Distinct Cellular Expression Pattern of Annexins in *Hydra vulgaris*

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Abstract. The annexins are a structurally related family of Ca^{2+} and phospholipid binding proteins whose function has not been clearly defined. Further investigations of annexin function may be enhanced by studying simpler organisms that express fewer annexin gene products.

We previously characterized annexin XII from the freshwater cnidarian *Hydra vulgaris* (Schlaepfer, D. D., D. A. Fisher, M. E. Brandt, H. R. Bode, J. Jones, and H. T. Haigler. 1992. *J. Biol. Chem.* 267:9529–9539). In this report, we detected one other hydra annexin (40 kD) by screening hydra cell extracts with antibodies raised against peptides from highly conserved regions of known annexins. The 40-kD protein was expressed at <1% of annexin XII levels. These biochemical studies indicate that hydra contain a very limited number of annexin gene products.

The cellular hydra annexin distribution was analyzed by indirect immunofluorescence. Using affinity-purified antibodies to annexin XII, the epithelial battery cells were stained throughout the tentacle. A lower level of annexin XII staining was detected in peduncle region epithelial cells. No other cell types showed detectable annexin XII staining. The anti-peptide antibody that specifically detected the 40-kD hydra annexin, maximally stained the cytoplasm of nematocytes. The immunofluorescent results showed that annexin XII and the 40-kD annexin were not co-expressed in the same cells. Since the hydra annexins localized to specific subsets of the total hydra cell types, it is likely that these proteins perform specialized biological roles, and not general "housekeeping" functions which are part of the essential molecular machinery of all cells.

ANNEXINS are a structurally related family of proteins that bind in a reversible Ca^{2+}-dependent manner to plasma membrane phospholipids (for reviews see Geisow et al., 1987; Crompton et al., 1988*a*; Crumpton and Dedman, 1990). Most mammalian annexins are abundant intracellular proteins and can compose up to 1% of the total cell protein (Schlaepfer and Haigler, 1990). Currently, sequence analyses have established the existence of nine distinct vertebrate annexins (Huang et al., 1986; Wallner et al., 1986; Weber et al., 1987; Crompton et al., 1988*b*; Kaplan et al., 1988; Pepinski et al., 1988; Burns et al., 1989; Hauptmann et al., 1989; Towle and Treadwell, 1992), two distinct annexins in *Drosphila melanogaster* (Johnston et al., 1990), and one distinct annexin in *Hydra vulgaris* (Schlaepfer et al., 1992*b*). In addition, homologues to annexins originally characterized in vertebrates have been found in lower organisms such as the slime mold *Dictyostelium discoideum* (Döring et al., 1991; Gerke, 1991; Greenwood and Tsang, 1991) and in the sponge *Geodia cydonium* (Robitzki et al., 1990). Experiments performed to date have not detected annexin expression in yeast or bacteria.

Presently, the most pressing issue in annexin research is to obtain a clear understanding of the annexin function(s) within cells. The exact biological role is not known for any of the annexins, but proposed functions under investigation include: the regulation of membrane traffic and exocytosis (Drust and Creutz, 1988; Ali et al., 1989; Nakata et al., 1990; Sarafian et al., 1991; Wu and Wagner, 1991), the regulation and inhibition of protein kinase C activity (Schlaepfer et al., 1992*a*), the mediation of cytoskeletal-membrane interactions (Gerke and Weber, 1984; Glenny et al., 1987; Powell and Glenny, 1987; Ikebuchi and Waisman, 1990), the mediation of mitogenic signal transduction (De et al., 1986; Pepinski and Sinclair, 1986; Haigler et al., 1987), and the formation of ion-specific channels (Pollard & Rojas, 1988; Burns et al., 1989; Rojas et al., 1990).

Preliminary indications that individual annexins have specialized physiological roles come from observations that different annexins have unique tissue and cell distributions (Gould et al., 1984; Pepinski et al., 1988; Kaetzel et al., 1989). Within a given individual cell type, the expression level of certain annexins has been shown to undergo large changes in response to cell proliferation (Schlaepfer and Haigler, 1990) or in response to the processes of cell differentiation (William et al., 1988; Horseman, 1989; Isacke et al., 1989; Schlaepfer and Haigler, 1990). Although

insights into protein function can sometimes be deduced from specific expression patterns, all vertebrate cells examined to date express more than one annexin. Thus, sorting functional insights out of patterns of annexin expression might be difficult because individual annexins might have separate, overlapping, complementary, or antagonistic cellular roles. In addition, gene disruption experiments as a way to elucidate annexin function may be complicated or hindered by the presence of two or more annexins within each cell.

Because of these annexin expression pattern complexities in vertebrate systems, further investigations of annexin function might be facilitated by studying a more simple organism with fewer annexin gene products. Since all known annexins exhibit the property of reversible Ca^{2+}-dependent phospholipid binding, cell extracts can be efficiently screened for putative annexin proteins that exhibit this activity. Although, no annexin expression was detected in *Escherichia coli* and *Saccharomyces cerevisiae* extracts, two or more different annexins were detected in *Xenopus laevis*, *D. melanogaster*, or *D. discoideum* extracts as Coomassie blue-stained proteins that reversibly bound to phospholipid vesicles (Johnston et al., 1990; Gerke et al., 1991; D. D. Schlaepfer and H. T. Haigler, unpublished results). When *H. vulgaris* cell extracts were screened by these methods, only a single Coomassie blue-stained annexin protein was detected (Schlaepfer et al., 1992*b*). We chose to pursue the characterization of this annexin in *H. vulgaris*, annexin XII, to establish the foundation of using this animal as a simple model system in which to investigate annexin function.

A hydra has a simple body plan. It is essentially a tube with a head at one end and a foot at the other. The head consists of the hypostome, a domelike mouth structure surrounded by a ring of tentacles. The foot is a disc of cells that secrete a sticky substance which anchors the animal to surfaces. The body consists of a gastric cavity that is surrounded by two concentric epithelia, the ectoderm and endoderm which are separated by a basement membrane, the mesoglea. Specialized stinging (nematocyte), nerve, gland and their respective interstitial cell precursors are interspersed among the epithelial cells of the animal.

An adult animal is ~5-mm long consisting of ~100,000 cells distributed among 15 different cell types. The regional distribution, cell lineages, and cell cycle characteristics of the different cell types are well defined (David and Campbell, 1972; Bode et al., 1973; Campbell and David, 1974; David and Gierer, 1974; Bode et al., 1987, 1990). Additionally, many of the hydra cell types have distinct morphological characteristics and can be readily identified within a single cell macerate suspension or within the intact animal (David, 1973; Campbell and Bode, 1983). Further, the animal is amenable to a variety of cellular experimental manipulations which can lead to alterations in the cell composition of the animal (Diehl and Burnett, 1964; Campbell, 1976; Sacks and Davis, 1979; and Wanek et al., 1980).

Previously, we identified and characterized a novel annexin in *H. vulgaris*, annexin XII, which was expressed at 0.3% of total hydra protein (Schlaepfer et al., 1992*b*). The expression level of annexin XII was similar to that of other annexins in vertebrate systems. In this report, we complete a thorough screening of hydra extracts for the presence of other annexin proteins. With antibodies made to conserved annexin peptide sequences, we detected a second putative hydra annexin (40 kD) that was expressed at <1% of annexin XII levels. Indirect immunofluorescent results showed that annexin XII and the 40-kD annexin were selectively and separately expressed within distinct cell types of the epithelial and interstitial lineages, respectively.

Materials and Methods

Materials

H. vulgaris stock cultures were maintained as described (Lenhoff and Brown, 1970). *Hydra oligactis* and *Hydra magnipapillata* animals were kindly provided by Dr. Lynne Littlefield (University of California, Irvine, CA). Hydroxyurea treatment of *H. vulgaris* to create "epithelial" animals was performed as described (Littlefield, 1985). *H. oligactis* "stingless" mutants were generated by a sexual cross (Dr. Lynne Littlefield, personal communication).

Affinity purified polyclonal antibodies made to the annexin-specific sequence MKGAGTDEDVLIEILASRT, termed consensus peptide to annexin repeat 2 (CP2) or to the annexin-specific sequence RVMVSRSEIDLLDIR, termed consensus peptide to annexin repeat 4 (CP4) were kindly provided by Dr. Volker Gerke (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). The characterization of the affinity-purified CP2 antibodies has been documented elsewhere (Gerke, 1989). Affinity purified polyclonal antibodies made to the conserved annexin sequence (KAMKGLGTDE) were kindly provided by Dr. John Dedman (University of Texas Medical School, Houston, TX). As described and fully characterized, polyclonal antibodies were generated to purified human annexins I, II, IV, and V (Haigler et al., 1987; Kaplan et al., 1988).

The expression and purification of recombinant annexin XII in *E. coli* was performed as described (Schlaepfer et al., 1992*b*). The concentration of recombinant annexin XII was determined from A_{280} using a calculated extinction coefficient of 12,200 M^{-1}. Protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO) and molecular weight standards for polyacrylamide gel standardization were from Bio Rad (Richmond, CA).

Annexin XII Antibody Preparation

Polyclonal antibodies to annexin XII were raised in a rabbit by five injections of annexin XII purified from hydra as described (Schlaepfer et al., 1992b). Annexin XII-specific antibodies were affinity purified from rabbit serum (10 ml) using recombinant annexin XII attached to a solid support (Affi-Gel 10; Bio Rad). Annexin XII-specific IgGs were eluted from the recombinant annexin XII affinity column (0.2 M acetic acid, pH 2.7, with 0.5 M NaCl), immediately neutralized with 1 M Tris-HCl, pH 9.5, and stabilized with BSA addition (100 μg/ml). The annexin XII affinity-purified antibody fraction was dialyzed against PBS, concentrated by ultra-filtration to the original serum volume (10 ml), and was stored frozen at −70°C. By immunoblot analysis, the affinity-purified annexin XII antibodies could detect <25 ng of annexin XII and did not cross-react to any other hydra protein.

Preparation of a Hydra vulgaris Membrane Extract

An extract, enriched for the presence of annexin proteins, was prepared by using the reversible Ca^{2+}-dependent phospholipid binding properties of endogenous annexin proteins. Approximately 6,000 animals were Dounce homogenized with a motorized pestle in buffer H (20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM benzamidine, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) in the presence of 1 mM EGTA at 4°C. The homogenate was centrifuged at 1,000 *g*, the pellet containing nuclei and non-disrupted cells was discarded, and $CaCl_2$ was added to the supernatant to a final concentration of 2 mM. After 15 min, the homogenate was centrifuged (100,000 *g*, 30 min), the supernatant was discarded and the cellular membrane pellet was washed by resuspension with a Dounce homogenizer and motorized pestle in buffer H with 0.5 mM $CaCl_2$. After centrifugation (100,000 *g*, 30 min), proteins were eluted from the hydra membrane pellet by resuspension in buffer H containing 2 mM EGTA followed by centrifugation (150,000 *g*, 20 min.). The proteins that were extracted from the membranes with EGTA were concentrated by ultra filtration (YM-10; Amicon Corp., Arlington Heights, IL), and dialyzed against buffer B (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 20 mM $MgCl_2$, 2 mM

NaN_3). Hydra annexin XII was purified from this extract as previously described (Schlaepfer et al., 1992*b*). Total protein in the hydra extract was determined with the BCA Protein Assay micro protocol (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

Reversible Phospholipid Binding of Annexins Isolated from the Hydra Membrane Extract

Large thin-walled vesicles, formed from a 2:1 mixture of phosphatidylserine (bovine brain, #840032; Avanti Polar Lipids, Birmingham, AL) and phosphatidylcholine (egg yolk, #P7524; Sigma Chemical Co.) in the presence of 240 mM sucrose as described (Reeves and Dowben, 1969), were used to determine the lipid binding properties of the hydra annexins. The vesicles were suspended in buffer B and harvested by centrifugation (15 min, 20,000 *g*). For annexin binding, 1 mg of vesicles were added to 100 μg of the hydra membrane extract in buffer B containing 1 mM $CaCl_2$. After a 10 min incubation at room temperature, the solutions were subjected to centrifugation (10 min, 30,000 *g*) to separate the vesicles (pellet) from the soluble protein (supernatant). The vesicles and associated protein were washed by resuspension of the vesicles in buffer B containing 0.25 mM $CaCl_2$ followed by centrifugation (10 min, 30,000 *g*). Reversible annexin binding to the vesicles was assayed by washing the vesicle pellet isolated in the presence of Ca^{2+} (0.25 mM) in buffer B containing EGTA (1 mM). The resuspended vesicles in EGTA were subjected to centrifugation (10 min, 30,000 *g*) and 80% of endogenous annexin XII was solubilized into the supernatant fraction.

