CALCIUM-BINDING PHOSPHOPROTEIN: THE PRINCIPAL ACIDIC PROTEIN OF MAMMALIAN SPERM*

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SUMMARY: A calcium-binding phosphoprotein previously found only in brain and adrenal medulla has been isolated from the adult bovine testis. The testicular protein is electrophoretically indistinguishable from the protein of adult bovine brain and adrenal medulla in 15% polyacrylamide gels at pH 8.3 and 4.7. Crude homogenates and acidic protein fractions of adult testis, fetal testis and epididymal spermatozoa were examined electrophoretically for the presence of this calcium-binding protein. The protein was present in homogenates of adult testis and epididymal spermatozoa but only to limited extent in the homogenate of fetal testis. It was the major acidic protein of spermatozoa. It appears likely that the calcium-binding protein evident in adult testicular tissue is contributed largely by the developing spermatozoa.

INTRODUCTION

A variety of physiological studies have demonstrated the essential role of extracellular calcium in neurotransmission (1). On the basis of such studies, previous efforts in this laboratory were directed toward the isolation of calcium-binding proteins which might function as the putative neuronal calcium receptor whose existence has been postulated by Katz and Miledi (2). Two such proteins were isolated from adult pig brain and one of these was found to be a phosphoprotein (3). Extracts of bovine adrenal medulla, a tissue specialized for catecholamine secretion, contained a calcium-binding protein which was found to be identical to the phosphoprotein of bovine brain (4).

No significant calcium-binding activity was found when a variety of non-nervous tissues, including adrenal cortex, were examined by these same techniques. However, we have recently found that extracts of bovine testis contain

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large amounts of calcium-binding activity. It was of particular interest to examine testis because of the observation that brain and testis contain a number of common antigenic determinants not present in other tissues (5). We report here the isolation of a calcium-binding protein from bovine testis which is identical to the calcium-binding phosphoprotein of bovine brain and adrenal medulla and trace its presence to the developing spermatozoa of adult testis.

MATERIALS AND METHODS

Calcium-binding protein was prepared from bovine adrenal medulla as previously described (4) except that the final purification step involving gel filtration on Sephadex G-50 was replaced by chromatography on hydroxylapatite. For this chromatography the protein sample from Sephadex G-100 was concentrated to 2 ml on a Dia-Flo PM-10 membrane, dialyzed against one liter of 20 mM potassium phosphate buffer at pH 6.8, and applied to a 15 x 0.9 cm column of hydroxylapatite equilibrated with the same buffer. The column was developed with gravity flow and 2 ml fractions were collected. The calcium-binding protein did not adsorb under these conditions and eluted as a homogenous protein (3).

Adult bovine testes were kept in ice during transport from the slaughter house to the laboratory. The connective-tissue capsule was slit lengthwise with a scalpel and the soft testicular tissue removed. This tissue was divided into two portions and frozen at -70° until used. Calcium-binding protein was prepared from the testicular tissue exactly as described for adrenal medulla (4). For some of the experiments reported here, the crude tissue homogenate was heated for 3 min at 90° and centrifuged at 5° for 60 min at 95,500 x g. Calcium-binding protein was prepared from the supernatant by gel filtration on Sephadex G-100 and chromatography on hydroxylapatite.

Testes were obtained from fetal animals 15-40 cm in length. The gubernaculum was removed and the entire testis used for the preparation of calcium-binding protein. Epididymal spermatozoa were collected as described by Henle

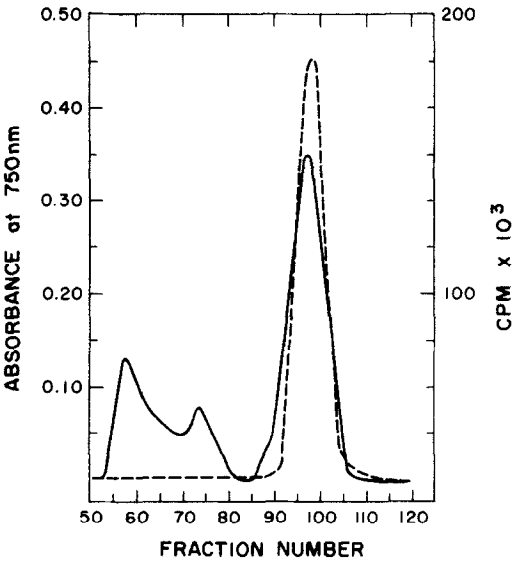
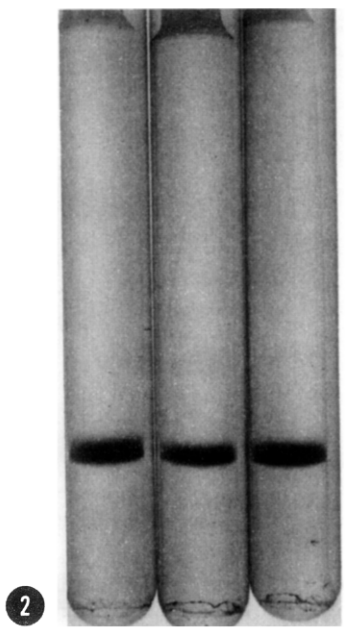


