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A MOLECULAR BASIS FOR SYNEXIN-DRIVEN, CALCIUM-DEPENDENT MEMBRANE FUSION

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Summary

Membranes of secretory vesicles fuse with each other and with plasma membranes during exocytosis in many different cell types. The probable role of calcium in the process is now widely accepted, and it is possible that at least one cytosolic mediator of calcium action is synexin. Synexin is a 47000 M_r calcium-binding protein, initially discovered in the bovine adrenal medulla, which binds to granule membranes and to inner aspects of chromaffin cell plasma membranes. Synexin causes chromaffin granules to aggregate, and such aggregates can be caused to fuse in the additional presence of arachidonic acid. Synexin also mediates the direct fusion of liposomes and chromaffin granule ghosts. To understand better the mechanisms of membrane fusion promoted by synexin we have attempted to define the primary sequence of the protein. Our initial efforts were directed towards purification of bovine synexin in sufficient amounts to allow us to sequence tryptic peptides. However, as the project progressed we also directed our attention to human synexin, preparing peptides from this protein as well. From analysis of bovine peptides we learned that the synexin molecule might be closely related to a class of proteins including lipocortin I, calpactin (p36), endonexin II, protein II and calelectrin 67K. Complete analysis of a human synexin cDNA clone revealed strong homology with bovine synexin. The analysis also showed that synexin contained a unique, long, highly hydrophobic *N*-terminal leader sequence followed by a characteristic four-fold repeat homologous with those found in other members of the synexin gene family. The highly hydrophobic character of synexin seems consistent with information previously obtained that synexin is able to insert directly into the interior of bilayers prepared not only from purified phosphatidylserine but also from biological membranes. The evidence for such insertions is a dramatic increase in the capacitance of the membrane, formed at the tip of a patch pipette, when calcium-activated synexin is applied to the bilayer. Additional evidence is the fact that synexin also forms calcium-selective channels when the protein is applied to the cytosolic aspect of the plasmalemma when that side is also exposed to calcium at sub-millimolar concentrations. Thus, the synexin molecule not only enters the membrane, but also spans it. From these and other data we have developed the concept that the fusion process may involve synexin forming a 'hydrophobic bridge' between two fusing membranes. Lipid movement across this bridge may then be the material basis for final fusion. We

Key words: human synexin, membrane fusion, calcium channels, sequence.

have termed this concept the 'hydrophobic bridge hypothesis', and have developed the predictive properties of this hypothesis in considerable detail.

Introduction

Secretion by exocytosis involves fusion of a secretory vesicle membrane with the plasma membrane of the secreting cell (for a summary see Pollard *et al.* 1985). In many endocrine cells, including chromaffin cells, beta cells and mast cells, this simple exocytosis event is followed by contact and fusion of more deeply situated secretory vesicles with the initially fused secretory vesicle membranes. The latter process is called compound exocytosis, and presumably allows for additional secretion without moving secretory granules long distances through the cytoskeleton to reach the plasma membrane.

The immediate signal for the membrane fusion events described here may involve calcium. Indeed, historically, both simple and compound exocytosis have usually been closely associated with a requirement for extracellular calcium. More recently, fluorescent methods have been developed to monitor intracellular calcium, and data obtained in this way have been interpreted to indicate that secretion in many types of cells depends upon an increase in the intracellular free calcium ion concentration. For this reason, studies on the mechanism of membrane contact and fusion during exocytosis have tended to focus on how calcium might promote the process.

A decade ago we began a concerted search for proteins that could mediate calcium-dependent contact and fusion of chromaffin granules, and discovered synexin in the process (Creutz, Pazoles & Pollard, 1978). Preliminary studies with 'conventional' calcium-binding protein such as actomyosin, tubulin and calmodulin had proved fruitless, and led us to search the adrenal medulla, a classical secretory tissue, for endogenous membrane fusion factors. Synexin was the initial fruit of this search. The remainder of this review is a summary of what ensued over the next 10 years as we pursued a better understanding of the mechanism of synexin action.

Synexin causes chromaffin granule aggregation and fusion

Synexin was initially characterized as a protein which caused chromaffin granules to aggregate in a *calcium-dependent* manner. For measuring synexin activity we developed a precise and quantitative method in which granule aggregation could be followed by changes in turbidity at 540 nm. Other divalent cations including Mg^{2+}, Sr^{2+} and Ba^{2+} were tested, but only calcium proved effective. The $K_{1/2}$ of calcium for granule aggregation was found to be $200\,\mu mol\,l^{-1}$, and we later learned that synexin self-associated in the presence of calcium with the same $K_{1/2}$ (Creutz, Pazoles & Pollard, 1979). Thus the calcium effect seemed clearly to be on synexin, and not exclusively on some separate membranes process. By electron microscopy the aggregated granules were found

to be connected by quite close 'pentalaminar' membrane contacts. Since these contacts were quite similar to those observed in secreting cells by Palade and others (Palade, 1975), we were somewhat encouraged in our expectation that synexin action might actually have something to do with exocytosis.

However, for years we (and others, e.g. Morris, Hughes & Whittaker, 1982) puzzled over the physiological meaning of the rather substantial value of the $K_{1/2}$ for calcium-dependence for granule aggregation. In the late 1970s the free calcium ion concentration in resting chromaffin cells was believed, and later shown, to be in the range of 50–150 nmol l^{-1} (see Pollard *et al.* 1985, for a summary). Stimulation caused the calcium concentration to rise perhaps two- to three-fold, a value well below the $K_{1/2}$ for synexin, and substantially below the threshold calcium ion concentration needed to activate synexin (approx. 5 μmol l^{-1}).

How then could synexin be involved in exocytosis? Over the next few years studies with better indicators of cytosolic free calcium concentration revealed that peak calcium concentration values were transiently quite high following stimulation. Indeed, the change in calcium concentration was found to be possibly in the range of 10–100 μmol l^{-1} in the volume immediately beneath the plasma membrane (Simon & Llinas, 1986). Furthermore, Creutz & Sterner (1983) found that the calcium-dependence for *binding* of synexin to granule membranes could be moved to the low micromolar range by raising the pH of the assay from the optimum pH of 6·0 to the less than optimum pH of 7·0. Nonetheless, the magnitude of granule aggregation under these conditions remained lower, indicating that the processes mediating synexin binding to membranes and granule aggregation could be distinct.

In studies with intact chromaffin granules, fusion *per se* could only be observed if relatively low concentrations (approx. 5 μmol l^{-1}) of arachidonic acid were added to the aggregated granules (Creutz, 1981; Creutz & Pollard, 1982*a*,*b*). Arachidonic acid was not exclusively required, however, since any other fatty acid possessing at least one cis-unsaturated double bond could support fusion. In addition, although calcium was required for the synexin-driven granule aggregation step, removal of calcium by addition of EGTA left the granules still aggregated and still susceptible to fusion by added fatty acids. Thus the fusion step itself was not calcium-dependent.

The possible physiological relevance of this process is indicated by the fact that chromaffin cells synthesize easily measurable amounts of free arachidonic acid when stimulated by physiological secretagogues (Hotchkiss, Pollard, Scott & Axelrod, 1981; Frye & Holz, 1984). A possibly more compelling argument for physiological relevance is the nature of the fusion structures formed by arachidonic acid treatment of synexin-aggregated granules. The granules fuse into vacuolar structures nearly identical in character to compound exocytotic structures observed in stimulated chromaffin cells (Pollard, Creutz &Pazoles, 1981; Pollard *et al.* 1984; Pollard *et al.* 1982; Ornberg, Duong & Pollard, 1986).