SDS Gel Electrophoresis and Western Immunoblot Analysis

Hydra cell protein aliquots were subjected to SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to Immobilon PVDF membranes (Millipore, Bedford, MA) as described (Towbin et al., 1979). After staining with Coomassie Blue, the Immobilon membranes were incubated for 2 h at 37°C in 2% powdered milk (wt/vol) in a TBS solution (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.2% NaN_3) to saturate the Immobilon membrane binding capacity. The membranes were washed in TBS (5 min) and then exposed to specific affinity-purified rabbit polyclonal antiserum (1:100 dilution) in a 0.25% gelatin-TBS solution for 2 h at 23°C. The membranes were sequentially washed with TBS, TBS containing 0.05% NP-40 (twice), and then TBS again. After washing, the membranes were exposed to ^{125}I-labeled protein A (37.8 μCi/μg) (ICN, Irvine, CA) at 5×10^5 cpm/ml in 0.25% gelatin-TBS solution for 1 h at 23°C. The membranes were washed with TBS and TBS containing NP-40 as before, dried, and autoradiography was performed with XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −70°C.

Whole Animal Immunocytochemistry

The distribution of annexin XII or the other hydra annexins in intact animals was visualized with affinity-purified rabbit antiserum to annexin XII, or the CP2 peptide, using a whole-mount technique as described (Koizumi and Bode, 1986) with minor modifications. Live animals were relaxed in 2% urethane (wt/vol) in hydra medium (1 mM $NaHCO_3$, 1 mM $CaCl_2$, and 250 nM $MgCl_2$) at 22°C for 2 min and then fixed for 16 h at 4°C (2% paraformaldehyde [wt/vol], 0.2% picric acid [wt/vol], and 0.1 M sodium phosphate buffer, pH 7.2). The animals were washed (PBS with 0.05% Tween 20, 1 h at 22°C) and then incubated in 0.4 M glycine, pH 7.0, for 1 h to block any remaining fixative. The cells of the animal were permeabilized by incubation in PBS containing 0.25% Triton X-100 (1 h at 22°C). Non-specific binding was minimized by incubation in PBS with 0.25% Triton X-100 containing 10% calf serum (Irvine Scientific, Tustin, CA). The fixed animals were allowed to incubate overnight at 4°C in affinity-purified annexin XII or CP2 primary antibodies. All antibody dilutions calculated from the original serum volume (within a range of 1:50 to 1:500 per experimental point) were made in PBS containing 0.5% BSA and 0.1% Triton X-100. Washing steps were performed between all antibody incubations (PBS with 0.1% Triton X-100, 1 h at 22°C). Visualization of the primary antibody binding was accomplished by incubating the fixed animals with biotin-conjugated goat anti-rabbit IgG secondary antibody diluted 1:100 (6630; Tago Immunologicals, Burlingame, CA) for 1 h at 22°C. This was followed by incubation (1 h at 22°C) with a streptavidin-fluorescein isothiocyanate conjugate diluted 1:100 (6267; Tago Immunologicals). The animals were mounted on glass slides in PBS/glycerol (3:1) with 50 mg/ml *N*-propyl gallate. Control incubations were performed without the addition of primary antibody.

Single Cell Immunocytochemistry

Whole animals were macerated into a suspension of fixed single cells as described (David, 1973). Briefly, whole animals were macerated in a solution containing glycerol/acetic acid/water (1:1:13) for 1 h at 22°C and fixed with the addition of paraformaldehyde (2% wt/vol). The cell suspension was spread onto poly-L-lysine–treated glass slides (200 μg/ml in H_2O, dried at 40°C) and allowed to air dry overnight. The cells attached to the glass slides were postfixed (2% paraformaldehyde in PBS, 30 min), washed (PBS, 0.1% Triton X-100, and 0.1 M glycine, 30 min), blocked (1% BSA in PBS), and incubated in a humid chamber at 22°C with affinity-purified annexin XII or CP2 primary antibodies for 3 h (diluted within a range of 1:50 to 1:500 per experimental point). All antibody dilutions were made in PBS with 0.25% BSA. Visualization of the primary antibody binding was accomplished by incubating the cells with biotin-conjugated goat anti-rabbit IgG secondary antibody diluted 1:100 (6630; Tago Immunologicals) for 1 h at 22°C. This was followed by incubation (1 h at 22°C) with a streptavidin-fluorescein isothiocyanate conjugate diluted 1:100 (6267; Tago Immunologicals, CA). The slides were washed extensively in PBS and mounted in PBS/glycerol (3:1) containing 50 mg/ml *N*-propyl gallate. Control incubations were performed without the addition of primary antibody.

Results

The Characterization of Other Annexins in Hydra vulgaris

All annexins possess a well-defined structure consisting of a highly conserved core domain and distinctive amino-terminal domains of varying size. The homologues of different annexins have been highly conserved between species. To search for new annexins, one of the most sensitive methods available is to screen cell extracts for proteins that exhibit the property of reversible Ca^{2+}-dependent phospholipid binding; an activity common to all known annexins. Additionally, new annexins can be detected by using anti-peptide antibodies that have been made to the highly conserved annexin core domain sequences (Gerke, 1989; Kaetzel and Dedman, 1989). Although most characterized annexins are abundant cellular proteins, the use of these highly sensitive annexin-specific anti-peptide antibodies can assist in the detection of annexins that are expressed at low levels.

Previously, annexin XII was isolated by reversible Ca^{2+}-dependent binding to hydra membranes (Schlaepfer et al., 1992*b*). Like many of the vertebrate annexins, annexin XII is an abundant protein expressed at ~0.3% of total hydra protein. When hydra protein extracts, enriched for the presence of annexins, were analyzed by Coomassie blue protein staining, annexin XII was the only annexin detected. But, to complete a comprehensive evaluation of annexin expression in hydra, we re-screened hydra membrane extracts with annexin-specific anti-peptide antibodies for other proteins that exhibited reversible Ca^{2+}-dependent phospholipid binding activity. Two different anti-peptide antibodies made to conserved annexin core domain sequences, KAMKGLGTDE (Kaetzel and Dedman, 1989) and RVMVSRSEIDLLDIR (Gerke, 1989), were tested and found to recognize only annexin XII in the hydra extracts (data not shown). These antibodies have been shown to cross-react with many different vertebrate and Drosophila annexins. Another anti-peptide antibody made to a conserved region of the annexin 2nd core domain repeat (MKGAGTDEDVLIEILASRT), termed con-

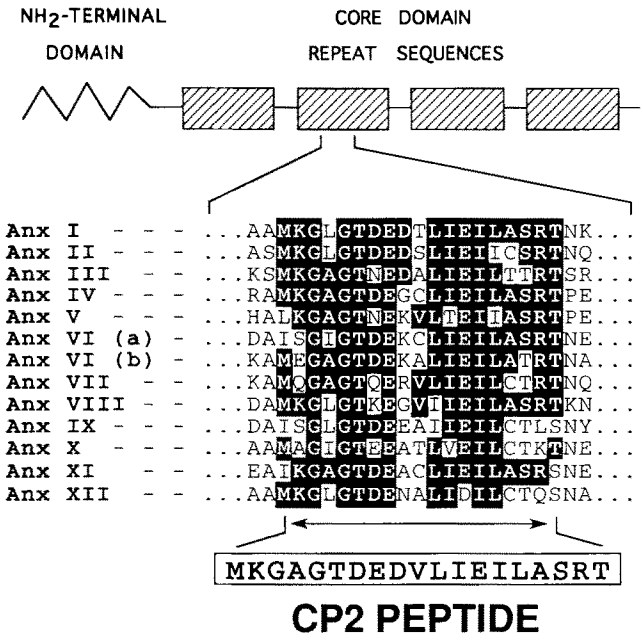


Figure 1. Core domain sequence alignments of the annexins; comparison with the CP2 peptide. A schematic illustration of the annexin amino-terminal and core domain repeat structure is shown. The single-letter amino acid code is used to compare the annexin sequences within the second core domain repeat with a peptide made to conserved residues within this domain, termed consensus peptide 2 or CP2 (Gerke, 1989). The CP2 peptide and affinity purified antibody to the peptide were developed and originally characterized by Dr. Volker Gerke (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). The amino acids outlined in black indicate the identical residues with respect to the CP2 sequence. The sequences shown are from human annexin I (Wallner et al., 1986), II (Huang et al., 1986), III (Pepinski et al., 1988), IV (Grundmann et al., 1988), V (Kaplan et al., 1988), VI (Crompton et al., 1988*b*) separated into the first four repeats (*a*) and the second four repeats (*b*), VII (Burns et al., 1989), VIII (Hauptmann et al., 1989), Drosophila annexin IX (Johnston et al., 1990), Drosophila annexin X (Johnston et al., 1990), bovine annexin XI (Towle and Treadwell, 1992), and hydra annexin XII (Schlaepfer et al., 1992*b*).

sensus peptide 2 or CP2 and developed by Dr. Volker Gerke (Gerke, 1989), was used in the current study to identify a new hydra annexin protein that was expressed at <1% of annexin XII levels (see below).

The residues of CP2 peptide are a combination of the most highly conserved amino acids shared between all known annexins within this region of the core domain (Fig. 1). The affinity purified CP2 polyclonal antibody has been shown to specifically recognize annexin II and annexin IV in porcine intestinal cell extracts, as well as two or more different annexin proteins in *X. laevis* and *D. melanogaster* cell extracts (Gerke, 1989). Immunoblot analysis performed in our laboratory showed that the CP2 antibody reacted strongly with human annexin I and human annexin IV, moderately with human annexin V, and very weakly with hydra annexin XII (data not shown). The identical residues found within the annexins tested, with respect to the CP2 consensus peptide sequence, have been highlighted (Fig. 1). Annexin I and IV contained 17 of 19 identities with the CP2 peptide sequence, annexin V had 14 of 19 identities, and annexin XII had only 11 of 19 identities. Not surprisingly, the annexins with most sequence identities with the CP2 peptide reacted the strongest with the CP2 antibody.

Since all known annexins undergo reversible Ca^{2+}-dependent binding to membrane phospholipids, total hydra membranes were isolated in the presence of Ca^{2+} and then extracted with EGTA to remove the bound annexins (see Materials and Methods). This EGTA membrane extract, enriched for the presence of annexin proteins, was analyzed with the CP2 antibody by SDS-PAGE and immunoblot analysis (Fig. 2). The CP2 antibody detected a 40-kD protein in the EGTA membrane extract, but the immunoreactive protein was not present at a high enough level with respect to other proteins in the extract to be detected directly by Coomassie blue staining (Fig. 2, lane *a*, 100 μg of EGTA extract protein). As expected from previous studies, annexin XII was readily identifiable by Coomassie blue staining in the EGTA membrane extract (Fig. 2, lane *a*, *Coomassie Blue*) and by annexin XII immunoblot analysis (data not shown). Even though a very large amount of annexin XII was present in the EGTA extract (25 μg per 100 μg protein in the EGTA extract), over-exposure of the CP2 immunoblot showed only very weak reactivity to annexin XII (Fig. 2, lane *a*).

