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Topical Review

Synexin (Annexin VII): A Cytosolic Calcium-Binding Protein which Promotes Membrane Fusion and Forms Calcium Channels in Artificial Bilayer and Natural Membranes

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Introduction

Synexin, a cytosolic Ca^{2+}-binding protein, was discovered in 1978 during a search for cytosolic proteins which could support Ca^{2+}-dependent membrane fusion [4]. Such fusion occurs during exocytotic secretion from chromaffin and many other cell types and involves intimate contacts between the membranes of the secretory granules and the plasma membrane. In contrast to this process of "simple" exocytosis, many cells also exhibit "compound" exocytosis. Compound exocytosis involves the fusion of the membranes of intact secretory granules with those of previously fused granules still attached to the plasma membrane. The consequence of this type of exocytosis is the formation of numerous tunnels made up of fused granule membranes, leading to the exterior of the cell. Our initial hypothesis was that specific proteins in the cytosol might mediate contact and fusion mechanisms for both processes, and we therefore looked for proteins which would promote Ca^{2+}-dependent aggregation and fusion of chromaffin granules. At the outset the reader should be warned that the field of synexin is historically heavily idiosyncratic, in that relatively few scientists have contributed to the primary literature. This is now rapidly changing and is the basis for this topical review.

Returning to our narrative, we subsequently found that the first step in granule aggregation appeared to require the binding of Ca^{2+} to synexin and subsequent polymerization of synexin monomers into characteristic rod-shaped structures [5]. We interpreted these data to indicate that the site of action of granule aggregation was on the synexin molecule. We also found that aggregated granules could be induced to fuse into large vacuolar structures by the addition of small amounts of arachidonic acid, or any other *cis*-unsaturated fatty acid [3, 7]. Furthermore, arachidonic acid production is increased in stimulated intact chromaffin cells [17, 11], and similar vacuolar structures are frequently observed in chromaffin cells fixed during the secretory process [28, 25]. Thus, these observations lent support to the concept that synexin might be promoting a process also occurring in vivo. We have interpreted this last observation to mean that arachidonic acid fusion of granule aggregates might eventually prove to be of physiological relevance to the exocytosis process.

Further support for involvement of synexin in true exocytosis has also come from pharmacological studies. Synexin-driven granule aggregation is blocked by low doses of phenothiazine drugs such as trifluoperazine (TFP) and promethazine (PMTHZ) [32, 6]. These phenothiazines, in low concentrations, also block secretion from chromaffin cells [32], as well as from islets of Langerhans [42]. TFP is a rather good inhibitor of calmodulin [22], effector of local anesthesia [38, 39], and inhibitor of virus-cell fusion [33]. By contrast, PMTHZ is equally poor for all these modalities, thus suggesting that, in this context, the action of PMTHZ would seem to be relatively selective for synexin.

In prospect, if synexin were also involved in simple exocytosis then it should also have affinity for the inner aspect of the plasma membrane. To test this hypothesis we prepared inside-out chro-

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maffin cell plasma membrane on poly-L-lysine coated beads. We found that synexin indeed interacts in a calcium-dependent manner with the inner aspect of chromaffin cell plasma membrane, but not with the extracellular surfaces [15]. These data thus indicate that synexin could also mediate the initial interactions between granules and plasma membranes. While circumstantial, the evidence certainly lends support to the concept of synexin involvement in simple exocytosis.

However, the fact remains that these phenomenological parallels between synexin action *in vitro* and processes occurring in secreting chromaffin cells in no way prove that synexin guides exocytotic membrane fusion and secretion. Nonetheless, what these results clearly show is that whatever synexin does in vitro, quite similar processes do occur in secreting cells. Therefore, knowing the details of the molecular mechanism by which synexin interacts with and fuses membranes might prove interesting if only for the hints it might provide for how the process might be otherwise accomplished. In this regard, some specific and useful information about the mechanism by which synexin interacts with membranes has come from recent studies with purified phospholipids.

Several years ago Papahadjopoulos and colleagues [13–16] showed that synexin could fuse liposomes prepared from phosphatidylethanolamine (PE) and phosphatidylserine (PS). However, synexin could only bind to and aggregate phosphatidylinositol (PI) liposomes. By contrast, synexin did not appear to interact with phosphatidylcholine (PC) liposomes. These workers further interpreted kinetic studies of synexin-driven liposome fusion to indicate that the primary action of synexin was to promote adherence between liposomes and that fusion occurred by some other process [15]. These results on lipid specificity seem to be consistent with synexin having calcium-dependent affinity for acidic lipids.

Interestingly, acidic phospholipids are the type found on the outer aspect of granule membranes and on the cytosolic aspect of plasma membranes. By contrast, PC is found on the inner face of chromaffin granule membranes and on the extracellular face of plasma membranes, sites to which synexin does not appear to bind [37]. More recently, Zachowski et al. [46], using spin labeled phospholipids, have reported that the transmembrane lipid asymmetry in chromaffin granules is due to a specific ATP-dependent carrier that seems to be inverted with respect to the aminophospholipid translocase of plasma membranes.

These conclusions regarding lipid specificity for synexin have been confirmed and extended in our

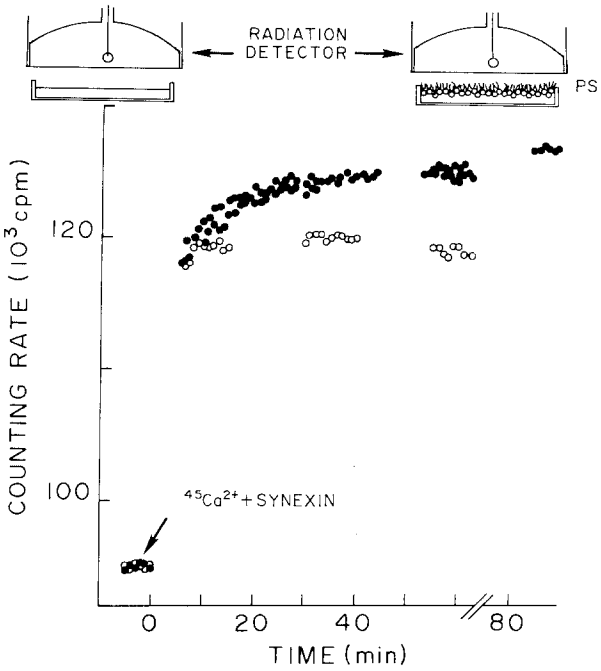

Fig. 1. Influence of synexin (ca. 18 μg/5 ml) on recruitment of 45-[Ca] (200 μM) to monolayer. Drawings above the graph represent geometry of the detector. Open circles represent calcium alone. Filled circles represent synexin + calcium. Counts include all isotopes from the interface to 660 μm below the interface. All surfaces were coated with bovine serum albumin (1 mg/ml) dissolved in buffer prior to the experiment, and we ascertained that 14-[C]-BSA was inert to all experimental manipulations described here. (Reproduced exactly from reference 31)

