

In Vitro Interactions of Calmodulin with the Ovine Proacrosin-Acrosin System

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The authors studied the interaction of calmodulin (CaM) with proacrosin and acrosin from ram spermatozoa. CaM binding evaluated by the [¹²⁵I]-CaM overlay procedure was shown to occur preferentially with both proacrosin and acrosin in the presence of EGTA; in the presence of Ca²⁺, the interaction was less intense. Further studies with native proenzyme preparations showed that proacrosin activation at pH 7.1 or 8.0 was significantly accelerated in the presence of CaM and EGTA (t_{1/2} = 23 min vs. 55 min for EGTA alone at pH 7.1), but not in the presence of Ca²⁺ (t_{1/2} = 73 min). The enzymatic activity of acrosin towards benzoyl arginine paranitroanilide, however, was not significantly affected by CaM whether Ca²⁺ was absent or present. Finally, the authors demonstrated that acrosin hydrolyzed CaM rapidly and extensively in the presence of EGTA. These results indicate that CaM interacts *in vitro* with proacrosin and acrosin, and that acrosin can attenuate CaM activity through proteolysis. Whether these interactions also occur *in vivo* and are involved in some aspects of spermatozoa function remains to be determined.

Key words: acrosome, spermatozoa, proteolytic activity, calmodulin binding protein.

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High concentrations of calmodulin (CaM), the eucaryotic intracellular Ca²⁺ receptor (Means et al, 1982), are known to be present in the sperm head of several mammalian and nonmammalian species (Jones et al, 1978). More recently, CaM has been shown to be localized in the acrosome (Weinman et al, 1986; Camatini et al., 1986). Since an enhanced Ca²⁺ influx appears to be required for the acrosome reaction to take place (Yanagimachi and Usui, 1974), it is logical to suppose that this reaction involves not only the calcium mediator CaM but also CaM binding proteins. Recently, Camatini and Casale (1987) have shown a close codistribution of calmodulin and actin in boar sperm. In the same study, they also demonstrated that a 45 kDa polypeptide bound CaM in the ¹²⁵I-CaM overlay procedure. The identity of the 45 kDa CaM-binding protein was not determined. A possible candidate for this CaM binding protein could be the activated form of acrosin which is abundant in the acrosome; it has a molecular mass of 45 kDa in the boar (Kaufmann et al, 1987), and apparently plays a key

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role in sperm physiology (Hinrichsen-Kohane et al, 1984; Polakoski and Siegel, 1986). We have undertaken this study to test the hypothesis that acrosin was indeed a CaM binding protein whose activity could be modulated by CaM and Ca^{2+} . For these experiments, we have used acrosin and proacrosin preparations from ram spermatozoa because acrosin can be easily prepared in large amounts from this species (Brown and Hartree, 1978) and because CaM concentrations in spermatozoa and spermatogenic cells vary from 59 to 4,675 μg per 10^9 cells in epididymal spermatozoa and pachytene spermatocytes, respectively (Weinman et al., 1986).

Materials and Methods

Chemicals

Tris, imidazole, NaCl, KCl, benzamidine HCl, EGTA, glycerol, dimethyl sulfoxide (DMSO), and benzamidine Sepharose 6B, *N* α -benzoyl-DL-arginine p-nitroanilide (BAPNA) were purchased from Sigma Chemical Corp., (St. Louis, MO); spectrapor 2 membrane, acetic acid, and chlorhydric acid from Fisher Scientific (Montreal, Canada); N,N-dimethyl formamide (DMFA) from Anachemia (Montreal, Canada); and $CaCl_2$ standard solution from Orion Research Inc. (Cambridge, MA).

Sperm Collection

Ram semen was obtained from La Section d'Épreuve de Béliers de La Pocatière. After ejaculation, the semen was diluted to 1.6×10^9 spermatozoa with a skimmed milk solution (11%) containing 0.5% Pen-di-strep (Rogar/STB Inc.) and 0.3% Linco-Spectin (Upjohn). The semen was then progressively cooled to 15 C and transported to our laboratory.

Preparation of Proacrosin and Acrosin

The semen was centrifuged over a 1M sucrose cushion containing 10 mM benzamidine at $6,000 \times g$ for 20 min (Goodpasture et al, 1980). The pellet was extracted for 16–18 h at 4 C in 0.1 M acetate (pH 2.7) containing 10% glycerol and 50 mM benzamidine. After extraction, the suspension was centrifuged at $30,000 \times g$ for 20 min. The supernatant was concentrated with Amicon CF-25 cones, applied on a Sephadex G-100 column (55 cm \times 1.5 cm) and eluted with a 34 mM acetate buffer, pH 3.0, and 145 mM NaCl (Mack and Zaneveld, 1986). An aliquot of each fraction was tested for activatable proteolytic activity (proacrosin) using BAPNA as substrate (see below). The activation was usually done by elevating the pH from 3.0–8.0 using Tris HCl buffer (50 mM final concentration), pH 8.0, during 30 min. An aliquot of each fraction was analyzed by SDS gel electrophoresis according to Laemmli (1970). The proacrosin fractions contained two major protein bands (45 and 43 kDa) and minor contaminants of higher and lower molecular weights. These fractions were used for studies of CaM interactions and as a material for further purification of acrosin by affinity chromatography. For that purpose, they were first dialysed against 1 mM HCl in a Spectrapor

