant pressure when substance P was introduced into the subphase. They observed insertion into monolayers containing the anionic lipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (25–100%, in POPC). No insertion into uncharged monolayers at concentrations of substance P between $6.5 \times 10^{-6}$ and $1.25 \times 10^{-4}$ M was observed. For the charged monolayers the level of insertion increased linearly with the content of anionic lipid, and diminished with increasing surface pressure. At constant pressures and anionic lipid content, it appeared to follow a simple partitioning process between the aqueous and hydrophobic phases. The insertion became negligible at pressures at which the monolayers are claimed to 'mimick bilayer membranes' (32–35 mN/m), and the authors conclude that their "...data strongly suggest that substance P does not penetrate into neutral bilayer membranes in this concentration range", i.e., $6.5 \times 10^{-6}$–$1.25 \times 10^{-4}$ M. They further conclude that the best estimate for the intrinsic association constant to the POPG monolayer is about 1 $M^{-1}$.

There are major differences between our measurements and those of Seelig and MacDonald [12]. These authors measure changes of surface area ($\Delta A/A$) of between '0' and 43% upon addition of substance P to the aqueous subphase. The latter value corresponds to a substance P/lipid ratio of 0.14, which is at odds with the binding capacity determined by Schäfer et al. [11] (maximum binding capacity of about 0.008). The uncertainty in the area measurement would appear to be about $\Delta A/A = 1$–2%. Our measurements indicate saturation of binding at about $10^{-2}$ molecules/nm$^2$. Assuming an average area for the inserted $\alpha$-helix of about twice that of the lipid, i.e., approx. 1.6 nm$^2$/molecule *, our saturating bound concentration would yield a change in area of 1.6%, (which is below, or at best near, their limit of resolution. Thus, it is not surprising that their measurements, made at effective surface concentrations of substance P of up to 0.1 M (with 100% POPG), reveal nothing of the interaction we have measured with much higher sensitivity at maximal surface concentrations of 1 mM.

Conversely, one can address the question of whether we should have detected the reported partitioning reaction with our technique. Taking the intrinsic association constant to be 1 $M^{-1}$, as given in ref. 12, and assuming the area per lipid molecule to be about 0.7 nm$^2$, the concentration of substance P expected to undergo partitioning into the membrane at the aqueous concentrations we have used is indicated on fig. 4 (lower straight line). It can be seen that the partitioning reaction only reaches a significant fraction of the total binding at the highest concentration employed (1 mM). Nevertheless, it is conceivable that this reaction may be responsible for the tail of the binding curve. Subtracting the partitioning curve from the total bound concentration yields the middle curve of fig. 4. Again, both the Langmuir and Volmer isotherms give satisfactory fits to the corrected data, the $K_d$ and $\sigma_m$ values being slightly lower than those for the uncorrected curve ($K_d^{L'} = (1.5 \pm 0.3) \times 10^{-4}$ M, $\sigma_{max}^{L'} = (0.77 \pm 0.05) \times 10^{-2}$ molecules of substance P per nm$^2$ surface; $K_d^{V'} = (1.5 \pm 0.1) \times 10^{-4}$ M, $\sigma_{max}^{V'} = (1.14 \pm 0.1) \times 10^{-2}$ molecules/nm$^2$). While it would not be justified to infer the presence of the partitioning reaction from our data, there is no contradiction between the partitioning parameters reported by Seelig and MacDonald and our measurements. Further work

* Seelig and MacDonald assume a value of about 3-times that of the lipid, based on a polyphenylalanine $\alpha$-helix, which will overestimate the average helix cross-sectional area.

is necessary to determine whether partitioning into neutral membranes does occur.

Our results are consistent with the model proposed earlier for the hydrophobic interaction of substance P with lipid membranes [3,7–9]. The model has the C-terminal domain inserted perpendicularly into the hydrophobic layers of the membrane as an $\alpha$-helix and the charged N-terminal segment remaining in contact with the aqueous phase as a random coil. The prediction of the hydrophobic association constant [3,7] was based solely on the hydrophilic-hydrophobic partitioning of a C-terminal segment of substance P: no specific polypeptide-lipid head group or electrostatic interactions were assumed. The validity of this derivation is strengthened by the observed correspondence between the amount of substance P bound to both neutral POPC bilayers and anionic PS/DOPC bilayers when referred to the effective concentration of substance P (table 1). This effect is also the justification for the relevance of studies in the millimolar range of a regulatory peptide for which the biological potency is in the nanomolar range. Many biological membranes have negative fixed-charge surface potentials of the order of 30–40 mV [12,33,34], so that the Boltzmann accumulation factor on such membranes will be similar to that for the PS/DOPC bilayers, where the substance P-bilayer interaction is measurable in the range of $10^{-7}$ M.

A complementary study in a biological system revealed a close correlation between the Boltzmann accumulation factor for dynorphin A-(1–9)-nonapeptide and the influence of salts on the interaction of this peptide with the $\kappa$-receptor sites [35,36], which have been proposed to be sequestered in a hydrophobic membrane compartment [2]. Both the dynorphin studies and the present results demonstrate the physical interplay between electrostatic and hydrophobic interactions that has been postulated to be a major element of membrane-catalyzed peptide-receptor interactions [4].

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