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Voltage dependent binding of annexin V, annexin VI and annexin VII-core to acidic phospholipid membranes

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Abstract

Annexin V, VI and VII-core (Δ1–107) are members of the annexin protein family and bind to acidic phospholipid membranes in a calcium dependent manner. They also show ion channel activity under certain conditions. As annexins bind peripherally to lipid membranes, ion channel formation must consist of at least two steps: An adsorption reaction regulating the binding of annexin to the membrane surface and the opening and closing of the active species controlling the channel activity. By using the baseline current through the patch clamp seal as a probe for unoccupied binding sites at the membrane, we show that the adsorption of annexins to membranes is not only calcium dependent but also strongly voltage dependent. Whereas the free transfer energies at low calcium concentrations are similar for all three annexins, the binding of annexin V becomes much tighter with higher calcium levels, compared to annexin VI and VII-core. This correlates with the finding that annexin VI and VII-core display channel activity much more often than annexin V if one assumes that a high coverage of the membrane surface with annexins stabilizes the bilayer. At higher protein concentrations weaker binding is observed in agreement with the previously reported anti-cooperativity of membrane binding. © 1997 Elsevier Science B.V.

Keywords: Lipid bilayer; Electric field; Membrane electrophysiology; Lipid-protein interaction; Patch clamp; Adsorption

1. Introduction

Annexins are a family of calcium binding proteins consisting of at least 13 members. They share the property of calcium dependent binding to phospholipid membranes; their physiological role, however, is not yet clear (for reviews see [1,2]). Some members of the annexin protein family are involved in the aggregation and fusion of lipid vesicles as well as endo- and exocytosis [3,4]. Their anti-inflammatory and anti-coagulatory effects might be explained by the depletion of membrane substrate, as annexins compete with phospholipase A_2 and blood coagulation factors for binding to the membrane surface [5,6]. Annexins also bind to components of the cytoskeleton [7] and are targets for intra-cellular kinases in vivo [8].

Since the calcium binding sites are located in exposed loops of the *C*-terminal core [9,10], this part of the protein is responsible for membrane binding. In contrast, the variable *N*-terminus, which is unique

Abbreviations: anx, annexin; $c(X)$, concentration of X; F, Faraday's constant; HEPES, *N*-(2-hydroxyethyl)-*N'*-ethane sulfonic acid-piperazine; I, current; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; R, resistance; T, temperature; V, voltage; WT, wildtype

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for each annexin, supposedly carries the distinctive function of each annexin, like protein–protein-interaction [11] and harbours the phosphorylation sites in annexin I and II [7,12].

The calcium dependent adsorption of annexins to membranes has been studied from different point of views, particularly its dependence on the different phospholipid composition (for a review see [13]). All reported data agree that annexins bind preferentially to acidic phospholipids [14]. Whereas the dissociation constants for annexin V–Ca^{2+} and PS–Ca^{2+} are about 10^{-3}–10^{-4} M, the dissociation constant for the ternary complex annexin V–Ca^{2+}–PS is in the range of 10^{-9}–10^{-11} M [15,16]. The most common parameter determined is the calcium concentration for half-maximal binding of annexin to membranes, $c_{1/2}(Ca^{2+})$. This parameter is slightly different for different annexins. For annexin V, values of $c_{1/2}(Ca^{2+})$ of 50 and 30 μM are reported whereas annexin VI shows a $c_{1/2}(Ca^{2+})$ of 140 μM [14,17]. Energies of binding events have been obtained by enthalpy measurements with isothermal titration calorimetry [17].

The influence of the transmembrane potential on membrane binding has not been determined so far, but is important for the in vivo functions in the cell and for the ion channel function in patch clamp experiments. Voltage dependent binding to membranes involves all charged components, calcium ions, protein and phospholipid. One has to consider two main interactions of annexins on membranes: adsorption (peripheral binding) and ion channel activity.

There have been several attempts to understand protein adsorption to membranes recently [18–20]. Generally, the free energy of transfer for the process:

$$\text{protein}_{\text{dissolved}} \rightleftharpoons \text{protein}_{\text{bound}} \quad (1)$$

consists of various contributing energy terms:

$$\Delta G_{\text{transfer}} = \Delta G_{\text{bind}} + \Delta G_{\text{imm}} + \Delta G_{\text{lip}} + \Delta G_{\text{el}} + \Delta G_{\text{inter}} \quad (2)$$

with ΔG_{bind}, the binding energy of the protein to the membrane consisting of an ionic (ΔG_{ionic}) and a hydrophobic (ΔG_{phob}) energy, ΔG_{imm}, which is due to the immobilisation of protein at the membrane, ΔG_{lip}, an energy describing the perturbation of the lipids, ΔG_{el}, an electrostatic energy resulting from the interaction of the protein dipole with the transmembrane potential and a term ΔG_{inter}, which describes possible protein–protein-interactions on the membrane.

Recently, Ben-Tal et al. [19] investigated the binding of basic peptides to acidic phospholipid vesicles and pointed out that the electrostatic energy is composed of two terms, a long range attractive interaction and a short range repulsion.

We note that in case of living cells and in voltage clamp experiments a third term contributes to ΔG_{el} representing the transmembrane potential.

For some annexins ion channel activity with selectivity for cations was found [21–26]. The experiments are done with synthetic lipids either in planar lipid bilayer or patch clamp experiments. Also, variable ionic conditions were used in the past with different types of cations on each side of the membrane bilayer. Ion channel experiments with annexins are characterized by the rare activity of these proteins. Additionally, the activity suddenly gets lost in many preparations while the membrane seal remains intact (unpublished results).

