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ARTICLE

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Calcium-dependent association of annexins with lipid bilayers modifies gramicidin A channel parameters

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Abstract In order to examine whether calcium-dependent binding of annexin to acidic phospholipids could change the lipid bilayer environment sufficiently to perturb channel-mediated transmembrane ion-transport, gramicidin A channel activity in planar lipid bilayers was investigated in the presence of calcium and annexins II, III or V. The experiments were performed with membranes consisting of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in 300 mM KCl solution buffered to pH 7.4 and with either 0.1 or 1 mM calcium added to the solutions. Annexin (1 μM) was subsequently applied to the *cis* side of the membrane. All three annexins (II, III and V) when tested at 1 mM calcium decreased the gramicidin single-channel conductance. Annexins II and III increased the mean lifetime of the channels whereas annexin V seemed to have no influence on the mean lifetime. Since the lifetime of gramicidin A channels is a function of the rate constant for dissociation of the gramicidin dimer, which is dependent on the physical properties of the lipid phase, binding of annexins II and III seems to stabilize the gramicidin channel owing to a change of the bilayer structure.

Key words Annexins · Gramicidin · Lipid bilayer · Calcium

Introduction

Annexins are a family of cytosolic, calcium-binding proteins, which are different from the EF-hand family. The annexins (apart from annexin VI) possess four repeats, each having a calcium-binding site which in the presence of acidic phospholipids shows increased calcium affinity, resulting in association with bilayers through formation of calcium bridges between the annexin molecule and the acidic lipid headgroups (Swairjo et al. 1995). Biophysically, these proteins are well characterized with regard to structure (Huber et al. 1992) and binding properties (Blackwood and Ernste 1990) and they have been shown, in experiments with lipid bilayers in vitro, to form calcium-selective channels (Burns et al. 1989; Pollard et al. 1990; Rojas et al. 1990). Physiologically, a number of diverse effects of the annexins have been demonstrated, among these, modulation of ion channels in cell membranes (Chan et al. 1994; Kaetzel et al. 1994; Jørgensen et al. 1997).

The cell membrane is a dynamic structure with an asymmetric composition of lipids in the outer and inner leaf of the bilayer, which is actively maintained. The inner leaf of the cell bilayer membrane, which faces the cytosol where the annexins are present, is enriched with regard to acidic phospholipids. Since oscillations in cytosolic ionized calcium is part of many cellular processes, this raises the possibility that annexins could participate in regulatory processes by association/dissociation with the inner membrane in concert with the Ca^{2+} oscillations and thereby influence the function of other membrane constituents. This could either be through substrate protection as has been proposed with regard to the inhibition of phospholipase C, which is due to binding to phosphatidylinositol and PIP_2 (Machoczek et al. 1989), or by a direct change of the properties of or access to the bilayer. In order to elucidate possible interactions between annexins and ion channels, gramicidin-doped bilayers were used as a model system, and the influence of annexins on the channel behavior was investigated in the presence of Ca^{2+}. Gramicidin channels form by association of two gramicidin molecules, one from each layer of the lipid membrane creating a transmembrane pore. Consequently it could be expected that binding of annexin to the membrane surface could affect either the geometry of the membrane or the lateral

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diffusion rates, and thereby the lifetimes of the observed gramicidin open and closed states, as well as total activity and conductance.

Materials and methods

Annexins

The annexins tested originated from different sources. Swine lung tissue was used for the isolation of annexins II and V; from ox lung and murine Ehrlich ascites cells, annexins II, III and V were purified. After homogenization and centrifugation of tissue or cells in an EDTA-containing buffer, the soluble fraction was saturated to 50% with ammonium sulfate and the precipitate removed by centrifugation. The supernatant contained the annexins. A further purification of the annexins was performed utilizing their reversible calcium-dependent binding to membrane phospholipids using inside-out vesicles of the human red cell (Kristensen and Kristensen 1989; Kristensen et al. 1993). Separation of the individual annexins from these crude preparations was performed by DEAE-Sepharose anion exchange chromatography (Pharmacia). Annexin II eluted in the flow through and annexins III and V were eluted in a linear NaCl gradient at different salt concentrations, annexin III being eluted at a lower NaCl concentration (0.15 M) than annexin V (0.25 M). Ox lung annexin V exists in two forms with apparent molecular weights of 34 and 32 kDa and a slight difference in isoelectric points (Boustead and Walker 1991). Separation of the two annexins V from ox lung was achieved by using a flat NaCl-gradient elution profile during the ion exchange chromatography.

