Annexins V and VI: Major Calcium-Dependent Atrial Secretory Granule-Binding Proteins

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Atrial natriuretic peptide is stored by atrial myocytes in secretory granules, known as atrial specific granules, and is released from these granules by exocytosis. We have isolated a group of atrial proteins by affinity chromatography that bind to atrial specific granules in a calcium-dependent manner. The two major proteins isolated (32.5 kd and 67 kd) are calcium-binding proteins and have been identified as annexins V and VI by immunoblotting with specific antisera. The calcium dependence of their binding to atrial specific granules has been characterized in vitro and indicates that this interaction takes place at micromolar levels of calcium. In addition, the group of proteins isolated includes another calcium-binding protein of 20 kd, as well as GTP-binding proteins of 22 to 26 kd. Membrane interactions during exocytosis are presumably mediated by the interaction of specific proteins with the granule membrane. The properties of the proteins described here, and their ability to bind to atrial specific granules in a calcium-dependent manner, make them likely candidates in the search for regulatory proteins mediating atrial natriuretic peptide secretion. (Hypertension 1991;18:648-656)

Atrial natriuretic peptide (ANP), a potent natriuretic, diuretic, and vasorelaxant factor, is secreted from the mammalian heart.1 Electron-dense granules present in atrial myocytes,2 known as atrial specific granules (ASG), are now recognized as the intracellular storage sites for ANP.3 These secretory granules therefore form part of the regulated secretory pathway for ANP.

In other endocrine organs, such as the adrenal medulla, calcium plays an important role in the control of the secretory pathway.4,5 Various studies have indicated that calcium is also involved in the regulation of ANP secretion from the heart.6-10 The regulatory role of calcium during hormone secretion in many noncardiac endocrine organs is mediated by calcium-binding proteins.11 Calmodulin, the ubiquitous calcium-binding protein, has been extensively studied in this regard.12 Although ANP is present in cardiac tissue,13 it appears that regulation of its secretion is not mediated by calmodulin.6,14 Members of the annexin calcium-binding protein family, such as synexin15 and calpactin,16 have also been considered as regulatory factors during exocytosis. The calcium-dependent, lipid-binding properties of annexins suggest that these proteins could regulate membrane interactions during exocytosis.17 Although immunologic data indicate that some members of the annexin family of calcium-binding proteins are present in cardiac tissue,18-21 nothing is known about their possible involvement in the regulation of ANP secretion.

In this study, calcium-dependent affinity chromatography with immobilized ASG was used to isolate atrial cytosolic proteins that may interact with atrial secretory granules during the regulated secretion of ANP. Proteins isolated include annexins V and VI, and their calcium-dependent binding to ASG has been characterized.

Methods

Granule Isolation

Atria were obtained from female Sprague-Dawley rats (weight 250-275 g) after decapitation. Granules were isolated essentially as reported earlier.3 Batches of 30 atria (from 15 rats) were processed and pooled. They were rinsed, minced, and homogenized in 0.25 M sucrose, 10 mM tris (hydroxymethyl) aminomethane...
Preparation of Postmicrosomal Supernatants

Atria from 75 rats (approximately 5 g tissue) were rinsed, minced, and homogenized in 0.3 M sucrose, 2 mM ethylene glycol-bis (aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), and 5 mM (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES) (pH 7.3). After centrifugations at 10,000g for 90 minutes, the supernatant was finally spun at 27,000g (N-\(\text{N},\text{N},\text{N}',\text{N}'\)-tetraacetic acid (EGTA), and 0.5 M NaCl, 0.1 M sodium acetate (pH 4), and 0.5 M NaCl, 0.1 M Tris (pH 8), respectively, before packing into a 10 ml column.

