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Evidence for the presence of calmodulin in fish mucus

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Calmodulins are ubiquitous calcium-dependent activators of various enzymes in eukaryotic cells. The regulatory functions of calmodulins have been established for fundamental cell processes like cyclic nucleotide metabolism, transmembrane Ca\(^{2+}\)-transport, cell division, and cell motility in a variety of organisms. It generally functions as a second messenger in the cytosol [1–4].

In this paper we report evidence for the presence of calmodulin on an extracellular location, the mucus layer covering the body of three species of fish. In our studies on fish gills and gut we could identify calcium-binding proteins, including typical calmodulins, in homogenates of branchial and intestinal epithelial cells [5]. However, when examining the mucus covering the body surface we also discovered calmodulin-like activity. Since calmodulins are considered intracellularly operating activator proteins, we decided to characterize this activity in more detail. Calmodulins can be distinguished from other calcium-binding proteins, like troponin C or parvalbumin, by a molecular weight of 16800 and a pi of about 4, by a typical Ca\(^{2+}\)-dependent shift in electrophoretic mobility as calmodulin.

Evidence for the presence of calmodulin in fish mucus was collected carefully from the body surface by rinsing it with buffer solution containing 0.0137 M Tris, 0.12 M NaCl and 0.003 M KCl (pH 7.4). Insoluble particles were removed by centrifugation at 1000 \times \text{g} for 10 min at 4 °C (Beckman TJ 6). The supernatant was centrifuged at 20000 \times \text{g} for 10 min at 4 °C (Sorvall RC 5B; SM 24).

Partial purification of mucus of tilapia, catfish and rainbow trout. The 20000 \times \text{g} supernatant was purified after Teo et al. [11]. Saturated ammonium sulphate solution (adjusted to pH 7.4 with NaOH) was added to the supernatant to obtain final saturation of 55%. The solution was left on ice for 2 h and centrifuged at 25000 \times \text{g} for 20 min at 4 °C (Sorvall RCM 5B, SM 24). The supernatant was adjusted to pH 4 with H\(_2\)SO\(_4\), and centrifuged at 100000 \times \text{g} for 30 min at 4 °C (Beckman L-8-80, 70 Ti). The pellet was resuspended in distilled water and desalted by dialysis against distilled water (Spectrogon dialyzing tubing, molecular cut off 3.5 kDa). This preparation was heated for 5 min at 100 °C and centrifuged at 10000 \times \text{g} for 10 min at 4 °C (Beckman Minifuge). The supernatant containing 200–400 \text{µg protein \cdot ml}^{-1} was used for gel electrophoresis, phosphodiesterase assay or radioimmunoassay.

Crude mucus preparations. The 20000 \times \text{g} supernatant was dialyzed (Spectrogon dialyzing tubing, molecular cut off 3.5 kDa) against distilled water, and lyophilized. The lyophilizate was suspended in a small volume of distilled water and heated for 5 min at 100 °C. After centrifugation at 10000 \times \text{g} for 5 min at 4 °C (Beckman Minifuge) the supernatant (50–200 \text{µg protein \cdot ml}^{-1}) was used for radio-immunoassay or phosphodiesterase assay.

Skin homogenates. Parts of the skin were dissected from the lateral body wall of tilapia, and homogenized after careful removal of the mucous. After centrifugation at 1000 \times \text{g} for 10 min at 4 °C (Beckman TJ 6), the supernatant was processed as for partial purification of mucus. The protein content of the final solution amounted to about 100 \text{µg protein \cdot ml}^{-1}.

Electron microscopy. Freshly collected tilapia mucus was fixed according to Wendelaar Bonga and Van der Meij [12]. The particulate material was collected by centrifugation at 1000 \times \text{g} for 3 min (Beckman Minifuge), embedded in Spurr’s resin and examined in a Philips 200 electron microscope.