2 membrane. The dialysed material was activated as described above and applied immediately on a benzamidine Sepharose 6B column (10 ml syringe) equilibrated in buffer A (0.2 M NaCl in 50 mM Tris, pH 8.0). The column was washed with 10–15 vol of buffer A, and acrosin was eluted with 0.1 M acetate buffer, pH 2.7, containing 200 mM NaCl. Eluted acrosin was dialysed against 1 mM HCl and kept frozen at -80 C. The purified material gave a single band of 40 kDa on SDS gel electrophoresis and thus resembled the preparation of ram acrosin of Brown and Hartree (1978) prepared by a similar procedure. Polyclonal antibodies against the 40 kDa acrosin were developed in rabbits using standard procedures.

Determination of Acrosin Activity

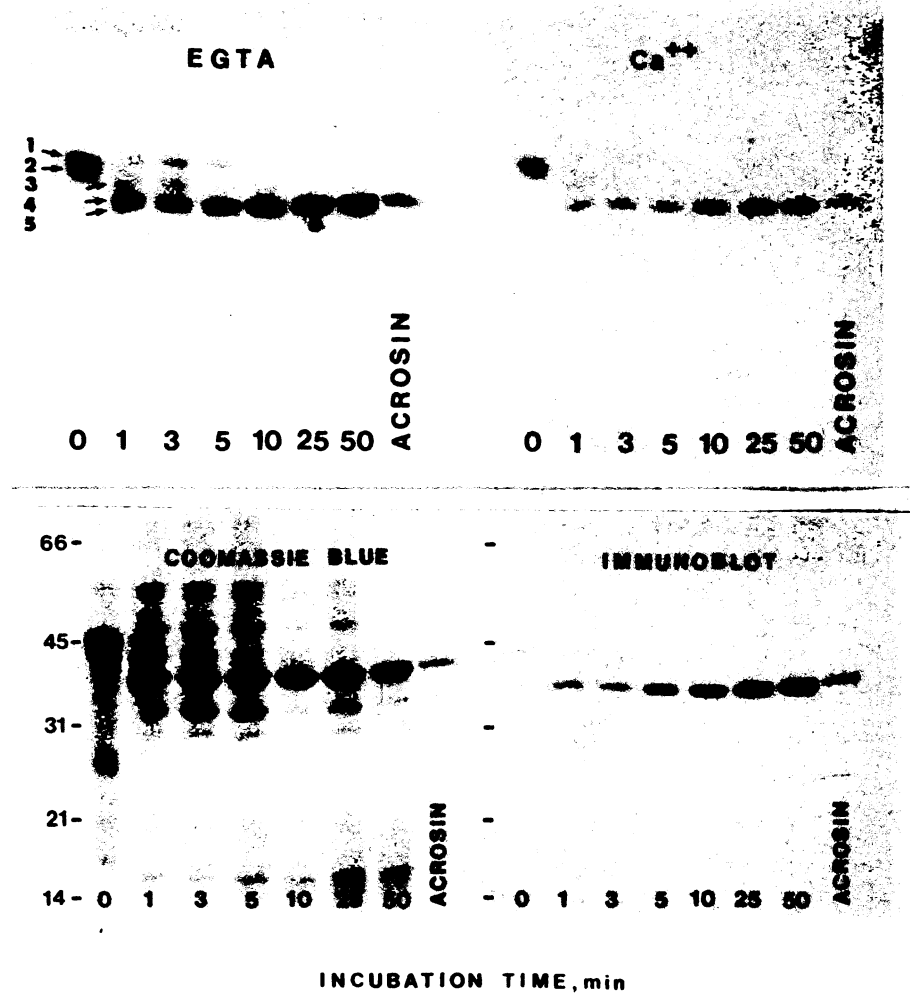
The amidase activity of acrosin was determined with BAPNA as substrate (Erlanger et al, 1961). The reaction mixture contained 50 mM Tris HCl pH 8.0, 2 mM BAPNA and 2% dimethyl sulfoxide (DMSO) at 25 C in a total volume of 1.0 ml. The reaction mixture was preincubated for 5 min and the enzymatic reaction was started by the addition of either an aliquot of a chromatography fraction, a proacrosin preparation, or purified acrosin. The reaction was stopped by adding 0.1 ml of 1 M benzamidine. The release of p-nitroanilide was evaluated with a spectrophotometer at 412 nm.

For the hydrolysis of CaM, we used initial concentrations of acrosin and calmodulin of 10 and 90 μg , respectively, in a total volume of 0.4 ml of reaction mixture and various concentrations of Ca^{2+} . At various time intervals, samples of 50 μl were withdrawn and mixed with 50 μl of SDS gel electrophoresis sample buffer (twice the normal concentration) of Laemmli (1970) to stop the enzymatic reaction. The whole sample was subjected to 10% polyacrylamide slab gel electrophoresis (Laemmli, 1970). The proteins were stained with Coomassie blue and the relative concentration of remaining CaM at each time was quantitated by densitometry using the Research Analysis System from Amersham. CaM was purified from rat testis by a procedure described previously by Chafouleas et al (1982).