The annexins for the electrophysiological recordings were dialyzed, freeze dried and dissolved in distilled water to make a stock solution of 1–2 mg/mL.

Gramicidin A

Gramicidin D was obtained from Sigma and resolved by reversed phase chromatography (Perkin-Elmer solvent delivery system 140B and UV detector 785A). The sample was applied in 66% methanol onto a Zorbax C-8 column (4 × 250 mm) and eluted at a rate of 0.5 mL/min, with the following profile. From 0 to 5 min, isocratic at 66% methanol in water, increasing between 5 and 6 min to 83% methanol, and thereafter isocratic at this methanol concentration. The resulting chromatogram was qualitatively identical to that obtained by Koeppe and Weiss (1981). The peaks corresponding to gramicidin A and C were collected manually. Rechromatography of gramicidin A showed one peak only.

Bilayers

Planar lipid bilayers were formed by painting, with a glass rod, lipid dissolved in *n*-octane on a 0.2 mm hole in a partition separating two Teflon chambers. The lipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-l-serine (PS) (Avanti Polar-Lipids) in the ratio 0.4:0.4:0.2. Bilayer formation was detected by monitoring the membrane capacitance, which typically stabilized around 300 pF.

Electrophysiological measurements

All experiments were performed at room temperature at a potential difference of 60 mV, *cis* side negative, using symmetrical solutions containing 300 mM KCl, 5 mM HEPES and 5 μM EGTA, pH 7.4, at varying Ca^{2+} concentrations. All reagents were analytical grade. Small amounts, 0.5–2.5 μL, of appropriately diluted gramicidin A were added to the *cis* and *trans* aqueous solutions, resulting in a final concentration in the range 10^{-11}–10^{-10} M, and stirred until channel activity appeared. When channel activity had been recorded for a period of 15–30 min, depending on the number of events, annexin was added to the *cis* side of the chamber to a final concentration of 1 μM. The solutions were stirred again and recording of channel activity was resumed. Single-channel currents were recorded with the PC501A patch/whole cell clamp (Warner Instrument) mounted with the bilayer headstage. The current output was filtered at 30 Hz, and sampled on-line at 100 samples/s on a PC using a DT2811 AD card from Data Translation and locally developed programs. Current amplitude and channel open times were calculated off-line.

Calculations

The baselines of all experimental traces were offset to zero and a current sample distribution calculated. The number of events was found from downward current transitions in the experimental trace exceeding a threshold defined as the mean distance between the peaks in the current histogram. The mean open time was calculated as the summed open time divided by the number of transitions.

In order to obtain a sufficient number of events within a reasonable time, considering the actual mean lifetime for the individual openings, it was necessary to use a gramicidin concentration where multiple openings were frequent. Consequently, it was not attempted to calculate an open time distribution. Furthermore, it was not possible to calculate a mean closed time, since the membrane-bound gramicidin did not attain equilibrium relative to the baths. However, since the channels are supposed to be identical and independent, the mean lifetime does not depend on stationarity.

In order to follow changes in activity with time, the mean currents in sequential datablocks of 10 s duration were calculated for the experimental traces and plotted against time. The slope determined from linear regression on these curves was used as an indicator for non-stationarity.

Results

The influence of annexin association with the bilayer was investigated using gramicidin A single-channel conductance, mean lifetime and total activity as estimators at two different Ca^{2+} concentrations. The activity was recorded in a control period, typically 15–30 min, before addition of annexin.

Ca^{2+} control experiments

The single-channel currents through gramicidin pores in planar lipid bilayers were measured at a holding potential of −60 mV using symmetric 300 mM KCl solutions containing 0.1 mM or 1 mM Ca^{2+}. The single-channel conductance was found to be higher in 0.1 mM Ca^{2+} solution (19.9 ± 2.2 pS, $n = 17$) than in 1 mM Ca^{2+} solution (17.5 ± 0.75 pS, $n = 13$), whereas the channel mean open time was unchanged. The channel mean lifetime was found to be 183 ± 39 ms ($n = 17$) in 0.1 mM and 175 ± 23 ms ($n = 13$) in 1 mM calcium solution. See Fig. 1, which shows typical current amplitude histograms with experimental traces inserted.

