Membrane capacity, measurements suggest a calcium-dependent insertion of synexin into phosphatidylserine bilayers

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Membrane capacity measurements suggest a calcium-dependent insertion of synexin into phosphatidylserine bilayers

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The mechanism by which synexin mediates calcium-dependent aggregation of medullary cell chromaffin granules and fusion of granule ghosts involves specific interactions with the lipid component of the membrane. To study the details of these interactions we measured synexin-induced changes in capacitance of phosphatidylserine bilayers formed at the tip of a patch pipet using the double-dip method. Provided calcium was present in the solution filling the pipet (10–50 mM) stable phosphatidylserine bilayers were easily formed. Addition of synexin (0.1 μg/ml) to an external medium lacking added calcium induced no measurable changes in either bilayer resistance (10–30 GΩ) or displacement current across the membrane. However, addition of calcium (0.1–2.5 mM) in the presence of synexin in the external solution caused a marked increase in the size and time constant of decay of the displacement current. From the steady-state value of the current we calculated a 5-fold decrease in resistance and from the charge displaced during the voltage-clamp pulses we calculated a 10-fold increase in membrane capacitance (from 20 to 200 fF). The size of the synexin-specific charge displacement in one direction during a pulse was always equal to the charge returning to the original configuration after the pulse. The synexin-specific transfer of charge reached saturation when the pipet potential was taken to a sufficient positive or negative value. These properties of the extra charge movement support our view that in the presence of calcium the cytosolic protein synexin penetrates into the bilayer. It is possible that these properties may be related to the mechanism by which synexin promotes membrane fusion in natural membranes.

Synexin; Displacement current; Membrane capacity; Membrane fusion

1. INTRODUCTION

Synexin is a soluble calcium-binding protein [1] which causes chromaffin granules [1–5], chromaffin granule ghosts [6] and acidic liposomes [7,8] to aggregate and fuse. Furthermore, chromaffin granules fused by synexin and small amounts of arachidonic acid form structures very similar to fusion structures observed by electron microscopy in secreting chromaffin cells [5,9]. Finally, while the specific mechanisms by which synexin causes membrane fusion remain to be determined, several studies indicate that calcium-activated synexin binds to a variety of acidic phospholipids including phosphatidylserine but not to neutral phospholipids such as phosphatidylcholine [10]. Indeed, the relative binding affinity of synexin to different phospholipids is paralleled by the ability of synexin to fuse liposomes prepared from different phospholipids [7,8]. These phospholipid-binding studies therefore suggest that synexin could act as a simple bridge between otherwise fusing membrane surfaces [8,10]. Alternatively, a more intimate interaction might also occur between synexin

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and the specific lipids involving direct insertion of synexin molecules into the bilayer [10]. Presumably such an event could be the reason for bilayer destabilization at the point of contact during the fusion event.

Here, we have measured synexin-induced changes in the electrical capacity of phosphatidylserine bilayers. These changes can come about as a consequence of insertion of protein dipoles into the substances of the bilayer, and this method constitutes a more direct approach to the problem of protein insertion than alternative chemical and spectroscopic methods. We therefore interpret these data to indicate that in the presence of calcium synexin can change from a water-soluble form to one that can penetrate into the substance of the bilayer, thus modifying the dielectric properties of the membrane. We anticipate these properties may also be relevant to the mechanism by which synexin promotes fusion of natural membranes.

2. MATERIALS AND METHODS

2.1. *Electrical measurements*

Pipets were prepared from micro-hematocrit capillary tubes (i.d. 1.1–1.2 mm, wall thickness 0.2 mm) using a BB-CH microelectrode puller (Mecanex, Geneva). These pipets had a total capacity of 3–10 pF, the lower value being for pipets coated almost to the tip with Sylgard, as described by Sakman and Neher [11]. The open tip resistance ranged from 0.5 to 10.0 MΩ and when bilayers were prepared by the double tip-dip method [12] the resistance rose to 10–30 GΩ.

Current transients were recorded under voltage-clamp conditions using a List EPC-7 amplifier (List-Electronic, Darmstadt-Eberstadt). The amplifier was used without compensation for the series resistance, with the capacity null-bridge switch off, and the 3-pole Bessel filter removed to allow recordings to be made at maximum bandwidth.