Preparation of Affinity Column

Granules isolated from 75 rats (approximately 5 g tissue) were dialyzed overnight at 4°C against coupling buffer (0.5 M NaCl, 0.1 M NaHCO\(_3\), pH 8.3). CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was prepared according to the manufacturer's recommendations. The granules, suspended in 15 ml coupling buffer, were added to 3 g activated Sepharose 4B, and coupling was allowed to continue overnight at 4°C. Activated groups were then blocked overnight by 1 M ethanolamine (pH 9) at 4°C. The affinity gel was washed for three alternating cycles with 0.5 M NaCl, 0.1 M sodium acetate (pH 4), and 0.5 M NaCl, 0.1 M Tris (pH 8), respectively, before packing into a 10 ml column.

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Chromatography

Before sample application, the affinity column was washed with 10 vol column buffer (0.24 M sucrose, 30 mM KCl, 1 mM MgCl\(_2\), 4 mM CaCl\(_2\), 2 mM EGTA, and 25 mM HEPES; pH 7.3), then with 2 vol column buffer with 0.1 \(\mu\)M calcium, and finally with another 5 vol column buffer. The cytosolic sample (postmicrosomal supernatant) and buffers were applied at 2 ml/min, using a Gilson peristaltic pump (Gilson, Middleton, Wis.). Thereafter, the column was washed with 12 vol column buffer. Bound protein was eluted by decreasing the calcium concentration of the column buffer to 0.1 \(\mu\)M. The first 50 ml eluted from the column was concentrated, using Centriprep-10 concentrators (Amicon, Danvers, Mass.), resuspended in 10-mM Tris (pH 7.4), and again concentrated before division into 50-\(\mu\)g aliquots and lyophilization. Protein amounts were determined according to the method of Bradford, using bovine serum albumin (BSA) as the standard.

Electrophoresis and Electroblotting

Electrophoresis was performed according to Laemmli's technique \(^2\) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a Mini-PROTEAN II system (Bio-Rad, Richmond, Calif.). Proteins were stained with silver according to the method of Sammons et al \(^24\) or with 0.1% Coomassie-R250 stain.

Proteins were blotted from polyacrylamide gels onto 0.1 \(\mu\)m nitrocellulose (Schleicher and Schuell, Keene, N.H.), using a Mini Trans-blot system (Bio-Rad). The transfer buffer was composed of 25 mM Tris (pH 8.5), 192 mM glycine, and 20% methanol.

Calcium-45 Binding

Calcium-45 (Amersham, Oakville, Canada) binding was determined essentially by the same method as that used by Maruyama et al. \(^25\) Proteins were separated by SDS-PAGE, transferred to nitrocellulose, rinsed in labeling buffer (60 mM KCl, 5 mM MgCl\(_2\), and 10 mM imidazole, pH 6.8), and incubated in the same buffer containing 1 mCi/l of \(^{45}\text{Ca}\) (0.645 \(\mu\)M) for 10 minutes. After rinsing and drying, the membrane was autoradiographed (72-120 hours) using Kodak XRP 6 film (Eastman Kodak Company, Rochester, N.Y.). For molecular mass determination, protein standards were electrophoresed and transferred to nitrocellulose in parallel with the samples. Nitrocellulose was stained for 1 minute with 0.03% Coomassie stain and destained with methanol:acetic acid:water (5:1:5).

\(\text{[}^{32}\text{P}\text{]}\text{GTP Binding}

\(\text{[}^{32}\text{P}\text{]}\text{GTP binding was carried out essentially according to the methodology of Bhullar and Haslam, except for first incubating the nitrocellulose for 30 minutes in GTP labeling buffer (50 mM Tris (pH 7.5), 2 \(\mu\)M MgCl\(_2\), 0.3% Tween 20) containing 0.3% BSA. The nitrocellulose was then incubated in 1 ml of \(\text{[}^{32}\text{P}\text{]}\text{GTP for 30 minutes.} \)\(\text{[}^{32}\text{P}\text{]}\text{GTP was detected by autoradiography (12-72 hours) using Kodak XRP 6 film. For molecular mass determinations, protein standards were run in parallel as for \(^{45}\text{Ca} binding.}