own laboratory with studies on phospholipids immobilized on hydrophobic solid surfaces [30] and on phospholipid monolayers at the air/water interface [31]. We found that in the presence of calcium ^{125}I-synexin exhibits no detectable interaction with PC but does interact strongly with PE, PS, PI and phosphatidic acid (PA). Furthermore, from studies on monolayers with $^{45}Ca^{2+}$ in the hypophase *we know that synexin raises the surface tension of a PS monolayer confined to a fixed area and brings calcium physically into close proximity with the bilayer* (Fig. 1). By contrast, synexin does bring calcium into proximity with a PI monolayer but has little effect on the surface tension. Thus both the liposome studies and the monolayer studies indicate that synexin can interact with PI, but to a less intimate degree than with PS.

At a more biological level of analysis, Stutzin [40] from our laboratory has developed a technique to measure volume-mixing during synexin-driven fusion of freeze-thawed chromaffin granule ghosts. The technique involves loading the ghosts with fluorescein-dextran (FITC-D) at self-quenching concentrations by a freeze-thaw technique. Loaded membrane vesicles are then allowed to fuse with empty

ghosts, the process being followed by relief of quenching caused by the dilution of the FITC-D. One interesting phenomenological result is that volume mixing appears to be a slower process than the mixing of membrane components, if one uses the octadecylrhodamine method to measure the latter process [40]. In this system fusion is characterized by a rapid leak (<5% of total signal), complete within 5 sec; a membrane mixing event, complete within 50 sec (τ = 38 sec); and a volume mixing event, complete with ca. 300 sec. The membrane mixing event appears to be composed of two processes: a fast one with τ = ca. 8 sec, and a slow one with τ = ca. 90 sec. Further kinetic analysis reveals that the rate-limiting event in this case is fusion *per se* rather than the initial membrane-membrane aggregation process [24]. While many caveats must be invoked, this mechanistic difference may distinguish liposome fusion from granule ghost fusion.

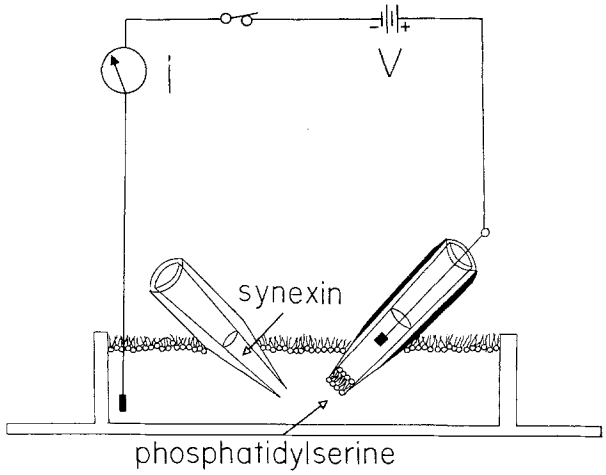

Fig. 2. Experimental arrangement for treating bilayer (right pipette) with synexin (left pipette). PS was added as a solid to the surface of the bath and a monolayer formed. Right pipette was double dipped through the monolayer to form the bilayer. Synexin was administered to the bilayer from a second pipette using a pico spritzer device. Only if the bath contained calcium could synexin dipoles be found to enter the bilayer

Calcium-Activated Synexin Inserts Itself into Artificial and Natural Membranes

From the previous discussion it seems certain that synexin can interact in a calcium-dependent manner, with either liposomes or monolayers formed from phosphatidylserine (PS). However, the nature of the interaction cannot be deduced from simple binding or absorption data. For example, synexin could simply adsorb to the surface of the bilayer. Indeed, Papahadjopoulos and colleagues [16] have proposed that this is the mechanism of synexin action and that fusion occurs as a consequence of a separate, calcium-driven dehydration process. Alternatively, synexin might itself drive both the membrane contact and fusion processes by more intimate insertional interaction with the bilayer membranes [34].

One way to distinguish these two processes would be to compare the capacitance of a bilayer membrane before and after exposure to Ca^{2+}-activated synexin. For example, if synexin were simply adsorbed to the surface, the capacitance of the membrane would not change. The reason would be that neither the overall dielectric constant of the membrane, the surface area, nor the thickness of the low dielectric region would be affected by this type of interaction. However, if the synexin molecules could penetrate deeply into the core of the bilayer, at a very minimum the relative dielectric constant, e/e_o, would increase and the capacitance would thereby increase. The relevant equation for thinking about this process is

$$C = e/e_o[A/d] \quad (1)$$

where C is capacitance, A is the area of the bilayer, and d is the thickness of the bilayer.

It is possible to estimate the increase in capacitance caused by the insertion of a molecule with an apparent dipole moment p from the Clausius-Mosotti relationship

$$(\varepsilon - 1)/(\varepsilon + 2) = (4\pi/3)\, N\, (p)^2/3kT \quad (2)$$

where ε represents the dielectric constant, N the number of dipoles inserted in the bilayer and kT equals 25.3 meV at 20°C [9]. Assuming that only 10,000 synexin molecules with a dipole moment, p, of 250 Debye are inserted per μm^2 of membrane, then the relative membrane capacity should increase by at least 10%.

We tested this prediction directly, and a diagram of the experimental arrangement for this study is shown in Fig. 2. A bilayer made at the tip of a patch pipette (right-hand side) is exposed for a brief period to a stream of solution containing synexin. A second pipette (left side) is used in this operation to act as a synexin source. The medium contains calcium, through which the synexin must move when it is puffed onto the bilayer. The consequence of puffing synexin onto the bilayer was that the capacitance dramatically rose *only* if calcium were in the bath [34]. The data used to estimate the changes in capacitance are shown in Fig. 3.