MATERIALS AND METHODS

Mucus collection. Adult tilapia (Sarotherodon mossambicus), catfish (Clarias lazera) and trout (Salmo gairdneri) were used. Fish were kept in tanks with tapwater. Some tilapia were adapted for one month to freshwater containing 0.2 mM dodecyl sulphate/polyacrylamide gel electrophoresis, comigrates with bovine brain calmodulin and shows the same calcium-dependent shift in electrophoretic mobility as calmodulin.

1. Partly purified mucus collected from the skin of three species of fish contains a protein that, on sodium dodecyl sulphate/polyacrylamide gel electrophoresis, comigrates with bovine brain calmodulin and shows the same calcium-dependent shift in electrophoretic mobility as calmodulin.

2. Fish mucus contains a heat-stable activator of cyclic nucleotide phosphodiesterase; activation is concentration dependent and sensitive to the specific calmodulin inhibitor calmidazolium (R 24571).

3. The presence of calmodulin in fish mucus is further indicated by means of a specific radioimmunoassay.

4. A drop in the calcium concentration of the water induces an increase in the immunoassayable calmodulin concentration of mucus, which indicates that the function of calmodulin in mucus is related to control of permeability of the skin epithelium to water and ions.
Protein assay. In the partly purified preparations, protein content was measured by a fluorescent method with Fluram Roche (Hoffmann-La Roche), and in crude preparations with a Bio-Rad protein kit. Bovine serum albumin was used as reference.

SDS-PAGE. SDS-PAGE was performed in 12% polyacrylamide slab gels [13], that were silver-stained [14]. Samples were run in the presence of 1 mM Ca$^{2+}$ or 1 mM EGTA, after Gitelman and Witman [9]. Bovine serum albumin, chick egg albumin, chymotrypsinogen and cytochrome c were used as markers for molecular mass (all from Boehringer). Bovine brain calmodulin was obtained from Fluka.

cAMP-dependent phosphodiesterase. Stimulation of cAMP-dependent phosphodiesterase was determined by a modification of the one-step procedure of Teo et al. [11]. Phosphate release was measured following the conversion — at pH 7.5 for 30 min at 30°C — of cAMP (Sigma A 6885) to adenosine by calmodulin-dependent phosphodiesterase (Sigma P 0520) and bovine alkaline phosphatase (Sigma P 5521; 1.5 U/assay; [8]). Assays were run at pH 7.5 for 30 min at 30°C. Inorganic phosphate was determined by the Malachite Green method [15], with a Zeiss PM 2 DL Spectrophotometer. The phosphodiesterase-concentration could be reduced 8-fold in comparison with the method from Teo et al. [11]. The inhibition of the phosphodiesterase-stimulating activity was studied by preincubation of the reaction mixtures in the presence of 1 µM calmidazolium (R24571; Janssen Pharmaceutica), for 30 min at 30°C, before starting the reaction by the addition of cAMP [8]. Assays run with distilled water instead of phosphodiesterase solution were used as blanks ('phosphodiesterase-blanks'). To determine endogenous phosphate release, the reaction mixture was incubated without mucus. The mucus sample was added after stopping the reaction (by adding the Malachite Green mixture).

Calmodulin radioimmunoassay. Crude and partially purified mucus preparations were assayed for calmodulin by a commercial $^{125}$I-radioimmunoassay kit (New England Nuclear, NEK-18), with an antibody raised in sheep against non-derivatized rat testis calmodulin. Other calcium-binding proteins such as troponin C and parvalbumin have been shown to be virtually unreactive to this antibody.