Since annexins also undergo reversible Ca^{2+}-dependent binding to phosphatidylserine containing vesicles, an in vitro binding assay was performed to test whether the 40-kD CP2 immunoreactive protein also exhibited this annexin-like activity. Phospholipid vesicles and Ca^{2+} were added and incubated with the hydra EGTA extract as described in Materials and Methods. The mixture was subjected to centrifugation to pellet the phospholipid vesicles, and the soluble (Fig. 2, lane *b*) or vesicle-associated (Fig. 2, lane *c*) hydra protein was resolved by SDS-PAGE and evaluated by Coomassie blue staining or by CP2 immunoblot analysis. The majority of annexin XII and >90% of the 40-kD protein associated with the vesicles in the presence of Ca^{2+} (Fig. 2, lane *c*). Without the addition of phospholipid vesicles to the reaction mixture, annexin XII and the 40-kD protein remained soluble in the presence of Ca^{2+} (data not shown). When the vesicle pellet was resuspended (Ca^{2+} reduced to 0.25 mM) and washed by repeated centrifugation, only a minor amount of annexin XII and no detectable 40-kD protein were solubilized (Fig. 2, lane *d*). When the vesicles were resuspended in EGTA (1 mM), ~80% of the annexin XII and 80% of the CP2 immunoreactive 40-kD protein were solubilized (Fig. 2, lane *e*). A faint 40-kD band corresponding to <0.1 μg protein as well as >15 μg of annexin XII protein were visualized by Coomassie blue staining in the EGTA wash fraction (Fig. 2, lane *e*).

The 40-kD protein exhibited the property of reversible Ca^{2+}-dependent binding to phospholipid vesicles; a characteristic of all known annexins. Whereas hydra annexin XII was abundant (~0.3% of the total protein), the 40-kD hydra protein was expressed at <1% of annexin XII levels. No increased levels of the 40 kD or other putative annexin proteins were detected by immunoblot analysis when total hydra protein was extracted directly into Laemmli SDS sample buffer (Laemmli, 1970) (data not shown). Thus, the low abundance of the 40-kD protein was not due to proteolytic degradation during the preparation of the hydra EGTA extracts.

In summary, the above experiments have identified a 40-

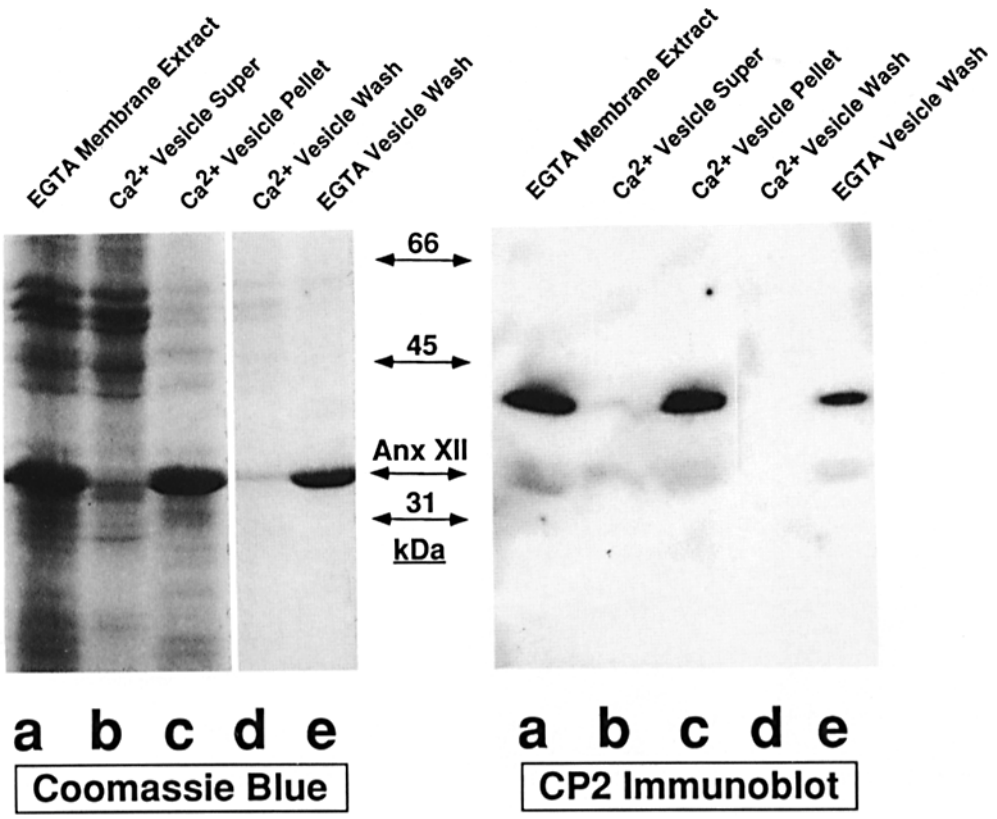


Figure 2. Ca^{2+}-dependent association of annexin XII and a 40-kD hydra annexin with phospholipid vesicles. Hydra membranes prepared in the presence of Ca^{2+} were extracted with EGTA as described in Materials and Methods section. Aliquots of the hydra proteins solubilized by EGTA treatment (lane *a*, 100 μg total protein) were resolved by SDS-PAGE and either stained with Coomassie blue or transferred to Immobilon PVDF membrane and analyzed by immunoblot analysis with the CP2 peptide antibody. Phosphatidylserine containing vesicles and Ca^{2+} (1 mM final concentration) were added and incubated with duplicate aliquots of the EGTA membrane extracted proteins (100 μg protein each aliquot) in an in vitro phospholipid vesicle binding assay as described in Materials and Methods section. After a 10-min incubation, the reactions were subjected to centrifugation to pellet the phospholipid vesicles and the soluble hydra protein (lane *b*) or vesicle associated protein (lane *c*) were resolved by SDS-PAGE and either stained with Coomassie Blue or analyzed by CP2 immunoblot analysis. Duplicate aliquots of the vesicle associated protein were washed (0.25 mM Ca^{2+}) by resuspension and repeated centrifugation. The solubilized protein from the Ca^{2+} wash step was resolved by SDS-PAGE (lane *d*). The vesicles were washed with EGTA (1 mM) by the same procedure and the solubilized protein was resolved by SDS-PAGE (lane *e*). The CP2 immunoblot image was purposely over-exposed to show the very low level of annexin XII cross-reactivity.

kD protein that appears to be a novel hydra annexin. Of course, there may be other hydra annexins which may have escaped the limits of annexin-specific anti-peptide immunoblot detection, but their total expression level must be quite low in hydra. No hydra annexin homologues were detected with polyclonal antibodies made to human annexins I, II, IV, and V (data not shown). As a comparison, over eight different annexin proteins have been detected by reversible Ca^{2+}-dependent binding to phospholipid vesicles when analyzing extracts of human placental tissue (D. D. Schlaepfer and H. T. Haigler, unpublished results). If other hydra annexins were present in significant amounts, they should have been detected by Coomassie blue staining or by immunoblot analysis as proteins in the EGTA membrane extract that would have undergone reversible Ca^{2+}-dependent association with added phospholipid vesicles (Fig. 2, lane *e*).

Cellular Localization of Annexin XII in Intact Hydra

Since the regional distribution, morphological characteristics, and tissue layer location is known for the 15 different hydra cell types (David, 1973; Bode et al., 1973), the hydra annexin cellular distribution can be determined in whole animals with indirect immunocytochemical staining.

Using affinity-purified polyclonal antibodies to annexin XII, staining for annexin XII protein expression was found to be maximal in the tentacles of mature adult and budding animals (Fig. 3). Annexin XII expression also was detected in epithelial cells of the peduncle region, the lower end of the body column above the foot (Figs. 3 and 4 *A*). No other regions in the intact animal showed detectable amounts of annexin XII immunofluorescent staining. Other control experiments performed showed that the affinity-purified annexin XII antibodies did not cross-react with other hydra proteins by immunoblot analysis, that the specific immunofluorescent signal could be blocked by excess recombinant annexin XII addition, that no staining was observed when the annexin XII antibody was omitted, and that the annexin XII antibody pre-immune serum did not stain the animal (data not shown).

In hydra, the tentacles are composed of terminally differentiated epithelial cells (Dübel et al., 1987; Dübel, 1990) and these cells showed the greatest amount of annexin XII expression. Intense annexin XII staining was constant throughout the length of tentacle and ended abruptly at the tentacle/head junction (Fig. 4 *B*). The annexin XII staining in the tentacle was localized to the battery cells which are the differentiated epithelial cells of the tentacle ectoderm. Analysis of the annexin XII expression pattern in two other species of hydra, *H. oligactis* and *H. magnipapillata*, showed the same intense battery cell staining (data not shown).

As the battery cell is complex, a brief description is useful to better understand the precise location of the annexin protein within the battery cells. Associated with each battery

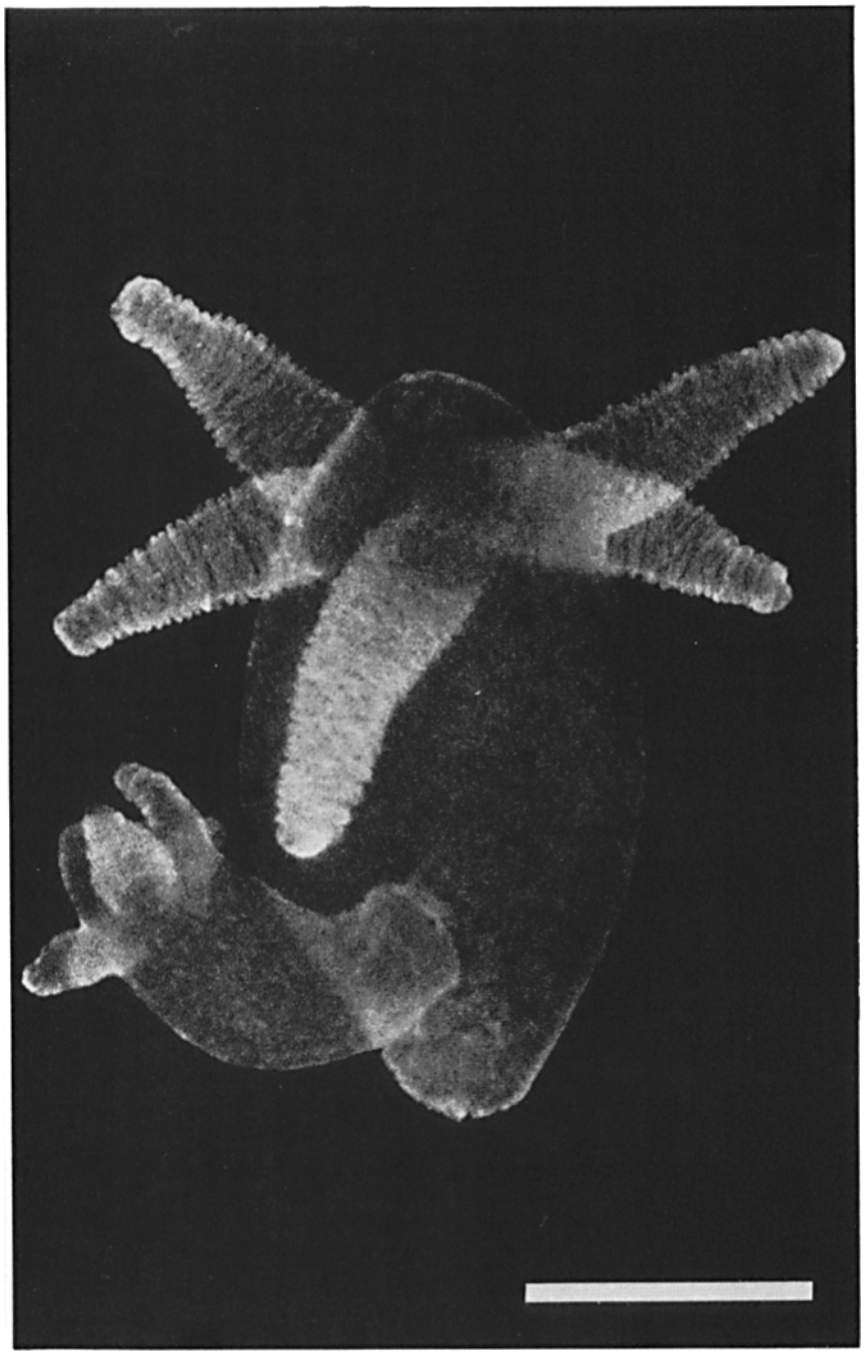


Figure 3. Annexin XII antibody indirect immunofluorescent staining of a whole animal. View of an adult *H. vulgaris* with a single bud. Bar, 1 mm.

cell are 15–20 nematocytes, which are cells involved in the capture and immobilization of prey as well as in defense. A few nematocytes are shown in the battery cell illustration in Fig. 5. Each nematocyte is enveloped by, but not within, the battery cell. Each nematocyte effectively resides in a "pocket" extending down from the apical surface of the battery cell facing the environment to just above the mesoglea at the basal surface of the battery cell (Campbell, 1987). In a similar fashion, each battery cell envelops a single epidermal sensory cell. Processes from the sensory cell contact individual nematocytes, and again these are enveloped by, but external to, the battery cell (Hobmayer et al., 1990).