Influence of annexins

The effect of 1 μM annexin II, annexin III and annexin V on the single-channel conductance and mean open time

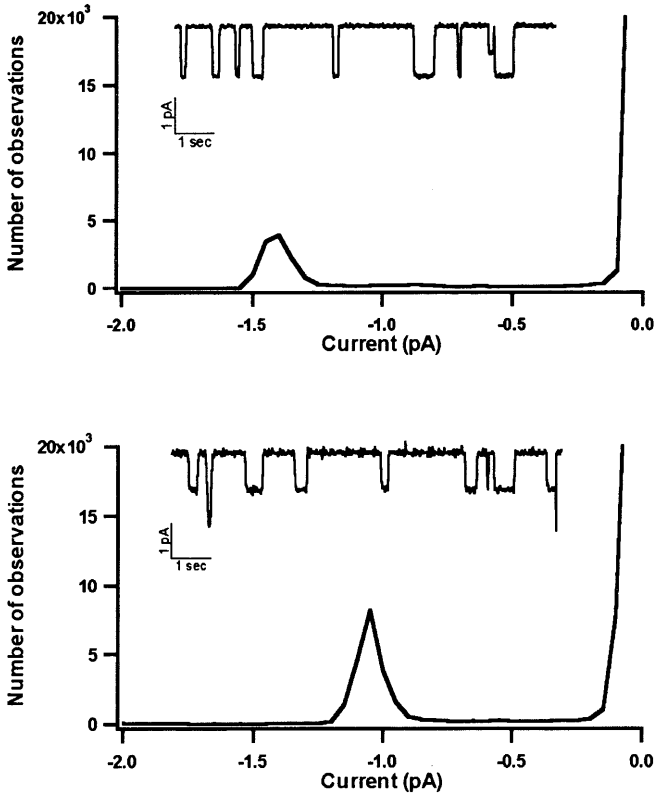

Fig. 1 The single-channel currents and current amplitude histograms of gramicidin A in planar lipid bilayer membranes measured at a potential difference of –60 mV and with 100 μM Ca^{2+} (*upper panel*) or 1 mM Ca^{2+} (*lower panel*) in the solutions. The single-channel conductance in the presented experiments is 23 pS at 0.1 mM Ca^{2+} and 17.5 pS at 1 mM Ca^{2+}

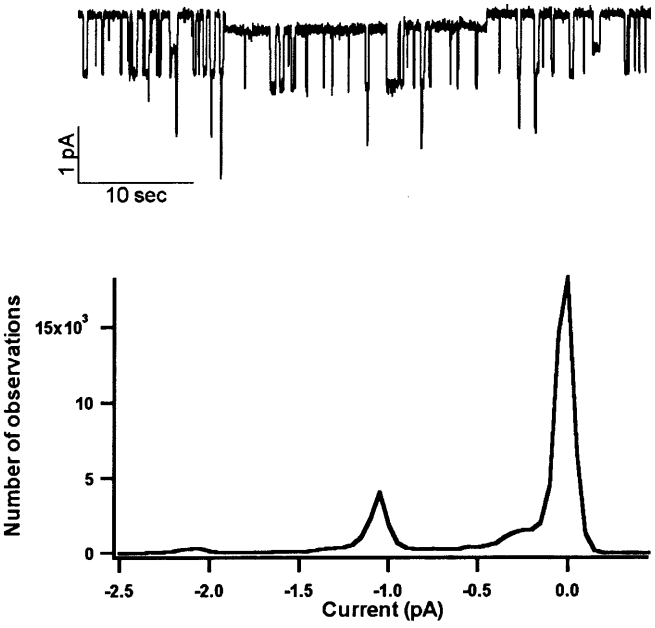

Fig. 2 Single-channel currents and current amplitude histogram of gramicidin A in the presence of annexin III at 0.1 mM Ca^{2+}, holding potential –60 mV. Note the long-duration low-conductance state in the sample trace. The shoulder to the left of the closed state distribution is due to this type of opening

was examined in the presence of 0.1 or 1 mM Ca^{2+} at a holding potential of −60 mV. Annexin II, isolated from pig lung, appeared to increase channel lifetime of gramicidin A both at 0.1 mM and 1 mM Ca^{2+}, with an average increase of 84% and 45%, respectively (see Table 1). Beside the effect on channel open time, annexin II decreased the channel conductance from 17 pS to 13.9 pS with 1 mM Ca^{2+} in the solution. At 0.1 mM Ca^{2+}, no effect on the conductance was observed.

