Chromaffin cells were prepermeabilized for 10 min and then incubated without or with anti-Ac-calpactin (1-15)-NH₂ next 20 min was then determined. Anti-Ac-calpactin had no effect on basal catecholamine secretion in the absence of Ca²⁺, but increased the extent of secretion due to the addition of arachidonic acid. Activation of chromaffin cells stimulates release of free arachidonic acid

which has been implicated in exocytosis. We have recently found, however, that arachidonic acid release is not required for Ca²⁺-dependent exocytosis in digitonin-permeabilized cells [21]. It may be that calpactin acts to link secretory granules to the plasma membrane following a rise in [Ca²⁺], and that an additional component at the exocytotic site then acts as a membrane fusogen to result in exocytotic fusion and release of vesicle contents.

Work in the authors' laboratory was supported by grants from the M.R.C.


Received 29 June 1990

Annexin 1 is secreted by the human prostate

HARRY T. HAIGLER and PETER CHRISTMAS
Department of Physiology and Biophysics, University of California, Irvine, CA 92717, U.S.A.

Introduction
Annexins are a structurally related family of Ca²⁺- and phospholipid-binding proteins [1, 2]. Each protein contains two structural domains: a small N-terminal domain and a core domain that is formed by either a 4- or 8-fold repeat of a conserved segment containing approximately 70 amino acids. Different annexins share little sequence similarity in the N-terminal domain, but have approximately 50% sequence identity in the core domain. The Ca²⁺-binding sites reside in the core domain. The N-terminal domain is the site of phosphorylation of annexin 1 [3–5] and annexin 2 [6] by tyrosine kinases, and the site of phosphorylation of several annexins by protein kinase C [7]. Neither the biological function of these proteins nor the role of phosphorylation is known. Recent studies have focused on their potential roles in intracellular Ca²⁺-mediated processes. It has been proposed that annexins also have an extracellular site of action, but additional studies are required to resolve this issue. The primary translation products predicted from annexin cDNA sequences do not contain a hydrophobic signal sequence [8]. Structural analysis of purified mammalian annexin proteins indicate that their N-termini are blocked and, when determined, the blocking group was identified as an acetylation. These structural
characteristics are consistent with annexins being intracellular proteins. However, certain of the annexins have been detected in low concentrations in peritoneal exudates [8]. In fact, some of the pivotal studies on annexins have been on proteins purified from this source [9]. It is not known if these extracellular annexins were secreted by novel cellular pathways or were released from lysed cells. In addition to the annexins, a number of other proteins such as interleukin 1 and fibroblast growth factor have been detected in extracellular fluids, although their primary structures indicate that they should be intracellular [10].

To investigate possible physiological roles for extracellular annexins, and to define a system with which to study the cellular mechanism by which annexins reach an extracellular location, we screened extracellular fluids for the presence of annexin 1. We found that the human prostate gland secretes high concentrations of annexin 1.

Results and discussion

Seminal plasma contains high concentrations of annexin 1. Immunoblot analysis of human semen identified two protein bands that reacted with polyclonal antiserum specific to annexin 1, but not with preimmune serum or immune serum adsorbed with placental annexin 1. One immunoreactive band had the same apparent M, (35 000) as annexin 1 and the second had a lower apparent M, (32 000). Experiments presented below showed that the bands were proteolytic cleavage product of annexin 1 missing the first 29 amino acids. After centrifugation of semen, greater than 99% of immunoreactivity was in the supernatant, thereby indicating that the majority of these proteins were in the seminal plasma and not within sperm or other cells. This conclusion is supported by the observation that seminal plasma from vasectomized patients contained the same amount of these immunoreactive proteins. These data are not inconsistent with a previous study that detected low concentrations of annexin 1 in sperm cells [11]. Our studies extend this previous report and show that there is more than 100 times more annexin 1 in the seminal plasma than in sperm.

The concentrations of annexin 1 and des(1-29)-annexin 1 in seminal plasma were estimated by comparing the immunostaining intensity of immunoreactive bands with known standards of placental annexins. Seminal plasma from five different individuals contained 10-40 μg/ml (mean 27; s.e.m. 4.8) annexin 1 and 20-50 μg/ml mean 33; s.e.m. 5.4) des(1-29)-annexin 1. Addition of EGTA to semen (final concentration 10 mM) before centrifugation did not alter the recovery of immunoreactive proteins in seminal plasma. Seminal plasma also was assayed for annexins 4 and 5. Immune serum specific to annexin 5 identified a single immunoreactive band (apparent M, 33 000) and its concentration in seminal plasma was approximately 20 μg/ml. Only trace amounts (<0.1 μg/ml) of annexin 4 were detected.

