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# Chicken Gizzard Calcyclin—Distribution and Potential Target Proteins

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The distribution of calcyclin in some chicken tissues was studied by Western blotting using polyclonal antibodies raised against calcyclin purified from chicken gizzard. The protein was found in gizzard muscle and in a lesser amount in skeletal and cardiac muscle. No immunological reaction was observed in chicken liver. Immunohistochemical studies of chicken gizzard tissue revealed the presence of calcyclin only in muscle fibers. $Ca^{2+}$-dependent interaction of chicken gizzard calcyclin with potential protein targets was also examined. By gel overlay method it was found that calcyclin bound to three proteins with molecular masses of approximately 35 kDa, 25 kDa and 15 kDa present in the cytosolic fraction derived from chicken gizzard muscle. The chicken gizzard calcyclin was also shown to interact with lysozyme. 

Calcyclin is a calcium binding protein belonging to the S-100 family (1)(2). It has been shown that S-100 proteins are expressed in a cell and tissue specific manner and that they might be involved in many cellular processes (3)(4). Calcyclin was originally purified from Ehrlich ascites tumor (EAT) cells (5)(6) but later it was found in other mammalian cells and tissues (7)(8)(9)(10). Recently a calcyclin isoform from smooth muscle of chicken gizzard has been isolated and partially sequenced (11).

Studies on mammalian calcyclin have generated many interesting data regarding the distribution and possible function of this protein (12). Immunohistochemical studies have shown that mammalian calcyclin is mainly present in the fibroblasts and epithelial cells (13). Affinity chromatography and gel overlay experiments showed that calcyclin interacted with several proteins in the presence of $Ca^{2+}$. It has been found, for example, that mouse calcyclin interacted with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and annexin II (14). The binding of GAPDH, annexin II and annexin VI was shown later in the independent studies on bovine heart calcyclin (15). Specific, $Ca^{2+}$-dependent interaction with annexin XI was described for rabbit lung calcyclin (16). The interaction of chicken gizzard calcyclin with caldesmon and a possible involvement of this complex in smooth muscle contraction have also been discussed (11).

The aim of the present work was to investigate the distribution of the avian isoform of calcyclin using specific polyclonal antibodies and to examine its localization in smooth muscle by immunohistochemical methods. In the search of a possible function of chicken gizzard calcyclin its interaction with potential target proteins present in chicken gizzard cytosol was also studied.

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EAT, Ehrlich ascites tumor; EGTA, ethylene glycol bis($\beta$-aminoethyl ether); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.



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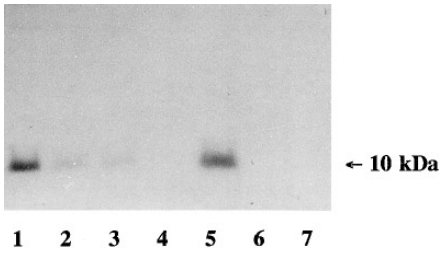


**FIG. 1.** Immunoblot of proteins probed with polyclonal antibodies against chicken gizzard calcyclin. Cytosolic fraction derived from chicken tissues: 1, gizzard; 2, skeletal muscle; 3, cardiac muscle; 4, liver. Equal amount (100 $\mu$g) of each protein fraction was applied on the SDS-gel. Purified proteins (4 $\mu$g): 5, chicken gizzard calcyclin; 6, EAT calcyclin; 7, S-100 protein from bovine brain.

## MATERIAL AND METHODS

*Proteins.* Chicken gizzard calcyclin was isolated by a procedure described by Filipek et al. (11). Lysozyme preparation was purchased from Sigma. Protein concentration was estimated according to the procedure of Lowry (17) with BSA as a standard protein.

*Tissue extracts.* Chicken tissues were homogenized using Polytron at 6000 rpm in the solution containing 1 mM EDTA, 1 mM DTT, 10 mg/l leupeptin, 20 mg/l soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride and 40 mM Tris adjusted to pH 7.5 with HCl. The extracts were centrifuged for 1 hr at 100,000 $\times$ g and the cytosolic fractions were collected for further analysis.