However, when synexin was used to promote fusion of candidate membranes such as liposomes (Hong, Duzgunes & Papahadjopoulos, 1981, 1982*b*; Hong,

Duzgunes, Ekert & Papahadjopoulos, 1982*a*) or chromaffin granule ghosts (Stutzin, 1986; Nir, Stutzin & Pollard, 1987; Stutzin, Cabantchik, Lelkes & Pollard, 1987) arachidonic acid was not required. Initial studies from Papahadjopoulos's laboratory revealed that liposomes prepared from acidic phospholipids such as phosphatidylserine (PtdS), phosphatidic acid (PtdA) or phosphatidylethanolamine (PtdE), or mixtures of these, would fuse directly if calcium and synexin were added to them in solution. Synexin was completely ineffective when the liposomes were prepared from phosphatidylcholine (PtdC), and liposomes prepared from phosphatidylinositol (PtdIns) were only aggregated (Hong *et al.* 1982*b*). The latter behaviour was reminiscent of intact chromaffin granules, in that they too only aggregated in the presence of calcium and synexin (Pollard *et al.* 1985). Thus, it was clear that liposome aggregation and eventual fusion seemed to be dependent on the specific lipid used, and that acidic phospholipids seemed to be preferred to neutral phospholipids. Consistently, Hong *et al.* (1983) showed in a kinetic study that the rate-limiting step for synexin-induced fusion of liposomes was the liposome *aggregation* step.

Chromaffin granule ghosts, prepared by a freeze/thaw technique, were also found to fuse spontaneously upon the addition of synexin. As illustrated in Fig. 1, this process can be followed by a volume-mixing assay in which some ghosts are loaded with self-quenching concentrations of FITC–dextran and allowed to fuse with empty ghosts. This process is, however, quantitatively different from liposome fusion in several regards. In contrast with the liposome experiments, the freeze/thaw ghosts fused in a manner only partially (40 %) dependent on calcium. Furthermore, membrane carboxyl groups, possibly on protein, seemed important for the process. Finally, Nir *et al.* (1987) showed that the rate-limiting step was the fusion event itself, rather than the membrane aggregation event as shown for liposomes.

These data therefore indicate that synexin can indeed cause contact and fusion of membranes that are relevant to the exocytotic process. Chromaffin granules possess relevant membranes simply because they are the central actors on the exocytotic stage in the chromaffin cell. However, the fact that acidic phospholipid liposomes are also substrates indicates that synexin may not only be directing its attention towards specific membranes in the secreting cell. Nonetheless, it is a fact that acidic phospholipids such as PtdS, PtdE, PtdA and PtdIns are primarily localized in the inner leaflets of plasma membranes and in the outer leaflets of granule membranes. Neutral phospholipids, such as PtdC and sphingomyelin, are in contrast localized in the outer leaflets of plasma membranes and the inner leaflets of granule membranes. Thus calcium-activated synexin may aggregate and fuse the membranes involved in exocytosis merely because of proximity of the appropriate phospholipid substrates at the sites where calcium concentration is highest.

This interpretation of how synexin might be directed to act during exocytosis is also consistent with our experiments on inside-out plasma membranes from chromaffin cells. In these experiments, chromaffin cells were attached to poly-

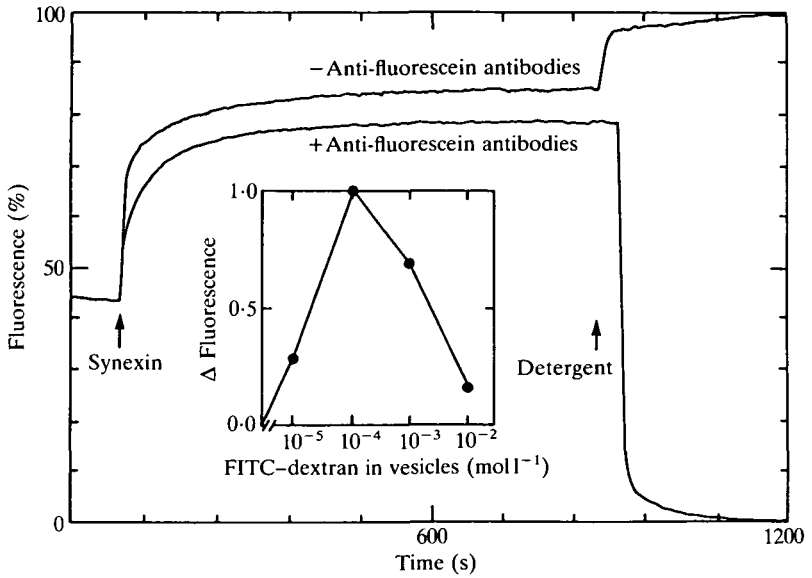

Fig. 1. Synexin induces fusion of frozen/thawed chromaffin granule ghosts. The increase of fluorescence due to addition of synexin (34·08 μg, first arrow) is shown in the absence and presence of anti-fluorescein antibody. The medium is 140 mmol l^{-1} KCl and 20 mmol l^{-1} Hepes-K, pH 6·03, pCa = 7, at 37°C. The detergent NP-40 is added at the second arrow in the presence or absence of the antibody to calibrate the signal. An increase in fluorescence of the system indicates fusion of the vesicle containing self-quenching concentrations of FITC–dextran with an empty vesicle. Inset: the self-quenching curve for FITC–dextran. Granules were loaded with different concentrations of FITC–dextran by freezing and thawing in liquid nitrogen. For the experiment demonstrated in the main figure, granule ghosts were loaded in approx. 30 mmol l^{-1} FITC–dextran. Data are a composite from Stutzin (1986).

L-lysine-coated beads and the cells then broken (Scott, Creutz, Pollard & Ornberg, 1985). In these experiments we found that synexin would bind in a calcium-dependent manner to the inner leaflets of chromaffin cell plasma membranes, but not to the neutral lipid-rich outer leaflets of intact chromaffin cells, otherwise also attached to the beads.

Synexin inserts into and spans the bilayer

During the brief time that synexin has been available for analysis, two distinct views have been pursued as to how the molecule might interact with target membranes. We had previously been of the opinion that calcium interacted with individual synexin molecules, thereby modifying the conformation, and thus rendering synexin able to interact with the membrane in some way (summarized in Pollard *et al.* 1985). Alternatively, workers from Papahadjopoulos' laboratory suggested that synexin acted by causing close approach of membrane pairs, and

that calcium *independently* induced fusion through a dehydration step (Hong *et al.* 1982*a*).

To help distinguish between these possibilities, and perhaps to discover others, we turned to the technique of membrane capacitance measurement. We prepared PtdS bilayers at the tip of a patch pipette and added calcium-activated synexin to the bilayer. If synexin merely adhered to such membranes, capacitance would be relatively unaffected. In contrast, if synexin actually inserted into the membrane one would expect the capacitance of the membrane to rise. The reason is that capacitance of a membrane (C) is given by the equation:

$$C = (E/E_0)\,(A/d)\,,$$

where the ratio E/E_0 is the dielectric constant, A is the area and d is the thickness of the membrane. The dielectric constant of a phospholipid bilayer is approximately 2, and if protein dipoles were to be inserted into the membrane the value of the dielectric constant would rise. Obviously, mere adherence of the synexin molecules to the membrane would not affect capacitance since adherence would neither expand the area of the low dielectric region (A) nor change its thickness (d).