Extracellular proteins in semen are either secreted by the accessory sex glands or transudated from the blood. The seminal plasma to blood plasma concentration ratios of albumin provides a marker for transudation, and proteins with higher concentration ratios than albumin are assumed to be locally secreted [12]. Blood plasma was assayed for annexins by immunoblotting and was found to contain approximately 0.05 μg of annexin 1/ml and 0.5 μg of annexin 5/ml. No proteolytic cleavage products of annexin 1 were detected in blood plasma. The seminal plasma to blood plasma concentration ratios of annexins 1 and 5 are greater than the concentration ratio of albumin by a factor of more than 104. This suggests that the proteins are actively secreted by the accessory sex glands.

Characterization of secreted annexins. The characteristics of seminal plasma annexins were compared with those of annexins purified from intracellular sources by two-dimensional gel analysis, Ca2+-dependent phospholipid-binding activity, amino acid composition analysis and partial amino acid sequencing.

Seminal plasma was analysed by two-dimensional gel electrophoresis followed by immunoblotting with antisera against either annexin 1 or 5. Two spots that reacted with antisera against annexin 1 were observed. These spots had apparent M, values and pI values that are indistinguishable from those of placental annexin 1 and des(1-29)-annexin 1. A single spot that reacted with antisera against annexin 5 was observed at a location expected for placentental annexin 5. The two-dimensional gel system has been optimized for separating forms of annexin 1 that have undergone post-translational modification [7]. It can readily separate forms of placentental annexin 1 differing by one unit charge. Since the secreted and intracellular annexins had indistinguishable migrations on this sensitive gel system, their structures appear to be very similar or identical.

Seminal plasma annexins were purified by exploiting their reversible Ca2+-binding dependency to phospholipids. A phospholipid affinity column [13] quantitatively removed annexins 1 and 5 and des(1-29)-annexin 1 from seminal plasma. After washing the column with Hepes buffer containing 1 mM-CaCl2, the adsorbed annexins could be recovered by elution with 5 mM-EGTA. The affinity column gave a yield of approximately 10 μg of annexin/ml of seminal plasma.

The M, 32 000 protein that reacted with anti-annexin 1 was further purified from the EGTA eluate of the phospholipid affinity column by cation exchange on an f.p.l.c. mono S column. Five cycles of automated Edman degradation on the purified protein yielded the sequence Gly-Gly-Pro-Gly-Ser. This corresponds to the N-terminal sequence of intracellular annexin 1 following a proteolytic clep by plasmin [14].

Purified annexin 1 from seminal plasma was subjected to structural analysis. Its amino acid composition was within experimental error of that of placentental annexin 1, but it did not reveal any Edman degradation products. It, therefore, appears to have a blocked N-terminal. Structural analysis of annexin 5 purified from seminal plasma by this method yielded an identical conclusion.

Tissue source of secreted annexins. The extracellular location of annexins in seminal plasma and their high seminal plasma to blood plasma concentration ratios suggest an active secretion by the accessory sex glands. The secretions that make up seminal plasma are predominantly from the seminal vesicles (approximately 75%) and prostate (approximately 25%) [15]. The epididymis is excluded as a possible source because the concentrations of annexins in seminal plasma are not changed by vasectomy (see above).

Seminal vesicle tissue, prostate tissue, and secretory fluids from both tissues were analysed by immunoblotting using antisera against annexins 1, 4 and 5. Only annexin 5 was found in appreciable quantities in seminal vesicle tissue, and none of the annexins was detected in seminal vesicle fluid. However, all three annexins were present at very high levels in prostate tissue. The concentration of each annexin was approximately 200-300 μg/g wet wt. prostate tissue. This corresponds to 0.4-0.6% of the total protein in the tissue homogenate. Anti-annexin 1 stained a single band with apparent M, 35 000 in prostate tissue. In contrast to seminal plasma, no lower M, bands were detected. Prostate fluid contained high concentrations of both the M, 35 000 (60 μg/ml) and M, 32 000 (80 μg/ml) bands with annexin 1 immunoreactivity. It also contained high concentrations of immunoreactive annexin 5 (60 μg/ml). Interestingly, only trace amounts (less than 0.1 μg/ml) of immunoreactive annexin 4 were detected in prostate fluid, despite its high level of expression in prostate tissue. It is unlikely that annexin 4 is
secreted then degraded by endogenous proteases, because
placental annexin 4 was not degraded when added to pro-
state fluid and incubated at 37°C. The collective concen-
trations of annexin 1, des-(1-29)-annexin 1, and annexin 5 in
prostate fluid and seminal plasma were 1.3% and 0.2% of the
total protein, respectively. This is consistent with their secri-
tion by the prostate and subsequent dilution of seminal vesicle fluid.