When annexin III, isolated from ox lung, was tested on the gramicidin A channel behavior, events which deviate from the normal channel characteristics appeared. These channels have very long open times, with single-channel currents lower than the most frequently observed channel events (see Fig. 2, upper panel). In some of the experiments they were visible as an additional peak in the current histogram (see Fig. 2, lower panel). These types of channels were detected especially

Table 1 The effect of 1 μM of annexin II, annexin III or annexin V on single-channel conductance (pS) and mean open time (ms) measured at a holding potential of 60 mV in the presence of 0.1 or 1 mM Ca^{2+}. Mean and standard deviation is calculated assuming normal distribution. The number of events $n \geq 4$

	$[Ca^{2+}]$ (mM)	Conductance (pS)[a]	Mean duration (ms)[a]	$[Ca^{2+}]$ (mM)	Conductance (pS)[a]	Mean duration (ms)[a]
Control	0.1	20.4 ± 1.6	216 ± 30	1	17.0 ± 0.7	181 ± 23
Annexin II	0.1	19.6 ± 0.9	397 ± 118	1	13.9 ± 0.8	263 ± 21
	–	n.s.	–[b]	–	–[d]	–[c]
Control	0.1	18.9 ± 0.7	148 ± 30	1	17.3 ± 0.7	161 ± 13
Annexin III	0.1	17.4 ± 0.4	290 ± 101	1	–	320 ± 124
	–	–[d]	–[c]	–	–	–[b]
Control	0.1	20.6 ± 3.6	194 ± 25	1	18.0 ± 0.6	182 ± 29
Annexin V	0.1	21.1 ± 3.7	220 ± 25	1	14.9 ± 0.6	192 ± 29
	–	n.s.	n.s.	–	–[d]	n.s.

[a] A two-sample *t*-test has been used to estimate the probability that the means of the control and of the corresponding annexin experiments are equal

[b] Designates that the probability is less than 5%
[c] The probability is less than 1%
[d] The probability is less than 0.1%

in the 0.1 mM Ca^{2+} solution and only occasionally (in one out of four experiments) in the 1 mM Ca^{2+} solution. The lifetime was typically between 1 and 10 s. However, values up to 230 s were observed. The channels with long-lasting open times most frequently had single-channel currents in the range 0.2–0.4 pA at −60 mV, corresponding to a conductance of about 4–7 pS, but values both lower and higher were also recorded. Occasionally, with gramicidin in annexin-free solution, low conductance channels were observed, but with channel lifetimes in the order of milliseconds and not seconds as recorded with annexin III.

As shown in Table 1, the conductance of the standard gramicidin channel in the presence of 0.1 mM Ca^{2+} is reduced from 18.9 pS to 17.4 pS with addition of annexin III and the mean open time is increased from a control value of 148 ± 30 ms to 290 ± 28 ms ($n=6$).

In salt solutions with 1 mM Ca^{2+}, annexin III reduces the current amplitudes of single channels from 1.04 pA (17.3 ± 0.7 pS, $n=4$) to channels with currents varying between 0.65 and 0.9 pA (Fig. 3A and C). Beside measuring channel currents of different amplitudes, the channels appeared to be modified in the open state since the single-channel current attained different values without intermittent closure, as shown in Fig. 3B. The current amplitude histograms from these experiments are broad compared to the corresponding controls (see Fig. 3B and D). The mean open time at 1 mM Ca^{2+} increased from 161 ± 13 ms to 320 ± 124 ms ($n=4$) with annexin III, indicating an increased channel stability in the lipid bilayer.

The annexin V tested for effect on gramicidin A channel activity was isolated from ox lung, pig lung and Ehrlich ascites tumor cells. There was no significant increase in the lifetime nor any change in the single-channel conductance of the gramicidin channel with annexin V in the 0.1 mM Ca^{2+} solution (see Table 1). Neither was there an effect on the lifetime at 1 mM Ca^{2+}. However, in the 1 mM Ca^{2+} solution, significant reduction in single-channel conductance, from 18.0 ± 0.6 pS to 14.9 ± 0.6 pS ($n=5$), is measured with annexin V. Furthermore, annexin V appeared to reduce the number of gramicidin A channel events.