The results showed the synexin could profoundly change the capacitance of the membrane by nearly 10-fold if calcium were also in the bath (Rojas & Pollard, 1987). An example of such data is shown in Fig. 2, where capacitance is measured using a time-based method. In the figure, different voltages have been applied to the membrane and the capacity currents of the membrane before synexin addition subtracted from that of the synexin-supplemented membrane. The traces are therefore *synexin-specific* capacity currents. The advantage of this method is that it allows all frequencies to be sampled and the increase in the charge on the bilayer to be quantitatively determined. Thus, synexin is clearly able to enter the bilayer, and does not merely adhere to it. It is worthwhile noting here that these results do not exclude the possibility that some synexin molecules might also adhere since we cannot detect them by this method.

In addition we also found that the synexin-dependent capacitance change was voltage-dependent. This voltage-dependence could be fitted to a Boltzmann's distribution, as shown in Fig. 3. The possible meaning of this result is that the synexin dipole(s) can be moved within a reaction coordinate that is defined in some way by the membrane, but that the dipole(s) cannot be moved *out* of the confines of the membrane. This structural limitation is our interpretation of the saturation of change in capacitance at negative and positive extremes of the applied voltage. In the case of sodium channels, this voltage-dependent capacity current has been called 'gating current'. The conventional interpretation of gating current has been that part or all of the channel protein moves within the membrane to allow the channel to open (Rojas, 1976; Armstrong & Bezanilla, 1976).

The analogy between voltage-dependent capacity currents for synexin and gating currents for sodium channels led us to examine synexin more closely for

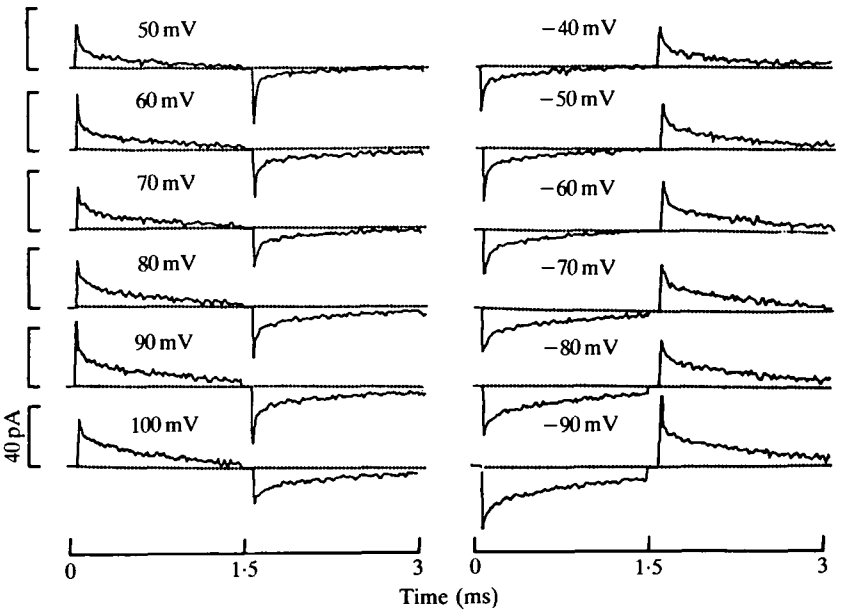

Fig. 2. Synexin-specific displacement currents at different transmembrane potentials. The traces are differences between displacement current records calculated by subtracting current transients in PtdS bilayers from current transients obtained from bilayers supplemented with synexin. The left-hand side of the figure displays net displacement current records for positive pipette potentials. The right-hand side displays those for negative pipette potentials. The data are from Rojas & Pollard (1987).

channel activity. We focused our attention on calcium as a possible conducting ion and found, as shown in Fig. 4, that synexin could indeed exhibit exquisitely selective calcium channel activity in PtdS bilayers (Pollard & Rojas, 1988). To observe these channels we had to increase the chemical driving force (50 mmol l^{-1} Ca^{2+} in the pipette and 1 μmol l^{-1} Ca^{2+} in the bath) and to make the pipette potential (V_p) positive. These channels proved to be highly selective for calcium, being virtually impermeant to barium, strontium or magnesium. Similar calcium channel activity could be elicited when the target membrane was an inside-out patch of chromaffin cell plasma membrane (Fig. 5). In this case the chemical gradient was exactly as described for the PtdS bilayers. Thus the membrane active properties of synexin were not simply due to unusual properties of pure phospholipid bilayers.

In addition, the phenothiazine drugs, trifluoperazine (TFP) and promethazine (PMTHZ) proved to be quite effective blockers of the channel activity (Pollard & Rojas, 1988). This was an important observation because both drugs, at low micromolar concentrations, also block synexin-driven chromaffin granule aggregation (Creutz, Pazoles & Pollard, 1982; Pollard, Scott & Creutz, 1983), nicotine- and veratridine-driven exocytosis from chromaffin cells (Pollard *et al.* 1983) and

glucose-driven insulin secretion from rat or *Psommys obesus* islets of Langerhans (Sussman *et al.* 1983). In contrast, TFP has no reported effect on conventional calcium channels (L, T or N). Finally, neither cadmium nor nifedipine were able to block synexin channels at pharmacologically relevant doses ($<10\,\text{mmol}\,l^{-1}$ and $<300\,\mu\text{mol}\,l^{-1}$, respectively). These properties taken together effectively exclude synexin channel activity from being due to inadvertent contamination of the preparation by conventional membrane calcium channels (see Miller, 1987 for detailed pharmacology of conventional calcium channels).

The physiological significance of synexin channels remains to be determined, and we will not offer any of the more obvious speculations here. However, the *operational* meaning of the synexin channel observation is clearly that calcium allows all or part of the synexin molecule not only to *enter* the bilayer but also to *span* the bilayer. This conclusion has had important significance for our thinking about the mechanism by which synexin might cause fusion of target membranes.

The hydrophobic bridge hypothesis for synexin-driven membrane fusion

These data, together with preliminary studies on the sequence of bovine synexin, recently led us to propose the hydrophobic bridge hypothesis for synexin-driven membrane fusion (Pollard, Rojas & Burns, 1987; Pollard, Rojas, Burns & Parra, 1988). As summarized in Fig. 6, and in detail in the legend to the figure, we proposed that calcium would cause synexin to undergo significant conformational

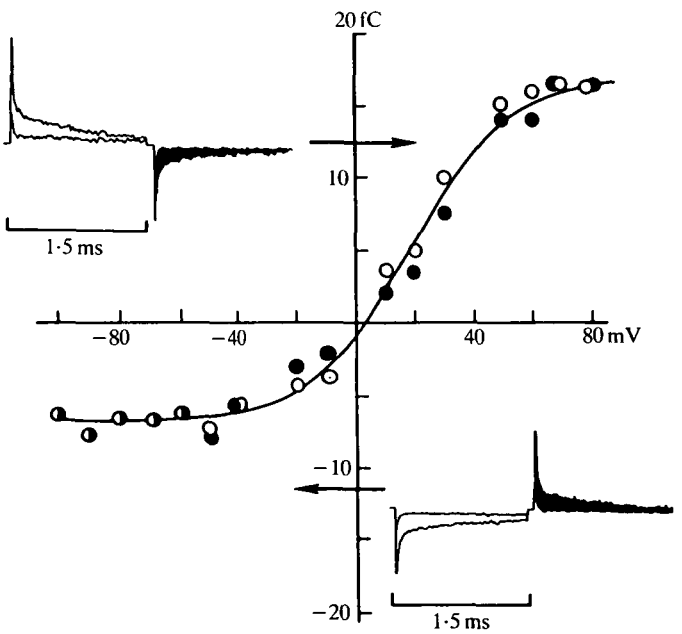

Fig. 3. Boltzmann's distribution of charge movement as a function of voltage difference pulses applied across a synexin-supplemented PtdS bilayer. The data are from Rojas & Pollard (1987).