\(\text{Liposome Binding}

A modification of the method published by Genge et al \(^27\) was used. Phosphatidyl serine (Sigma Chemical Co., St. Louis, Mo.), obtained as a chloroform solution, was evaporated under \(\text{N}_2\), resuspended in liposome buffer (10 mM Tris (pH 7.5), 100 mM KCl, 1 mM dithiothreitol), and sonicated to give a liposome stock suspension containing 4 mg lipid/ml. Ten microliters of calcium-binding proteins were then added to liposomes (400 \(\mu\)g/ml) in an incubation mixture containing 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 2 mM EGTA, and 2.5 mM MgCl\(_2\), either in the absence or presence of free calcium concentrations of 10 \(\mu\)M or 1 mM, respec-
cally. The samples were incubated at room temperature for 20 minutes and were spun in a Beckman Airfuge (Beckman Instruments, Fullerton, Calif.) at 28 PSI for 20 minutes to sediment the liposomes and associated proteins. Equivalent portions of the supernatant and pellet were assayed by SDS-PAGE.

Isolation of Other Membranes

Cardiac (nongranule) membranes were obtained during the preparation of ASG. These membranes were separated from granules as a low density band in a Percoll gradient as described previously. The preparation consisted mainly of broken cell membranes, microsomes, mitochondria, and lysosomes with very few granules.

Noncardiac membranes were prepared as inside-out vesicles from rat red blood cells as described by Garcia-Sancho and Alvarez.

Quantification of Membrane Content

Membrane amounts were determined by quantitating their phospholipid content. Total lipid content was extracted according to Bligh and Dyer. Extracted lipids were dried under nitrogen and hydrolyzed in H2SO4 at 200°C for 2 hours. The phosphate content was then measured according to the method of Lanzetta et al.

Protein Iodination

Iodine-125 was obtained from Amersham. The eluted proteins were either iodinated as a mixture of the various proteins using the lactoperoxidase method, or individually, in the case of the 32.5 kd and 67 kd proteins. For this purpose the proteins were separated by ion-exchange, high-pressure liquid chromatography on a Spherogel-TSK DEAE 5PW column (7.5 mm x 7.5 cm) (Beckman Instruments, Altex Division, San Ramon, Calif.). The 32.5 kd protein was iodinated by the lactoperoxidase method, and the 67 kd protein by using Iodobeads (Pierce, Rockford, Ill.). Iodinated proteins were separated from free iodine-125 on a Sephadex G25 column (Pharmacia).

Binding to Atrial Specific Granules and Other Membranes

ASG, cardiac (nongranule) membranes, and noncardiac membranes (inside-out red blood cell vesicles) were prepared as described above. All preparations were washed three times in 20 mM Tris (pH 7.4), 0.25 M sucrose, 30 mM KCl, 0.5 mM dithiotreitol, and 15 mM EDTA. To allow accurate comparison of binding to the various membranes, equal amounts of each membrane, as determined by their phospholipid content, were used in each experiment. Isolated granules (or other membranes) were allowed to react with labeled protein (1,000 cpm/μl) at room temperature for 20 minutes in a reaction mixture containing 20 mM Tris (pH 7.4), 0.25 M sucrose, 30 mM KCl, 0.5 mM dithiotreitol, and 5 mM EDTA, either in the absence or presence of various calcium concentrations. Calcium concentrations were calculated by the use of a computer program as described by Moreland and Murphy. All tubes were precoated with BSA to minimize nonspecific binding. After incubation, membrane-bound radioactivity was collected by centrifugation in an Airfuge for 20 minutes at 28 PSI. Equal portions of supernatant and pellet were then subjected to SDS-PAGE and transferred to nitrocellulose, and iodinated proteins were detected by autoradiography with Kodak XRP 6 film (12-48 hours). Alternatively, binding was evaluated by counting membrane-bound radioactivity and expressing binding as counts per minute per picomole phospholipid. Statistical analysis was carried out using the nonparametric Wilcoxon signed rank test.