*Antibodies.* The polyclonal antibodies were raised in rabbits by injecting 1 mg of purified chicken gizzard calcyclin with complete Freund's adjuvant and, after four weeks, 1 mg of the antigen emulsified in incomplete Freund's adjuvant. Serum was collected one week after the second injection and fractionated by ammonium sulfate precipitation to 50% saturation. After dialysis against PBS the soluble proteins were applied to the affinity column with chicken gizzard calcyclin coupled to CNBr-Sepharose 4B (Pharmacia). Calcyclin specific antibodies were eluted with a buffer containing 0.1 M glycine pH 2.6 and 0.15 M NaCl. Immediately after elution the pH was adjusted to about 7.0 using 1 M Tris base and the sample was dialyzed against PBS and stored at $-20$°C.

*Immunohistochemistry.* The immunohistochemical studies were performed on chicken gizzard muscle. The tissue was fixed by immersion in 4% formaldehyde in PBS, pH 7.4. Paraffin blocks were cut in 10 $\mu$m thick sections which were placed on slides covered with poly-L-lysine. Sections were incubated with affinity purified antibodies against chicken gizzard calcyclin for 2 hrs at room temperature. After washing, the distribution of calcyclin was visualized by incubation with a second antibody coupled to horseradish peroxidase. Color development was performed with diaminobenzidine (1mg/ml from DAKO) and 0.01% $H_2O_2$ in 50 mM Tris buffered with HCl, pH 7.5. Control sections were incubated with non-immune serum or with PBS. The slides were than stained with hematoxylin and covered by glycerogelatin.

*Other methods.* Gel electrophoresis with 15% polyacrylamide containing 0.1% SDS was performed according to the method of Laemmli (18). Gels were stained with Coomassie brilliant blue R 250 (Sigma). Immunoreactivity was analyzed by blotting the proteins from the polyacrylamide gels on to nitrocellulose sheets. Calcyclin was detected with specific polyclonal antibodies. Staining of the blots was developed with chloronaphtol and $H_2O_2$. Gel overlay experiments were performed as described by Filipek et al. (19).

## RESULTS

### *Distribution of Chicken Gizzard Calcyclin*

Antibodies raised against chicken gizzard calcyclin reacted with their proper antigen but not with mouse EAT cells calcyclin or with S-100 protein purified from bovine brain (Fig. 1, lane 5, 6 and 7). These antibodies detected a single protein band in the cytosolic fraction derived from chicken gizzard muscle. The band stained with antibodies on the immunoblots corresponded to the monomer of calcyclin (Fig. 1, lane 1 and 5). When some other chicken tissues were examined by immunoblotting calcyclin was detected in the cytosolic fraction derived from skeletal and cardiac muscle (Fig. 1, lane 2 and 3). The strongest signal, as it was shown on Fig. 1, was observed for smooth muscle and no immunological reaction was seen for chicken liver.