Shown in Fig. 3 (traces A, B, and C) are two superimposed displacement current transients in response to voltage-clamp pulses (50 mV) recorded

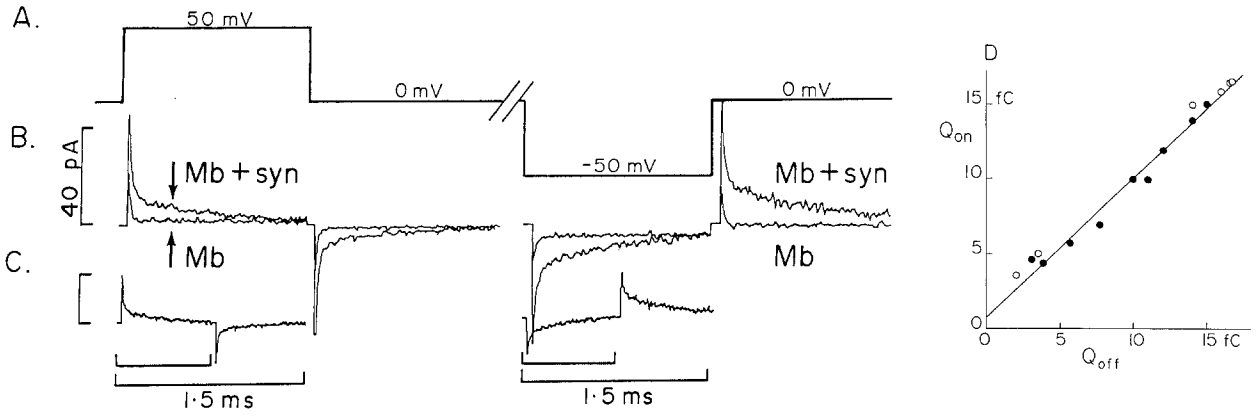

Fig. 3. Subtraction protocol to measure the synexin-specific displacement (*A–C*), and demonstration of charge conservation (*D*). (*A*) Pipette potentials (mV) are indicated over the voltage record. Between the pulses (interval marked by cut marks equivalent to 500 msec) the pipette potential was held at 0 mV. (*B*) *Left*: 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*: displacement currents in response to −50 mV pulses. (*C*) Net synexin-specific displacement currents obtained by subtracting the record labeled *Mb* from that labeled *Mb + syn*. Calibrations: vertical, 40 pA; horizontal, 1.5 msec. (*D*) Charge displaced during the voltage pulses, Q_{on}, *versus* charge displaced after the pulses, Q_{off} (the data are reproduced exactly from reference 34)

first in the absence of synexin (*Mb*), and then after exposure of the bilayer to synexin (*Mb + Syn*). As shown, there is a dramatic increase in the size of the displacement current across the bilayer after exposure to synexin (*compare* records labeled *Mb + Syn* and *Mb*). To further verify that the currents were indeed displacement or capacity currents, we tested for conservation of charge by estimating the charge transferred during and after the pulses from the difference between the two current transients in Fig. 3*C*.

Figure 3*D* shows a graph of the synexin-dependent, extra charge transferred during voltage-clamp pulses of increasing amplitude as a function of the charge transferred after the pulses. The slope of the line is close to unity, indicating that during the pulses membrane charge was displaced in one direction and, after the pulses, the same charge moved back in the opposite direction. We interpreted these results to indicate that synexin molecules had been incorporated into the core of the bilayer.

Figure 4 shows the characteristics of the charge movements for a PS bilayer alone (Fig. 4*A*) and after incorporation of Ca^{2+}-activated synexin (Fig. 4*C*). The point-by-point calculation of the slopes of each charge/voltage plot is shown in the lower graph for each condition (*B* and *D*, respectively). The graph in *B* is the value of the slope at each voltage, showing that the capacity of the membrane, without synexin added, is essentially constant. However, when a synexin supplemented membrane is examined (Fig. 4*C*), the analogous plot for the synexin-supplemented membrane, Fig. 4*D*, shows that the capacity has become quite voltage dependent. Indeed, the maximum additional capacity is obtained when the bilayer is held at 0 mV.

To further study the mobility of the synexin molecules within the bilayer, we subjected bilayers containing synexin to a protocol in which the bilayer was polarized (negative potential for the *cis* side) and then pulsed to ever more positive potentials. We found, as shown in Fig. 5, that the steady-state distribution of the charge displaced by the electric field across the bilayer fit a Boltzmann's distribution. This indicated that synexin dipoles within the membrane could move in a reaction coordinate located within the membrane and normal to the plane of the bilayer.

Synexin Forms Voltage-Gated Calcium Channels in Phosphatidylserine Bilayers

From these data it seemed probable that synexin, indeed, entered into the substance of the bilayer. This conclusion also seemed consistent with the observation that synexin could increase the surface tension of PS monolayers [31]. Nonetheless, we still could not say with certainty what the limits of the spatial distribution of synexin might be within the bilayer.

To remove synexin molecules adsorbed to the *cis* side of the bilayer membrane, we lowered $[Ca^{2+}]$ in the *cis* side (bath solution). Application of positive potentials to the *trans* side of the membrane

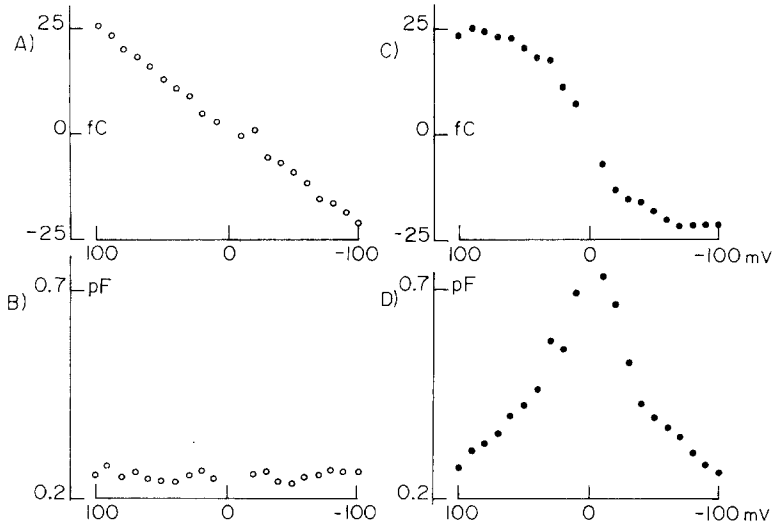

Fig. 4. Effects of Ca^{2+}-activated synexin on charge movements and bilayer capacity as a function of pipette potential. (*A*) Charge displacement (in fC) across the bilayer in the absence of Ca^{2+}-activated synexin. (*B*) Bilayer capacity (in pF) derived from the slope of the *Q-V* curve shown in *A*. (*C*) Charge displacement across the bilayer in the presence of Ca^{2+} activated synexin. (*D*) Bilayer capacity derived from the slope of the *Q-V* curve shown in *C*. Open symbols: in the absence of synexin; filled symbols: in the presence of synexin (3 μg/ml). Membrane resistance: is 12 GΩ for *A* and *B*, 8 GΩ for *C* and *D*. The pipette, which was coated with sylgard, had an open tip resistance of 20 MΩ