Battery cells arise through the terminal differentiation of body column epithelial cells and this event has been shown to occur within the span of one cell cycle (Dübel and Schaller, 1990). As shown in Fig. 4 *B*, the epithelial cells of the upper body column and head did not express significant levels of annexin XII. Dramatic increases in annexin XII expression occurred at the base of the tentacle in the newly formed battery cells (Fig. 4 *B*). This is especially interesting since body column epithelial cells are continuously being displaced toward the head, into the tentacle zone, and onto the tentacles as differentiated battery cells (Shostak and Globus, 1966; Bode et al., 1986). It is within the tentacle zone where the stimulus for the epithelial to battery cell differentiation event occurs (Dübel, 1989). Thus, increased annexin

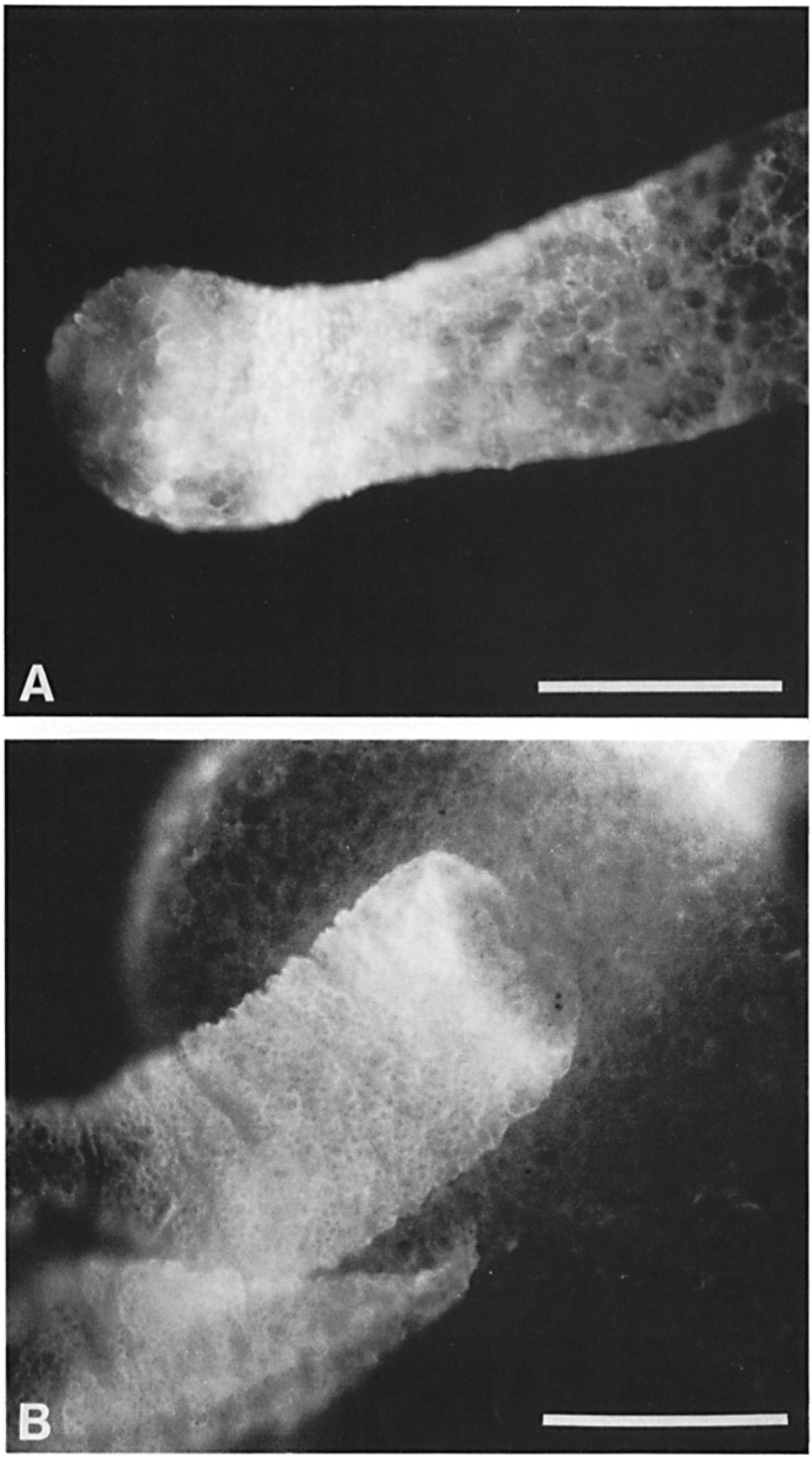


Figure 4. Annexin XII antibody indirect immunofluorescent staining of *H. vulgaris*. (*A*) View of the peduncle epithelial cell staining at the lower end of the body column. The foot of the animal is toward the left. (*B*) View of tentacle/head junction of an adult animal. Top of head is toward upper left, and the body column, which is nearly invisible due to the lack of staining, extends toward lower right. Bar, 250 μm.

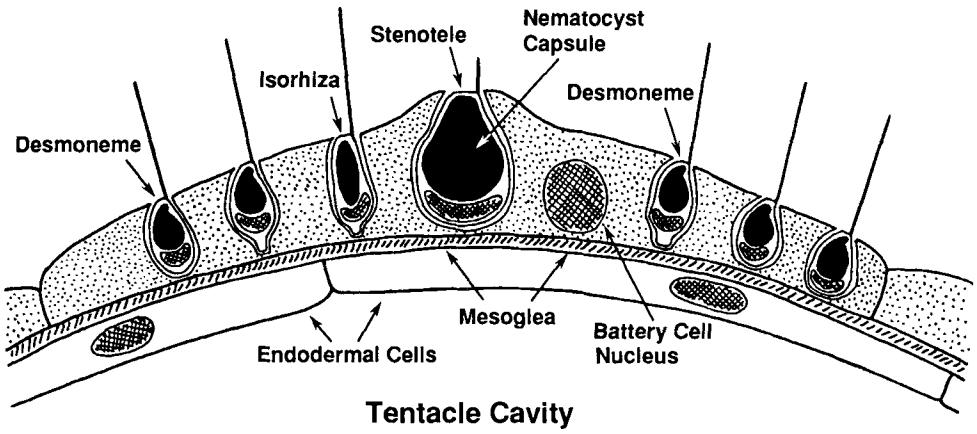


Figure 5. Illustrated cross-section of a tentacle; structure of an epithelial battery cell. The cytoplasm of the battery cell and two neighboring battery cells has been stippled. The mesoglea (*cross-hatched*) separates the battery cell from endodermal epithelial cells (*clear*) in the tentacle. The region below the cells is the cavity of the tentacle. The location of the different cell types that are embedded within the battery cell are indicated: *stenotele*, *isorhiza*, and *desmoneme* nematocytes (sensory neurons are also found within battery cells, but are not shown). Mature battery cells usually contain one stenotele, two isorhizas, and about 15–20 desmoneme nematocytes. The nematocyst capsule which contains the specialized projectile has been blackened for each nematocyte. The crescent structures (double cross-hatched) beneath the capsules are the nuclei of the nematocytes. The individual nuclei for the battery and endodermal epithelial cells also have been double cross-hatched.

XII expression in the newly formed battery cells may be intimately associated with or occurred as a direct result of the epithelial to battery cell differentiation process.

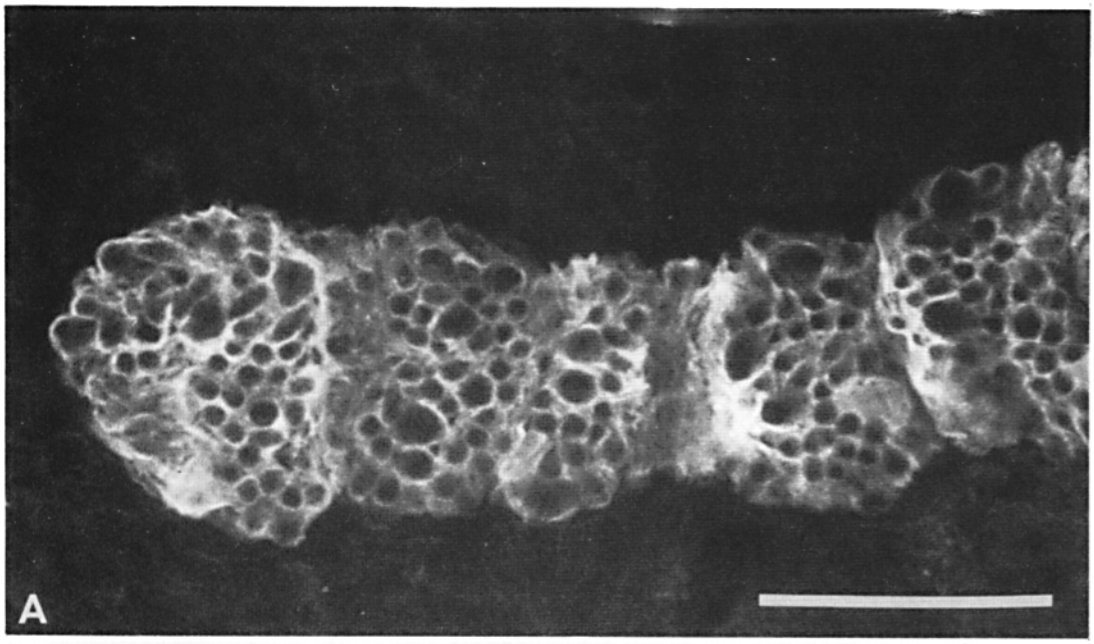
The intense annexin XII fluorescent staining within tentacle battery cells compared to body column epithelial cells was pictured best when using laser scanning confocal microscopy to optically section an intact tentacle (Fig. 6 *A*). The annexin XII stained tentacle in Fig. 6 *A* is positioned next to midsection of the animal and lies within a depression of body column epithelial cells as a result of the fixation process. The focal plane of the confocal image has been aligned to cut through the tentacle battery cells as well as through the surrounding epithelial cells in the animal's body column. A strong annexin XII fluorescent signal was detected in the tentacle battery cells whereas it was absent within the body column epithelial cells (Fig. 6 *A*). Additionally, no staining was observed in the endodermal epithelial cells of the tentacle as determined by laser scanning confocal microscopy (data not shown).