RESULTS

SDS-PAGE of partially purified tilapia mucus

Although typical calmodulin characteristics (an isoelectric point around pH 4; great heat-stability) were exploited to purify the mucus preparation, the SDS-gels showed many bands (Fig.1). This is mainly due to omission of DEAE-cellulose column chromatography, a usual step in calmodulin purification. This step was eliminated because of the limited amounts of mucus that were available. One of the bands visible on the SDS-gels comigrated with bovine brain calmodulin. This band further showed a similar mobility shift as bovine brain calmodulin during electrophoresis in the presence of 1 mM Ca$^{2+}$: the apparent molecular mass ($m$) changed from around 17 kDa to 16 kDa (Fig.1). Partially purified mucus from catfish or trout contained similar bands that showed the same Ca$^{2+}$-dependent shift in electrophoretic mobility (results not shown here).

Assay of 3',5'-cAMP-dependent bovine brain phosphodiesterase

In the phosphodiesterase assay of crude mucus preparations — only dialyzed and heat treated — the ratio of calmodulin-stimulated (calmidazolium-sensitive) phosphate release and non-specific phosphate release approximated 1, due to high phosphodiesterase blanks. In the phosphodiesterase assay of partially purified mucus samples this ratio amounted to 2.7 (Table 1). The non-specific phosphate release is unlikely to be caused by the presence of endogenous phosphodiesterase in mucus, since heat treatment inactivates this enzyme [16]. When alkaline phosphatase was omitted together with phosphodiesterase, hardly any phosphate release occurred. However, omission of cAMP had no effect on phosphate release. These findings point to the presence of a substrate for alkaline phosphatase that does not pass a dialysis membrane with a cut-off of 3.5 kDa and that is not eliminated by heat treatment.

Table 1. Phosphodiesterase-stimulating activity of tilapia mucus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Non-specific Pi release</th>
<th>Calmodulin stimulated Pi release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude preparations</td>
<td>52.5 ± 6.4</td>
<td>47.5 ± 6.4</td>
</tr>
<tr>
<td>Partially purified</td>
<td>27.3 ± 5.4</td>
<td>72.6 ± 5.5</td>
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Table 2. Effect of Ca\(^{2+}\) concentration of the water on the calmodulin concentration (means ± SD) of crude tilapia mucus preparations. The fish were adapted for 4 weeks to water with either 0.8 mM (controls) or 0.2 mM Ca\(^{2+}\). Calmodulin concentrations were determined by radioimmunoassay. n: number of fish.

<table>
<thead>
<tr>
<th>Water Ca(^{2+}) concentration</th>
<th>Calmodulin (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>0.2</td>
<td>1.6 ± 0.7(^*)</td>
</tr>
</tbody>
</table>

\(^*\) Significantly different from control (P < 0.02; Student's t-test for unpaired observations).

Partly purified tilapia mucus showed a distinct ability to stimulate calmodulin-free cAMP-dependent phosphodiesterase. This stimulation was concentration-dependent and analogous to the stimulation by bovine brain calmodulin (Fig. 2). The specific activity of this mucus preparation was equivalent to 0.84 ± 0.1 µg calmodulin/mg protein. The phosphodiesterase-stimulating activity of the mucus preparation could be inhibited completely by calmidazolium at a nominal concentration of 1 µM.

Calmodulin \(^{125}\)I-radioimmunoassay

The concentration of substances with calmodulin antigenic properties in partially purified tilapia mucus amounted to 0.7 µg/mg protein. In crude mucus preparations from tilapia, catfish and trout the concentrations amounted to around 0.5 µg/mg protein. These data pertain to fish from normal freshwater (Ca\(^{2+}\): 0.8 mM). Transfer to tilapia to freshwater with a low Ca\(^{2+}\) content (0.2 mM) led to a statistically significant increase in the radioimmunoassayable calmodulin concentration (Table 2).

To investigate the possibility that the calmodulins present in mucus were derived from desquamated epidermal cells that may be present in mucus, a homogenate of almost mucus-free tilapia epidermal cells was assayed. Although the protein content of the skin sample was similar to that of the crude tilapia mucus preparations, calmodulin was hardly detectable by radioimmunoassay.