(pipette solution) elicited, to our surprise, a flow of current, carried by Ca^{2+} from the *trans* to the *cis* side of the membrane [29]. Furthermore, under appropriate conditions, calcium channel activity could be observed, as shown in Fig. 6. As expected from the displacement current experiment (Fig. 5), the open channel probability was found to be significantly voltage dependent.

The chord conductance of the synexin channel was also found to be sensitive to the ion gradient across the bilayer. For example, with [Ca^{2+}] adjusted to about 25 mM in the *trans* side and to about 1 μM in *cis* side, the single synexin Ca^{2+}-channel conductance was 13 pS over a range of membrane potentials (±100 mV). Data for the *I/V* plot under this extreme calcium gradient condition are shown in Fig. 7. Consistently, human synexin [2] behaved in a quite similar manner. However, changing the calcium concentrations on the *cis* and *trans* sides to be more nearly equal (25 mM in the pipette and 42 mM in the bath) caused the chord conductance, in the specific case of a sample of human synexin [2], to rise to ca. 175 pS.

When viewed from the perspective of what we already know about conventional Ca^{2+} channels, synexin calcium channels prove to have a variety of unusual properties. However, these properties were consistent with the biochemical properties of synexin, such as chromaffin granule aggregating and fusing activity. For example, synexin-dependent granule aggregation is exclusively dependent on [Ca^{2+}]. Similarly, synexin channels seem profoundly selective for Ca^{2+}. Indeed, addition of Ba^{2+} or Mg^{2+} to either the *cis* or the *trans* side failed to reveal significant changes in the size of the single

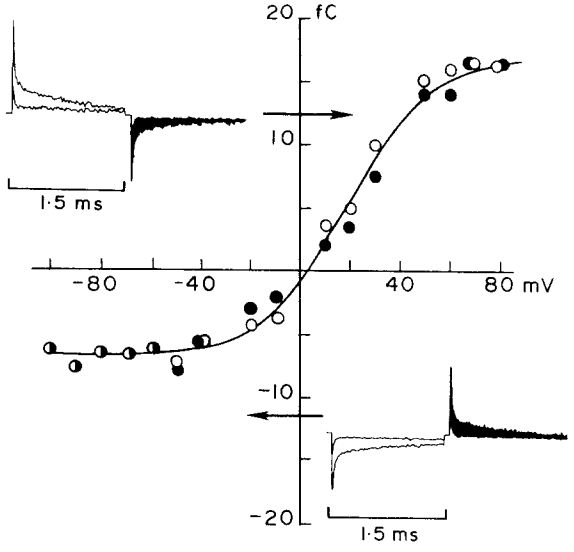

Fig. 5. Voltage dependence of the steady-state distribution synexin-specific mobile charges. The ordinate represents the size of the charge displaced during (○) and after (●) the pulses. The abscissa represents the pipette potential. Insets: superimposed current transients in response to positive (left) or negative (right) pipette potentials. Note that for the transients during the negative pulses and 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 fit to a Boltzmann's distribution, in which the effective valence a was −1.46 electronic charges, and a displacement of 16.7 mV (= kT/a) in membrane potential induced an e-fold change in the charge transferred at the midpoint (V_0 = 21 mV). (The experiment is reproduced exactly from Ref. 34)

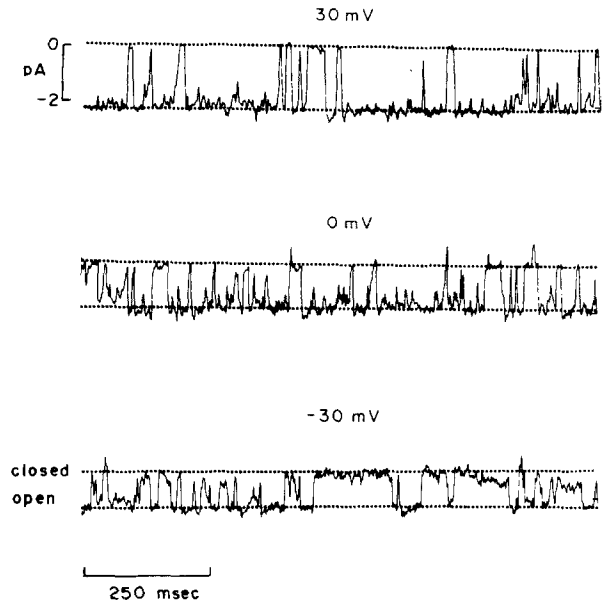

Fig. 6. Synexin calcium channel activity as a function of pipette potential (V_p). At positive values of V_p (+30 and 20 mV) channels are primarily open, although channels are less likely to be open at 20 than at 30 mV. At negative values of V_p (e.g., −30 mV) channels are primarily closed. The pipette contains 50 mmol · liter^{-1} Ca^{2+}, and the bath contains 1 μmol · liter^{-1} Ca^{2+}. Thus the chemical potential is large and constant in both cases, and the channels are clearly voltage dependent. These data are from ref. 29