Command voltage levels and pulses were generated through a digital-to-analog converter with a maximum linearity error of 0.002 mV over the range from −200 to 200 mV. The current signal was fed through a low-pass Bessel filter (Frequency Devices, model 902-LPF, Haverhill, MA) with the corner frequency set at 30 kHz to an analog-to-digital converter as described [13–15]. Voltage pulse protocols were under computer control and the current signal was digitized at 2 or 4 μs sampling intervals. The maximum linearity error of this data acquisition procedure was less than 1 mV in the voltage range from −1000 to 1000 mV. Saturation of the recording system during the fast capacity currents was avoided by selecting an appropriate gain (maximum gain set at 1.0 mV/pA). Thus, current samples could be taken immediately after a voltage displacement. The glass chamber, having a total volume of 300 μl, contained a buffer solution of the following composition (mM): 140 KCl, 1 $CaCl_2$ and 10 NaHepes at pH 6.5. The tip of the pipet was filled with a buffer solution containing 105 mM NaCl, 30 mM $CaCl_2$ and 10 mM NaHepes at pH 7.4. Phosphatidylserine (Avanti Polar Lipids, Birmingham, AL) was dissolved in chloroform (0.1 mg/ml) and applied directly to the surface (1 cm^2) of the chamber from a glass rod, following evaporation of the chloroform from the rod.

2.2. *Synexin*

Highly purified synexin was prepared from bovine liver by a previously described method involving ammonium sulfate fractionation, chromatography on Ultragel [1] and chromatofocussing [16]. Synexin was lyophilized and taken up in chamber buffer in the absence of added calcium.

3. RESULTS

3.1. *Membrane currents during and after a sudden displacement of the electric field across the bilayer*

A phosphatidylserine monolayer was spread on the surface (1 cm^2) of the glass chamber from a dry rod and a patch pipet dipped twice through the surface. Provided there was $CaCl_2$ in the pipet solution (1–50 mM), bilayer membranes were readily formed. These membranes had an intrinsic capacitance in the range 20–30 fF and could be broken by application of positive pressure inside the pipet and by a brief (1 ms) voltage jump exceeding 450 mV, presumably through dielectric breakdown. These were all properties to be expected from true bilayer membranes.

To measure the interaction of synexin with the bilayer membrane we used the voltage-jump method to change the electric field across the bilayer and followed an experimental protocol that consisted of three parts. Firstly, after a bilayer was formed, we recorded the current transients in response to voltage pulses of constant duration (3 ms) and increasing magnitude from a holding potential of 0 mV. Secondly, immediately after the first control in the absence of Ca^{2+}, a small volume (10 μl) of the Ca^{2+}-free buffer solution containing synexin (0.1 μg/ml) was added. After 3–5 min we recorded a second series of current transients. Finally, 1–3 min after the addition of Ca^{2+} (10 μl of 30 mM $CaCl_2$ solution) to the buffer in the chamber we recorded another series of current transients. We found that in the presence of synexin and 1 mM $CaCl_2$, the size and relaxation time constant of the current transients increased for all of the voltage pulses applied. This is shown in fig. 1 for one pair (50 and −50 mV) of the series. The order of addition of calcium and synexin could be reversed without affecting the final changes in current transients. However, if no calcium were present, or if $CaCl_2$ was replaced with either $MgCl_2$ or $BaCl_2$, we could not detect changes in the current transients recorded during the control and test runs.

3.2. *Charge movements associated with the incorporation of calcium-activated synexin into the bilayer*

The procedure used to estimate the charge movement across the bilayer is depicted in fig.1. [17]. The upper trace (A) represents the voltage levels applied to the solution in the pipet. The two superimposed records in the middle (B) represent the current transients in response to the pulses before (Mb) and 2 min after (Mb + Syn) addition of Ca^{2+} (1 mM) in the presence of synexin. The difference between the current record after synexin in the presence of Ca^{2+} (Mb + Syn) minus the current before synexin (Mb) is plotted beneath each pair of current transients (C).