Immunoblotting

Proteins separated by SDS-PAGE were electroblotted onto 0.1 μm nitrocellulose membranes as described above. The membranes were incubated at room temperature for 60 minutes in blotting buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 0.2% BSA, and 0.1% γ-globulin) supplemented with 5% nonfat, dry milk (Carnation brand). The membranes were then incubated with primary antibodies for 90 minutes in blotting buffer. Anti-calelectrin 67 (anti-annexin VI), a gift from Dr. Thomas Sudhof, was used at a dilution of 1:1,000; anti-lipocortins I, II, III, and V, a gift from Dr. Blake Pepinsky, were used at a dilution of 1:2,000, and antibodies to Protein II, a gift from Dr. Volker Gerke, were used at a dilution of 1:200. Finally, the membranes were incubated with secondary antibodies at a 1:2,000 dilution in blotting buffer (horseradish-peroxidase conjugated, goat anti-rabbit immunoglobulin G) (Bio-Rad). The color reaction uses diaminobenzidine-Ni and H2O2 as substrates.

Results

An affinity column was prepared by linking ASG to a Sepharose-4B matrix. After the application of atrial cytosolic proteins to the affinity column, the column was extensively washed. Optical density measurement and SDS-PAGE analysis of the wash fractions (Figure 1) indicate that virtually no protein is present in the final wash. A group of proteins remained bound to the column and were eluted by lowering the calcium concentration of the column buffer to 0.1 μM. No proteins could be eluted from the column by lowering the calcium concentration before sample application (Figure 1), indicating that proteins eluted after sample application do not represent "leaking" of granule proteins bound to the Sepharose column. Typically, 300 μg protein was recovered from the column, with 100 mg atrial cytosolic proteins as starting material. Coomassie staining identified two major bands (Figure 1) with apparent molecular masses of 67 kd and 32.5 kd, respectively. With silver staining (Figure 1), a number of additional minor bands could be observed, notably in the 15-20, 22-26, 30-36, and 40-58 kd ranges.
The ability of the eluted proteins to bind calcium was evaluated by $^{45}$Ca binding after their transfer to nitrocellulose. Figure 2 illustrates $^{45}$Ca binding by both the major bands, that is, the 67 kd and 32.5 kd bands. In addition, a minor band of 20 kd also bound $^{45}$Ca.

The synergistic role played by calcium and GTP in the regulation of some secretory models prompted an evaluation of the ability of the eluted proteins to bind GTP.34 We found that none of the major bands bound GTP, but a group of minor GTP-binding bands in the 22–26 kd range was detected (Figure 3).

The ability of the eluted proteins to bind to phospholipid membranes in a calcium-dependent manner was also evaluated. Figure 4 demonstrates the binding of eluted proteins to phosphatidyl serine liposomes at various calcium concentrations. With excess EGTA, most of the proteins remained in the supernatant, indicating that they do not bind to liposomes in the absence of calcium. At 10 µM calcium, more than 50% of the protein is liposome-bound, and at 1 mM calcium, most of the protein was bound to liposomes.

The experimental procedure used to isolate the proteins implies that eluted proteins bind ASG in a...
Calcium-dependent binding of eluted proteins to phosphatidyl serine (PS) liposomes. Eluted proteins incubated with PS liposomes, and proteins binding to liposomes were separated from unbound proteins by centrifugation. Lanes 2, 4, and 6 show proteins found in supernatants (S); lanes 1, 3, and 5 reveal proteins in pellets (P). Three different calcium concentrations were used: Excess EGTA in lanes 1 and 2 (a), 10 \( \mu \)M calcium in lanes 3 and 4 (b), and 1 mM calcium in lanes 5 and 6 (c).

calcium-dependent manner. This assumption was also tested directly to exclude the possibility of nonspecific binding to the Sepharose column. Initial experiments (with use of an iodinated sample of the mixture of eluted proteins and performed in a similar fashion to the experiment undertaken to evaluate calcium-dependent liposome binding) indicated that with excess EGTA, most of both the two major proteins remained in the supernatant; at 10 \( \mu \)M calcium, most of the protein was in the pellet, indicating granule binding; and at 1 mM calcium, almost no protein remained in the supernatant (data not shown).