This conclusion is drawn partly from inspection of the experiments where annexin V decreased the frequency of single-channel events compared to the frequency during the control period, and partly by calculating the mean current in sequential blocks of data. This is illustrated in Fig. 4, where the mean current in data blocks of 10 s duration is calculated for the control period and consecutively for the period with

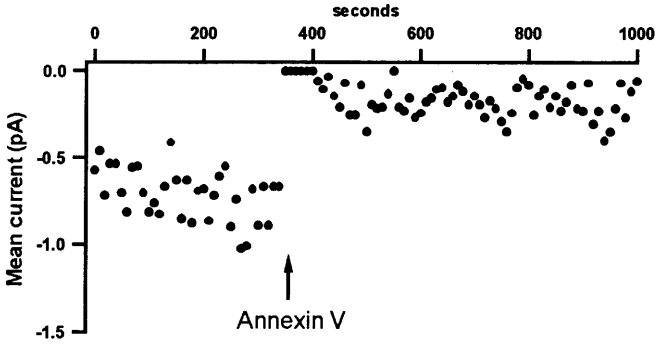

Fig. 4 Gramicidin channel mean current calculated for consecutive 10 s periods. The *arrow* indicates the addition of 1 μM annexin V, followed by a period of stirring during which measurements are impossible. Note that the mean current in the control period is increasing with time due to the non-equilibrium distribution of gramicidin between baths and the membrane. Since the mean lifetime of the channels are unchanged in the presence of annexin V and the mean current decreases more than corresponding to the lowering of the single-channel conductance (corresponding to a factor of 0.794), the depression of the current is due to a decreased number of events

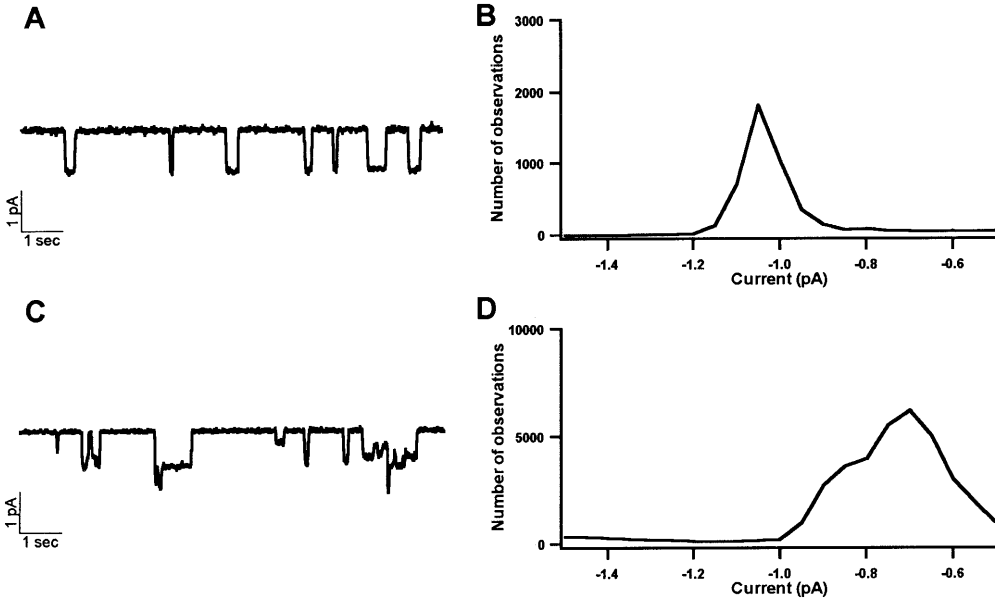

Fig. 3A–D Single-channel currents and current amplitude histogram of gramicidin A without (*upper panel*) and in the presence of annexin III at 1 mM Ca^{2+} (*lower panel*), holding potential –60 mV. The heterogeneity of the single-channel conductance induced by annexin III is apparent from the trace and the smeared amplitude distribution

annexin V. The channel current of the latter is multiplied by 1.26 to account for the difference in single-channel conductance. In the presented experiment the numerical value of the mean current increased and was higher than 0.5 pA. With annexin V the mean current was clearly reduced, giving a value numerically less than 0.4 pA. Reduction of the mean current of the data blocks was not observed with either annexin II or annexin III. Since the lifetime of the channels seems to be increased in those experiments, this may counteract the effect of channel inhibition on the mean current of the data blocks.