The common model behind these studies was an annexin-induced ion channel activity with the protein providing the ion pathway. There is no experimental evidence for insertion of annexins into lipid bilayers yet, but the electrophysiological data published indicate a symmetrical action of the channel active species. The channel opens (closes) with the same frequency for negative and positive holding potentials. The recorded current jumps during channel activity at a certain holding potential were assigned to a single channel with an activity for annexin V of 30 pS for symmetrical calcium concentrations [21,26].

Rojas and coworkers used a bath solution of 25 and 50 mM calcium as the ionic conditions for ion channel experiments with annexins [21]. Under these conditions one has to consider annexin dimerisation [27,28] and strong binding of annexins to acidic phospholipids. The model of single translocating annexin molecules is therefore not applicable and one has to expect extensive membrane coverage by these proteins.

In ion channel studies of annexins a parameter has to be considered which plays no role in transmembrane protein channels. These proteins reside within the membrane different from annexins, which are added to the patch preparation and bind only periph-

erally to the membrane [16,29,30]. As annexins carry a net charge which is altered by binding of calcium, this process is dependent on the electric field. Changing the holding potential in an electrophysiological experiment should therefore result in altered electrodiffusion behaviour of annexin molecules or the annexin–Ca^{2+} species, respectively. Accordingly, by varying the holding potential annexin is translocated from the bath solution to the membrane and vice versa to varying extents.

This paper describes for the first time the voltage dependent binding of annexins to membrane bilayers. The studies on the membrane adsorption of annexins presented here provide evidence that the view of single annexin molecules on the patch membrane surface displaying channel activity is too simple. In a patch clamp experiment with annexins four parameters come into play: The lipid composition of the membrane, the concentrations of both, protein and calcium and the applied transmembrane potential. Each of these four parameters has a strong influence on the actual state of the protein in the system and therefore the channel active species is affected by these, too. This work also introduces a new method of adsorption measurements applicable especially to peripheral binding proteins.

2. Materials and methods

2.1. Proteins

The proteins were purified as described elsewhere [10,31,32]. Annexin VII-core is a truncated form of human annexin VII (Δ1–107). Human annexin V and VI was used as recombinant material.

2.2. Seal forming

Electrophysiological recordings were carried out by using synthetic phospholipid bilayers formed by the double-dip technique [33]. Seals were formed from PS/PE (molar ratio 4:1) on patch pipettes (quartz, outer diameter 1.0 mm) with an opening diameter of about 1.0 μm. The lipid mixture was spread on the surface of a 150 μl drop of 50 mM $MgCl_2$, 10 mM HEPES (pH 7.4). After formation of a giga-seal (5–10 GΩ) the bath solution was exchanged once to remove excess lipid. The pipette solution in all experiments was 50 mM $MgCl_2$, 10 mM HEPES (pH 7.4). After correcting the offset potential to zero a control experiment was done with this preparation.

2.3. Pulse experiment

The membrane potential across the bilayer was stepped from −200 to +200 mV from a holding potential of 0 in 20 mV increments by clamping the pipette potential with respect to the bath using an EPC-7 amplifier (List Medical, Darmstadt, Germany) and the Strathclyde Electrophysiology Software (WCP v. 1.2, J. Dempster, Glasgow, Scotland). The voltage steps had a duration of 60 s; between the steps a holding potential of 0 mV was clamped for 60 s (Fig. 1). The currents were sampled at 2 Hz and analysed using the WCP software. All recordings were done at 19°C.

2.4. Calcium- and protein-titration

After recording a control current–voltage relation, $CaCl_2$ was added to the bath from highly concentrated stock solutions to the final bath concentration. In this way, the concentration of calcium in the bath was increased in steps from 0 to 50 mM. After waiting 5 min to allow for equilibration of the system, the next pulse experiment was started.

The appropriate amount of annexin (2 pM, 2 nM, 2 μM) was added with the first portion of calcium. Even at 2 pM, the number of annexin molecules present in the bath exceeds the number of possible binding sites at the membrane seal by a factor of 10^4 as estimated from the size of the membrane surface.

2.5. Data analysis

Each seal was tested for stability before performing the calcium–protein titration. Additionally, only seals without any channel activity after adding the protein were used. First, a control I–V relation was made in the absence of calcium and protein. The current amplitudes were normalized with respect to this maximal current amplitude and then averaged over three independent preparations for each experiment. The control experiment was fitted by a linear equation:

$$I_0 = \frac{1}{r}V + i \tag{3}$$

where I_0 is the current amplitude in the absence of

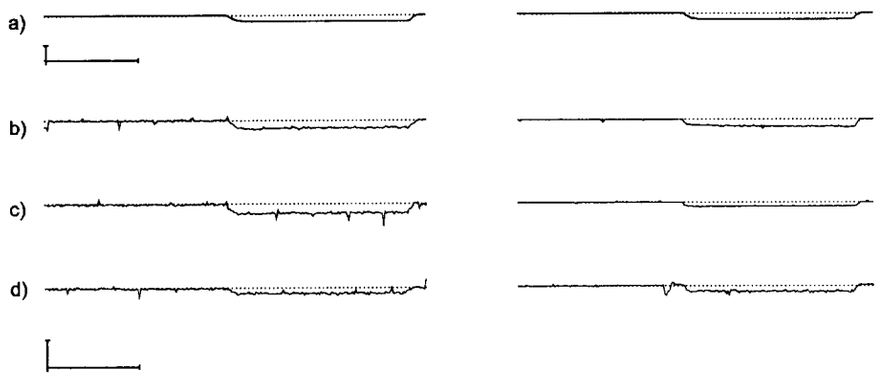

Fig. 1. Voltage–current recordings from a PS/PE (4:1) seal. (a) Applied voltage step; vertical bar: 200 mV, horizontal bar: 30 s. (b)–(d) Current recordings of the seal at different calcium levels. Without $CaCl_2$ (b), with 50 μM $CaCl_2$ (c), with 5 mM $CaCl_2$ (d). Vertical bar: 8 pA, horizontal bar: 30 s. Left panel: without annexin, right panel: with 2 pM annexin V in the bath. The transmembrane potential was stepped in the range from −200 to 200 mV in 20 mV increments. The holding potential was 0 mV. Current sampling was done at 2 Hz. The records shown refer to a voltage step 0–100 mV.