To enable more complete characterization of this calcium-dependent interaction, the 32.5 kd and 67 kd proteins were assessed individually at calcium concentrations ranging from 10\(^{-8}\) to 10\(^{-1}\) M (Figure 5). The binding profiles observed for the two proteins were similar. Very little binding occurred below 10\(^{-7}\) M. Binding was increased significantly as the calcium concentration was raised through 10\(^{-6}\) M to 10\(^{-5}\) M. Relatively little further increase in binding was seen when the calcium concentration was raised above 10\(^{-4}\) M. With both proteins, there was a tendency toward a biphasic pattern, with a slight dip in binding occurring at 10\(^{-3}\) M.

To evaluate further the specificity of the interaction of these two proteins with ASG, the binding to ASG membranes was compared with their binding to other nongranule membranes (Figure 6). This included binding to cardiac (nongranule) and noncardiac (inside-out red blood cell vesicles) membranes. Based on the binding profiles described above, binding to the three membranes was compared at a calcium concentration of 10\(^{-5}\) M. Although all three membranes examined did bind both of the proteins, the binding to ASGs was significantly higher \((p<0.05)\) than the binding to other cardiac and nongranular membranes for both proteins.

The two major proteins were then identified by immunoblotting, using specific antisera to candidate proteins (Figure 7). The 67 kd band reacted specifically with anti-callelectrin 67 (annexin VI) antibodies. The 32.5 kd band was recognized specifically by anti-lipocortin V (annexin V) antibodies. Some cross-reaction was observed with antibodies against lipocortin I (annexin I) and protein II (annexin IV) when using excess protein (20 times more protein than that needed for visualization by antibodies to lipocortin V).

**Discussion**

Chromatography, using isolated ASG as an affinity matrix, provides a powerful method for isolating soluble atrial proteins possessing calcium-dependent
Atrial Secretory Granule-Binding Proteins

**Figure 6.** Bar graph shows binding of 32.5 kd and 67 kd proteins to atrial specific granules, compared with binding to other membranes. Atrial specific granules, cardiac (nongranule) membranes, and noncardiac membranes (inside-out red blood cell vesicles) prepared as described in "Methods." Equal amounts of each membrane, as determined by phospholipid content, were incubated with one or the other of two proteins labeled with 125I (1,000 cpm/µl) for 20 minutes at calcium concentration of 10^{-5} M. Binding expressed as counts per minute per picomole phospholipid. Data for 32.5 kd protein represented by solid bars and for 67 kd protein by hatched bars. Each value represents mean±SEM (n=7). *p<0.05.

A prerequisite for applying the technique is a pure sample of isolated granules. The methodology we used in this study has been shown to yield granules of very high purity, with minimal lysosomal contamination. A notable difference between the results obtained in this study and those reported in the adrenal medulla investigation is the relatively small number of proteins isolated from cardiac tissue (Figure 1). This probably reflects differences in the methods we used, such as the more extensive washing of the column before eluting the proteins at a low calcium concentration, although the relative amounts of various proteins present in the two tissues obviously also play a role.

Two major granule-binding proteins were isolated from atrial tissue, with apparent molecular masses on electrophoresis of 67 kd and 32.5 kd, respectively. Experiments designed to characterize and identify these two major proteins provided valuable information about some of the minor bands as well. Calcium-dependent binding of soluble proteins to ASG is assumed to be due to calcium binding by the soluble proteins, leading to a conformational change in these proteins that promotes granule binding. However, granule binding can be equally well explained by a calcium-induced conformational change in a granule protein, increasing its affinity for a particular soluble protein that does not bind calcium itself. Alternatively, a soluble protein may be bound to ASG via a true calcium-binding protein that provides a calcium-dependent link between the soluble protein and the granule membrane, without the soluble protein or granule protein itself binding calcium. We therefore examined the ability of the isolated proteins to bind 45Ca (Figure 2). The results confirm that both the major bands isolated are true calcium-binding proteins. This calcium binding was demonstrated at a 45Ca concentration of 0.645 µM, suggesting that these proteins have an intermediate affinity for calcium. However, the respective $K_c$ values for calcium binding by the two proteins have yet to be accurately measured.