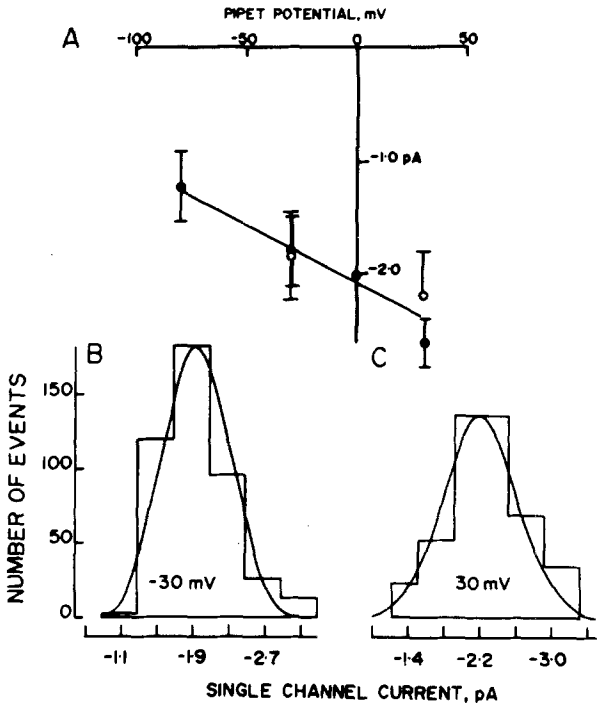

Fig. 7. Current-voltage relationship for the open synexin channel and amplitude histograms. (*A*) The fitted line corresponds to $I_{Ca} = \Gamma V_p + I^0_{Ca}$, where Γ is the single-channel conductance (10 pS) and I^0_{Ca} is the intercept [i.e., the value of the current at 0 mV pipette potential, V_p, (−2.09 pA)]. The reversal potential for the single-channel current was obtained from the extrapolated intercept on the V_p axis (*not shown*), −205 mV. Open circles correspond to the mean values for the histograms shown in *B* and *C*. Filled circles are data similar to that in *B* and *C*, but at different values of pipette potential. Error bars represent twice the SD. (*B*) Amplitude histogram for V_p = −30 mV. (*C*) Amplitude histogram for V_p = 30 mV. (Experiment is reproduced exactly from Ref. 29)

channel currents due to calcium. Consistently, synexin channel formation could be prevented by addition of phenothiazine drugs such as TFP or PMTHZ (<10 μM) to the bath prior to the puffing of synexin onto the bilayer. Granule aggregation, as described earlier, was similarly inhibited under the same conditions. Finally, synexin channels were profoundly insensitive to more standard calcium channel blocking substances such as Co^{2+}, Cd^{2+}, or nifedipine. In parallel, granule aggregation is similarly insensitive. These results thus clearly distinguish synexin channels from the more conventional Ca^{2+}-channels found in different types of plasma membranes, such as the *T*, *L* or *N* channels hitherto described [23].

From these data we might conclude that synexin channel activity and synexin-dependent granule aggregation and fusion might be mechanistically related. We will indeed return to this concept at a later part of this review. However, for the present we will simply conclude that the synexin Ca^{2+}-channel activity, at the very least, must mean that when synexin penetrates the membrane some or all of the molecule, or indeed polymers of the molecule, must span the entire bilayer.

Synexin Causes Capacitance Changes and Channel Activity in Inside-Out Patches of Chromaffin Cell Plasma Membrane

The fact that we can constitute synexin channel activity in artificial PS bilayers does make us confident in the intrinsic channel properties of synexin, *per se*. However, it was not necessarily obvious whether synexin could exhibit these properties in authentic biological membranes. To test this possibility we prepared inside-out patches of plasma membrane from intact, cultured chromaffin cells and applied synexin from a separate pipette onto the membrane surface. This orientation was of particular interest to us primarily because this was the surface with which synexin would putatively contact during exocytosis. Furthermore, the inner surface is enriched in the acidic phospholipids for

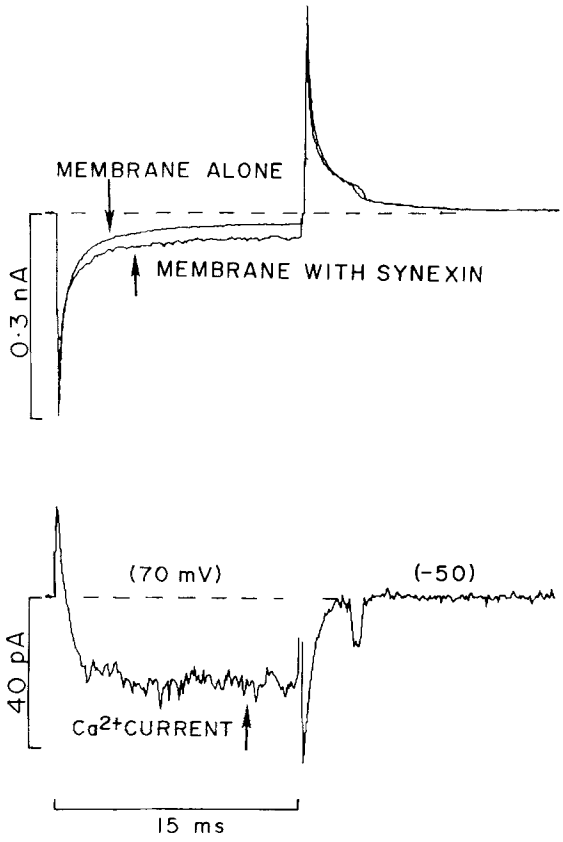

Fig. 8. Synexin channels in inside-out patches of chromaffin cell plasma membrane. Chromaffin cells were cultured for three days, and electrically silent, cell attached patches obtained. For patch-clamp experiments the cells were incubated first in a modified buffer containing (mM): 250 TEA-Cl, 1 $CaCl_2$, 10 TEA-HEPES, pH 6.8. After formation of a >20 GΩ seal the patch of membrane was excised and, prior to the application of synexin, voltage-clamp pulses of increasing size (−100 through +100 mV in 10-mV increments) were applied and the current transients recorded. If no current events were recorded during the application of depolarizing pulses (holding pipette potential was 50 mV), a stream of solution containing synexin was puffed onto the cytosolic aspect of the patch of membrane. [Ca^{2+}] was then lowered to less than 1 μM. Another series of pulses was then applied. Synexin-specific capacity and ionic current transients were then obtained by subtraction of the control current transients obtained in the absence of synexin from the current transients recorded after exposure of the patch to Ca^{2+}-activated synexin. Records below the zero current dashed line represent Ca^{2+} current flowing from the external to the internal side of the membrane

which synexin has substantial affinity in vitro. Accordingly, we also anticipated that little, if any, interactions would occur between synexin and the outer surface of the plasma membrane.

