IMMUNOELECTRON MICROSCOPIC LOCALIZATION OF CALMODULIN IN GUINEA PIG TESTIS AND SPERMATOZOA

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The localization of calmodulin in guinea pig testis and spermatozoa was determined by both immunoperoxidase labeling and immunocolloidal-gold methods on the electron microscopic level.

In spermatids, reaction deposits were scattered all over the cytoplasm, but hardly recognized in acrosome vesicle and Golgi complex. In the neck portion of elongated spermatids, anti-calmodulin immunoreactive sites were diffusely localized in cytoplasm, and also recognized along the axial filaments and near the connecting pieces. Mature spermatozoa exhibited the following distinct regions of anti-calmodulin immunoreactive sites: the cytoplasm between plasma and outer acrosomal membranes, between nuclear envelope and inner acrosome membrane, along the axial filaments, and outside of fibrous sheath.

These findings suggest that calmodulin may play a role in both acrosome reaction and tail movement.

Calmodulin, ubiquitous in eukaryotic systems, has been implicated as a multifunctional regulatory mediator of calcium in the cytoplasm of cells (5, 17, 23, 24).

By microscopic and biochemical methods, sites with increased amounts of calmodulin have been demonstrated in mitotic apparatus (13, 25–27), ciliary structures (15, 19), the microvillar structures of the intestinal brush border (4, 6, 8), and in the synapses (7, 14, 20).

The acrosome reaction of capacitated spermatozoa was induced in the presence of extracellular calcium ion (29), and relatively high levels of calmodulin were recognized in spermatozoa from mammals and sea urchins (9). By immunofluorescence technique, Jones et al. (10) observed that calmodulin was exclusively located in the head cap of spermatozoa.

The present study was undertaken to show the detailed distribution of calmodulin at the higher resolution level of electron microscopy in both guinea pig testis and spermatozoa, and to elucidate the biological role of calmodulin in spermatozoa. A part of this study has been reported previously (30).

MATERIALS AND METHODS

Calmodulin was purified from rat testis by the method of Kakiuchi et al. (11).
Anti-calmodulin antibody was raised in the rabbits and purified with affinity chromatography as described previously (30).

A guinea pig (body weight 350 g) was decapitated and the testis, excised quickly, was cut into small pieces in the PLP solution (the mixture of 2 g paraformaldehyde, 1.37 g L-lysine monohydrochloride, and 0.21 g sodium metaperiodate dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.4) (16), and then fixed in a fresh PLP solution at 4°C for 3 hr. After washing with 0.1 M phosphate buffer (pH 7.2) containing 100 mM lysine and 7% sucrose overnight at 4°C, one part was frozen in liquid nitrogen, and the other part was dehydrated in a series of graded ethanol and embedded in Lowicryl KM-4 resin. The resin was polymerized overnight on an ice-bath under ultraviolet-irradiation (irradiated in 30 cm distance with Tohshiba SHL-100 V ultraviolet lamp, Tokyo), and the polymerized blocks were stocked in a cold room (4°C) until use.

For immunoperoxidase labeling, the cryostat sections (6-10 μm in thickness) were first treated at 22°C for 30 min with 1% bovine serum albumin dissolved in 0.01 M phosphate buffer (pH 7.2) containing 8.5% NaCl (BSA-PBS). The pre-treated sections were incubated in specific anti-calmodulin IgG solution (25 μg protein/ml) for 60 min at 22°C, washed with BSA-PBS, and then exposed to horse-radish peroxidase (HRP)-labeled goat anti-rabbit IgG solution (prepared according to the method of Nakane and Kawaoi (18), and purified by using an affinity chromatography) for 30 min at 22°C. After washing the sections, HRP activity was visualized with 3,3′-diaminobenzidine (DAB; 0.25 mg/ml, purchased from Dohjin Chemical Co. Kumamoto) in 0.05 M Tris-HCl buffer (pH 7.6) in the presence of 0.01% hydrogen peroxide. In the control experiments, the sections pre-treated with BSA-PBS were primarily incubated in the anti-calmodulin IgG pre-adsorbed with an excess of purified calmodulin and then treated by the same manner as described above.

For immunocolloidal-gold method, the ultrathin sections embedded in Lowicryl KM-4 resin reacted with affinity purified anti-calmodulin IgG (25 μg protein/ml) for 60 min at 22°C, were washed well with BSA-PBS and then treated with protein A-gold complex (obtained from EY Labs Inc.) for 30 min at 22°C. After washing the sections with distilled water, they were treated with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 15 min and then exposed to 0.5% osmium tetroxide dissolved in 0.1 M phosphate buffer (pH 7.4) for 15 min. The sections were stained with uranyl acetate, and examined with a Hitachi HU-12A electron microscope. In control experiments, the sections were incubated primarily in anti-calmodulin antibody pre-adsorbed with an excess of purified calmodulin and then exposed to protein A-gold.