Figure 1. Gel-filtration on Sephadex G-100 of an acidic protein fraction from bovine testis in 50 mM Tris Cl, pH 7.4, in the presence of $^{45}CaCl_2$. Fractions of 2 ml were collected. The solid line represents protein determined by the Lowry procedure (10) and the broken line radioactivity.

(6), washed twice with collecting medium as described by Garbers et al. (7) and frozen as a pellet at -70° after the final centrifugation.

Homogenates prepared from equal amounts (10 gm wet weight) of adult testis, fetal testis and spermatozoa were compared for their content of calcium-binding protein. Thawed tissues were homogenized for 2 min at full speed in a Waring blendor with 15 volumes of 50 mM Tris Cl, pH 7.4 and the homogenate centrifuged for 60 min at 95,500 x g. Solid ammonium sulfate (28 gm/100 ml) was added to the supernatant solutions and the mixtures allowed to stand for 5 hr. The suspensions were centrifuged for 10 min at 30,000 x g and the supernatants taken to 100% of saturation with ammonium sulfate. After standing overnight, the precipitates were obtained by centrifugation, redissolved in 2 ml of 50 mM Tris Cl, pH 7.4 containing 0.2 M NaCl and each dialyzed against 1 liter (one change) of this solution. The samples were added to 15 ml polycarbonate centrifuge tubes containing 2 ml of Whatman DE 32 cellulose equilibrated with the same solution. The mixtures were stirred briefly and centrifuged for 5 min at 1000 x g. The resin was resuspended and washed twice with



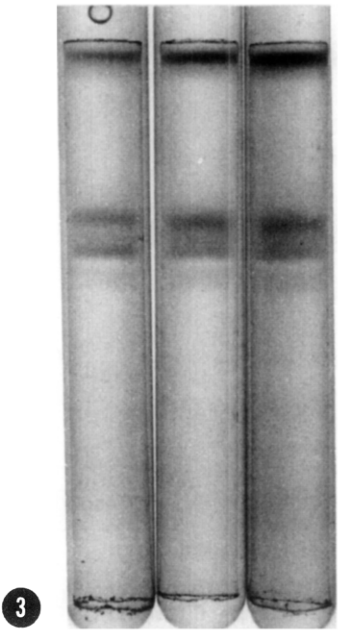


Figure 2. Co-electrophoresis experiment with calcium-binding proteins from adrenal medulla, testis and a mixture of both proteins in 15% polyacrylamide gels at pH 8.3. The samples of individual proteins contained 30 µg, and the mixture 15 µg of each protein. The anode is toward the bottom.

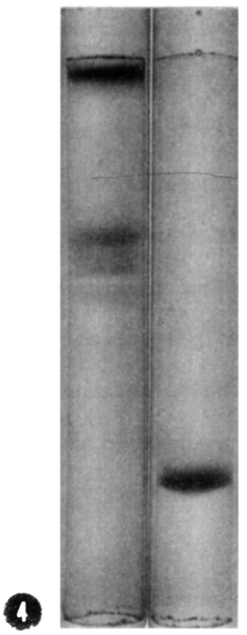
Figure 3. Co-electrophoresis as reported in Figure 2 except that electrophoresis in 15% polyacrylamide gels was performed at pH 4.7. The cathode is toward the bottom.

10 ml of the buffered-salt solution. Calcium-binding protein was eluted from the cellulose with two 10 ml washes of 50 mM Tris Cl pH 7.4 containing 0.32 M NaCl. This wash was referred to as the acidic protein fraction.

Procedures used for protein determination, preparation of chromatographic supports and electrophoresis at pH 8.3 have been described previously (4). Electrophoresis in 15% polyacrylamide gels at pH 4.7 was performed as described by Panyim and Chalkley (8). Samples for electrophoresis were dialyzed against 0.9% acetic acid. When 2-mercaptoethanol was used, it was added to the samples 2 hr prior to dialysis against 0.9% acetic acid.