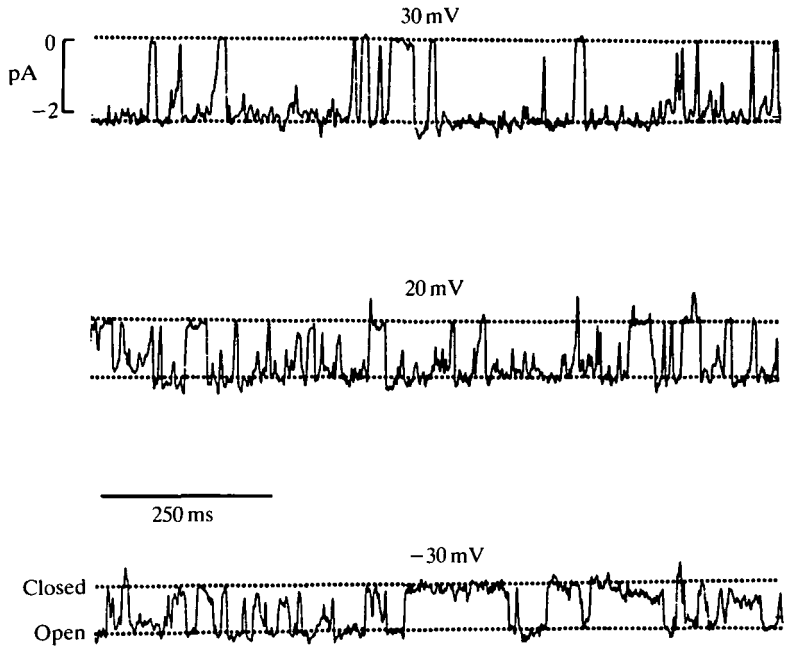

Fig. 4. Synexin calcium channel activity as a function of pipette potential (V_p). At positive values of V_p (+30 mV and +20 mV) channels are primarily open, although channels are less open at 20 mV than at 30 mV. At negative values of V_p (e.g. −30 mV) channels are primarily closed. The pipette contains 50 mmol l^{-1} Ca^{2+}, and the bath contains 1 μmol l^{-1} Ca^{2+}. Thus the chemical potential is large and constant in both cases, and the channels are clearly voltage-dependent. These data have not previously been published, but are from the data used in the preparation of the paper by Pollard & Rojas (1988).

changes, thus exposing hydrophobic residues to the aqueous environment. The consequence would be polymerization of the molecule, as in fact observed (Creutz *et al.* 1979), and *simultaneous insertion* of the synexin polymer into two adjacent target membranes. The capacitance and channel data support the concept that synexin can do this to a single membrane. The only additional suggestion here is that synexin can also do this to two membranes at once. Finally, fusion ensues when the hydrophobic bridge destabilizes both bilayers and provides a pathway for lipids on facing leaflets of both membranes to cross and mix.

Substantial evidence exists that membrane mixing indeed occurs either immediately before or simultaneously with synexin-driven membrane fusion. For example, self-quenching concentrations of octadecylrhodamine (R 18) have been used to label the outer leaflets of chromaffin granule ghosts. When synexin is added to such labelled ghosts in the presence of unlabelled ghosts fusion occurs, as measured by acquisition of a fluorescence signal (Stutzin, 1986). Separate evidence using the FITC–volume mixing assay verifies that the same ghosts are undergoing true fusion. True fusion, as defined by volume mixing, appears to occur slightly

more slowly than membrane mixing, at least as defined by the R 18 assay (see Fig. 7). The meaning of these slightly different rates must be considered carefully, since the reporter molecules are vastly different in molecular weight and are probably diffusing through media with vastly different viscosities.

The concept of a hydrophobic defect being the driving force for membrane fusion is a common proposal in many hypothetical mechanisms for this process (e.g. see Blumenthal, 1987, for a cogent summary). In addition, once this defect is in place several possible pathways have been proposed to achieve final fusion. The contribution of synexin is to provide at least one concrete biological example of a *bona fide* driving force and direction for the hydrophobic defect.

The remainder of the sequence in Fig. 6 (stages IV, V and VI) merely outlines one possible pathway that is consistent with our knowledge of synexin. We do not consider this pathway to be exclusive, since there is little compelling evidence for the proposed details. However, the concept that synexin might depolymerize or otherwise move about within the bilayer is certainly consistent with our capacitance data, as well as with time constants (of the order of milliseconds) determined for opening and closing of synexin channels.

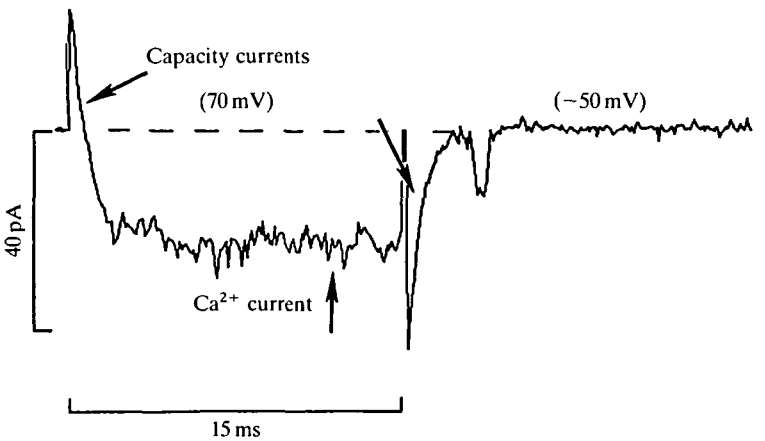

Fig. 5. Synexin calcium channel activity observed on inside-out patches of chromaffin cell plasma membranes. Chromaffin cells were cultured for 3 days, and electrically silent, cell-attached patches obtained. The cells were incubated in a modified buffer containing 250 mmol l^{-1} TEA-Cl, 1 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} Hepes-TEA, pH 6·8. After formation of a giga-ohm seal, the patch of membrane was excised and synexin was puffed onto the cytosolic aspect of the patch. Prior to the application of synexin a set of voltage-clamp pulses was applied and the current transients recorded. Calcium in the bath was then lowered to approx. 1 μmol l^{-1}, and the holding potential, V_m, adjusted to −50 mV. Capacity transients were then measured during voltage pulses up to +70 mV. Synexin-specific capacity transients were then obtained, and calcium-specific currents observed to ride upon the capacity current transient. In the figure these calcium currents are flowing down the concentration gradient out of the pipette, but are somewhat opposed by the electrical gradient which is negative in the pipette.