Ultrastructural examination of tilapia mucus

Electron microscopic analysis of particulate material collected from mucus did not reveal intact cells, fungi or protozoa. Only a few membrane fragments and some fibrous material of cellular origin were found, probably originating from the epithelium of the skin.

DISCUSSION

In this paper evidence is reported for the presence of calmodulin in the mucus secreted by the skin of fish. This claim is substantiated by the following observations: (a) fish mucus contains a substance with an apparent molecular mass similar to that of calmodulin; (b) this substance shows a calcium-dependent shift in electrophoretic mobility that is characteristic for calmodulin, and not for other calcium-binding proteins related to calmodulin, like troponin C or parvalbumin [7]; (c) mucus displays heat-stable cAMP-dependent phosphodiesterase stimulating activity that can be inhibited by the specific calmodulin antagonist calmidazolium [8]; (d) radioimmunoassay with a highly specific antibody against calmodulin shows the presence of substances with calmodulin antigenic determinants.

We suggest that the calmodulin in fish mucus is secreted by the mucocytes of the skin. The possibility can be excluded that the calmodulin in mucus has an intracellular location: freshly collected mucus was virtually free from protozoa, fungi or other viable cells, as judged by electron microscopy, and cell remnants were scarce. Moreover, the low calmodulin content of the skin epithelium makes it unlikely that the calmodulin in mucus is derived from desquamated epithelial cells.

In the mucus layer covering the body of freshwater fish, calmodulin is exposed to Ca\(^{2+}\) levels that may be more than a factor of 1000 higher (0.1 — 1 mM) than the basal Ca\(^{2+}\) levels in the cytosol (0.01 — 0.1 µM [1]). Intracellularly, calmodulin exerts its function as a protein activator by binding Ca ions and, subsequently, forming a complex with a calmodulin-dependent enzyme [1]. Ca-binding occurs at concentrations slightly above the basal cytoplasmic Ca\(^{2+}\) levels (0.1 — 1 mM). At the high levels present in fish mucus, the calcium-binding sites of calmodulin will be permanently occupied, and any association with an enzyme will have a permanent nature.

We can only speculate about the functional significance of calmodulin in mucus, since the mucus layer covering the fish integument has multiple functions: reduction of water drag, protection against pathogenic organisms, or a function connected with water and ion regulation [17]. The latter function may be of primary importance, since in tilapia like in many other fish, mucus secretion is controlled by prolactin, an important osmoregulatory hormone in freshwater fish [18]. In tilapia, mucus secretion is enhanced under conditions that increase the permeability of the integument for water and ions, like a low calcium concentration of the water [17, 19]. Our observation that the calmodulin content of the mucus increases in low-calcium freshwater points therefore to involvement of calmodulin in the control of the permeability of the skin for water and ions. The permeability of epithelia is determined, inter alia, by the amount of calcium bound to membrane phospholipids [20] and to the tight junctions connecting the
epithelial cells [21]. The presence of calmodulin in mucus suggests an enzymatic involvement in the control of integumental permeability. However, adaptation of fish to low calcium water not only leads to enhanced permeability of the skin, but also to an increase of the active uptake of ions, like Na\(^+\) or Ca\(^{2+}\), as a response to the high passive losses of ions across the skin. Mucus has been suggested to concentrate ions from the water and thus facilitate the active ion uptake by the ionocytes in the gills [22]. Part of the mucus covering the skin originates from mucocytes located in the gill chambers. A role for extracellular calmodulin in the process of active uptake of ions can therefore not be excluded.

The only other report known to us of calmodulin-like activity from an extracellular location concerns the finding of a factor present in cell-free human seminal plasma, with the capacity to stimulate calmodulin-dependent (Ca\(^{2+}\) - Mg\(^{2+}\))-ATPase from sperm cell plasma membranes [23]. The authors suggest that this factor may influence sperm cell development. Thus, the presence of calmodulin in secretory fluids may have wider occurrence.

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REFERENCES