Discussion

It is well known that annexins bind to acidic phospholipids in the presence of Ca^{2+}. A multitude of effects on the physical properties of the lipid membrane owing to the presence of calcium and association of annexin have been described or proposed, including changes of the lateral mobility of the phospholipids (Rojas et al. 1990), changes in membrane stiffness (Lundbæk et al. 1996), phase separation of the lipid mixture constituting the membrane, interference with flip-flop mechanisms in the bilayer and alteration of the electrostatic energy profile at the bilayer solution interface, to mention some.

In order to test the possibility that the interaction between annexin, Ca^{2+} and phospholipids could modify the behavior of integral proteins, primarily ion channels, the influence of annexins on a model system consisting of gramicidin A doped bilayers was investigated. The characteristics of the single channel formed, channel lifetime and single-channel conductance, when two molecules of this peptide associate were very sensitive to changes of the physical properties of the bilayer. Furthermore, it has been shown that changes of the lipid environment modifying the gramicidin single-channel behavior can also influence conformational changes of the integral membrane-spanning proteins and accordingly modify transport processes across cell membranes (Lundbæk et al. 1996).

The bilayers were formed from a mixture of PC, PE and PS to imitate partly the composition of the inner leaf of the cell membrane, with the actual composition determined to obtain maximum stability of the bilayers under the experimental conditions used and with a pH of the solutions in the physiological range. However, association of annexins occurs over a wide range of lipid compositions, and the general conclusions should not be dependent on a specific lipid composition.

The effect of annexins was tested at bulk Ca^{2+} concentrations of 0.1 and 1 mM, but not without Ca^{2+}, since the present bilayers lost stability in calcium-free solutions. Without annexins, the K^+ single-channel conductance is lower at 1 mM than at 0.1 mM Ca^{2+}, but with identical mean lifetimes. This decrease of the conductance is partly due to a lowering of the surface potential caused by the negative surface charges from PS and PE with increasing calcium concentration (McLaughlin et al. 1981), resulting in a redistribution of K^+ in the vicinity of the bilayer, and partly to a blockade of the channel by the impermeable Ca^{2+} ion (Gambale et al. 1987). However, the reduction of the single-channel conductance was less than that observed by Gambale et al. (1987), which might be due to differences both in lipid composition and bulk solution.

It has previously been demonstrated that Ca^{2+} in itself can induce lateral phase separation in mixtures of phospholipids consisting of acidic and neutral phospholipids (Knoll et al. 1986; Mittler-Neher and Knoll 1993), creating domains with varied lipid composition in the bilayer which causes changes in the observed channel mean lifetime. However, this does not seem to happen with the lipid mixture used in the present experiments, since the mean lifetimes at the two Ca^{2+} concentrations were not significantly different.

The two features of gramicidin channels, lifetime and channel conductance, which reflect changes in physical properties of the bilayer, and the bilayer/solution interface appear to be modulated by association of annexins with the bilayer. However, although all annexins (except annexin VI) associate with the membrane by way of up to four Ca^{2+} bridges, the annexins used in the present experiments show differential effects on the gramicidin parameters.

Common for all three annexins tested was a decrease of the single-channel conductance. This effect is probably due to heavy coating of the membrane surface with annexin, causing either a change in surface charge by screening (change of concentration profile), since each annexin molecule covers up to 40 lipid head-groups (Andree et al. 1990), or to increased diffusion resistance up to the channel mouth.

In contrast to annexin V, annexins II and III increased the average channel lifetime at Ca^{2+} concentrations of both 0.1 and 1 mM. It is known that the mean lifetime and conductance of the gramicidin A channel depend on the polar groups of the phospholipids (Neher and Eibl 1977) present in the bilayer and as well as the membrane thickness (Lundbæk et al. 1996), in that the gramicidin dimer is shorter than the membrane thickness. As a consequence, changes in curvature and stiffness of bilayers owing to deformation of the bilayer will influence the channel lifetime (Lundbæk et al. 1996). The increase in channel lifetime seen in the presence of annexins II or III could thus be caused by a deformation which lowers the deformation energy necessary for formation of a gramicidin dimer by creating areas where the membrane thickness has decreased.