annexin and calcium, V the applied step potential, $1/r$ the slope and i the intercept, respectively.

Current amplitudes were measured at the end of the voltage pulse, $I = I(60\ \text{s})$, and the calculated resistance $R(60\ \text{s})$ is used, assuming Ohmic behaviour.

2.6. Theoretical models: The Langmuir adsorption isotherm

To model the adsorption phenomenon we used the standard Langmuir isotherm [34]. The basic reaction is written as:

$$\text{annexin}_{\text{dissolved}} + n\ \text{lipid} \overset{K}{\rightleftharpoons} [\text{annexin} - \text{lipid}_n] \quad (4)$$

where K is the association constant in 1 mol^{-1}. Since I/I_0 represents the fraction of still available surface area, the coverage Γ can be computed by:

$$\Gamma = 1 - \frac{I}{I_0} \quad (5)$$

The adsorption isotherm can then be expressed as:

$$\Gamma = \frac{Kc(\text{anx})}{1 + Kc(\text{anx})} \quad (6)$$

The association constant K is available according to:

$$K = \frac{I_0 - I}{Ic(\text{anx})} \quad (7)$$

where I and I_0 are the currents in presence or absence of annexin and $c(\text{anx})$ is equal to the starting concentration, $c_0(\text{anx})$.

Applying the Langmuir adsorption isotherm requires some general assumptions:

(1) The adsorption constants for annexins are not voltage dependent. As a consequence the current in the presence of annexin is a linear function of the voltage if we assume that the blank seal can be represented as an Ohmic resistance:

$$I = V\frac{1}{R_{\text{seal}}[Kc(\text{anx}) + 1]} \quad (8)$$

(2) Maximal adsorption corresponds to a monomolecular layer of annexins on the membrane with a degree of coverage $\Gamma = 1$ and a related current of $I = 0$ mV.

(3) The binding sites in this model are treated as being independent and the adsorbed species do not interact with each other.

2.7. Theoretical models: The Woodhull model

Voltage dependent block of transmembrane channels is often quantified using the Woodhull model [35,36]. In terms of a chemical reaction one can express the basic process as:

$$\text{bp} + \text{bs} \rightleftharpoons [\text{bp} - \text{bs}] \quad (9)$$

where 'bp' stands for blocking particle and 'bs' for a binding site.

Current amplitudes in the presence (I) and in the absence (I_0) of the blocking particle are related by the equation:

$$I = \frac{I_0}{1 + \frac{c(\mathrm{bp})}{k_d(V)}}; \quad k_d(V) = k_d(0\ \mathrm{mV})\exp\left(\frac{z\delta VF}{RT}\right) \tag{10}$$

where $k_d(0\ \mathrm{mV})$ is the constant for half-maximal block at 0 mV and δ is the portion of the membrane electric field sensed by the blocking site. z usually refers to the charge of the blocking particle. F and T have their normal meanings, R is the general gas constant.

As the current response of a seal to the different concentrations of calcium and protein in voltage pulse experiments is similar to that observed in blocking experiments we can apply that model to the adsorption phenomenon. I and I_0 are now the current amplitudes (baseline current) in the presence and in the absence of annexin, respectively. δ is the fraction of the effective field sensed by the adsorption site and should therefore be constant if we assume a monomolecular layer of annexins on the bath side of the seal. To determine $k_d(0\ \mathrm{mV})$ we fitted a linear equation to the linear part of $\ln[I/(I_0 - I)]$ plotted against the step potential:

$$\ln\frac{I}{I_0 - I} = \frac{z\delta F}{RT}V + \ln\frac{k_d(0\ \mathrm{mV})}{c(\mathrm{anx})} \tag{11}$$

$c(\mathrm{anx})$ was set to be equal to the starting concentration $c_0(\mathrm{anx})$.

3. Results

3.1. Adsorption of annexins to a bilayer membrane increases the seal resistance

Seal preparations for single channel investigations of annexins often display significant changes of seal resistance upon addition of the protein. The starting hypothesis was that the increasing resistance is due to the coverage of the bilayer by annexin molecules. However, one has to assure that no other parameter influences the seal resistance during a typical patch clamp experiment. As described in Section 2, every seal was tested for stability before and during any adsorption measurement. A seal was regarded as stable when the baseline was stable in the absence of protein and no channel activity occurred in the presence of protein. Under these conditions, we can neglect the influence of the transmembrane potential on the seal resistance. Furthermore, the influence of calcium on the membrane has to be considered. Adding different amounts of calcium to the preparation varies the normalized seal resistance by factors of 2 and 4. On the other hand, with annexin present in the bath the normalized seal resistance reaches values of up to 14-fold at high calcium concentrations. Additionally, the resistance becomes strongly voltage dependent with higher values at more negative transmembrane potentials (Fig. 2a–b).