The localization of annexin XII expression within individual tentacle battery cells was determined by comparing the Nomarski and annexin XII–stained fluorescent images of the same cells in tentacles (Fig. 6, *B* and *C*). The black lines inserted in Fig. 6 *B* outline two individual battery cells. The annexin XII–stained fluorescent image (Fig. 6 *C*) reveals a honeycomb pattern of circular non-fluorescent holes in an overall fluorescent cell. Comparison of the two images indicates that the larger "holes" correspond to the location of individual stenoteles (Fig. 6, *B* and *C*, *arrows*), while the smaller ones represent the location of desmonemes (Fig. 6, *B* and *C*, *arrowheads*).

The distribution of annexin XII staining within battery cells was visualized best when whole animals were macerated into a suspension of fixed single cells. Since the maceration procedure does not affect the integrity of individual cells, and since each cell type has a distinct morphology, each macerated cell can be identified as to type (David, 1973). Macerates processed for annexin XII indirect immunofluorescence showed uniform cytoplasmic staining throughout the battery cell (Fig. 7). Additionally, 5 to 10% of epithelial cells also exhibited annexin XII staining (data not shown). This sub-fraction of epithelial cells that express annexin XII may account for the ectoderm staining in the peduncle region as observed in intact animals (Figs. 3 and 4 *A*). No annexin XII expression was detected in any other isolated hydra cell type (Fig. 7). The same annexin XII staining pattern was observed when single cell macerate preparations were analyzed with a HRP-catalyzed color detection method (data not shown).

The annexin XII staining within the battery cell cytoplasm was punctuated by non-fluorescent holes (Fig. 7). The "holes" correspond to nematocysts, the interior organelle of the nematocytes which are embedded within the battery cell (see Fig. 5 for an illustration). The nematocytes arise through differentiation from interstitial cells in the body column (David and Gierer; Bode and Flick, 1976). Upon completion of differentiation, the mature nematocytes migrate between the cells of the ectoderm into the tentacles and are subsequently mounted in the battery cells (Campbell, 1967; Herlands and Bode, 1974). Three isolated mature nematocytes are shown in Fig. 7 *A*. Two are isorhizas (Fig. 7 *A*, *i*) and one is a stenotele (Fig. 7 *A*, *s*). The cytoplasm of these isolated nematocytes did not stain for the presence of annexin XII (Fig. 7 *B*). Examination of many migratory

Figure 6. Annexin XII antibody indirect immunofluorescent staining of intact tentacle battery cells. (*A*) Laser scanning confocal microscopic image of annexin XII indirect immunofluorescent staining. The tentacle was positioned against the midsection body column epithelial cells of the animal. The image focal plane was aligned through the battery cells of the tentacle and through the surrounding epithelial cells of the body column. (*B* and *C*) The same location on a tentacle observed with (*B*) Nomarski optics, and (*C*) with fluorescence using indirect immunofluorescence to visualize the antibody against annexin XII. Two individual battery cells have been outlined (*B*). Stenotele nematocyst capsules (*arrows*) or desmoneme nematocyst capsules (*arrowheads*) have been indicated. Bars: (*A*) 250 μm; (*B* and *C*) 100 μm.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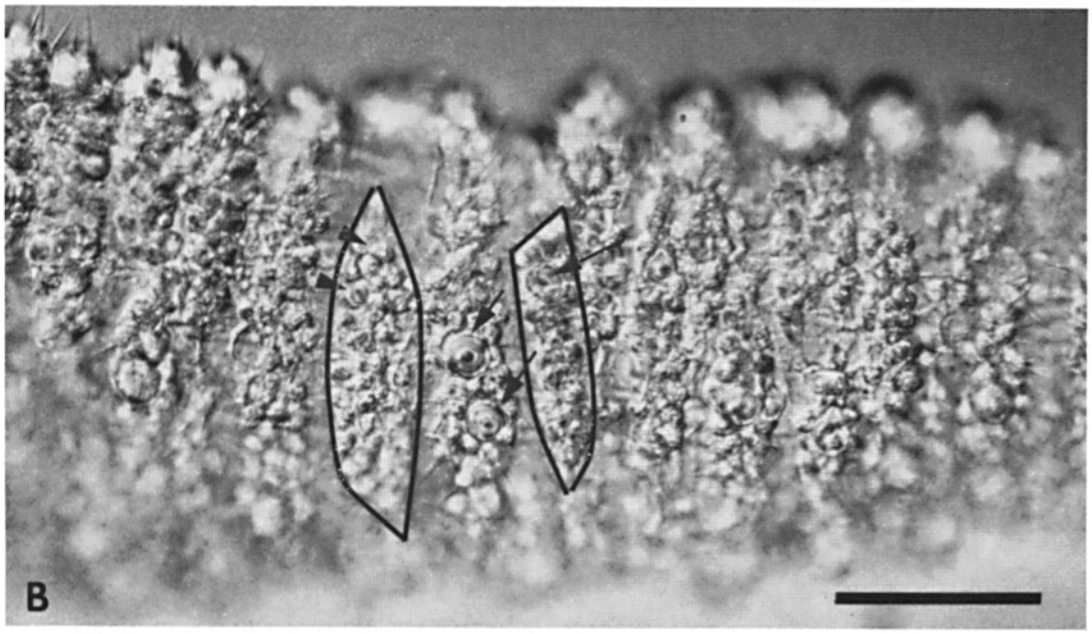
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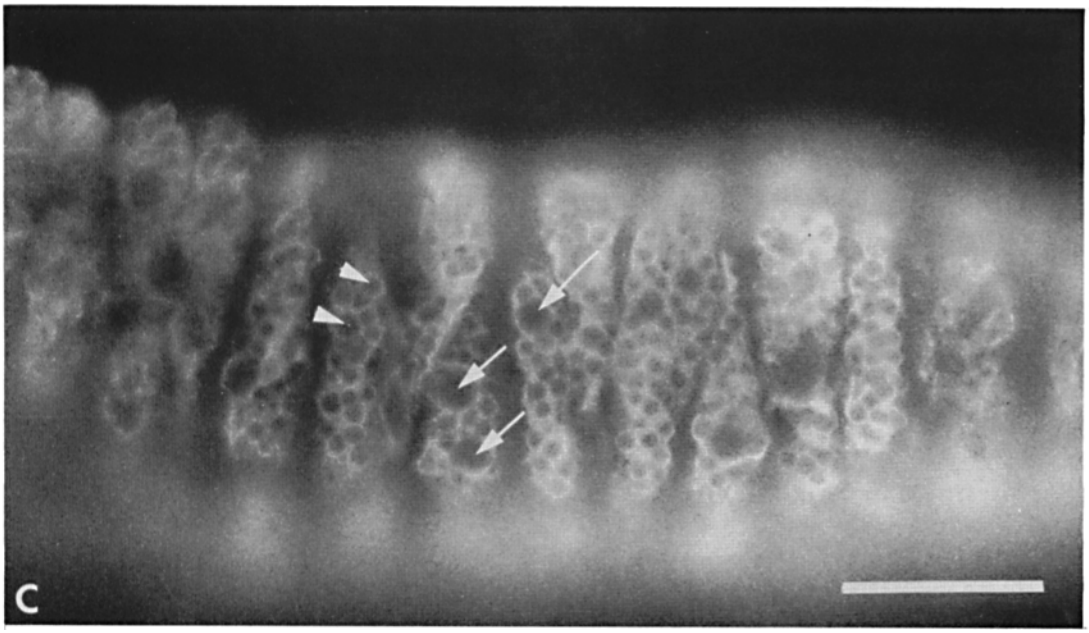
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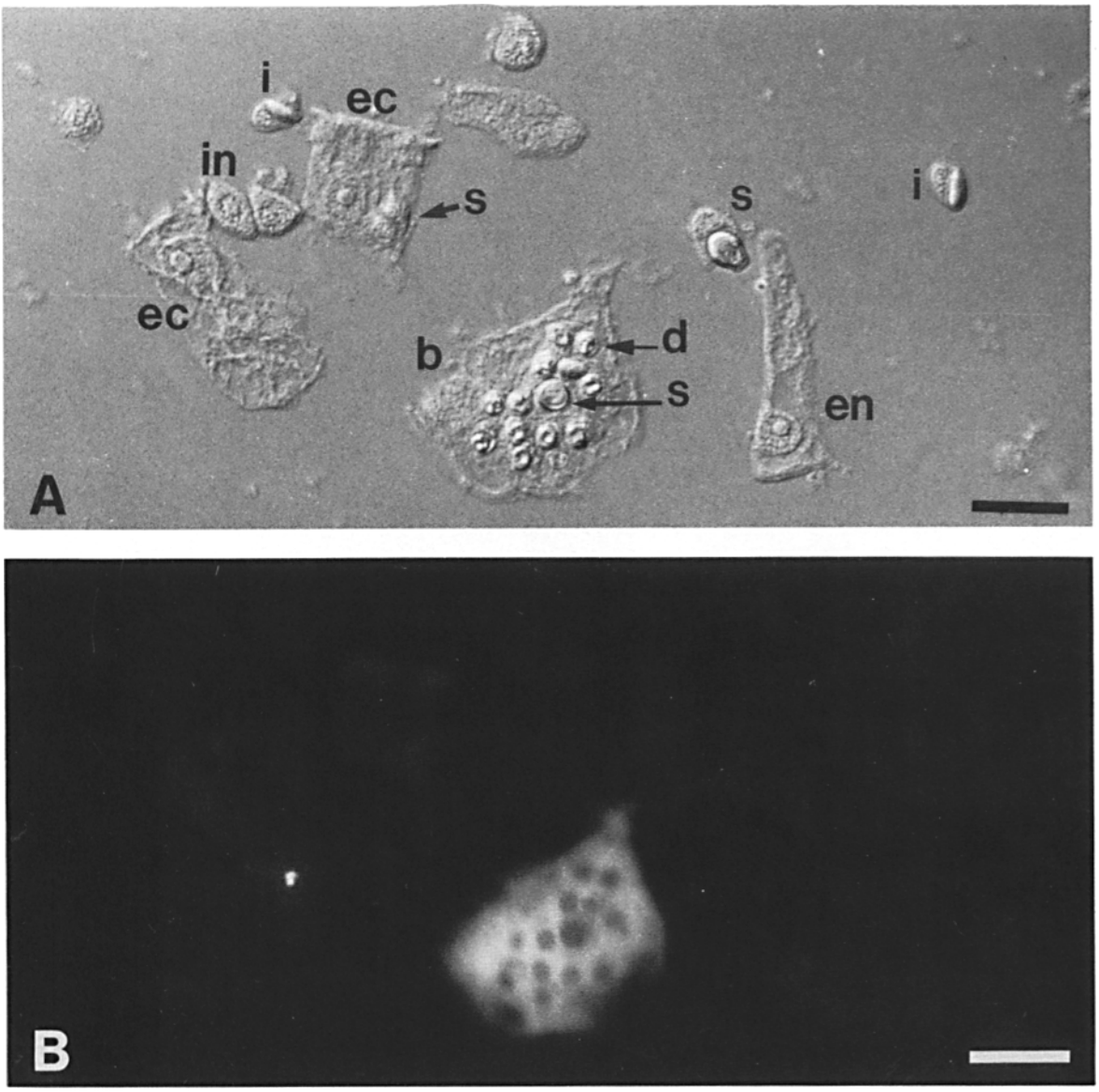

Figure 7. Annexin XII antibody indirect immunofluorescent staining of battery cells within a fixed single cell macerate preparation. Nomarski phase-contrast of single cells isolated from a whole animal (*A*) or annexin XII indirect immunofluorescent staining (*B*) of the same cells. The different hydra cell types present are indicated: *b*, tentacle battery cell; *ec*, ectodermal epithelial cell; *en*, endodermal epithelial cell; *in*, interstitial cell (pairs); *s*, stenotele nematocyte; *d*, desmoneme nematocyte; and *i*, isorhiza nematocyte. Bar, 25 μm.

nematocytes of all types indicated that none were stained (data not shown). Although it could not be directly determined from the battery cell staining whether annexin XII was expressed in the thin shell of nematocyte cytoplasm surrounding the nematocysts, it is unlikely that the nematocytes embedded in the battery cells expressed annexin XII due to the lack of annexin XII staining in the isolated nematocytes (Fig. 7 *B*).