As shown in Fig. 8, addition of synexin in the presence of Ca^{2+} indeed leads to a significant elevation in membrane current. Furthermore, single-channel events similar to those observed with bilayers were often seen (Fig. 8, lower trace). These results strongly support the idea that exogenously added synexin is the origin of the activity. Even so, we cannot entirely exclude the possibility, no matter how unlikely, that some of the synexin-dependent activity observed in these inside-out plasma membrane patches could be due to interaction of synexin with some plasma membrane component.

As a further control for the specificity of the interaction of synexin with the inner leaflet of the plasma membrane, we applied synexin to outside-out patches of chromaffin cell plasma membrane. Previous chemical studies [37] had shown little or no calcium-dependent binding of synexin to the membranes of intact chromaffin cells. By contrast, calcium-dependent synexin binding to inside-out chromaffin cell plasma membranes had been easily observed. Consistently, our newer studies showed no detectable electrical consequences of adding synexin to outside-out patches.

Synexin Is a Member of the Annexin Gene Family of Calcium-Dependent Membrane Binding Proteins

Calcium-dependent membrane binding proteins include a set of proteins, also known variously as annexins, lipocortins, lipocortin/calpactins, or endonexin-fold proteins (*see* Refs. 20 and 26 for reviews), of which synexin was the first member to be chemically isolated. Indeed, synexin cDNA has only recently been identified in our laboratory by cloning [2]. The derived amino acid sequence reveals that synexin has ca. 45% homology with five other family members in the conserved C-terminal domain. Historically, efforts here and elsewhere with bovine libraries did not yield synexin clones. We assume that bovine synexin cDNAs are either significantly underrepresented or absent from these libraries, since no synexin cDNAs could be identified using oligonucleotide or cDNA probes. Successful cloning was achieved, however, when human libraries were screened using oligonucleotide probes constructed on the basis of peptide sequences from human synexin [2]. Interestingly, the derived human and bovine amino acid synexin sequences, where known [27], are highly conserved (ca. 93%), as described in a later paragraph.

Like other members of the annexin family, synexin is constructed from a conserved C-terminal tetrad repeat, and a unique N-terminal region. These data are shown in Fig. 9 and compared with sequences from other family members. The other members of the family include endonexin II (E2 [36, 19]); lipocortin I (L1 [44]), calpactin heavy chain/p36 (C1 [12, 18, 21, 35]), protein II (p2 [45]), and

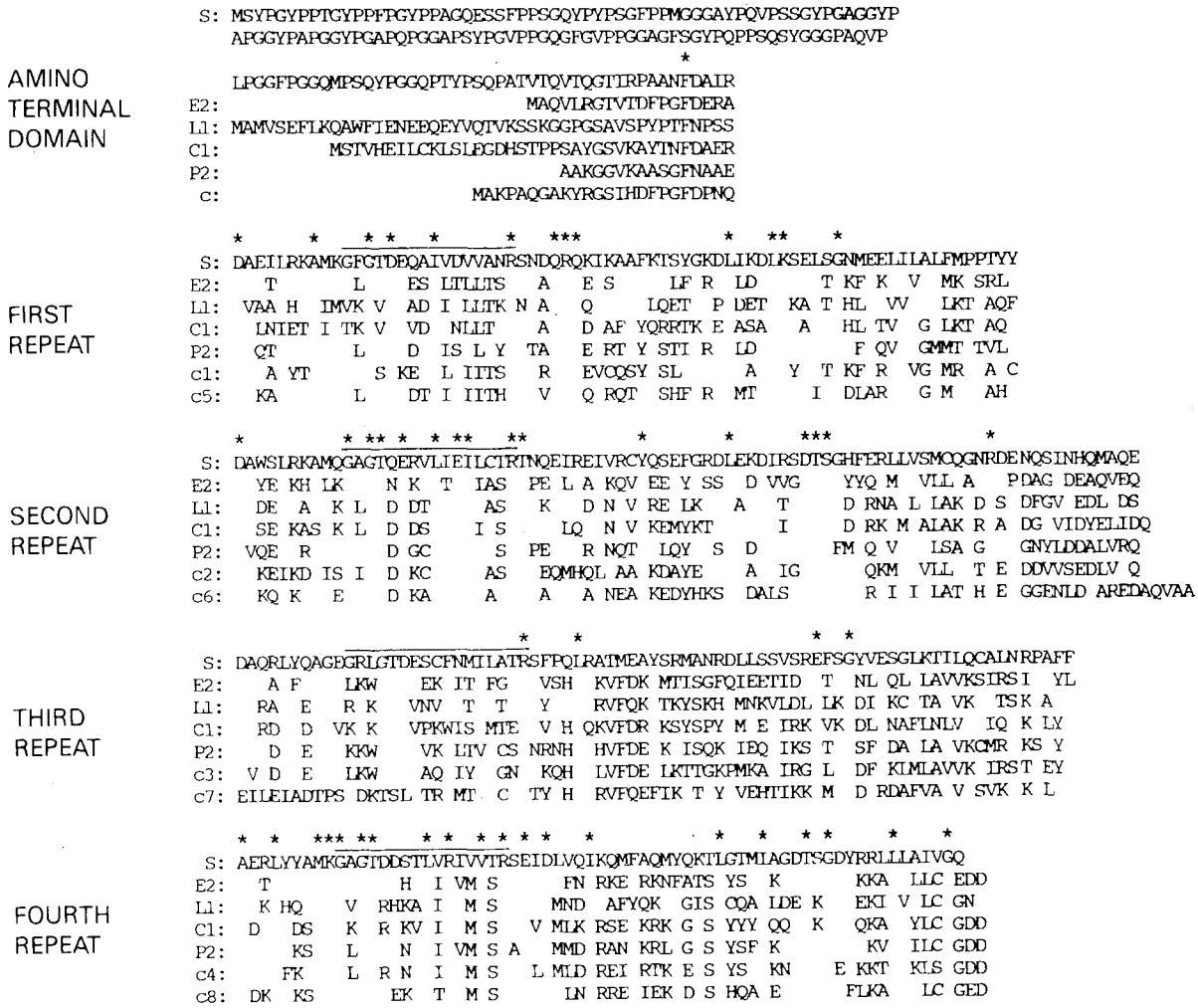

Fig. 9. Comparison of human synexin with other members of the family of calcium-dependent membrane binding proteins. Abbreviations are: *s* (synexin); *E2* (endonexin 2); *L1* (lipocortin 1); *C1* (calpactin 1); *P2* (protein 2); *c* (calelectrin, with numbers 1–8 corresponding to specific repeats; *c1–c4* occur in the most N-terminal tetrad, while *c5–c8* occur in the subsequent tetrad). Asterisks represent exactly conserved residues. Horizontal lines delineate the location of the highly conserved core repeats (α for I; β for II; γ for III; and δ for IV)

calelectrin 67K (c [41]). Uniquely, calelectrin 67K has a double tetrad repeat, in which the two tetrads are joined by a unique segment.