An interesting fact in this connection is the asymmetric behaviour of the changes of seal resistance. The annexin-induced increase in resistance is only observed with negative transmembrane potentials whereas positive potentials seem to be indifferent (Fig. 2c).

3.2. High calcium concentration suppresses the voltage dependence of annexin adsorption

The influence of calcium on the annexin membrane system is crucial but complex: On the one hand, calcium is responsible for the membrane binding of annexins, on the other hand the calcium influx into phospholipid vesicles is increased upon annexin addition [37,38]. When preparing a patch clamp experiment one has to be aware of possible calcium-induced changes in annexin binding mode. In fact, most annexin treated seals are inactive under high calcium conditions in patch clamp experiments, whereas it is known from the macroscopic calcium-influx assay, that annexins are able to induce this ion influx. Our hypothesis was that the reason for this apparent discrepancy is the ratio of protein, calcium and the membrane surface. Therefore we tested the influence of different calcium concentrations on the seal resistance of annexin treated seals.

Calculating the degree of coverage according to Eq. (5) shows that higher calcium concentrations increase the degree of coverage (Fig. 3). This is not surprising because this experiment is the basic proce-

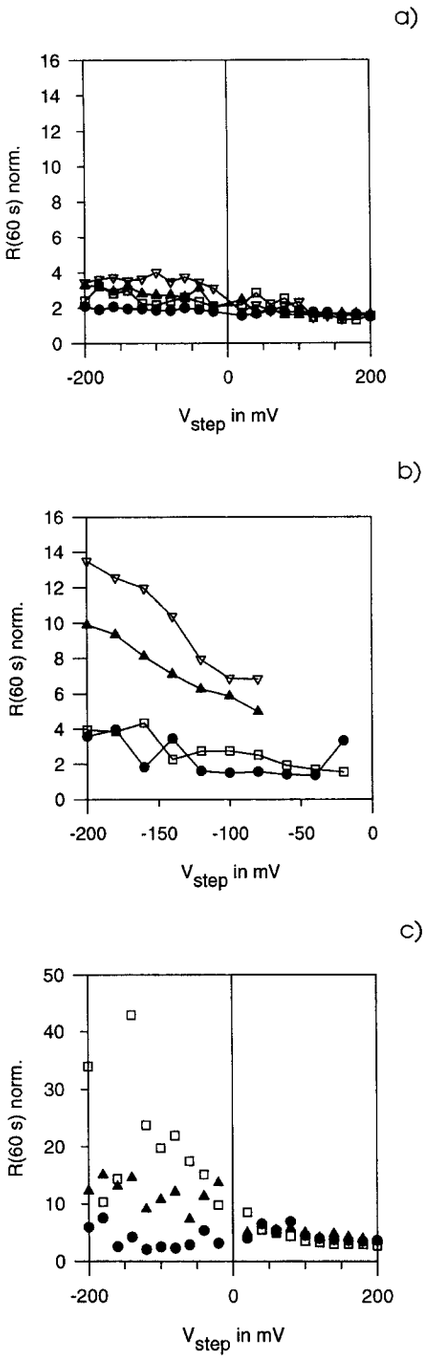

Fig. 2. Normalized seal resistance vs. applied step potential. Influence of (a) calcium, (b) annexin VII-core (2 pM) and (c) annexin V (2 pM) on a PS/PE (4:1) seal. 50 μM $CaCl_2$ (filled circles), 500 μM $CaCl_2$ (open squares), 5 mM $CaCl_2$ (filled triangles), 50 mM $CaCl_2$ (open triangles). Whereas calcium shows only a small effect on the seal resistance, annexin addition causes a dramatic increase which occurs only with negative step potentials.

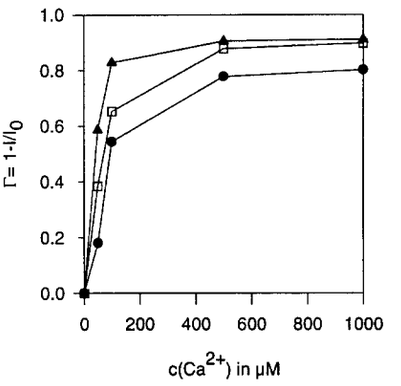

Fig. 3. Degree of coverage vs. calcium concentration at different step potentials. The step potentials applied to a PS/PE (4:1) seal are: −100 mV (filled circles), −120 mV (open squares), −140 mV (filled triangles); c(anx V) = 2 pM. Higher voltages shift the degree of coverage to higher values, the binding of annexin to the membrane is thus voltage dependent.

dure of any binding assay where annexin is translocated to the membrane in a calcium dependent fashion. One has to be aware that at 25 mM/50 mM Ca^{2+} in the bath (used as standard solution in former patch clamp experiments [21,26]) the membrane is fully covered with annexin molecules. Under these conditions one should not expect a few single channel events.

The second aspect to be mentioned is the voltage dependence of the binding of annexins to the membrane. An appropriate parameter is provided by the

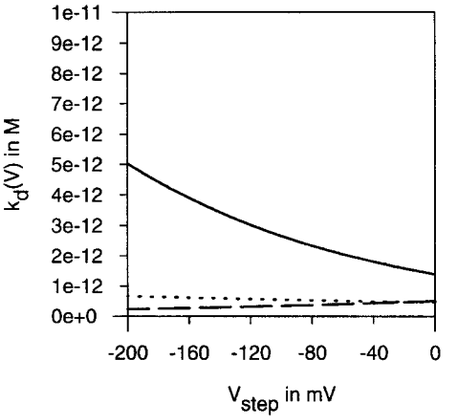

Fig. 4. Constant for half-maximal current reduction, k_d(0 mV), vs. applied step potential. 2 pM annexin V at different calcium concentrations: 50 μM (solid line), 500 μM (dashed line), 5 mM (dotted line). An increase in the calcium concentration reduces the voltage dependence of the adsorption process.