Since one of the distinct battery cell characteristics is the presence of many embedded nematocytes, experiments were performed to determine whether the nematocyte association with the battery cells was the signal for increased annexin XII expression within these cells. It has been shown that treatment of hydra with hydroxyurea eliminates the interstitial cell population (Sacks and Davis, 1979). Due to the tissue dynamics of the animal, the loss of the interstitial cell population results in the reduction and eventual loss of all interstitial differentiation products from the animal, including the nematocytes (Wanek et al., 1980). Such animals consist only of epithelial cells. In these "epithelial" animals lacking nematocytes, annexin XII was still expressed in high amounts in the tentacle battery cells (Fig. 8 *A*). Additionally, a mutant strain of *H. oligactis*, "stingless," also showed intense annexin XII battery cell staining (Fig. 8 *B*). The tentacle battery cells in the "stingless" animals are completely devoid of all nematocytes (Dr. Lynne Littlefield, personal communication).

The above results show that battery cells express annexin XII in the absence of nematocyte association. Additionally, stenotele nematocytes also are found embedded within ectodermal epithelial cells of the body column (Bode and Flick, 1976). Within the single cell macerate preparation, an

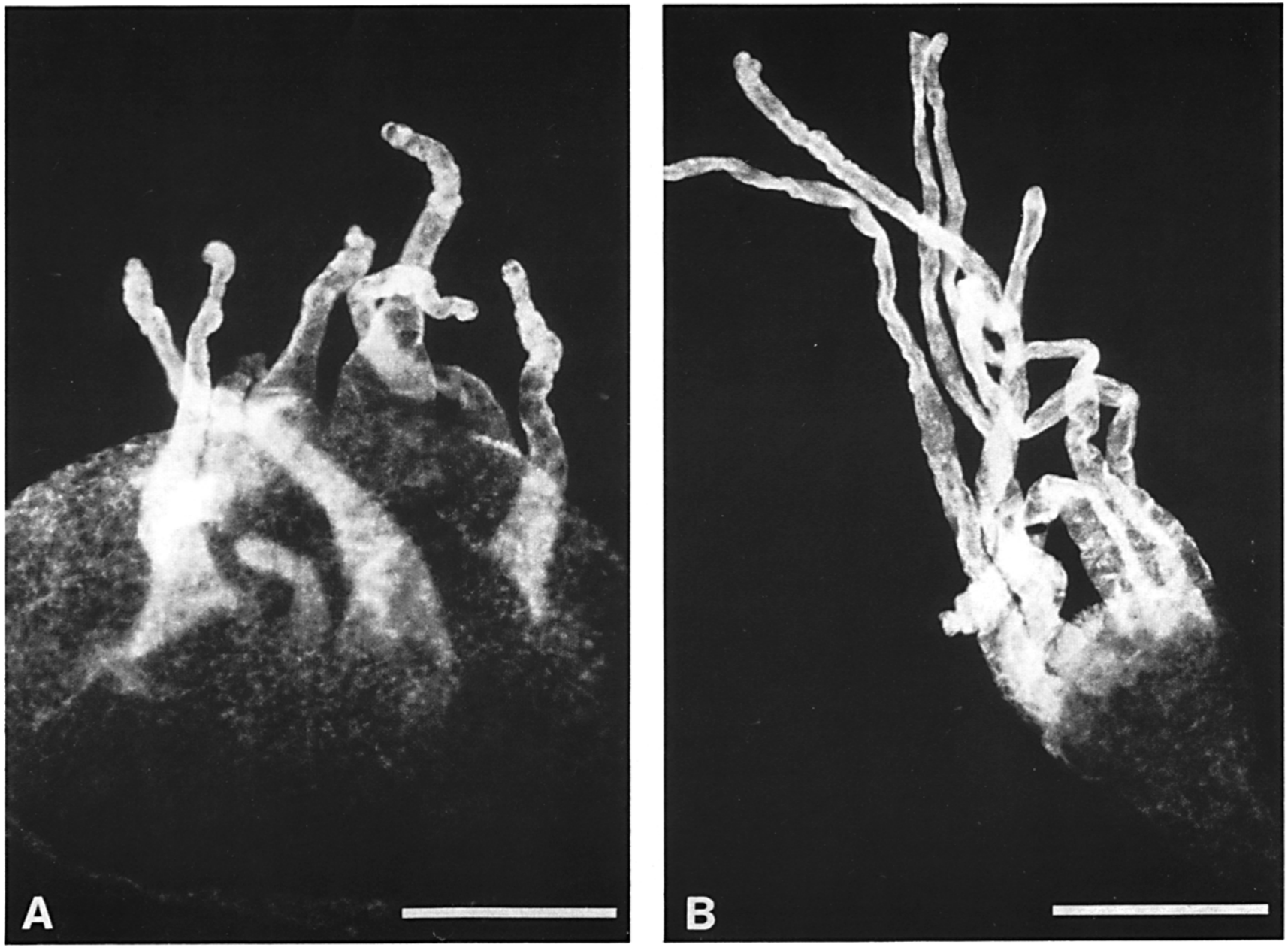

Figure 8. Annexin XII antibody indirect immunofluorescent staining of tentacle battery cells without nematocytes. (*A*) *H. vulgaris* treated with hydroxyurea; view of hypostome and tentacles. (*B*) *H. oligactis* "stingless" mutant; view of hypostome and tentacles. Bars, 1 mm.

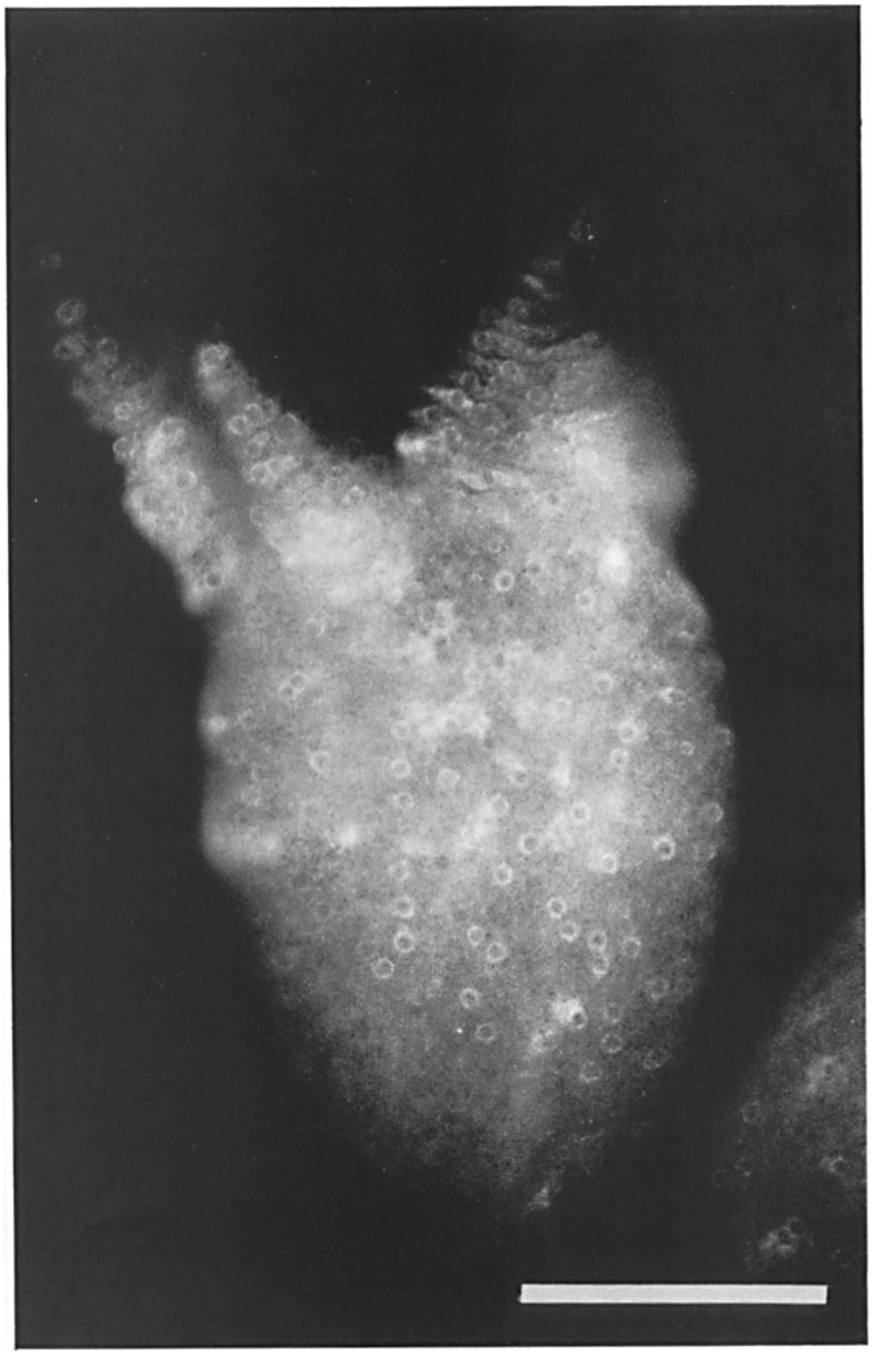

Figure 9. CP2 antibody indirect immunofluorescent staining of *H. vulgaris*. View of ectodermal body column cells. Recombinant annexin XII (1 mg/ml) was added to the primary antibody incubation mixture. Bar, 500 μm.

isolated epithelial cell with characteristic cuboidal shape of a body column ectodermal cell is shown to contain an embedded stenotele nematocyte (Fig. 7, *short arrow*) and did not stain for the presence of annexin XII (Fig. 7 *B*). None of the many body column epithelial cells with an associated nematocyte were stained nor was significant annexin XII expression detected within body column epithelial cells in whole animal preparations (Figs. 3 and 6 *A*). Clearly, the event of nematocyte association with epithelial cells is not the stimulus for increased annexin XII expression as seen in the tentacle battery cells.