Application of a rectangular voltage pulse to the bilayer alone (Mb) induced a fast current surge followed by a rapidly declining current transient (relaxation time constant <0.1 ms). The explanation for this behavior is that before the application of the pulse the bilayer capacity was uncharged

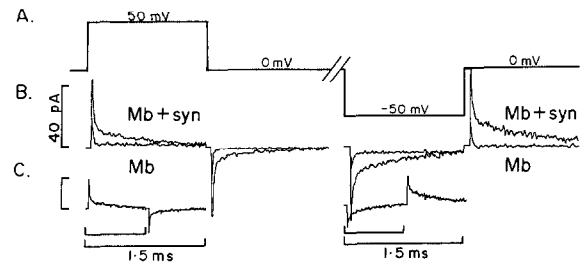

Fig.1. Subtraction protocol to measure the synexin-specific displacement currents. (A) Pipet potentials (mV) are indicated over the voltage record. Between the pulses (interval marked by cut marks equivalent to 500 ms) the pipet potential was held at 0 mV. (B) Left side: two superimposed current transients in response to 50 mV pulses in the absence (Mb) and presence of synexin plus calcium (1 mM) in the solution (Mb + syn). Right side: displacement currents in response to −50 mV pulses. (C) Net synexin-specific displacement currents obtained by subtracting the record labelled Mb from that labelled 'Mb + syn'. Calibrations: vertical, 40 pA; horizontal, 1.5 ms.

and therefore the initial current flow at the onset of the pulse was large. As the bilayer capacity was charged the current flow decreased rapidly. When the membrane was treated with calcium-activated synexin the initial current surge was larger and the

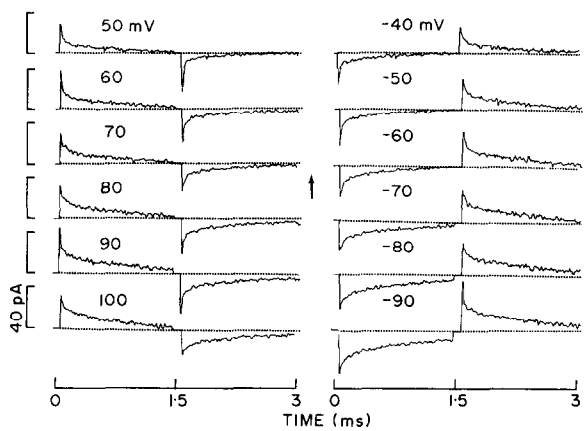

Fig.2. Synexin-specific displacement current at different transmembrane potentials. Left and right sides: differences between displacement current records calculated as explained in fig.1C. Left side: positive pipet potentials as indicated next to the corresponding net displacement current record. Right side: negative pipet potentials.

current decreased more slowly towards a steady level (see records labelled Mb + Syn). We were able to evaluate the increase in current due to synexin alone by subtracting the transient for the membrane alone (Mb) from that of the membrane plus synexin (Mb + Syn) as illustrated in fig.1C. Note that for the positive pulse (pipet potential 50 mV) the area between the two current transients (or the charge displaced) is positive during the pulse and negative after the pulse.

We interpret these currents (fig.1C) to represent displacement currents due to the incorporation of synexin into the substance of the bilayer. The synexin-specific additional displacement current can thus be used to estimate the associated capacity increase caused by the incorporation of synexin into the bilayer.

3.3. *Steady-state characteristics of the charge movements in bilayers with incorporated synexin*

Fig.2 shows a family of derived records obtained as outlined in fig.1. These records confirm the conclusions of section 3.2 in showing that the difference between the capacity transients has at least three components: (1) during the application of a positive pulse (fig.2, left side) there is an extra positive current which rises quickly to a peak and then decays towards a basal level; (2) immediately following the end of the pulse there is a membrane current transient in the opposite direction decaying to zero with a similar time course; (3) the first component is superimposed on another component

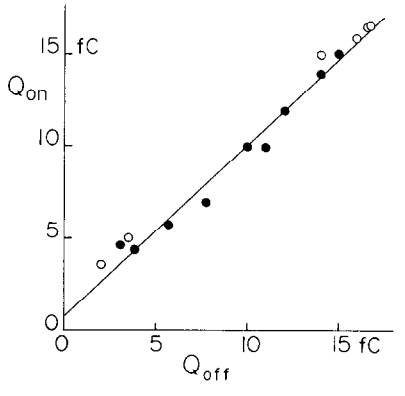

Fig.3. Charge displaced during the pulse as a function of charge displaced after the pulse. The size of the charge displaced during (Q_{on}) and after (Q_{off}) the pulses was calculated by numerical integration of the records representing net displacement current (see fig.2). The line is the linear regression through the points (m = 0.94; n = 0.07; r^2 = 0.98). (●) Charge displaced during and after the application of positive potentials; (○) charge displaced with negative potentials.