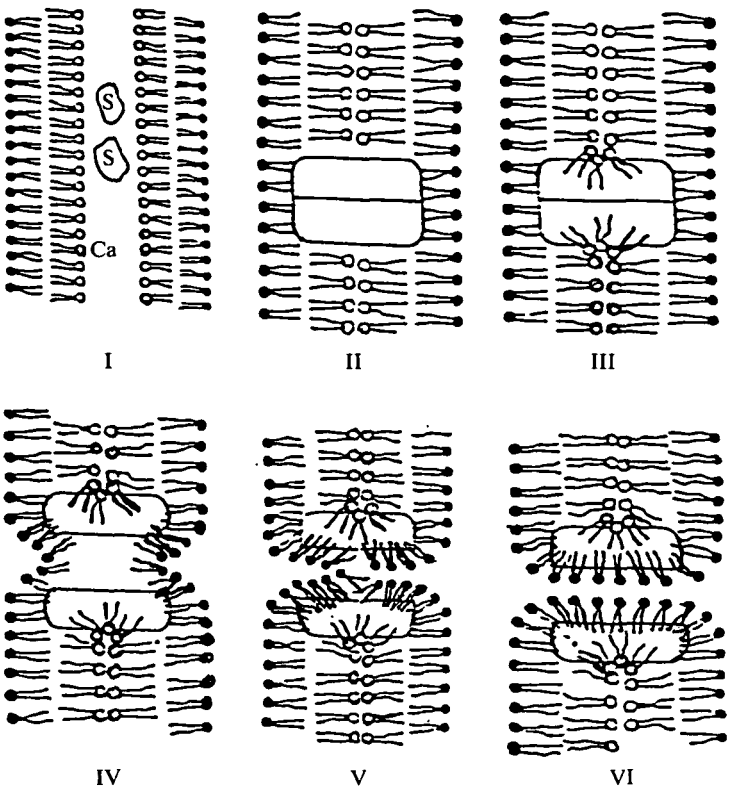

Fig. 6. Hydrophobic bridge hypothesis for synexin-driven membrane fusion. (I) Two membranes are poised to fuse. Open circles represent acidic phospholipids on the cytoplasmic (cis) leaflet of a membrane. Closed circles represent phospholipids (e.g. PtdC or sphingomyelin) on the opposing (trans) leaflet. Calcium causes synexin monomers (S) to polymerize. (II) Calcium-activated synexin polymer binds to the acidic phospholipids on both cis surfaces, penetrates both bilayers and crosslinks the membranes. (III) Phospholipids cross the hydrophobic bridge created by synexin, thereby allowing the cis leaflets of the fusing membranes to mix. (IV) Synexin polymer dissociates in the low dielectric environment, thereby providing a hydrophobic pathway for the trans leaflets to approach. (V) Trans leaflets complete their reorientation, leaving (VI) synexin in the bilayer of the newly fused membranes. Some or all of the synexin may leave the membrane, but we have little information as yet on this process. (This model is taken from our drawings in Pollard, Rojas, Burns & Parra, 1988, modified from those in Pollard, Rojas & Burns, 1987.)

Cloning and sequencing of human synexin

At the time we proposed the hydrophobic bridge hypothesis we were certain that we were being as faithful as possible to our knowledge regarding the structure and function of synexin. The limitations of our knowledge, however, were painfully obvious. Most importantly, we lacked information on the complete primary sequence of synexin, and thus did not know to what extent the structure might indeed express the profound hydrophobic character predicted by our model.

Indeed, capacitance changes and channel activity did not necessarily demand participation by the entire molecule, as implicit in the two-dimensional model. In fact, the observation that synexin has channel activity indicates that at least some parts of the molecule must be in virtual contact with the aqueous phase. However, structures involved with channel activity need not be coincident with structures involved in fusion activity.

For these reasons we have been devoting substantial efforts to solving the problem of the sequence of synexin, using the techniques of molecular biology and protein chemistry (Pollard *et al.* 1987). Until recently we had devoted all our efforts to bovine synexin. We prepared mRNA and derived cDNA libraries from bovine liver and adrenal medulla, and successfully prepared tryptic peptides from bovine liver synexin. We sequenced a number of these peptides and used the sequences to prepare oligonucleotide probes for detecting synexin-specific cDNAs in our various libraries. In addition, we searched bovine adrenal medulla and liver libraries prepared in lambda gt11, using monoclonal and polyclonal anti-synexin antibodies as probes.

However, over the course of this study it became clear that a complete bovine clone might remain elusive for some time. In fact, an apparent partial bovine synexin clone, identified by immunoreactivity using a monoclonal antibody, proved to be a false positive (Pollard *et al.* 1987). We therefore broadened our search for synexin cDNAs in other tissues and organisms. Using the goat anti-bovine synexin antibody (Creutz, Pazoles & Pollard, 1980) in a Western blot of human liver, we noted a single immunoreactive band very close to the molecular

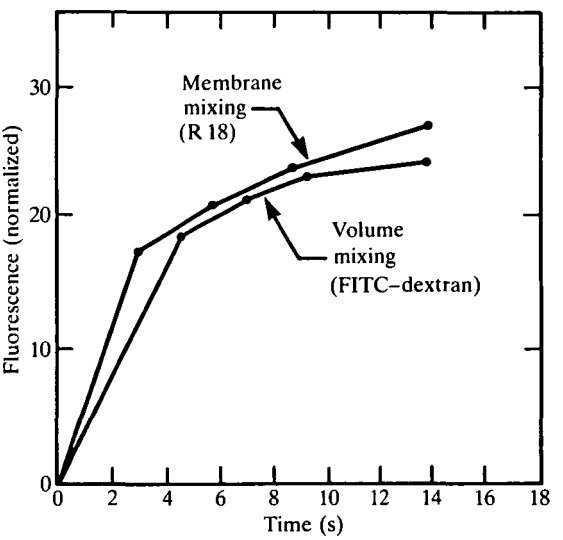

Fig. 7. Comparison of rates of synexin-driven chromaffin granule ghost fusion measured by membrane mixing (R 18) and volume mixing (FITC–dextran) methods. Composite data are from Stutzin (1986), calculated and redrawn by A. Stutzin (personal communication to HBP).

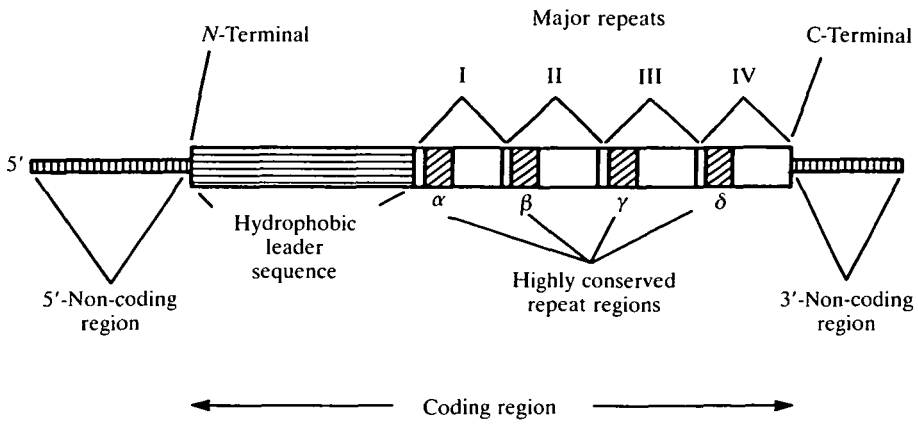

Fig. 8. Structure of the human synexin cDNA clone. Details are given in the text and are taken from Burns *et al.* (1988). The *N*-terminal represents a unique, hydrophobic sequence comprising about 30 % of the molecule. The remainder is homologous with a four-fold repeat observed in other members of the synexin gene family.

weight of bovine synexin. We therefore initiated a successful effort to purify synexin from human liver, and then purify and sequence tryptic peptides. From these peptides we prepared oligonucleotide probes, and successfully searched human lung, liver and retina libraries for synexin-specific clones (Burns *et al.* 1988).