An additional calcium-binding protein of 20 kd was also demonstrated. This protein was much less abundant in the sample than the 67 kd and 32.5 kd proteins, yet the 45Ca signals of the 67 kd and 20 kd proteins were comparable. This suggests that the calcium affinity of the 20 kd protein is considerably higher than that of the two major bands. The low quantity in which it was isolated has so far prevented us from identifying the 20 kd protein, and the possibility that it represents one of the known high-affinity calcium-binding proteins, such as calmodulin, has not yet been excluded.

Interest in the involvement of GTP-binding proteins in the regulation of the secretory apparatus of various cells has recently grown considerably, mainly due to the study of secretion-deficient mutants of the yeast *Saccharomyces cerevisiae*. These investigations have led to the detection of various GTP-binding proteins that regulate specific steps in vesicle trans-
port in secretory cells, including the transport of secretory vesicles to the cell surface. The term $G_\text{G}$ has been proposed to describe a guanine-nucleotide-binding protein putatively present on secretory granule membranes with a capacity to direct granule transport. Recently, Burgoyne and Morgan reported the presence of GTP-binding proteins on adrenal medulla secretory granules. The involvement of both calcium and GTP-binding proteins in the regulation of secretion has been proposed in various studies. This concept prompted us to investigate the GTP-binding abilities of the isolated granule-binding proteins. A group of GTP-binding proteins in the 22–26 kd range was detected (Figure 3). It is interesting to note that the GTP-binding proteins known to regulate vesicle transport in yeast are all in the 18–26 kd range, as are the GTP-binding proteins detected on adrenal medullary secretory granules. This leads us to speculate that the soluble GTP-binding proteins detected here may represent the cardiac equivalent of a $G_\text{G}$ protein involved in regulating ANP secretion.

The ability of the two major proteins to bind to phosphatidyl serine liposomes (Figure 4) is shared by many members of the annexin family of calcium-binding proteins. They characteristically bind to membranes composed of acid phospholipids. The cytosolic surfaces of cell membranes are enriched in these phospholipids. This provides a potential mechanism whereby interaction between the secretory granules and the plasma membrane can be mediated by annexins. The demonstration of ASG binding by the isolated proteins (Figure 5) implies that the correct phospholipid composition, or proteins that bind the isolated soluble proteins, or both, must be present in the granules. It also supports the fact that the proteins were isolated by using their ability to bind to ASG in a calcium-dependent fashion, and that their isolation is not due to nonspecific calcium-dependent binding to the column. The specificity of the interaction of the two major proteins with granules is further supported by the findings of the comparative binding studies shown in Figure 6. Although both the 32.5 kd and 67 kd proteins interacted with noncardiac membranes and cardiac nongranule membranes, in addition to ASGs, the binding to granules was significantly higher than the binding to other membranes.

The calcium dependence of ASG binding by the 32.5 kd and 67 kd proteins shown in Figure 5 illustrates some important points. Substantial increases in binding are seen as the calcium concentration is raised through $10^{-6}$ M to $10^{-4}$ M, with near-maximal binding occurring at $10^{-3}$ M. The normal intracellular calcium concentration in cardiac myocytes probably varies between $10^{-7}$ and $10^{-5}$ M. Allowing for in vivo factors that may further modify this interaction with ASG and for spatial heterogeneity of intracellular calcium concentration, which can provide microdomains with relatively high calcium concentrations, it seems reasonable that these in vitro observations are compatible with a physiological role for this calcium-dependent binding to ASG. In contrast, the tendency toward a biphasic response observed as calcium concentrations are increased further is unlikely to reflect a physiological event. The very high calcium concentrations needed to observe the second phase of the response make it unlikely that this response is of physiological significance. These high calcium concentrations were included in this study due to the marked biphasic response reported in a study of the binding of annexins to chromaffin granules.