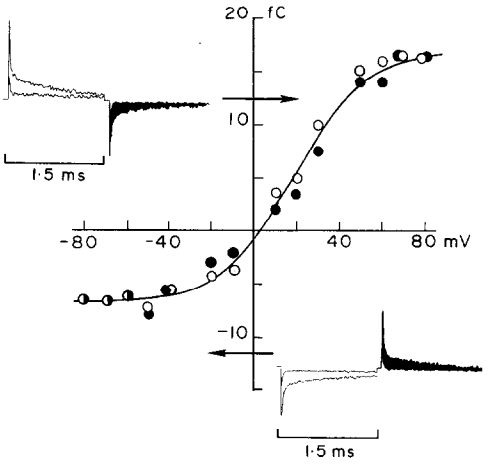

Fig.4. Voltage dependence of the steady-state distribution of synexin-specific mobile charges. The ordinate represents the size of the charge displaced during (○) and after (●) the pulses. The abscissa represents the pipet potential. Insets: superimposed current transients in response to positive (left) or negative (right) pipet potentials. Note that for the transients during the negative pulses the membrane current did not return to the zero baseline. Since the charge transferred was calculated by numerical integration of the difference between the two records (see inset, right), it is likely that the charge in the negative quadrangle represents the sum of a synexin-specific and a leakage current component. The curve was drawn to fit the points after correcting for this leakage component (◑). The curve was computed as the least-squares best fit (r^2 = 0.98) with the equation

$$Q = Q_{max}\{1/(1 + \exp[a(V-V_0)/kT])\}$$

giving a/kT = −0.06 and V_0 = 21 mV. Taking kT as 24.4 meV, the effective valence a becomes −1.46 electronic charges. Thus, a displacement of 16.7 mV (= kT/a) in membrane potential induces an e-fold change in the charge transferred at the midpoint (V_0 = 21 mV) [19]. Note: (i) the corresponding membrane potential inside a secretory cell would be −21 mV. (ii) V_0 results from the difference in surface potentials (i.e. potential of the side facing the high [Ca^{2+}] solution in the pipet minus that facing the low [Ca^{2+}] medium in the chamber).

which is usually time-independent and is sometimes referred to as leakage current.

Components 1 and 2 of the synexin-specific current may be identified with the displacement of charged portions of the synexin molecules that are integral parts of the membrane. However, if this were true the total transfer of charge in one direction at the beginning of the pulse should be exactly balanced by the transfer of charge in the other direction at the end of the pulse. This is indeed the case, as shown in fig.3 where we compare Q_{on} and Q_{off}. The filled circles are for positive pulses, while the empty circles are for negative pulses, over the range −120 to 120 mV. The slope is 0.94, statistically indistinguishable from the theoretically perfect slope of 1.0 (r^2 = 0.99). We therefore conclude from these data that the synexin-specific charge movement observed in phosphatidylserine bilayers is indeed nearly exclusively capacitative in character.

Another criterion must also be satisfied to establish that the records shown in fig.2 indeed represent synexin specific displacement currents. This criterion is that the charge displacement should exhibit saturation when large enough pulses are applied to the membrane. Since the dipole cannot move outside the confines of the membrane, progressively increasing the voltage should eventually be ineffective at causing additional charge movement. As shown in fig.4, increasing the size of the pulses from 10 to 50 mV increased the charge transferred by the electric field. However, increasing the potential beyond 50 mV (upper right-hand quadrant) did not lead to any further charge displacement. Similarly, decreasing the voltage below −50 mV (lower left hand quadrant) did not lead to further changes in charge movement.

4. DISCUSSION

The present data lend unambiguous support to the concept that the specific current transients observed in membranes containing synexin are due to the displacement of a substantial portion of the synexin molecule inserted into the substance of the bilayer. Our data also show that the portion of the synexin molecule inserted in the bilayer behaves as a dipole which can be displaced by the electric field within the confines of the membrane.