Summary

The human prostate expresses very high concentrations of
annexins 1, 4, and 5 and secretes high concentrations of
annexins 1 and 5. Although the biological roles of these pro-
teins in prostate secretions are not known, these studies emphasize the need to consider extracellular sites for physi-
ological functions of annexins. The clear demonstration of
secretion of proteins that have blocked N-termini and lack
hydrophobic signal sequences raises the possibility that novel
cellular secretory pathways exist. Preliminary immunohisto-
chemical experiments in collaboration with Dr James Fallon
indicate that both annexins 1 and 4 are expressed in prostate
ductal secretory epithelium. Since annexin 1, but not annexin
4, is secreted, a comparison of the cellular fate of these two
related proteins in the prostate may provide a useful model
system for determining the structural elements that direct
the secretion of proteins which lack conventional signal
sequences.

(Cambridge, Mass.) 55, 1-3

Tyrosine kinase substrate annexin II (p36) — biochemical characterization and conservation among species

VOLKER GERKE

Max Planck Institute for Biophysical Chemistry, Department of Biochemistry, D-3400 Göttingen, F.R.G.

Introduction

Annexin II (p36) is a member of the annexin family of
Ca2+- and phospholipid-binding proteins [for review see
[1-5]]. Like other proteins which belong to this multigene
family, annexin II shows a characteristic structural feature,
the so-called annexin repeat. This term describes a segment
70-80 amino acids in length which is repeated four times
along the annexin II polypeptide chain. The annexin repeats
exhibit homologies with one another not only within annexin
II but also with other members of the family [1-5]. Typically,
given a repeat shows a higher degree of conservation between distinct members of the family than to other repeats in
the same protein. The sum of the four repeats in annexin II
forms the so-called protein core, which is resistant to mild
proteolytic treatment. In the primary structure, this core is
preceded by a proteinase-sensitive N-terminal tail, some 30
residues in length. Biochemical characterization of the
proteolytic derivatives revealed that the protein core harbours binding sites for Ca2+, phospholipids and cyto-
skeletal proteins, such as F-actin and non-erythroid spectrin.
Since the ability to interact with phospholipids in a Ca2+-de-
pendent manner is shared with all other annexins, it is believed that a highly conserved stretch of 17 amino acids
(the so-called endonexin fold), which is found in each
annexin repeat of every member of the family, is involved in
the interaction with these ligands. The N-terminal tail of
annexin II is not required for Ca2+-, phospholipid- or
F-actin-binding, but harbours different phosphorylation sites and thus is believed to represent an important regulatory
region of the molecule. Tyr-23 is phosphorylated by pp60crk,
while Ser-25 is a site for protein kinase C phosphorylation.
Consequently, annexin II was originally identified as a major
cytosplasmic substrate for the src-encoded tyrosine kinase
[for review see [6]].

Annexin II is so far the only annexin which has been shown to interact with a cellular protein ligand, p11, both in vitro and in vivo [for review see [3]]. Interaction leads to the
formation of a tetrameric complex, which comprises two
annexin II and two p11 chains. p11 binding is probably an
important regulatory event, since it modulates properties dis-
played by annexin II. While monomeric annexin II shows
50% binding to liposomes at 10 μM-free Ca2+, annexin II
complexed with p11 only needs 10 nm-Ca2+ for the same
interaction [7]. Similarly, the annexin II-p11 complex is able to induce chromaffin granule aggregation and even fusion of
these vesicles at much lower Ca2+ concentrations than
monomeric annexin II [8]. The p11-binding site is also
located within the N-terminal tail of annexin II. However,
while the phosphorylation sites cluster between residues 20
and 30, only the 12 N-terminal amino acids are required for
p11 binding. This region of the molecule forms an amphi-
pathic helix with the hydrophilic face of this helix probably
being the actual p11 contact site [9].

p11 itself belongs to a different multigene family showing
sequence homologies to the S100 proteins and other related
polypeptides like calsecin or the calgranulins [for review see
[10]]. Members of this protein family are characterized by

Received 13 July 1990