In synexin, as with other members of the family, there is within each repeat a more highly conserved core segment, which has been offered by some as a possible calcium-binding site [43]. These regions, for which exact homology between synexin and endonexin II is 56.3%, are: in repeat I, (GFGTDEQAIVDVVANR); in repeat II, (GAGTQERVLIEILCTR); in repeat III, (RLGTDESCFNMILATR); and in repeat IV, (GAGTDDSTLVRIVVTR). In three of four core repeats where bovine and human synexin sequences are known, there is agreement for 40 out of 41 amino acids (97.6%). The single difference, however, is a conservative replacement of isoleucine (bovine) by valine (human). A detailed analysis of these core repeats is given by ourselves in Ref. 27.

The unique N-terminal domain in synexin is highly hydrophobic, and contains 167 amino acids (*see* Fig. 10). Only two amino acids in this segment are charged, glu-123 and arg-158. According to Chou and Fasman secondary structure predictions (reported by ourselves in Ref. 2), the region is entirely beta pleated sheet and beta turn, with no evidence for amphipathic structure. Indeed, the region is prominently marked by a characteristic GYP (gly-tyr-pro) motif, repeated eight times, with variations occurring seven times as GXP (where X is hydrophobic) and six times as GXP (where X is neutral). In addition, GG, PP and PG doublets occur 10, 8

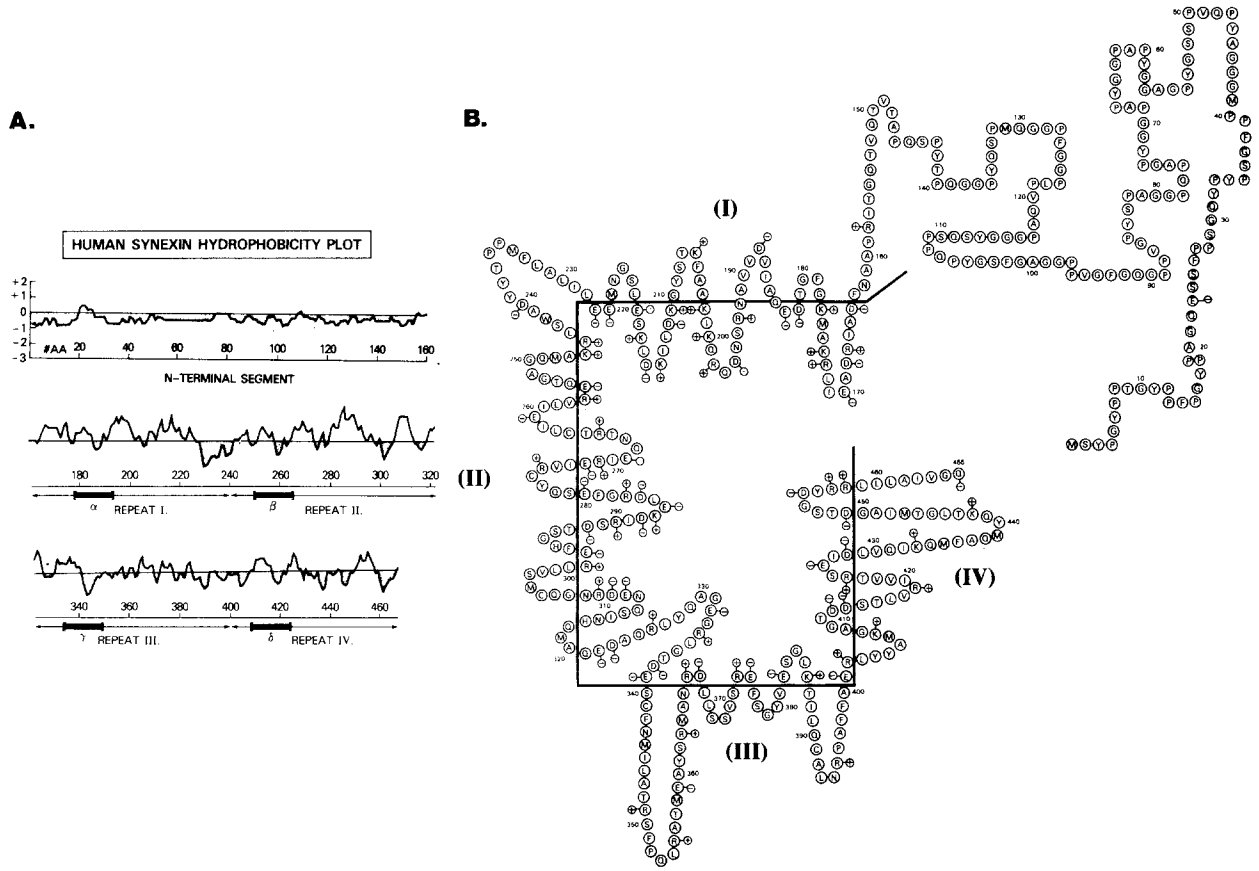

Fig. 10. (*A*) Hydrophobicity plot of human synexin. Lengthy hydrophobic domains (negative values) are stippled. The location of each repeat is marked off by double-headed arrows. The highly conserved core repeats are marked by heavy black lines and are marked α, β, γ, and δ. The secondary structure of the N-terminal segment is predicted to be entirely β-sheet or β-turn. (*B*) Two-dimensional (face-on-view) model of the synexin calcium channel inserted into a membrane. The hydrophobicity plot in *A* serves as the basis of this model, in which the four major repeats (*I*, *II*, *III*, and *IV*) in the C-terminal region form the sides of a box and the highly hydrophobic N-terminal segment presumably protrudes into the membrane. Amino acid stretches of hydrophobic character in the repeat regions that were above the line in the hydrophobicity plot are now placed outside the lines of the box and likely interact with the hydrophobic elements of the membrane. Conversely, the hydrophilic amino acid areas were placed inside of the lines. The inside of the box has a net charge of −7 and is consistent with calcium ion specificity of the channel. (The figure is reproduced exactly from Ref. 2)

and 13 times, respectively. Although this N-terminal region has slight similarities to collagen, glutinin, keratin, synaptophysin and gliadin [8], the function of this hydrophobic domain remains to be determined.