Cellular Localization of CP2 Immunoreactivity in Intact Hydra

Since annexin XII was expressed only in the battery and peduncle epithelial cells, it was important to determine whether the 40-kD hydra annexin expression would overlap in the same cell types as annexin XII. By using the antibody made to the CP2 peptide, the cellular expression of the 40-kD hydra annexin was determined in intact animals through indirect immunofluorescent staining. As shown in Fig. 9, a distinctly different fluorescent staining pattern was observed with the CP2 antibody as compared to annexin XII staining. Cellular "rings" of fluorescent staining ($\sim$10 μm in diameter) were observed throughout the body column and in the tentacles of intact animals. No specific staining was observed when the CP2 antibody was omitted (data not shown). Since the CP2 antibody showed weak reactivity to annexin XII on immunoblots (Fig. 2), recombinant annexin XII protein (1 mg/ml) was added to all CP2 antibody immunofluorescent experiments to block any signal from endogenous annexin

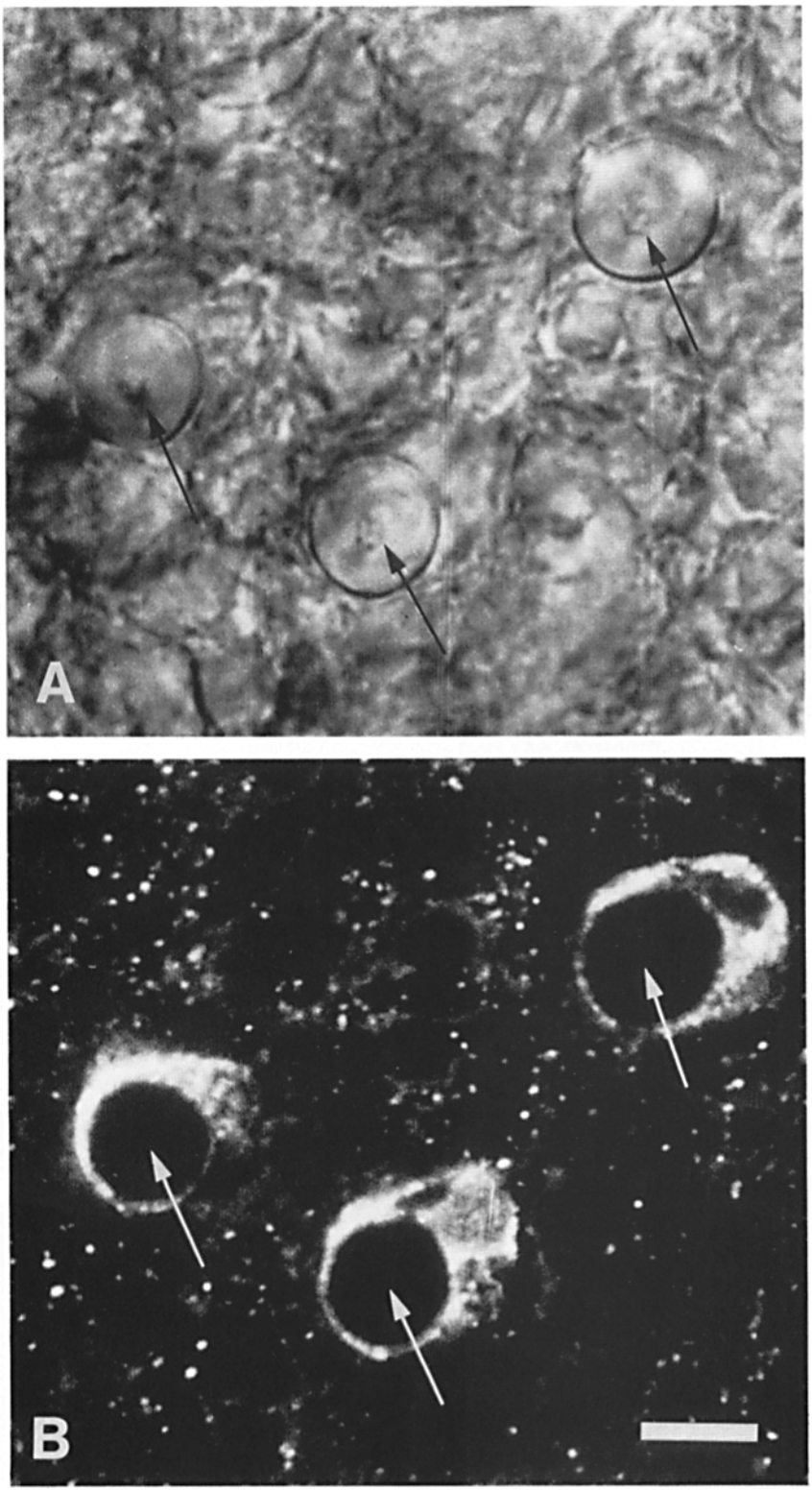

Figure 10. Laser scanning confocal microscopic image of CP2 antibody indirect immunofluorescent staining within body column stenotele nematocytes. Phase contrast image (*A*) or CP2 antibody immunofluorescent staining (*B*) of the same midsection body column region. The distinct stenotele nematocyst capsules present within the hydra body column have been indicated (*A*, *arrows*). Small (<1 μm) spots of immunofluorescent staining were artifacts of the preparation. Recombinant annexin XII (1 mg/ml) was added to the primary antibody incubation mixture. Bar, 10 μm.

XII in the animals. The addition of excess soluble recombinant annexin XII did not affect the CP2 antibody reactivity to the 40-kD hydra annexin as determined by immunoblot analysis when hydra EGTA extracts were resolved by SDS-PAGE (data not shown).

The CP2 cellular "ring-like" fluorescent staining in the body column of intact animals (Fig. 9) was further analyzed using laser scanning confocal microscopy at high magnification (Fig. 10). Three stenotele nematocytes with intact nematocyst capsules (Fig. 10, *arrows*) are shown to be embedded within the epithelial cells of the body column. These body column stenotele nematocytes have a similar structure as those found in the battery cells (see Fig. 5 for a schematic illustration). The "ring-like" CP2 immunofluorescent staining was found within the stenotele nematocytes and was formed due to the absence of CP2 staining in the inner nematocyst capsules (Fig. 10 *B*, *arrows*). Although the outline of the stenotele nematocytes within the body column ep-

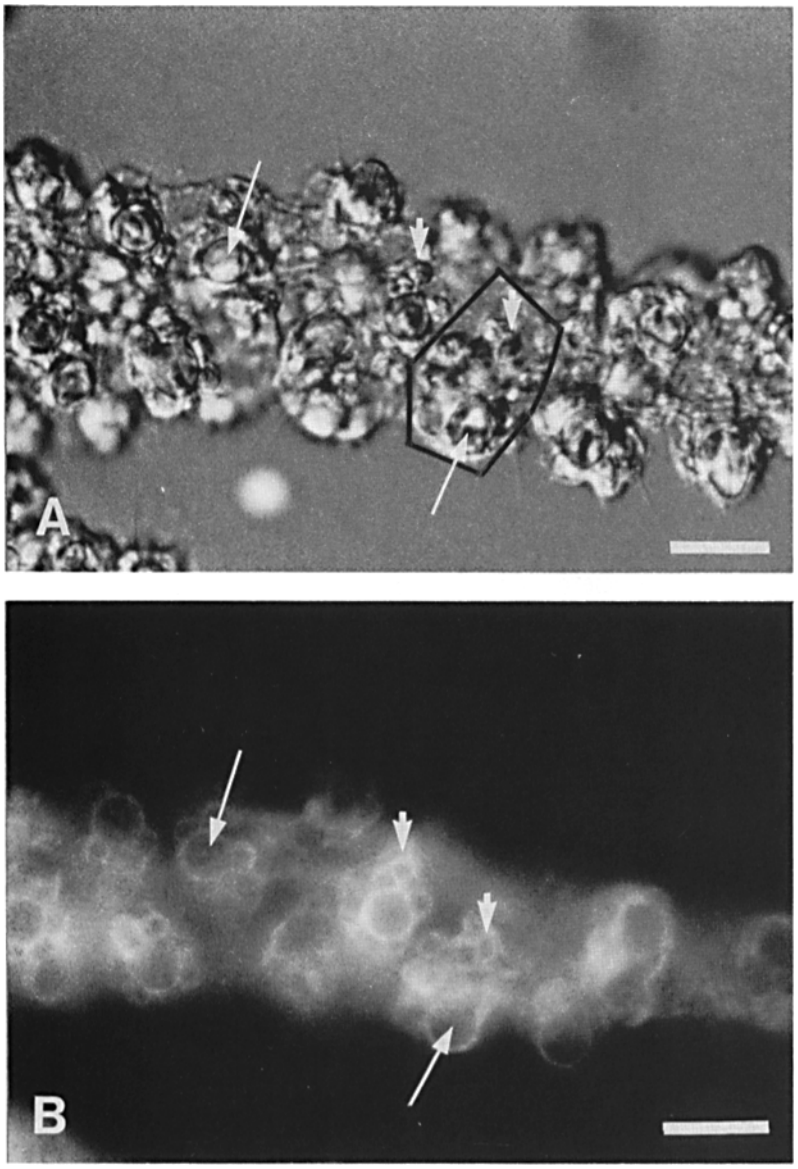

Figure 11. CP2 antibody indirect immunofluorescent staining of nematocytes mounted within tentacle battery cells. The same location on a tentacle observed with (*A*) Nomarski optics, and (*B*) with fluorescence using indirect immunofluorescence to visualize the CP2 antibody. An individual battery cell has been outlined in black (*A*). Stenotele nematocyst capsules (*arrows*) or desmoneme nematocyst capsules (*arrowheads*) have been indicated. Recombinant annexin XII (1 mg/ml) was added to the primary antibody incubation mixture. Bar, 100 μm.

ithelial cells is not readily apparent (Fig. 10 *A*), the CP2 staining pattern appears to be localized within the cytoplasm of the stenotele nematocytes. No specific CP2 staining was detected in the surrounding body column epithelial cells.

The localization of CP2 "ring-like" fluorescent staining to nematocytes mounted within battery cells of intact tentacles was determined by comparing the Nomarski and CP2-stained fluorescent image of the same cells in tentacles (Fig. 11). The black lines inserted in Fig. 11 *A* outline a single battery cell. The location of nematocyst capsules for the mounted stenoteles (Fig. 11, *arrows*) and desmonemes (Fig. 11, *arrowheads*) within the battery cells revealed that the CP2 staining immediately encircled these nematocyst structures (Fig. 11 *B*). This CP2 "ring-like" staining pattern was distinct from the annexin XII honeycomb staining pattern of battery cells (Fig. 6, *B* and *C*). Although the resolution of the Nomarski and CP2-stained fluorescent images (Fig. 11) were not as sharp as those obtained by confocal microscopy (Fig. 10), the "ring-like" nematocyte CP2 staining in the battery cells corresponded to the cytoplasmic region within the individual mounted nematocytes (Fig. 11 *B*). Significantly, no CP2 staining was detected within the cytoplasm of the epithelial battery cells where annexin XII expression was localized.

The absence of CP2 staining within the cytoplasm of battery cells was supported by experiments with hydroxyurea treated "epithelial" *H. vulgaris* animals and with the *H. oligactis* "stingless" mutants. Both of these animals lack nematocytes in the body column and tentacles and neither of these animals exhibited detectable CP2 "ring-like" staining patterns (data not shown). Thus, in the tentacles, the 40-kD hydra annexin expression was localized to the cytoplasm of mounted nematocytes as determined by CP2 staining whereas annexin XII expression was localized to the tentacle battery cell cytoplasm.