Woodhull model which allows the calculation of constants for a half-maximal reduction of current. From Eq. (11), the $k_d(0\ \text{mV})$, i.e., the constant for half-maximal reduction of current at 0 mV, can be obtained by fitting. The actual $k_d(V)$ at a certain voltage V is computed by multiplying the $k_d(0\ \text{mV})$ with the exponential factor. The variation of $k_d(V)$ with the applied transmembrane potential gives an impression about the voltage dependence of the current-reduction phenomenon. Increasing the calcium concentration reduces the voltage dependence as can be seen from Fig. 4. As adsorption is a dynamic process, it is not surprising that the voltage dependence is decreased with higher calcium concentrations. As calcium favours the adsorbed state an increase in calcium concentration should suppress the voltage dependence by depletion of the population of the dissolved state. With calcium concentrations well above values of the concentration needed for half-maximal binding ($c(Ca^{2+}) > 250\ \mu M$) the voltage dependence of the adsorption is rather small.

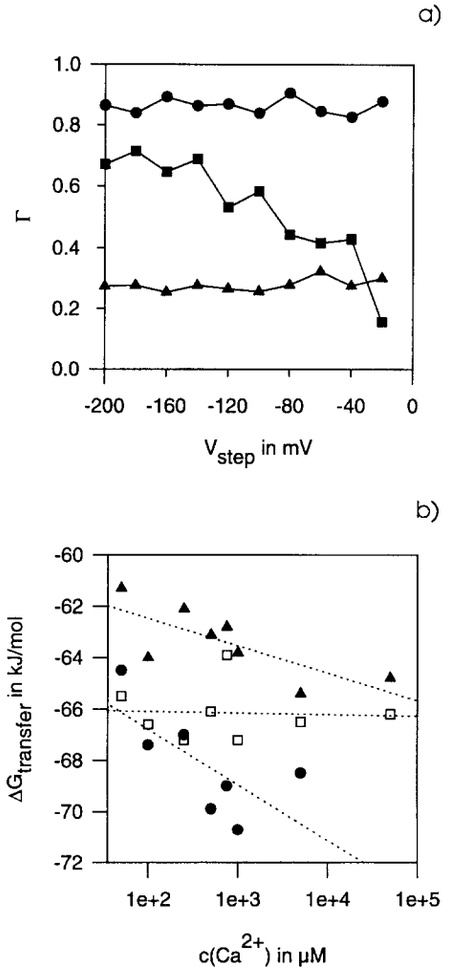

Fig. 5. Anti-cooperativity of annexin binding to membranes. (a) Degree of coverage with different concentrations of annexin V at 500 μM $CaCl_2$. (b) Free transfer energies according to the Langmuir isotherm vs. calcium concentration. A PS/PE (4:1) seal was treated with 2 pM (circles), 2 nM (squares), 2 μM (triangles) annexin V. High amounts of annexins weaken the strength of binding; there is a loss in the free transfer energy and the degree of coverage is lower with higher concentrations of protein. Addition of calcium decreases the free transfer energy, as expected.

Table 1
Adsorption data for annexin V as derived from the Langmuir isotherm and the Woodhull model, respectively

$c(CaCl_2)$	c(anx V)	$\Delta G_{transfer}$ /kJ mol^{-1}	k_d(0 mV)/M
50 μM	2 pM	−64.5	1.4×10^{-12}
	2 nM	−65.5	15×10^{-9}
	2 μM	−61.3	4.6×10^{-6}
500 μM	2 pM	−69.9	0.5×10^{-12}
	2 nM	−66.1	6.4×10^{-9}
	2 μM	−63.1	5.0×10^{-6}
5 mM	2 pM	−68.5	0.5×10^{-12}
	2 nM	−66.5	4.4×10^{-9}
	2 μM	−65.4	4.0×10^{-6}

3.3. Annexin adsorption on the membrane is anti-cooperative with respect to annexin

With annexin V we examined the influence of the protein concentration on the adsorption. The voltage pulse experiments were done with 2 pM, 2 nM and 2 μM of annexin V in the bath and different calcium concentrations. The degree of coverage is shifted to lower values drastically, as seen from Fig. 5a. Data analysis according to the Langmuir model shows that there is a decrease in the free transfer energy with rising protein concentrations; additionally, the dependence on the calcium concentration is lowered (Fig. 5b). The data derived from the Woodhull model show in analogy an increase of the half-maximal dissociation constant (Table 1). This means that at higher protein concentrations the binding of annexins to the

membrane becomes much weaker. This cannot be explained with a simple adsorption reaction.

4. Discussion

In electrophysiological investigation of the ion channel activity of annexins one is confronted with the rarity and irregularity of open events (unpublished results). In order to learn more about the mechanisms involved in single-channel-behaviour of annexins, we studied the adsorption of annexins to membrane bilayers in a patch clamp preparation. An important observation in single channel experiments was the change in seal resistance upon annexin addition to the bath. We interpret this change in seal resistance in terms of annexin adsorption to the membrane where the density of packing of the lipid molecules to the glass pipette increases. This explanation seems reasonable because Mukhopadya and coworkers report an increase in the surface pressure of membrane bilayers after annexin addition [39]. The denser packing of the lipid molecules reduces the leakage (baseline) current through the seal. The method described here uses the baseline current as a probe for the unoccupied adsorption sites on the membrane. Control experiments with pure seals and calcium titrated seals were used to ensure that the seal itself was stable for all transmembrane potentials and that calcium had only minor effects on the seal resistance. The resistance change in the presence of annexin therefore must be due to the protein.