In the case of mature sperm, spermatozoa were collected from the guinea pig epididymis and washed twice with calcium-free Tyrode solution. After incubation in the calcium-free Tyrode solution overnight at 37°C, the spermatozoa were treated with the Tyrode solution containing 3 mM calcium chloride for 7 min at 37°C. Calcium-triggered acrosome reaction occurred in this condition (29). The intact spermatozoa and the sperm that had undergone the acrosome reaction were fixed in PLP solution for 30 min at 4°C. The fixed sperm were washed well with calcium-free Tyrode solution containing 1% BSA, and applied to slide glasses. After treating the slide glasses with cold acetone (−20°C) for 3 min, anti-calmodulin immuno-
reactive sites were localized with immunoperoxidase labeling by the same method as described above. For immunofluorescent localization of calmodulin, acetone-treated preparations were primarily incubated in anti-calmodulin antibody for 60 min at 22°C, followed by labeling with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG goat IgG (obtained from Seikagaku Co. Ltd., Tokyo) for 30 min at 22°C. In the case of electron microscopic observations, PLP- and acetone-treated sperm were incubated at 22°C for 30 min in BSA-PBS to prevent subsequent non-specific binding of immunoglobulin. The spermatozoa were incubated in anti-calmodulin antibody (10 μg protein/ml) overnight at 4°C. After washing them with BSA-PBS, they were reacted with HRP-labeled anti-rabbit IgG goat IgG for 60 min at 22°C, followed by washing with BSA-PBS. After visualization of HRP-activity by a DAB solution, spermatozoa were post-fixed with 1% osmium tetroxide dissolved in 0.1M phosphate buffer (pH 7.4), dehydrated in a series of graded ethanol and embedded in epoxy resin. Ultrathin sections were examined with a Hitachi HU-12A electron microscope without any electron staining.

RESULTS

Light microscopic observations of anti-calmodulin immunoreactive sites:

The frozen sections of guinea pig testis fixed with PLP solution were stained with immunoperoxidase labeling. The anti-calmodulin immunoreactive sites were clearly observed in the cytoplasm of spermatocytes and spermatids (Fig. 1A). The reaction deposits were also seen in the nuclei of spermatids (shown by the thin arrows in Fig. 1A). The acrosome cap were not stained (indicated by the thick arrows in Fig. 1A). Calmodulin was hardly recognized in the cytoplasm of spermatogonia and in the nuclei of both spermatogonia and spermatocytes.

In intact spermatozoa, the anti-calmodulin immunoreactive sites were observed in the head cap and in the tail (Figs. 2A, C). In the sperm that had undergone the acrosome reaction, the fluorescence in the anterior part of the head disappeared, but remained in the tail (Fig. 2D).

Immunoelectron microscopic localization of calmodulin:

At the beginning of spermiogenesis, several granules (proacrosomal granules) are formed within small vesicles of the Golgi apparatus. These vesicles and the granules within Golgi apparatus coalesce to form a single large vesicle and granule (acrosome), and the membrane of vesicle adheres to the nuclear envelope. The Golgi apparatus remains closely associated with the surface of the acrosomal vesicles. At this stage, the calmodulin-immunoreactive sites were scattered all over the cytoplasm (Fig. 3A). Calmodulin was absent in acrosome vesicle and Golgi complex. Calmodulin-immunoreactive sites were not observed in the nuclei of spermatocytes (Fig. 1A), but recognized in the nucleus of spermatid at this stage.

In a more advanced stage of spermiogenesis, the limiting membrane of the acrosomal vesicle increases into the area of adherence to the nuclear envelope, and forms a head cap. Simultaneously, a condensation of the nucleoplasm and an elongation of spermatid occur. In this (or a later) stage, the calmodulin-immunoreactive sites in the neck portion and head cap region are shown in Figs. 4A and 4C.
FIG. 1A. Light micrographs showing the localization of calmodulin in guinea pig testis. Reaction deposits are densely seen in the cytoplasm of spermatocytes and spermatids, and also recognized in the nuclei of spermatids (shown by thin arrows). The acrosome cap of spermatids are not stained (indicated by the thick arrows). Reaction deposits are absent in the cytoplasm of spermatogonia and in the nuclei of spermatogonia and spermatocytes. × 750

FIG. 1B. Control specimens, treated primarily with anti-calmodulin antibody pre-adsorbed with an excess of the purified calmodulin and then stained by immunoperoxidase labeling method. The reaction deposits are not observed anywhere. × 750
Gold particles were clearly observed along the cytoplasmic surface of the plasma membrane in both perinuclear and neck portions (Fig. 4A). Furthermore, the anti-calmodulin immunoreactive sites were diffusely recognized in the cytoplasm and slightly seen near the connecting piece and inside the dense fiber. However, gold particles were hardly seen in the paracentriolar body and on both the basal plate and the dense fiber. In the head cap portion (Fig. 4C), the calmodulin-immunoreactive sites were clearly recognized in the space between the nuclear envelope and inner acrosomal membrane. Gold particles were also slightly observed in the space between the plasma membrane and the outer acrosomal membrane.