Using specific antisera, the identities of the two major proteins were determined. Both proteins displayed calcium-binding and calcium-dependent, lipid-binding properties (Figures 2 and 4) compatible with being annexins. Antibodies against calelectrin confirmed that this protein represents the high molecular weight member of the annexin family, which we refer to as annexin VI (Figure 7). After cloning of the complementary DNA (cDNA) for calelectrin, it has become evident that calelectrin and calcimedin are probably all derived from the same gene. However, it must be noted that protein III was reported to be specifically absent from cardiac tissue and the possibility of cardiac-specific isotypes of annexin VI must be considered.

In trying to identify the 32.5 kd protein, many more members of the annexin family have to be considered. On the basis of molecular weight, annexin IV (protein II) and annexin V (lipocortin V) are the most likely candidates, but other annexins in the 30–36 kd range, such as annexin I (lipocortin I), annexin III, chromobindin II, lipocortin VI, and calphobindin II are probably all derived from the same gene. cDNA for both rat and human annexin V has been cloned and confirms that lipocortin V and endonexin II, PAP, PP4, and calphobindin I are all products of the same gene. We refer to this protein as annexin V.

With higher amounts of protein, some cross-reaction with antibodies to lipocortin I (annexin I) and protein II (annexin IV) was observed. Annexin I is known to have a narrow tissue distribution, and has not been found in cardiac tissue. Protein II also has been reported to be absent from cardiac tissue or present only in very low amounts. It is very likely that the cross-reaction with antibodies to annexins I and IV seen at higher protein concentrations reflects nonspecific cross-reaction that one could expect when dealing with a family of proteins sharing a high degree of structural homology, such as the annexins.
group is still controversial, regulation of exocytosis has been suggested to be one of their main functions in secretory cells. Functions as diverse as regulation of cell differentiation in response to growth factors, mediation of the anti-inflammatory effects of steroids, anticoagulation, and control of cytoskeletal organization have also been attributed to the annexins. The calcium-dependent association with ASG demonstrated in this study makes it attractive to speculate that they are involved in the secretory control of ANP.

However, we have isolated the same two annexins from ventricular cytosol (unpublished observations from our laboratory). Ventricular myocytes contain only small secretory vesicles and have been reported to secrete ANP in a constitutive rather than a regulated manner. Therefore, the fact that ventricular cytosol contains the same major annexins as atrial cytosol may reflect involvement of these proteins in the basic secretory process in both the regulated and constitutive pathways, rather than a specific role in mediating calcium-regulated secretion of ANP from the heart. Recent observations of the cellular distribution of the two cardiac annexins suggests another explanation. Immunocytochemical analysis (unpublished observations from our laboratory) has shown that in atrial tissue, immunoreactivity is found mostly in myocytes, but in ventricular tissue, significant immunoreactivity localizes to vascular tissue. Therefore, the finding of significant amounts of these annexins in both atrial and ventricular tissue does not necessarily reflect a common function in the two tissues.

In conclusion, a group of atrial proteins that manifest a calcium-dependent interaction with ASG has been isolated. The 67 kd and 32.5 kd proteins isolated are members of the annexin family of calcium-binding proteins. Immunologic data indicate that the 67 kd protein is annexin VI, and the 32.5 kd protein is annexin V. These two annexins bind to ASG at calcium concentrations compatible with a physiological role for this interaction. An additional calcium-binding protein of 20 kd and GTP-binding proteins in the 22-26 kd range were also isolated. Further study of these proteins will provide insight into their functional role for this interaction. An additional calcium-concentration compatible with a physiological role for this interaction. An additional calcium-binding protein of 20 kd and GTP-binding proteins in the 22-26 kd range were also isolated. Further study of these proteins will provide insight into their functional role for this interaction.

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