Applying the Langmuir adsorption isotherm and a Woodhull model, respectively, we are able to derive three basic properties of annexin adsorption to membranes:

(1) The membrane coverage by annexins is voltage dependent increasing with more negative potentials.

(2) The coverage of the membrane is calcium dependent and increases with the calcium concentration. This is in agreement with binding assays. Increasing the calcium concentration lowers the voltage dependence of the adsorption process.

(3) The adsorption process is influenced strongly by the annexin concentration. The membrane binding is much weaker at higher annexin levels.

(2) and (3) are well-known by other experiments; thus the results obtained by our method agree with those from binding assays (e.g. [15]) and calorimetric experiments [17]. However, one has to be aware that binding assays or the calcium influx assay (FURA-2 assay) take place under very different conditions with a much larger membrane surface. Additionally, we show that the state of the system investigated by patch clamp methods is influenced by the calcium concentration, the membrane composition (e.g. [16,17]), the amount of annexin present and, most importantly, the transmembrane voltage. The voltage dependent binding has severe implications for the interpretation of channel measurements: The adsorption process is overlayed over the pure channel dynamics and the voltage influences both, the channelling and the binding behaviour.

4.1. The free transfer energy suggests large entropy changes upon annexin adsorption

As the Langmuir model assumes independence of the association constant K with respect to the voltage the assertions derived from this model are only valid for step potentials of zero. Additionally, the calculated value of the free energy is based on the assumption that total coverage of the membrane ($\Gamma = 1$) corresponds to an actual current of $I = 0$, as mentioned above. In practice, zero current was only measured once. There are two possible explanations. Either the membrane current can not be lowered to zero even if there is complete coverage of the membrane or there is never complete coverage of the membrane surface. The first possibility is more likely due to other leakage currents of the seal. It is important to notice that the degree of coverage obtained by this method is therefore only a lower limit of the real value and the reported values of $\Delta G_{transfer}$ an upper limit. The free transfer energy $\Delta G_{transfer}$ of the tested annexins is shown to be equal for a calcium concentration of 50 μM. Increasing the calcium leads to increased free transfer energies of annexin V but only to small changes in case of annexin VII-core and annexin VI. Therefore annexin V seems to be bound more tightly to the membrane at higher calcium concentrations with respect to a starting concentration of 50 μM and also in comparison with other annexins (Fig. 6). This is consistent with the finding that channel activity with annexin VI and VII-core appears more frequently than with annexin V. The

strong binding of annexin V seems to oppose channel activity.

According to Plager and Nelsestuen enthalpies of -201.1 and -142.5 kJ mol^{-1} are measured with annexins VI and V, respectively (membrane: PS/PC/PE = 18:32:50, 450 μM $CaCl_2$) [17].

The values of the association constants found here are in the range of 10^{11}–10^{13} M^{-1} corresponding to free transfer energies of about -65 kJ mol^{-1}. For 500 μM $CaCl_2$, the association constants K for annexin V–WT and annexin VI are 3.2×10^{12} and 3.6×10^{11} M^{-1}, respectively. Despite the distinct differences of the two experiments and the fact that the reported values of $\Delta G_{transfer}$ represent an upper limit there is a large difference between the enthalpy and the free energy which can only be explained by large entropy changes.

A large negative contribution by the entropic term has to be expected from the 2D-protein crystal formation and by the possibly accompanying membrane rigidification. Annexin crystallization on membrane surfaces is well-documented by electron microscopy [29].

4.2. Anti-cooperative binding of annexins to the membrane: A self-regulating process?

Bazzi and Nelsestuen already noted that there are some indications for an anti-cooperative effect in annexin-membrane binding [40]. It is therefore not surprising that there is a slight decrease of transfer energy as the annexin concentration is raised (Fig. 5b). The k_d(0 mV) values show the same tendency (Table 1).

A number of effects may cause the anti-cooperativity related to concentration-dependent properties of annexins in the dissolved phase and membrane-bound, respectively. Also, membrane properties could be involved. Annexins are oligomeric in solution [27,28]. If the aggregates are unable to associate with the membrane the concentration of the active membrane binding species is reduced. The effective electrostatic field at the membrane surface may be lowered with coverage. Additionally, the membrane properties seem to be varied by annexin binding severely. Changes in the fluid phase structure and the overall properties of the lipid bilayer upon binding of annexins were discussed by Gilmanshin et al. [41]. Recently, Mukhopadya and coworkers showed that annexin binding is dependent on the surface pressure of phospholipid monolayers [39]. Hence, binding of annexin molecules to the membrane is a self-regulating process which might include domain formation within the membrane induced by annexin. In summary, most terms contributing to the free transfer energy (Eq. (2)) vary with the amount of annexin molecules on the phospholipid layer.

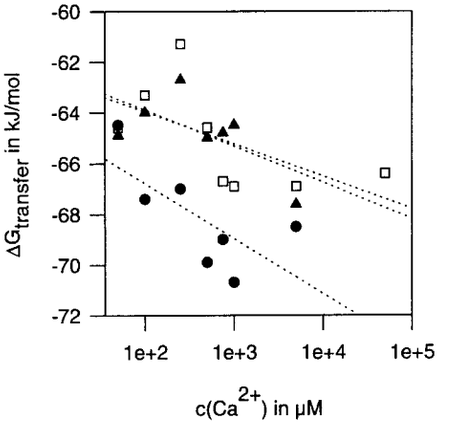

Fig. 6. Transfer energies of different annexins vs. calcium concentration. Annexin V (filled circles), annexin VI (open squares) and annexin VII-core (filled triangles) at 2 pM concentration. The free transfer energy is lower for annexin V than it is for the other two annexins. Thus, the binding of annexin V to the membrane is much tighter under the same circumstances.