Finally, two regions of homology have been detected in the N-terminal domains of synexin and calpactin I heavy chain, and to a lesser degree of the other annexins. The first two amino acids for both synexin and calpactin I heavy chain are methionine followed by serine, whereas the others have serine in the second position. Secondly, all the annexins possess a similar joining sequence at the C-terminal end of the N-terminal domains. In the case of synexin this is (FDAIR). In the whole set of annexins a phenylalanine (F) occurs at the fifth amino acid from the junction, while aspartic acid or asparagine occur as the fourth amino acid from the junction. We have summarized these sequences as follows: S (FDAIR); E2 (FDERA); L1 (FNPSS); C1 (FDAER); P2 (FNAAE); and c (FDPNQ).

The C-Terminal Tetrad Repeat in Synexin May Be the Channel

From the forgoing analysis one must presume that part or all of the synexin molecule(s) must penetrate the bilayer to form the calcium channel. Although it is possible that the N-terminal region of synexin might be part of the channel, on practical grounds we have focused our attention on the tetrad repeat domain as the likely basis of channel function [2]. Perhaps the most compelling reason is our observa-

tion [34*a*] that both human placental and recombinant endonexin II behave as calcium channels under conditions similar to those used to study synexin channels. The N-terminal domain of endonexin is only 18 amino acids long, and, as illustrated in Fig. 9, is entirely different in character than the equivalent 167 amino acid domain in synexin. We have therefore attempted to understand how synexin might fit into and function within a membrane, with specific reference to the C-terminal domain.

By analogy with models of membrane resident ion channels, one obvious way to think about the relationship between synexin sequence and structure within the membrane would be to look for long stretches of alpha helix, anticipating highly hydrophobic or amphipathic character. However, this was not found [2]. Rather, the hydrophobicity plot, shown in Fig. 10*A*, revealed many segments of alternating hydrophobic and hydrophilic character. Furthermore, very little alpha helix was predicted by the Chou and Fasman program. Finally, where alpha helix was predicted, little or no amphipathic or hydrophobic character was predicted. In fact, the C-terminal domain of synexin has substantial beta sheet and beta turn character, if one uses the *standard default parameters* for the structure determination.

It was thus clear that synexin structure might be somewhat different from that of standard membrane resident channels hitherto modelled. Our alternative is shown in Fig. 10*B*, where we have used the hydrophobicity plot to create a two-dimensional channel-like structure with a view down the "mouth" of the channel. In other words, the view is face-on to the membrane, rather than the more common cross-sectional view. To prepare this view we used the axis of the hydrophobicity plot in Fig. 10*A* to generate the sides of a box, each side corresponding to each of four repeats (I to IV), and with a length corresponding to the number of amino acids in each repeat. We placed a string of amino acids on the interior of the box if hydrophilic or on the exterior of the box if hydrophobic. This approach frees us from any assumptions about secondary structure.

In this model, most of the charged residues are on the interior of the box, forming the walls of the putative channel. Most of the hydrophobic residues are on the exterior of the box, forming the surface adjoining the hydrophobic lipid phase. Using the hydrophobicity plot to assign strings of amino acids to one side or the other proved quite unambiguous and had the further advantage that no assumptions regarding conformation were needed.

Several properties of this simple model have proved potentially relevant to the behavior of the synexin channel. For example, the net charge on the interior of the model is −7, suitable to attract cations and repel anions. This does not, of course, explain the exquisite selectivity of the synexin channel for calcium, *per se*. Another property of the model is that the distribution of charges on the hydrophilic surface of the different repeats is asymmetric. We have considered whether this might be the basis of the voltage-gating properties of synexin. Finally, we have noted that the few charged amino acids on the hydrophobic surface of the model have a net charge of +6. We have therefore considered whether this might explain the preference of synexin for acidic phospholipids. Our present view of this model is that it at least has the advantage of internal consistency, is only slightly speculative since it is only a variation on the traditional hydrophobicity plot, and leads to specific predictions which can be tested experimentally.

Synexin May Promote Fusion by Forming a Hydrophobic Bridge Between Fusing Membranes

Several years ago, in the absence of the sequence data now available, we proposed that the mechanism by which synexin promoted membrane fusion might be by forming a hydrophobic bridge between fusing bilayers. We termed this our "hydrophobic bridge hypothesis," and suggested that as a first step in the fusion process phospholipids on nearby membranes might cross the hydrophobic bridge and mix together [30, 31]. The primary evidence for such a process was the fact that in the presence of calcium synexin could enter target bilayer membranes, as indicated by capacitance studies, and then span the bilayer, as indicated by channel studies. Our hypothesis was based on the possibility that if synexin could *simultaneously penetrate two neighboring membranes*, then the putative hydrophobic bridge might be formed. The fact that synexin can form polymers in the presence of calcium may lend further credence to this mechanism.

The sequence predicted by synexin cDNA certainly supports the concept that synexin could be such a hydrophobic bridge. It is evident that the C-terminal tetrad repeat domain of the synexin molecule, likely to be the material basis of the synexin calcium channel, must be able to assume profoundly hydrophobic character on its own. It is also evident that the unique N-terminal segment of synexin, consisting of 167 amino acids, is virtually entirely hydrophobic. It could therefore contribute to the intrinsically hydrophobic character of synexin. We can thus conclude that sequence information

does not exclude the hydrophobic bridge hypothesis as a basis for synexin-driven membrane fusion.

Finally, it is worth including here our expectation that, to the extent that other members of the synexin gene family express channel and membrane fusion properties similar to those of synexin, one might reasonably anticipate the mechanisms underlying these properties to be held in common. Other annexins potentially falling into this category may well include lipocortin I (annexin I, yet unpublished observations by G. Lee, M. de la Fuente, E.R. Haigler, E. Rojas, and H. Pollard).

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