The distribution of CP2 staining within other hydra cell types was visualized when whole animals were macerated into a suspension of fixed single cells (Fig. 12). A low level of specific CP2 staining (Fig. 12 *B*) was detected in large interstitial cell pairs (Fig. 12 *A*, *in*) as well as in gland cells

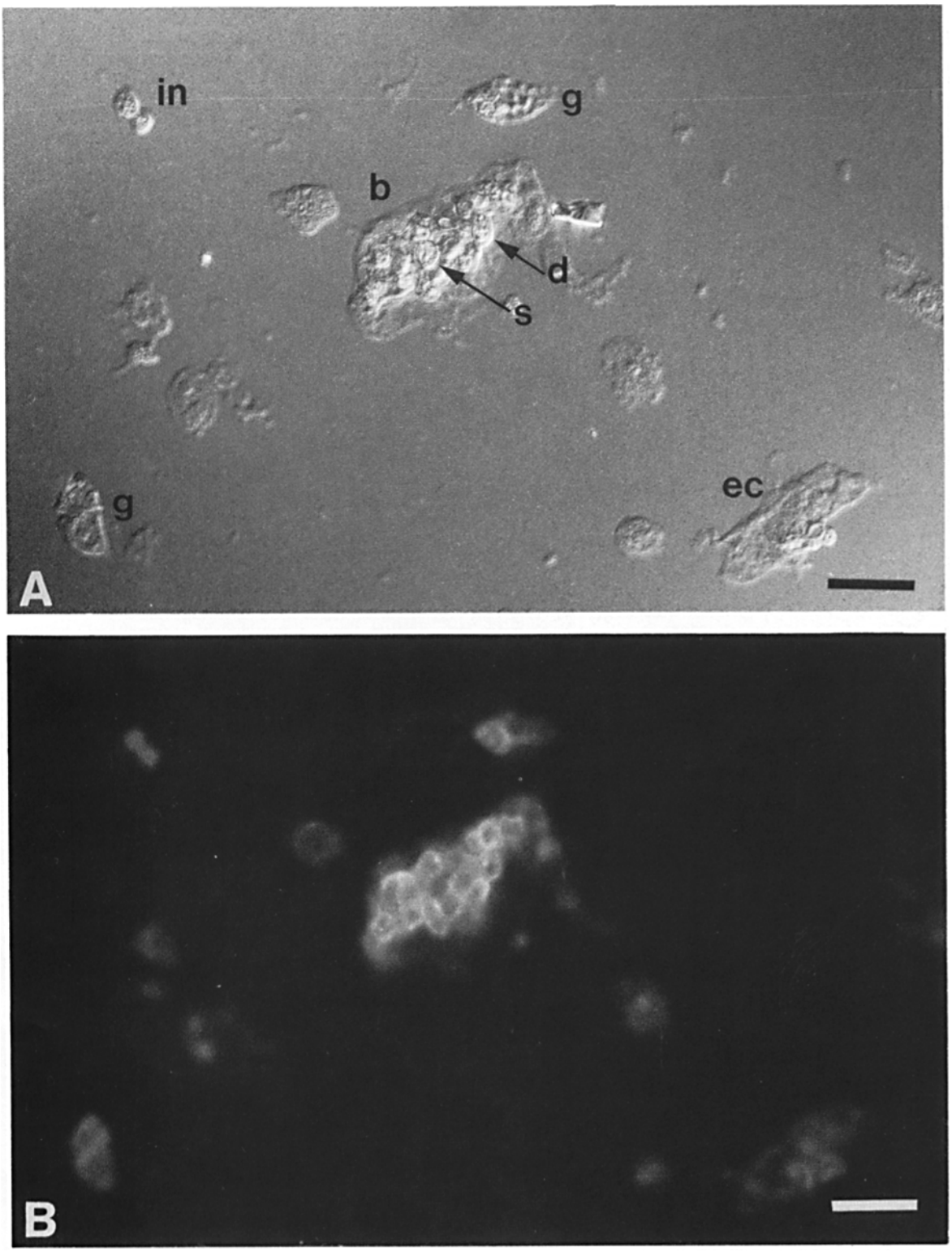

Figure 12. CP2 antibody indirect immunofluorescent staining of a single cell macerate preparation. Nomarski phase-contrast of single cells isolated from a whole animal (*A*) or CP2 antibody indirect immunofluorescent staining (*B*) of the same cells. The different hydra cell types present are indicated: *b*, tentacle battery cell; *ec*, ectodermal epithelial cell; *in*, interstitial cell (pairs); *g*, gland cell; *s*, stenotele nematocyte; and *d*, desmoneme nematocyte. Recombinant annexin XII (1 mg/ml) was added to the primary antibody incubation mixture. Bar, 25 μm.

(Fig. 12 *A*, *g*) which arise through interstitial cell differentiation (Bode et al., 1987). Maximal "ring-like" CP2 staining was detected within a 1–2-μm region surrounding the mounted nematocytes within the battery cells (Fig. 12 *B*). Isolated stenotele, isorhiza, and desmoneme nematocytes and their precursors also contained detectable CP2 staining (data not shown). No CP2 staining was found in the battery cell cytoplasm that surrounded the mounted nematocytes (Fig. 12 *B*). Additionally, no CP2 fluorescent staining was detected in the ectodermal epithelial cells, nerve cells, or in the endodermal epithelial cells that line the gastric cavity of the animal.

In summary, the distribution of CP2 fluorescent staining was localized to nematocytes, some interstitial cell pairs, and gland cells. Annexin XII expression was limited to tentacle battery cells and ectodermal peduncle cells. The immunofluorescent results showed that annexin XII and the 40-kD annexin were selectively and separately expressed within distinct cell types of the epithelial and interstitial lineages, respectively. Since the hydra annexins localized to specific subsets of the total hydra cell types, it is likely that these proteins perform specialized biological roles, and not general "housekeeping" functions which are part of the essential molecular machinery of all cells.

Discussion

As a first step toward understanding the physiological role of annexins in hydra, a comprehensive evaluation of annexin expression was completed. Previously, we identified and characterized annexin XII which comprises 0.3% of total hydra protein (Schlaepfer et al., 1992*b*). Annexin XII was the only hydra protein detected by Coomassie blue staining that underwent reversible Ca^{2+}-dependent phospholipid binding. In this report, we used anti-peptide antibodies against conserved annexin core domain sequences (Gerke, 1989; Kaetzel and Dedman, 1989) and antibodies against mammalian annexins to re-screen hydra membrane extracts for the presence of other hydra annexins.

Since the different antibodies to the conserved annexin peptide sequences have been shown to react with all annexins tested, the use of these antibodies can assist in the detection of putative annexin homologues and novel annexin species. The antibody made to the CP2 peptide sequence (Fig. 1) detected a 40-kD hydra protein that exhibited the property of reversible Ca^{2+}-dependent phospholipid binding (Fig. 2); an activity common to all known annexins.

As previously documented, most vertebrate annexins are abundant intracellular proteins and can compose up to 1% of the total cell protein in human fibroblasts (Schlaepfer and Haigler, 1990). Annexin XII was expressed at 0.3% of total hydra protein (Schlaepfer et al., 1992*b*). We were able to detect the 40-kD hydra annexin even though it was present at <1% of annexin XII levels (~0.003% of total hydra protein).Thus, it appears that hydra may only contain two different annexin gene products in contrast to the eight or more different annexins found in vertebrates. Previous studies of other gene products also showed that hydra contain less complex gene families than vertebrates (Bosch et al., 1989).

We did not pursue the search for other hydra annexins using methods such as low stringency Southern blotting techniques because previous studies with mammalian annexins have indicated that the nucleotide sequence similarities between annexin genes were not high enough for detectable cross-hybridization. The results from our immunoblot and reversible Ca^{2+}-dependent binding assays indicate that annexin XII and the 40-kD annexin may be the only annexins expressed in hydra. Of course, it is possible that other novel annexins were not detected with the various annexin-specific polyclonal antibodies used in our immunoblot assays (see Materials and Methods; Gerke, 1989; Kaetzel and Dedman, 1989), but their expression level must be quite low in hydra.

Interestingly, the size of the 40-kD hydra annexin indicates that this protein may be a novel annexin gene product not yet characterized within vertebrate systems. Of the different vertebrate annexins characterized, six vary in size from 33 to 37 kD, one is ~52 kD, and another is 68 kD. As a family, the annexins all contain a highly conserved core domain that mediates the Ca^{2+}-dependent phospholipid binding activity. Each different annexin possesses a distinctive amino-terminal domain of varying size. Homologues to the human annexins have been highly conserved between different species. Since the 40-kD hydra annexin reacted strongly with the antibody to the annexin-specific CP2 peptide sequence and since it exhibited reversible Ca^{2+}-dependent phospholipid sequence and since it exhibited reversible Ca^{2+}-dependent phospholipid binding activity (Fig. 2), the 40-kD hydra annexin most likely contains a structurally conserved core domain. In addition, the unique size of hydra 40-kD annexin indicates that this protein is not a homologue of a previously characterized annexin and that it may possess a novel amino-terminal domain not yet characterized in the annexin family.

Since hydra consist of only 15 morphologically distinct cell types (Campbell and Bode, 1983), the distribution of the two hydra annexins within the different cell types was determined in the intact animal and within isolated single cells. Indirect immunofluorescent detection of antibodies to the CP2 peptide was used to determine the cellular expression pattern of the 40-kD hydra annexin. The CP2 antibody strongly stained only the cytoplasm of the nematocytes, and to a lesser extent the cytoplasm of gland cells and large interstitial cells (Figs. 10, 11, and 12). In contrast, antibodies to annexin XII stained only the cytoplasm of the epithelial cells of the peduncle region and the tentacle battery cells (Figs. 4 *A*, 6, and 7). Not only are the two hydra annexins expressed in distinct cell types, they are expressed in separate and unrelated hydra cell lineages. The interstitial cells, gland cells and nematocytes are part of the interstitial cell lineage, while the battery and peduncle cells are part of the ectodermal epithelial cell lineage. Within the hydra, there is no interconversion between the interstitial and epithelial cell lineages.

This singular annexin expression pattern within particular hydra cell types is unique for eukaryotic organisms; annexin expression patterns in all other animals studied to date have shown that two or more different annexins are expressed as abundant proteins in all cell types. Another important aspect of the antibody immunofluorescent studies was that annexin expression was not detected in certain hydra cell types. Although one must keep in mind the limitations of the detection methods used, our results indicate that endodermal epithelial cells, nerve cells, and particular subsets of the interstitial cells in hydra may not express any annexin gene products at all. Overall, since the two hydra annexins localized to specific subsets of the total hydra cell types, it is likely that

these proteins perform specialized biological roles, and not general "housekeeping" functions which are part of the essential molecular machinery of all cells.

In hydra, the tentacles are composed of terminally differentiated epithelial cells and it was these cells that showed the greatest amount of annexin XII expression. The distinct and intense staining for annexin XII within the tentacle battery cells became apparent at the tentacle/head junction (Fig. 4 *B*). Although annexin XII expression within battery cells was not related to the event of nematocyte association with these cells (Figs. 7 and 8), increased annexin expression in the newly formed battery cells may be associated with or occurred as a direct result of the epithelial to battery cell differentiation process.

Surprisingly, the specific pattern of tentacle battery cell and ectodermal peduncle staining as documented with antibodies to annexin XII was the same as the cellular staining pattern previously elucidated with monoclonal antibodies to the TS-19 head specific antigen (Bode et al., 1988). The TS-19 antigen was characterized as a head formation pattern process marker because its expression in regenerating animals was found to precede the events of tentacle morphogenesis. Although the molecular processes involved in head formation and regeneration are not well understood, it has been shown that diacylglycerol treatment of hydra can result in the formation of multi-headed animals (Müller, 1989; Müller, 1990). The implication is that these diacylglycerol treatments may be stimulating the activity of an endogenous hydra protein kinase C species. Because annexin XII was shown to be phosphorylated by an endogenous hydra protein kinase C activity in vitro (Schlaepfer et al., 1992*b*), the potential linkage between annexin XII expression and a role in the head formation patterning process is interesting and will be pursued.

Presently, the most pressing issue in annexin research is to obtain a clear understanding of the annexin function(s) within cells. The elucidation of annexin function in vertebrates has been slow to progress because all vertebrate cells examined to date express more than one annexin and each may have overlapping physiological roles. As a result, gene disruption experiments have been complicated or hindered by the presence of two or more annexins within each cell. Compared to other eukaryotic systems, the expression of one annexin per hydra cell type will make future gene disruption experiments simpler to interpret in this animal because the resulting phenotypes will not be masked by the expression of other annexins within the same cells. In addition, since the development of all hydra cell types has been well characterized, cellular phenotypic changes resulting from annexin gene disruption and loss of function will be readily interpretable as has been shown previously by gap junction protein disruption experiments in hydra (Fraser et al., 1987).

In summary, hydra constitute a simple animal model system in which the biological role of annexins can be studied. It is the simple annexin expression pattern of one annexin per cell type that will set the foundation for the future elucidation and characterization of their specialized biological roles.

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