5. Conclusions

One of the most interesting features of annexins is the ion channel activity which is investigated mostly by patch clamp techniques. The observations described here provide evidence that the dynamic properties of the annexin-membrane complex complicate the interpretation of single channel kinetics. As annexins bind peripherally to lipid membranes ion channel formation of these proteins is accompanied by at least two steps, an adsorption reaction regulating the binding of annexin to the membrane surface and the opening and closing of the active species controlling the channel activity. It is difficult to decompose the measured channel activity into these two components and deduce intrinsic channel kinetics.

In this work, we report for the first time the voltage dependence of annexin binding to membranes which was modeled with a Langmuir adsorption isotherm and a Woodhull model. Whereas the Langmuir isotherm is only valid for step potentials of zero, the Woodhull model is applicable with all transmembrane potentials. Annexin V, VI and VII-core have similar free transfer energies at low calcium concentrations. With higher calcium levels the binding of annexin V becomes much tighter compared to annexin VI and VII-core. This correlates with the finding that the latter proteins display channel activity more often than annexin V. Additionally, the voltage dependence of binding is reduced by increased calcium concentrations. At high protein concentrations weaker binding is observed in agreement with the previously reported anti-cooperativity of membrane binding of annexins.

In a patch clamp experiment with annexins four components are to be taken into account: The membrane bilayer, annexin and calcium concentration and the transmembrane voltage. The view of some single annexin molecules on the seal displaying channel activity is too simple. Moreover, the adsorption process is not a simple one either, because it is not clear how many calcium ions are bound to the protein in each step of the adsorption reaction. The unknown effective charge prevents modelling the whole process accurately.

On the other hand, our results have implications on possible in vivo functions of annexins which may act as sensors for transmembrane potentials.

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References

[1] P. Raynal, H.B. Pollard, Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins, Biochim. Biophys. Acta 1197 (1994) 63–93.

[2] S. Liemann, A. Bentley, Annexins: a novel family of calcium- and membrane-binding proteins in search of a function, Structure 3 (1995) 233–237.

[3] C.E. Creutz, The annexins and endocytosis, Science 258 (1992) 924–931.

[4] V. Gerke, Annexins and membrane traffic, in: B.A. Seaton (Ed.), Annexins: Molecular Structure to Cellular Function, Springer-Verlag, Heidelberg, 1996, pp. 67–79.

[5] F.F. Davidson, M.D. Lister, E.A. Dennis, Binding and inhibition studies on lipocortins using phosphatidylcholine vesicles and phospholipase A_2 from snake venom, pankreas and a makrophage-like cell line, J. Biol. Chem. 265 (1990) 5602–5609.

[6] F. Russo-Marie, Annexins, phospholipase A_2 and the glucocorticoids, in: S.E. Moss (Ed.), The Annexins, Portland Press, London, 1992, pp. 35–46.

[7] V. Gerke, Evolutionary conservation and threedimensional folding of the tyrosine kinase substrate annexin II, in: S.E. Moss (Ed.), The Annexins, Portland Press, London, 1992, pp. 47–59.

[8] S.E. Moss, H.C. Edwards, M.J. Crumpton, Diversity in the annexin family, in: E.W. Heizmann (Ed.), Novel Calcium-Binding Proteins, Springer Verlag, Berlin, 1991, pp. 535–566.

[9] R. Huber, M. Schneider, I. Mayr, J. Römisch, E.P. Paques, The calcium binding sites in human annexin V by crystal structure analysis at 2.0 Å resolution. Implications for membrane binding and calcium channel activity, FEBS Lett. 275 (1990) 15–21.

[10] J. Benz, A. Bergner, A. Hofmann, P. Demange, P. Göttig, S. Liemann, R. Huber, D. Voges, The structure of recombinant human annexin VI in crystals and membrane-bound, J. Mol. Biol. 260 (1996) 638–643.

[11] W.S. Mailliard, H.T. Haigler, D.D. Schlaepfer, Calcium-dependent binding of S100C to the N-terminal domain of annexin I, J. Biol. Chem. 271 (1996) 719–725.

[12] D.D. Schlaepfer, H.T. Haigler, Characterization of Ca^{2+}-dependent phospholipid binding and phosphorylation of lipocortin I, J. Biol. Chem. 262 (1987) 6931–6937.

[13] P. Meers, Annexin binding to lipid assemblies, in: B.A. Seaton (Ed.), Annexins: Molecular Structure to Cellular Function, Springer-Verlag, Heidelberg, 1996, pp. 97–119.

[14] P. Meers, D. Daleke, K. Hong, D. Papahadjopoulos, Interactions of annexins with membrane phospholipids, Biochemistry 30 (1991) 2903–2908.

[15] J.F. Tait, D. Gibson, Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content, Arch. Biochem. Biophys. 298 (1992) 187–191.