Human synexin proved to be virtually identical to bovine synexin in terms of calcium-dependent chromaffin granule aggregation properties, as well as voltage-dependent calcium channel activity. Furthermore, derived amino acid sequences from a human synexin clone proved to have substantial homology with our known bovine sequences obtained by sequence analysis of tryptic fragments. Examples of these homologies are shown in Tables 1–4, and we will discuss these relationships further after describing the structure of our human synexin clone.

The general structure of our initial human synexin clone is shown in Fig. 8, and includes characteristic 5′- and 3′-non-coding regions. The 5′-non-coding region is relatively long, but is well within the range known for mammalian mRNAs. The coding region consists of a unique, highly hydrophobic *N*-terminal leader sequence comprising about 30 % of the entire molecule, followed by four repeating domains. We have labelled these I, II, III and IV, and have noted that although they are homologous they are by no means identical. However, within each of these repeating domains are characteristic core sequences with much higher homology. We have labelled these α, β, γ and δ, and have described them in detail in Tables 1–4. These repeats also have substantial regions of strong hydrophobicity, as well as regions of hydrophilicity.

We conclude from these data that the highly hydrophobic *N*-terminal segment might satisfy, at least in principle, the requirement for a highly hydrophobic structure able to provide the hydrophobic bridge between fusing membranes. Of

course, we cannot at present conclude that this hydrophobic *N*-terminal segment is or is not the bridge. In fact, the C-terminal segment containing the four-fold repeat could also be involved in the bridge or in the channel function (Burns *et al.* 1988).

A search of the protein data banks, and a timely personal communication from Dr Harry Haigler (University of California at Irvine) early in our analysis, revealed significant homology between human and bovine synexin and a unique set of calcium-dependent membrane binding proteins. These proteins include lipocortin I (Wallner *et al.* 1986), endonexin II (Schlaepfer, Mehlman, Burgess & Haigler, 1987; Kaplan *et al.* 1988), calpactin heavy chain/p36 (Glenney, 1986; Huang *et al.* 1986; Kristensen *et al.* 1986; Saris *et al.* 1986), protein II (Weber *et al.* 1987) and calelectrin 67K (Südhof *et al.* 1988). Data in Tables 1–4 summarize the homologies in the four 16-amino-acid core repeats. The key to the similarity is a characteristic GXGTDE sequence, found with varying degrees of fidelity in the different core repeats. This common repeat was first noted by Geisow *et al.* (1987). Clearly evident from these comparisons is the fact, borne out in our complete sequence, that bovine and human synexin share many common sequences. For these specific examples, the homology was α (15 of 16), γ (14 of 14) and δ (11 of 11) in the three of four sequences available for comparison.

The similarities between human and bovine synexin indicated to us that synexin varies less *across* species lines than it varies with regard to the other members of the synexin gene family *within* species lines. In fact, within the four highly conserved core repeats the other proteins did not give homologies of more than about 50%, except in the case of the β segment. The β segment, however, is

Table 1. *Highly conserved α-core segment in repeat I of human synexin: comparison with other members of the synexin gene family*

Protein	Sequences	Similarity to human synexin
Human synexin	G F G T D E Q A I V D V V A N R	
Bovine synexin*	G F G T D E Q A I I D V V A N R	15/16
Human endonexin II (N31-46)	G L G T D E E S I L T L L T S R	6/16
Human calpactin I (N48-63)	T K G V D E V T I V N I L T N R	7/16
Human lipocortin I (N58-73)	V K G V D E A T I I D I L T K R	7/16
Porcine protein II (N28-43)	G L G T D E D A I I S V L A Y R	9/16
Bovine calelectrin (67K) no. 1 (N24-39)	G F G S D K E A I L D I I T S R	8/16
Bovine calelectrin (67K) no. 5 (N378-393)	G L G T D E D T I I D I I T H R	8/16

* Sequence derived from peptide isolated from tryptic digest of bovine liver synexin.

substantially different from the others in the C-terminal half of the sequence, where a characteristic LIEIL sequence is found. All the proteins share part or all of this sequence to varying degrees.

The most common characteristic of the core repeats in the synexin gene family is the fact that peripheral portions of the 16-amino-acid sequence are quite

Table 2. *Highly conserved β-core segment in repeat II of human synexin: comparison with other members of the synexin gene family*

Protein	Sequences	Similarity to human synexin
Human synexin	G A G T Q E R V L I E I L C T R	—
Human endonexin II (N102-117)	G A G T N E K V L T E I I A S R	10/16
Human calpactin I (N121-136)	G L G T D E D S L I E I I C S R	10/16
Human lipocortin I (N130-145)	G L G T D E D T L I E I L A S R	10/16
Porcine protein II (N101-116)	G A G T D E G C L I E I L A S R	12/16
Bovine calelectrin (67K) no. 2 (N120-135)	G I G T D E K C L I E I L A S R	10/16
Bovine calelectrin (67K) no. 6 (N450-465)	G A G T D E K A L I E I L A T R	12/16

Table 3. *Highly conserved γ-core segment in repeat III of human synexin: comparison with other members of the synexin gene family*

Protein	Sequences	Similarity to human synexin
Human synexin	R L G T D E S C F N M I L A T R	
Bovine synexin*	– L G T D E S X F N M I L A T R	14/14
Human endonexin II (N187-202)	K W G T D E E K F I T I F G T R	9/16
Human calpactin I (N206-221)	R K G T D V P K W I S I M T E R	7/16
Human lipocortin I (N213-228)	R K G T D V N V F N T I L T T R	10/16
Porcine protein II (N185-200)	K W G T D E V K F L T V L C S R	7/16
Bovine calelectrin (67K) no. 3 (N191-206)	K W G T D E A Q F I Y I L G N R	8/16
Bovine calelectrin (67K) no. 7 (N539-554)	D K T S L E T R F M T I L C T R	6/16

* Sequence derived from peptide isolated from tryptic digest of bovine liver synexin.

Table 4. *Highly conserved δ-core segment in repeat IV of human synexin: comparison with other members of the synexin gene family*

Protein	Sequences	Similarity to human synexin
Human synexin	G A G T D D S T L V R I V V T R	
Bovine synexin*	G A G T D D S T L V R	11/11
Human endonexin II (N262-277)	G A G T D D H T L I R V M V S R	8/16
Human calpactin I (N281-296)	G K G T R D K V L I R I M V S R	8/16
Human lipocortin I (N288-303)	G V G T R H K A L I R I M V S R	6/16
Porcine protein II (N259-274)	G L G T D D N T L I R V M V S R	8/16
Bovine calelectrin (67K) no. 4 (N267-282)	G L G T R D N T L I R I M V S R	8/16
Bovine calelectrin (67K) no. 8 (N614-629)	G A G T D E K T L T R I M V S R	8/16

* Sequence derived from peptide isolated from tryptic digest of bovine liver synexin.

hydrophobic, whereas carboxylic amino acids occur at internal sites. In synexin, three negatively charged amino acids occur in the α core and two occur in the β, γ and δ cores. More characteristic is the DE or DD sequence in positions 5 and 6 from the *N*-terminal end of the core repeats. These characteristics are shared to a large extent by the other members of the synexin gene family, except in the case of the lipocortin I δ repeat.

This characteristic location of negative charges in a hydrophobic nest has been noted previously by Taylor & Geisow (1987) in an analysis of some of the previously known members of the synexin gene family. In that case, the repeats were interpreted in terms of their being calcium-binding sites, possibly analogous to the E–F bands in the parvalbumin/calmodulin gene family. Although possibly relevant to some members of the synexin gene family, the generalization of this concept must be viewed with caution. For example, as previously noted the lipocortin I γ repeat uniquely possesses cationic rather than anionic sequences. Thus, these regions cannot be symmetrical, homologous calcium-binding sites. Furthermore, in the specific case of synexin, calcium interaction sites cannot have too great an affinity for calcium, or the protein would be a calcium-binding rather than a calcium channel protein.