In the case of annexin III, association in the presence of 1 mM Ca^{2+} gave rise to multiple conductance states. Measured from individual openings the conductances were 11, 12, 13 and 14 pS, giving a very broad current amplitude histogram. This could indicate formation of channels in domains with varied composition of lipids. It is thus tempting to suggest that addition of annexin III at 1 mM Ca^{2+} induces a phase separation of the bilayer.

This is supported by the occurrence of a very stable low-conductance state when annexin III is added at 0.1 mM Ca^{2+}. These long-life channels were only observed in the presence of gramicidin, but infrequently and with shorter lifetimes in the absence of annexin. They are most probably gramicidin channels, stabilized by annexin III-induced changes of the bilayer organization. This is supported by the finding (Mobashery et al. 1993) that hydrophobic mismatch between the bilayer and gramicidin A gives rise to long-lived low-conductance states. These results could be interpreted to mean that annexin III induces more profound changes in the membrane geometry than annexins II and V, giving rise to areas with a decrease in membrane thickness as indicated by the prolonged lifetime of the normal states, and areas with increased thickness as indicated by the low-conductance state.

Some of the annexins (I, V, VI and VII) have been found to form low-conductance calcium or cation channels (Pollard et al. 1992) but not annexin III, and furthermore the low-conductance state is only observed when gramicidin is added to the solution.

Contrary to annexins II and III, annexin V did not increase the mean lifetime of the gramicidin A channels. However, addition of annexin V in 1 mM Ca^{2+} reduced the number of channel openings, which indicates either a channel block or channel block combined with inhibition of channel formation. Since the channel activity was not stationary in these experiments but increased as more and more gramicidin A dissolved into the bilayer, the number of channels open within a given period of time is therefore expected to be closely identical to or larger after addition of annexin than before, as found for annexins II and III (not shown). Under the present experimental conditions, cation channels that could be ascribed to channel forming by annexin V were never observed. Although it should be noted that, with the sample rate used, only events longer than 10 ms would be observed, it is tempting to speculate whether the reported annexin channels require conditions which are peculiar to lipid bilayers with a very high phosphatidylserine content formed on a pipette tip, and do not form in planar lipid bilayers as used in this work or in native cell membranes.

A wide range of calcium affinities for phospholipid-annexin binding are reported in the literature (Andree et al. 1990; Blackwood and Ernste 1990), but the values seem to depend on the experimental conditions. The decrease of the channel conductance in the presence of annexin II, III or V, going from 0.1 to 1.0 mM Ca^{2+}, indicates that the membrane surface is still unsaturated with protein at these calcium concentrations. This is in accordance with binding experiments with annexin V to planar membranes, where it has been shown that the Ca^{2+} half-saturation concentration depends on the amount of PS in the membrane, with a value of 0.22 mM at 20% PS (Andree et al. 1990) and of 0.3 mM for binding of annexin V to the inside-out vesicles from red cells (Kristensen and Kristensen 1989).

It is a characteristic for the different annexins that they have a highly conserved core and a rather short tail, with the calcium-binding sites located at the core. Although differences between annexins exist, exemplified in different Ca^{2+} affinities, it seemed reasonable to expect the different annexins to have identical effects on the gramicidin channel kinetics. There is, however, a qualitative difference between annexin V and annexins II and III. Apart from differences in tail length, with annexin V having the shortest tail, a comparison of the amino acid compositions indicates slightly more hydrophobic tails for annexins II and III than for annexin V. This points to the possibility that the tails of annexins II and III could be associated more closely with the lipid phase than annexin V, contributing to a deformation of the membrane, but further work is necessary to identify the molecular mechanism.

Conclusion

The present work demonstrates that although most biological effects are explained on the basis of specific receptor interaction, regulation on a purely physical basis cannot be excluded. Association/dissociation of annexins is fast and oscillations in cellular Ca^{2+} are common, creating the basis for dynamic changes in bilayer properties. Furthermore, it is noteworthy that even in a system as simple as the present, where the effects of annexins a priori could be expected to be dominated by the Ca^{2+}-lipid headgroup binding, a differentiation between individual annexins can be observed. The lipid bilayer setup seems well suited to demonstrate that type of interaction. It should however be borne in mind that this experimental system is ''more clean'' than the lipid membrane of the living cell, which is stabilized by integral proteins and associations such as the cytoskeleton.

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