[16] H.A. Andree, C.P. Reutelingsperger, R. Hauptmann, H.C. Hemker, W.T. Hermens, G.M. Willems, Binding of vascular

anticoagulant alpha (VAC alpha) to planar phospholipid bilayers, J. Biol. Chem. 265 (1990) 4923–4928.
[17] D.A. Plager, G.L. Nelsestuen, Direct enthalpy measurements of the calcium-dependent interaction of annexins V and VI with phospholipid vesicles, Biochemistry 33 (1994) 13239–13249.
[18] A. Ben-Shaul, N. Ben-Tal, B. Honig, Statistical thermodynamic analysis of peptide and protein insertion into lipid-membranes, Biophys. J. 71 (1996) 130–137.
[19] N. Ben-Tal, A. Ben-Shaul, A. Nicholls, B. Honig, Free-energy determinants of alpha-helix insertion into lipid bilayers, Biophys. J. 70 (1996) 1803–1812.
[20] Th. Heimburg, D. Marsh, Thermodynamics of the interaction of proteins with lipid membranes, in: K.M. Merz Jr., B. Roux (Eds.), Biological Membranes: A Molecular Perspective from Computation and Experiment, Birkhäuser, Boston, pp. 405–462.
[21] E. Rojas, H.B. Pollard, H.T. Haigler, C. Parra, A.L. Burns, Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes, J. Biol. Chem. 265 (1990) 21207–21215.
[22] N. Arispe, E. Rojas, B.R. Genge, L.N.Y. Wu, R.E. Wuthier, Similarity in calcium-channel activity of annexin-V and matrix vesicles in planar lipid bilayers, Biophys. J. 71 (1996) 1764–1775.
[23] H.B. Pollard, A.L. Burns, E. Rojas, Synexin (annexin VII): a cytosolic calcium-binding protein which promotes membrane fusion and forms calcium channels in artificial bilayer and natural membranes, J. Mem. Biol. 117 (1990) 101–112.
[24] R. Berendes, D. Voges, P. Demange, R. Huber, A. Burger, Structure-function analysis of the ion channel selectivity filter in human annexin V, Science 262 (1993) 427–430.
[25] A. Burger, R. Berendes, S. Liemann, J. Benz, A. Hofmann, P. Göttig, R. Huber, V. Gerke, C. Thiel, J. Römisch, The crystal-structure and ion-channel activity of human annexin II, a peripheral membrane-protein, J. Mol. Biol. 257 (1996) 839–847.
[26] A.L. Burns, K. Magendzo, A. Shirvan, M. Srivastava, E. Rojas, M.R. Alijani, H.B. Pollard, Calcium channel activity of purified human synexin and structure of the human synexin gene, Proc. Natl. Acad. Sci. 86 (1989) 3798–3802.
[27] N.C. Khanna, M. Hee-Chong, D.L. Severson, M. Tokuda, S.M. Chong, D.M. Waisman, Inhibition of phospholipase A_2 by protein I, Biochem. Biophys. Res. Comm. 139 (1986) 455–460.
[28] J.-M. Neumann, A. Sanson, A. Lewit-Bentley, Calcium-induced changes in annexin V behaviour in solution as seen by proton NMR spectroscopy, Eur. J. Biochem. 225 (1994) 819–825.
[29] D. Voges, R. Berendes, A. Burger, P. Demange, W. Baumeister, R. Huber, Three-dimensional structure of membrane-bound annexin V. A correlative electron-microscopy-X-ray crystallography study, J. Mol. Biol. 238 (1994) 199–213.
[30] C. Ravanat, J. Torbet, J.M. Freyssinet, A neutron solution scattering study of the structure of annexin-V and its binding to lipid vesicles, J. Mol. Biol. 226 (1992) 1271–1278.
[31] A. Burger, R. Berendes, D. Voges, R. Huber, P. Demange, A rapid and efficient purification method for recombinant annexin V for biophysical studies, FEBS Lett. 329 (1993) 25–28.
[32] S. Liemann, I. Bringemeier, J. Benz, P. Göttig, A. Hofmann, R. Huber, A.A. Noegel, U. Jacob, Crystal structure of the C-terminal tetrad repeat from synexin (annexin VII) of *Dictyostelium discoideum*, J. Mol. Biol. 270 (1997) 79–80.
[33] B. Suarez-Isla, K. Wan, J. Lindstrom, M. Montal, Single channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipettes, Biochemistry 22 (1983) 2319–2323.
[34] G. Wedler, Lehrbuch der Physikalischen Chemie, VCH, Weinheim, 1987.
[35] A.M. Woodhull, Ionic blockage of sodium channels in nerve, J. Gen. Physiol. 61 (1973) 687–708.
[36] B. Hille, Ionic Channels of Excitable Membranes, Sinauer, Sunderland, 1992.
[37] R. Berendes, A. Burger, D. Voges, P. Demange, R. Huber, Calcium influx through annexin V ion channels into large unilamellar vesicles measured with fura-2, FEBS Lett. 317 (1993) 131–134.
[38] E.L. Goossens, C.P. Reutelingsperger, F.H. Jongsma, R. Kraayenhof, W.T. Hermens, Annexin V perturbs or stabilises phospholipid membranes in a calcium-dependent manner, FEBS Lett. 359 (1995) 155–158.
[39] S. Mukhopadhyay, W.H. Cho, Interactions of annexin V with phospholipid monolayers, Biochim. Biophys. Acta 1279 (1996) 58–62.
[40] M.D. Bazzi, G.L. Nelsestuen, Highly sequential binding of Protein Kinase C and related proteins to membranes, Biochemistry 30 (1991) 7970–7977.
[41] R. Gilmanshin, C.E. Creutz, L.K. Tamm, Annexin IV reduces the rate of lateral lipid diffusion and changes the fluid phase structure of the lipid bilayer when it binds to negatively charged membranes in the presence of calcium, Biochemistry 33 (1994) 8225–8232.