However, there exists one possible interpretation of this concept with interesting structural consequences. If these core sequences did indeed bind or transmit calcium in or through the synexin molecule, then these regions would have to span the membrane, possibly protected from the low dielectric medium by hydrophobic

domains in neighbouring parts of the synexin molecule. A further consequence of this structural interpretation is that far more of the synexin molecule than the *N*-terminal leader sequence might be available to provide hydrophobic surfaces to drive membrane fusion processes. Answers to these questions will obviously await ongoing biophysical and genetic analysis of the synexin molecule.

The synexins and exocytosis

Synexin initially attracted our attention because it provided a mechanism for calcium-dependent membrane contact and fusion processes that we knew to occur during exocytosis (Pollard *et al.* 1979). For some years after its discovery, synexin was the only protein with the appropriate properties, and for this reason held our unwavering attention. We have now been rewarded by a quite detailed biophysical picture of how synexin interacts with membranes, and have had the opportunity to apply this knowledge to formulate the hydrophobic bridge hypothesis.

More recently, other proteins have been discovered which share with synexin a calcium-dependent affinity for acidic phospholipids, and in the cases of calpactin polymer ($p36_2$,$p10_2$, Drust & Creutz, 1988) and calelectrin 67K (Südhof *et al.* 1984) the ability to aggregate chromaffin granules. We might, therefore, have reasonably anticipated that some or all of these proteins might share other properties of synexin. But with the successful cloning and sequencing of human synexin we now appreciate that all these proteins are members of a common gene family, and that some common properties might be expected.

It follows that if any of these other synexin-related proteins, or other yet to be discovered members of the synexin gene family, also proves to have membrane fusion properties, we might reasonably expect it (them) to do so by mechanisms similar to those described above for synexin itself. In previous discussions we have only considered the synexin molecule for possible involvement in exocytosis. However, we may eventually have to consider some or all of the synexin gene family members as a class in this process. Time will tell.

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References

Armstrong, C. M. & Bezanilla, F. (1976). Properties of the sodium channel gating current. *Cold Spring Harb. Symp. quant. Biol.* **XL**, 297–304.

Blumenthal, R. (1987). Membrane fusion. *Current Topics Membr. Transp.* **29**, 203–254.

Burns, A. L., Magenzo, K., Srivastana, M., Cheung, B., Seaton-Johnson, D., Shirvan, A., Alijani, M. R., Rojas, E. & Pollard, H. B. (1988). Purification of human synexin calcium

channel protein and structure of the human synexin gene. *Proc. natn. Acad. Sci. U.S.A.* (in press).

CREUTZ, C. E. (1981). Cis-Unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. *J. cell Biol.* **91**, 247–256.

CREUTZ, C. E., PAZOLES, C. J. & POLLARD, H. B. (1978). Identification and purification of an adrenal medullary protein (synexin) that causes calcium dependent aggregation of isolated chromaffin granules. *Biol. Chem.* **253**, 2858–2866.

CREUTZ, C. E., PAZOLES, C. J. & POLLARD, H. B. (1979). Self-association of synexin in the presence of calcium: Correlation with synexin-induced membrane fusion and examination of the structure of synexin aggregates. *J. biol. Chem.* **254**, 553–558.

CREUTZ, C. E., PAZOLES, C. J. & POLLARD, H. B. (1980). Immunohistochemical and biochemical studies of synexins of diverse tissues. In *Calcium Binding Proteins and Calcium Function* (ed. F. L. Siegel, E. Casafoti, R. H. Kretsinger, D. H. MacLennon & R. H. Wasserman), pp. 79–80. New York: Elsevier North Holland.

CREUTZ, C. E., PAZOLES, C. J. & POLLARD, H. B. (1982). Further characterization of the aggregation and fusion of chromaffin granules by synexin as a model for compound exocytosis. *J. cell. Biochem.* **18**, 87–97.

CREUTZ, C. E. & POLLARD, H. B. (1982*a*). Development of a cell-free model for compound exocytosis using components of the chromaffin cell. *J. autonom. nerv. Syst.* **7**, 13–18.

CREUTZ, C. E. & POLLARD, H. B. (1982*b*). A model for protein–lipid interactions during exocytosis: Aggregation and fusion of chromaffin granules in the presence of synexin and cis-unsaturated fatty acids. *Biophys. J.* **37**, 119–120.

CREUTZ, C. E. & STERNER, D. C. (1983). Calcium dependence of the binding of synexin to isolated chromaffin granules. *Bioch. biophys. Res. Commun.* **114**, 355–364.

DRUST, D. S. & CREUTZ, C. E. (1988). Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. *Nature, Lond.* **331**, 88–91.

FRYE, R. A. & HOLZ, R. W. (1984). The relationship between arachidonic acid release and catecholamine secretion from cultured bovine adrenal chromaffin cells. *J. Neurochem.* **43**, 146–150.

GEISOW, M. J., FRITSCHE, U., HEXAM, J. M., DASH, B. & JOHNSON, T. (1987). A consensus amino acid sequence repeat in *Torpedo* and mammalian Ca-dependent membrane binding protein. *Nature, Lond.* **320**, 636–638.

GLENNEY, J. R. (1986). Two related but distinct forms of the M_r 36,000 tyrosine kinase substrate (calpactin) that interact with phospholipid and actin in a Ca^{2+} dependent manner. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4258–4262.

HONG, K., DUZGUNES, N. & PAPAHADJOPOULOS, D. (1981). Role of synexin in membrane fusion. Enhancement of calcium-dependent fusion of phospholipid vesicles. *J. biol. Chem.* **256**, 3641–3644.

HONG, K., DUZGUNES, N., EKERT, R. & PAPAHADJOPOULOS, D. (1982*a*). Synexin facilitates fusion of specific phospholipid vesicles at divalent cation concentrations found intracellularly. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4642–4644.

HONG, K., DUZGUNES, N. & PAPAHADJOPOULOS, D. (1982*b*). Modulation of membrane fusion by calcium-binding proteins. *Biophys. J.* **37**, 297–306.

HONG, K., EKERDT, R., BENTZ, J., NIR, S. & PAPAHADJOPOULOS, D. (1983). Kinetics of synexin-facilitated membrane fusion. *Biophys. J.* **41**, 31a.

HOTCHKISS, A., POLLARD, H. B., SCOTT, J. & AXELROD, J. (1981). Release of arachidonic acid from adrenal chromaffin cell cultures during secretion of epinephrine. *Fedn Proc. Fedn Am. Socs exp. Biol.* **40**, 256.

HUANG, K.-S., WALLNER, B. P., MATTALIANO, R. J., TIZARD, R., BURNE, C., FREY, A., HESSION, C., MCGRAY, P., SINCLAIR, L. K., CHOW, E. P., BROWNING, J. L., RAMACHANDRAN, K. L., TANG, J., SMART, J. E. & PEPINSKY, R. B. (1986). Two human 35 Kd inhibitors of phospholipase A_2 are related to substrates for $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. *Cell* **46**, 191–199.

KAPLAN, R., JAYE, M., BURGESS, W. H., SCHLAEPFER, D. D. & HAIGLER, H. T. (1988). Cloning and expression of cDNA for endonexin II, a Ca^{2+} and phopholipid binding protein. *J. biol. Chem.* (in press).