Two main conclusions regarding synexin interactions with membrane can therefore be drawn:

4.1. *Synexin not only binds to the surface of the acidic phospholipid bilayer but penetrates into its core*

We [10] and others [8] have shown that synexin could bind to the surface of the acidic phospholipid bilayers. However, our new data indicate that synexin also penetrates into the core of the bilayer. The evidence for this conclusion is the observed 7–10-fold (n = 4) increase in the capacitance of the bilayer. The membrane capacity is determined by

$$Cm = [E/E_0]A/d$$

where A represents the area of the bilayer, E/E_0 the dielectric constant and d the thickness. Assuming that A and d remain constant after penetration of the bilayer by synexin molecules, the insertion of dipolar molecules into the bilayer will cause an increase in the ratio E/E_0.

As to the mechanism for the synexin-induced increase, it should be emphasized that, in addition to the insertion of permanent dipoles, the synexin molecules may also undergo rapid polarization under the influence of the intense electric field in the bulk of the bilayer. This additional dipole moment of the synexin molecule will also contribute to the increase in E, the increase being proportional to the intensity of the electric field in the region of the membrane where the polarizable portion of the synexin molecule is located. Indeed, the bilayer is the locus where the intensity of the electric field is highest, and where the electrical effects on synexin molecules would be more manifest.

From the voltage dependence of the synexin-specific charge movement (see data in fig.4) we calculated the effective valence of the synexin dipoles to be equal to −1.5 electronic charges. Thus, a displacement of the pipet potential of 17 mV from the midpoint of 21 mV would induce an e-fold change in the synexin-specific membrane capacity. This value falls within the same range as other voltage-sensitive membrane processes [18]. This property may also relate to the mechanism by which synexin fuses membranes, a subject to which we will next turn.

4.2. *Penetration of synexin into the membrane may be relevant to the membrane fusion mechanism*

Indeed, the initial purpose of these physical studies on synexin was to find out how synexin caused fusion of biological membranes. With the data at hand, we may now be in a position to make a more accurate description of the process, which we have recently termed the 'hydrophobic bridge hypothesis' for membrane fusion [10]. This hypothesis predicts the following sequence of events in synexin-driven fusion:

(i) Synexin binds calcium [1], polymerizes [2], and is activated into a form that can bind to acidic phospholipids on the surface of a fusing membrane.
(ii) Synexin binds concomitantly to an adjoining membrane and cross-links the two together.
(iii) Synexin then penetrates into the hydrophobic substance (this work) of both membranes, providing a hydrophobic bridge linking both bilayers.
(iv) Phospholipids cross the bridge, allowing the outer leaflets of the fusing membranes to mix [6].
(v) Synexin provides an internal pathway for the inner (trans) leaflets of the fusing membranes to mix and yield volume continuity between the two originally separate membrane fusion partners [6,10].

The first four steps in this process now appear to be on firm experimental ground. Furthermore, quite explicit data [6] from kinetic studies on synexin-driven fusion of chromaffin granule ghosts show that membrane mixing must precede volume mixing. Therefore, step v, volume mixing, most probably occurs in the proposed sequence. We have previously shown how the mixing of the inner leaflets (step v) might precede by a restructuring of the hydrophobic bridge [10]. Since synexin dipoles can, in fact, move within the bilayer, such a process is clearly possible. However, in our opinion the details of the final step in synexin action remain to be fully explained in molecular terms.

Finally, although all of our capacitance studies have been performed on reconstituted bilayers, we have reasons to believe that this system is highly relevant to fusion events with real biological membranes. In cells, acidic phospholipids such as phosphatidylserine are localized on the membrane surface facing the cytosol. Indeed, synexin has little or no affinity for neutral phospholipids such as phosphatidylcholine, which are mainly localized on the opposite membrane surfaces [10]. Thus the membrane surfaces with which synexin must interact to promote fusion in vivo are enriched for the specific phospholipid we have used here for our in vitro studies. These facts are relevant to our previous observations that synexin has affinity for the inner aspect of the chromaffin cell plasma membrane, but none at all for the outer aspect [19]. Consistently, our preliminary studies with synexin applied to inside-out patches of chromaffin cell plasma membrane have revealed capacitance and other properties to be quite similar to those reported here for purified phosphatidylserine bilayers. We therefore feel confident that the hydrophobic bridge hypothesis is a useful model of how synexin goes about fusing membranes, and anticipate that the synexin mechanism might prove to be a prototype for fusion mechanisms in other biological systems.

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