RESULTS

A calcium-binding protein was found in homogenates of adult bovine testis, using the methods we have previously described for adrenal medulla (4). The presence of the protein was evident at the step involving gel filtration on



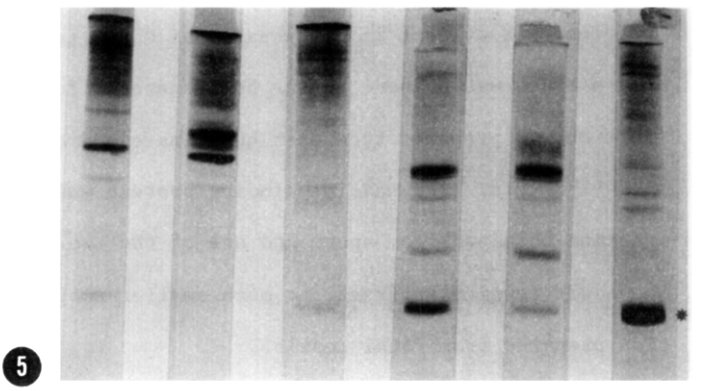


Figure 4. Electrophoresis of testicular calcium-binding protein in 15% polyacrylamide gels at pH 4.7 before and after treatment of the sample with 2-mercaptoethanol. Each gel contains 30 µg of protein; the gel on the right contains the protein treated with 2-mercaptoethanol.

Figure 5. Gels 1-3 represent, respectively, the electrophoresis of crude homogenate from adult testis, fetal testis and epididymal spermatozoa. The remaining gels contain samples of the acidic protein fraction prepared from these homogenates. The gels are 15% in acrylamide at pH 8.3; 100 µg of protein was applied to each gel. The location of the calcium-binding protein is indicated by an asterisk.

Sephadex G-100 in the presence of $^{45}CaCl_2$ (Fig. 1). A calcium-binding protein was prepared from the fractions containing protein-bound radioactivity by chromatography on hydroxylapatite. The yield of calcium-binding protein was 40 mg/kg, similar to that reported for adrenal medulla (4).

The calcium-binding protein from testis was compared electrophoretically with that from adrenal medulla. A single stained band was obtained after electrophoresis of the individual proteins and a mixture of both proteins in 15% polyacrylamide gels at pH 8.3 (Fig. 2). In a similar experiment at pH 4.7 four bands were observed (Fig. 3). This pattern was reduced to a single fast moving band if the samples were treated with 2-mercaptoethanol before electrophoresis (Fig. 4).

Samples of the crude homogenate of adult testis, fetal testis and spermatozoa were compared electrophoretically for the presence of calcium-binding protein (Fig. 5). This protein, which had the highest mobility of testicular proteins, was evident in homogenates of adult testis and spermatozoa while only a trace was present in the homogenate of fetal testis. An acidic protein fraction was prepared from each homogenate and again examined for the presence of the protein. The calcium-binding protein was the principal protein in the fraction obtained from sperm and one of the two major proteins obtained from the adult testicular fraction; much smaller amounts were evident in the fraction prepared from fetal testis.

DISCUSSION

We have previously examined extracts of a variety of mammalian tissues for an acidic calcium-binding protein using the technique of gel-filtration in the presence of $^{45}CaCl_2$ (4). Only brain and adrenal medulla showed significant calcium-binding activity under these conditions. Continued examination of mammalian tissues revealed large amounts of a calcium-binding protein in homogenates of adult bovine testis. We have determined that the same calcium-binding protein is present in each of these three tissues.

The electrophoretic experiments at pH 4.7 indicate the presence of sulfhydryl groups in the protein although we have found no evidence for cysteine from amino acid analysis (3). In the absence of 2-mercaptoethanol, the majority of the protein was present as an aggregate which barely entered the gel; in addition, three faint bands were present. Pretreatment of the sample with 2-mercaptoethanol resulted in only one band of protein following electrophoresis. We are currently investigating this 2-mercaptoethanol effect.

Since the calcium-binding protein has been isolated to date only from brain and adrenal medulla, it was reasonable to ask why it should be present in such quantity in testis, a tissue without neurosecretory function. It seemed most likely that the protein was associated with spermatozoa because of its possible participation in the contractile system or in the secretion of the

proteolytic enzymes involved in fertilization (9). Therefore, we examined fetal testis, which lacks spermatozoa, and epididymal spermatozoa for the calcium-binding protein. Levels of the protein in fetal testis were low while it was the predominant acidic protein in extracts of spermatozoa. Apparently, much of the calcium-binding protein of adult testis is contributed by the developing spermatozoa. The possibility that this protein serves a common specialized biochemical function in nervous tissues and spermatozoa is currently under investigation in this laboratory.

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