KRISTENSEN, T., SARIS, C. J. M., HUNTER, T., HICKS, L. J., NOONAN, D. J., GLENNEY, J. R., JR

& TACK, B. F. (1986). Primary structure of bovine calpactin I heavy chain (p36), a major cellular substrate for retroviral protein-tyrosine kinases: Homology with the human phospholipase A_2 inhibitor lipocortin. *Biochemistry* **25**, 4497–4503.

MILLER, R. J. (1987). Multiple calcium channels and normal function. *Science* **235**, 46–52.

MORRIS, S. J., HUGHES, J. M. X. & WHITTAKER, V. P. (1982). Purification and mode of action of synexin: A protein enhancing calcium induced membrane aggregation. *J. Neurochem.* **39**, 529–536.

NIR, S., STUTZIN, A. & POLLARD, H. B. (1987). Effect of synexin on aggregation and fusion of chromaffin granule ghosts at pH 6. *Biochim. biophys. Acta* **903**, 309–318.

ORNBERG, R. L., DUONG, L. T. & POLLARD, H. B. (1986). Intergranular vesicles: New organelles in the secretory granules of adrenal chromaffin cells. *Cell Tissue Res.* **245**, 547–553.

PALADE, G. (1975). Intracellular aspects of the process of protein synthesis. *Science* **189**, 347–358.

POLLARD, H. B., CREUTZ, C. E., FOWLER, V. M., SCOTT, J. H. & PAZOLES, C. J. (1982). Calcium-dependent regulation of chromaffin granule movement, membrane contact, and fusion during exocytosis. *Cold Spring Harb. Symp. quant. Biol.* **46**, 819–834.

POLLARD, H. B., CREUTZ, C. E. & PAZOLES, C. J. (1981). Mechanisms of calcium action and hormone release during exocytosis. In *Recent Progress in Hormone Research*, vol. 37 (ed. R. O. Greep), pp. 229–332. New York: Academic Press.

POLLARD, H. B., ORNBERG, R., LEVINE, M., HELDMAN, E., MORITA, K., KELNER, K., LELKES, P., BROCKLEHURST, K., FORSBERG, E., DUONG, L., LEVINE, R. & YOUDIM, M. B. H. (1985). Hormone packaging and secretion by exocytosis: A view from the chromaffin cell. In *Vitamins and Hormones*, vol. 42 (ed. G. Aurbach), pp. 109–196. Academic Press.

POLLARD, H. B., ORNBERG, R., LEVINE, M., HELDMAN, E., MORITA, K., LELKES, P. & HELDMAN, J. (1984). Ultrastructural and biochemical aspects of biosynthesis and secretion of hormones. In *Endocrinology* (ed. F. Labrie & L. Pronix), pp. 383–386. Amsterdam: Elsevier.

POLLARD, H. B., PAZOLES, C. J., CREUTZ, C. E. & ZINDER, O. (1979). The chromaffin granule and possible mechanisms of exocytosis. *Int. Rev. Cytol.* **58**, 159–197.

POLLARD, H. B. & ROJAS, E. (1988). Calcium-activated synexin forms highly selective, voltage gated calcium channels in phosphatidylserine bilayer membranes. *Proc. natn. Acad. Sci. U.S.A.* (in press).

POLLARD, H. B., ROJAS, E. & BURNS, A. L. (1987). Synexin and chromaffin granule membrane fusion: A novel 'hydrophobic bridge' hypothesis for driving and directing the fusion process. *Ann. N.Y. Acad. Sci.* **493**, 524–551.

POLLARD, H. B., ROJAS, E., BURNS, A. L. & PARRA, C. (1988). Synexin, calcium and the hydrophobic bridge hypothesis for membrane fusion. In *Molecular Mechanisms of Membrane Fusion* (ed. S. Ohki, D. Doyle, T. Flanagan, S. W. Hui & E. Mayhew). New York: Plenum Press (in press).

POLLARD, H. B., SCOTT, J. H. & CREUTZ, C. E. (1983). Inhibition of synexin activity and exocytosis from chromaffin cells by phenothiazine drugs. *Biochem. biophys. Res. Commun.* **113**, 908–915.

ROJAS, E. (1976). Gating mechanism for activation of the sodium conductance in nerve membranes. *Cold Spring Harb. Symp. quant. Biol.* **XL**, 305–320.

ROJAS, E. & POLLARD, H. B. (1987). Membrane capacity measurements suggest a calcium-dependent insertion of synexin into phosphatidylserine bilayers. *FEBS Letts* **217**, 25–31.

SARIS, C. J. M., TACK, B. F., KRISTENSEN, T., GLENNEY, J. R., JR & HUNTER, T. (1986). The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multi-domain protein with internal repeats. *Cell* **46**, 201–212.

SCHLAEPFER, D. D., MEHLMAN, T., BURGESS, W. H. & HAIGLER, H. T. (1987). Structural and functional characterization of endonexin II, a calcium- and phospholipid-binding protein. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6078–6082.

SCOTT, J. H., CREUTZ, C. E., POLLARD, H. B. & ORNBERG, R. O. (1985). Synexin binds in a calcium dependent fashion to oriented chromaffin cell plasma membranes. *FEBS Letts* **180**, 17–23.

SIMON, S. M. & LLINAS, R. R. (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* **48**, 485–498.

STUTZIN, A. (1986). A fluorescence assay for monitoring and analyzing fusion of biological membrane vesicles *in vitro*. *FEBS Letts* **197**, 274–280.

STUTZIN, A., CABANTCHIK, I., LELKES, P. I. & POLLARD, H. B. (1987). Synexin mediated fusion of bovine chromaffin granule ghosts: Mechanism of pH dependence. *Biochim. biophys. Acta* **905**, 205–212.

SÜDHOF, T. C., EBBECKE, M., WALKER, J. H., FRITSCHE, U. & BOUSTEAD, C. (1984). Isolation of mammalian calelectrins: A new class of ubiquitous Ca^{2+}-regulated proteins. *Biochemistry* **23**, 1103–1109.

SÜDHOF, T. C., SLAUGHTER, C. A., LEZNICKI, I., BARJON, P. & REYNOLDS, G. A. (1988). Human 67-KDa calelectrin contains a duplication of four repeats found in 35-KDa lipocortins. *Proc. natn. Acad. Sci. U.S.A.* **85**, 664–668.

SUSSMAN, K. E., POLLARD, H. B., LEITNER, J. W., NESHER, R., ADLER, J. & CERASI, E. (1983). Differential control of insulin secretion and somatostatin receptor recruitment in isolated islets. *Biochem. J.* **214**, 225–230.

TAYLOR, W. R. & GEISOW, M. J. (1987). Predicted structure for the calcium-dependent membrane-binding proteins p35, p36, and p32. *Protein Engineering* **1**, 183–187.

WALLNER, B. P., MATTALIANO, R. J., HESSION, C., CATE, R. L., TIZARD, R., SINCLAIR, L. K., FOELLER, C., CHOW, E. P., BROWNING, J. L., RAMACHANDRAU, K. L. & PEPINSKY, R. B. (1986). Cloning and expression of human lipocortia, a phospholipase A^2 inhibitor with potential anti-inflammatory activity. *Nature, Lond.* **320**, 77–81.

WEBER, K., JOHNSSON, N., PLESSMANN, U., VAN, P. N., SOLING, H.-D., AMPE, C. & VANDEKERCKHOVE, J. (1987). The amino acid sequence of protein II and its phosphorylation site for protein kinase C; the domain structure (of) Ca^{2+}-modulated lipid binding proteins. *EMBO J.* **6**, 1599–1604.