In the anterior portion of mature spermatozoa, the calmodulin-immunoreactive sites were clearly recognized along the cytoplasmic surface of plasma membrane (Fig. 5A).

By immunoperoxidase labeling, the reaction deposits were also observed along the plasma membrane (Fig. 6A). In the tail section at the principal pieces, the immunoreactive sites were localized between the outer dense fibers and axial
FIG. 4.
filaments, and in the outside of the fibrous sheath (Figs. 5B, 6C). Because the plasma membrane in these figures was obscure, it was not possible to judge whether the immunoreactive sites in the outside of the fibrous sheath were linked to the cytoplasmic surface of plasma membrane or not.

**DISCUSSION**

Lowicryl KM-4 resin was recently introduced as a low temperature embedding medium particularly well suited for applications in immunoelectron microscopy (1, 2). It has been reported previously (30) that the anti-calmodulin immunoreactive sites could be shown on the ultrathin sections embedded in Lowicryl KM-4 resin. In this study, the anti-calmodulin immunoreactive sites in guinea pig testis and spermatozoa recognized using the “post-embedding staining method” were in good accord with the sites observed by the immunoperoxidase labeling technique. Furthermore, the labeling sites of “post-embedding staining method” were clearly recognized, though the reaction deposits of immunoperoxidase labeling were relatively diffused. Therefore, the former method seems to be better for immunoelectron microscopic localization on calmodulin than the immunoperoxidase labeling.

The anti-calmodulin immunoreactive sites were recognized in the cytoplasm around acrosomal vesicles (Fig. 4A), but not observed on the membranes of acro-
Figs. 6A–D. Immunoperoxidase labeling of calmodulin in spermatozoon.

Fig. 6A. Anterior portion of acrosome cap is shown. The reaction deposits are observed along the plasma membrane. $\times 28,000$

Fig. 6B. Control specimen corresponding to the area shown in Fig. 6A. No reaction deposits can be recognized anywhere. $\times 25,000$

Fig. 6C. The cross- and longitudinal-sections of tail. The reaction deposits are observable on the cytoplasmic surface of plasma membrane and the area between the axial filaments and dense fibers. $\times 14,000$

Fig. 6D. Control specimen corresponding to the area shown in Fig. 6C. No reaction deposits are seen anywhere. $\times 14,000$
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somal vesicles and Golgi complex. With the elongation of spermatids, the cytoplasm between outer acrosomal and plasma membranes become smaller. Therefore, it is difficult to judge whether the anti-calmodulin immunoreactive sites in the anterior part of mature spermatozoa (Fig. 6A) are linked to the plasma membrane or located in the narrow cytoplasm. From the results obtained in this study, however, it may be that calmodulin is located in the cytoplasm between the outer acrosomal and plasma membranes, and in response to the calcium influx associated with acrosome reaction, calcium might bind to calmodulin and a portion of the calcium-calmodulin complex could then associate with the components of either the plasma or outer acrosomal membrane. It was shown that the calmodulin antagonist W-7 inhibited the acrosome reaction of sea urchin spermatozoa induced by egg jelly solution (21). Moreover, in preliminary experiments (31), the calmodulin antagonist W-7 inhibited considerably the acrosome reaction of capacitated guinea pig spermatozoa triggered by adding calcium ion. These results may support the hypothesis described above.

The anti-calmodulin immunoreactive sites are clearly recognizable along the axial filaments (Figs. 5, 6). Ohnishi et al. (19) showed that ciliary calmodulin in *Tetrahymena* was localized along the longitudinal axis of outer-doublet microtubules. Kumagai et al. (12) reported that *Tetrahymena* calmodulin bound to an affinity column of tubulin-sepharose 4B. However, they used porcine brain tubulin with microtubule-associated proteins (MAPs) to prepare their affinity column, so that it is not known whether calmodulin interacts with tubulin or MAPs. By the biochemical method, Sobue et al. (22) showed that calmodulin bound to tau factor, which was one component of MAPs. On the other hand, Blum et al. (3) reported that *Tetrahymena* calmodulin markedly stimulated dynein ATPase in the presence of calcium ion. The biological significance of specific localization of calmodulin near axial filaments in the tail remains to be elucidated, but the evidence described here may be useful in future for explaining how calmodulin is involved in tail movement.

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REFERENCES


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