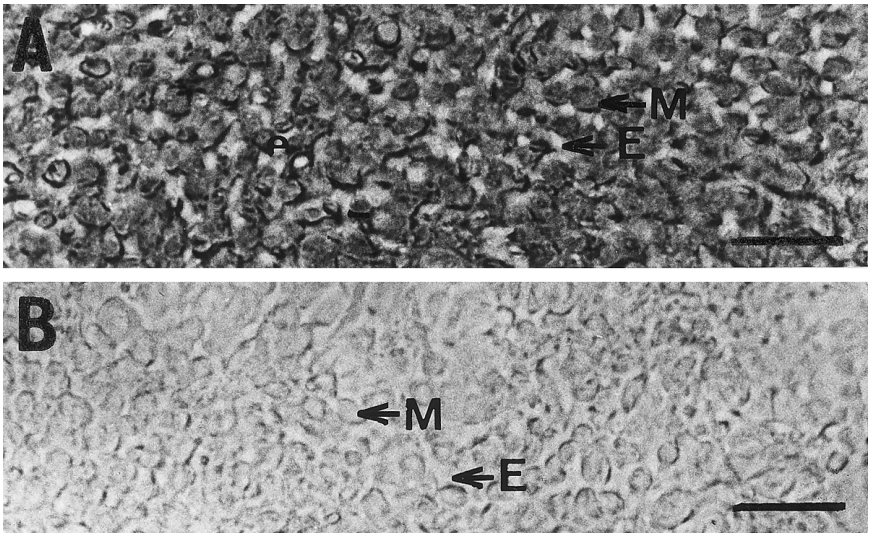


**FIG. 2.** Light micrograph of the section derived from chicken gizzard muscle. A, section stained with antibodies against chicken gizzard calcyclin; B, control section treated with PBS. M, muscle fibers, E, extracellular matrix. Bar = 0.025 mm.

When immunologically stained cross sections of smooth muscle from chicken gizzard were analyzed in the light microscope it was found that only the muscle fibers were stained with antibodies specific to chicken gizzard calcyclin (Fig. 2).

*Interaction of Chicken Gizzard Calcyclin with Potential Target Proteins*

$Ca^{2+}$-dependent interaction of chicken gizzard calcyclin with potential protein targets was studied by means of the gel overlay method. Fig. 3 (lane 1) shows that $^{125}$I-labeled calcyclin interacted with three proteins from the cytosolic fraction of chicken gizzard muscle. The major band of radioactivity corresponded to a protein of molecular mass of 15 kDa and weaker bands of radioactivity corresponded to targets with molecular masses of approximately 35 kDa and 25 kDa. In addition, the avian isoform of calcyclin was found to bind in a $Ca^{2+}$-dependent manner to lysozyme - a component of molecular weight standards (Fig. 3, lane 2). This interaction was confirmed using purified lysozyme preparation purchased from Sigma (not shown).

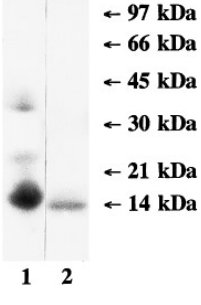


**FIG. 3.** Autoradiogram of proteins blotted on to nitrocellulose from the SDS-gel and incubated with $^{125}$I-labeled chicken gizzard calcyclin in the presence of 0.2 mM $CaCl_2$. 1, chicken gizzard cytosol (100 $\mu$g), 2, low molecular weight standards from BioRad.

## DISCUSSION

In the present work specific polyclonal antibodies against avian isoform of calcyclin were described for the first time. The lack of interaction of these antibodies with mouse calcyclin might confirm the structural differences between mammalian and avian isoforms of this protein (11). Using these antibodies it was shown that the highest amount of avian calcyclin was present in the smooth muscle of chicken gizzard and that this protein is localized in the muscle fibers, contrary to mouse calcyclin which is present in fibroblasts surrounding the muscle fibers (20).

$Ca^{2+}$-dependent interaction of chicken gizzard calcyclin with three proteins present in smooth muscle cytosol suggests that avian calcyclin, similarly to the mammalian isoform, might exert biological activity via its target proteins. However, when chicken gizzard calcyclin was replaced in the gel overlay assay with mouse calcyclin no protein from chicken gizzard cytosolic fraction nor lysozyme were labeled (not shown). This might suggest that mammalian and avian isoforms of calcyclin are also different in terms of interaction with their targets. While mouse [$^{125}$I]-calcyclin interacted with annexin II and GAPDH (19), the avian izoform bound in a $Ca^{2+}$-dependent manner mainly to the protein with molecular mass of 15 kDa.

An interesting finding was the observation that the chicken gizzard calcyclin bound to lysozyme. The specific, $Ca^{2+}$-dependent interaction between these two proteins prompted us to investigate the effect of avian calcyclin on lysozyme activity. In the *in vitro* assay calcyclin had no influence on the $V_{max}$ and $K_m$ of the enzyme when *micrococcus lysodeicitius* cell wall polysacharides served as substrate (not shown). Further studies are needed to identify protein targets of chicken gizzard calcyclin and to establish which of them are of physiological importance in smooth muscle.

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