^{125}I -CaM Overlay Procedure

To study the interaction of CaM with the proacrosin-acrosin system, the ^{125}I -CaM overlay procedure was used (Nelson et al, 1983). The proacrosin/acrosin preparations were first separated in 10% slab gels according to Laemmli (1970). The gel was fixed for 30 min in 40% MeOH: 10% CH_3COOH and rinsed in water. Then it was washed in 10% EtOH (18 h), rinsed in water, and incubated in 30 ml imidazole buffer (20 mM imidazole, pH 7.1, 0.2 M KCl, 0.1% BSA, 0.025% NaN_3 , and 1 mM CaCl_2 or 1 mM EGTA) containing 20×10^6 cpm ^{125}I CaM for 16 h at 4 C. The gel was rinsed several times in imidazole buffer. It was stained with Coomassie blue, dried, and exposed on a Kodak XR-2 film. For comparison, two other gels were run in identical conditions with aliquots of the same samples. One gel was stained with Coomassie blue and the other was transferred to nitrocellulose for Western blot analysis using antiacrosin antibodies developed in rabbits. The Western

Fig. 1. Binding of [125 I]-calmodulin to proacrosin and acrosin forms generated during autoactivation (0-50 min) at pH 7.1 of a proacrosin preparation from ram spermatozoa. After the activation step, the proteins were separated by polyacrylamide gel electrophoresis. Then, the gels were incubated with [125 I]-calmodulin (overlay procedure) either with 1 mM EGTA or 1 mM CaCl_2 as described in Materials and Methods. Upper panel: autoradiogram obtained after exposure of the dried gels over Kodak X-AR2 films for 3 days. The numbers 1-5 and the arrows indicate the proacrosin and acrosin forms having affinity for [125 I]-calmodulin. Lower panel: Coomassie blue staining and immunoblot of proteins from aliquots identical to those used in the [125 I]-calmodulin overlay procedure. For the immunoblotting procedure, the nitrocellulose filter was overlaid first with ram antiacrosin antiserum (1 in 100 final dilution) and then with protein A-peroxidase. The peroxidase complex was revealed with 4-chloro-1-naphthol in the presence of hydrogen peroxide. The numbers on the ordinate of the Coomassie blue stained gel represent the molecular mass in kDa of standard proteins.



blot conditions were identical to those described previously (Frenette et al, 1987).

Results

To ascertain the potential physical association between CaM and proacrosin/acrosin forms, we first separated these forms at various time intervals during the course of proacrosin activation. CaM binding was then studied by the overlay technique. Figure 1 (upper panel) shows that the proacrosin preparation contained two major CaM binding proteins of 45,000 and 43,000 daltons (bands 1 and 2) whose association with CaM appeared stronger in the presence of EGTA than in the presence of Ca^{2+} . During activation, three other CaM binding proteins of 42, 40, and 39 kDa (bands 3-5) were generated. The 40 kDa band 4 corresponded exactly to the migration of affinity purified ram acrosin,

which also bound CaM. In all cases, there was more [125 I]-CaM bound in the presence of EGTA than in the presence of Ca^{2+} . The antiacrosin polyclonal antibodies used in Western blot conditions also recognized the same proteins that had an affinity for CaM, i.e., 45 and 43 kDa proacrosin and 42, 40, and 39 kDa acrosin forms (Fig. 1, lower panel). However, proacrosin bands and 42 kDa acrosin were much less intense because the antibodies were preferentially directed against 40 kDa acrosin. All the bands recognized by CaM and by antiacrosin antibodies were major proteins on Coomassie blue staining. The interaction of CaM with proacrosin/acrosin forms could also be demonstrated with nitrocellulose blotted proteins. Under these conditions, we observed higher CaM binding in the presence of EGTA than in the presence of Ca^{2+} . However, this procedure was not convenient

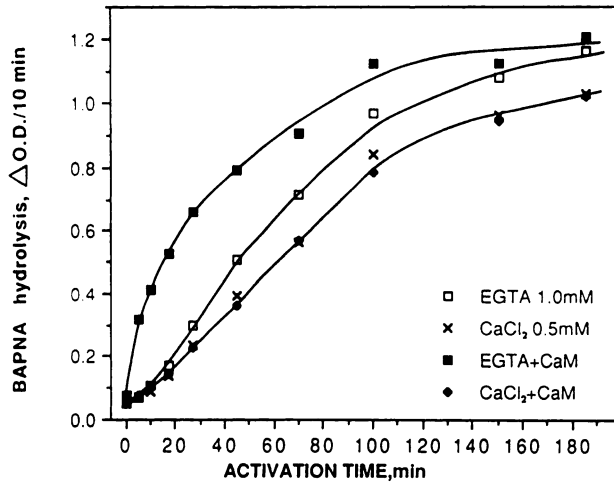


Fig. 2. Time-course of activation of proacrosin into acrosin in the presence or absence of CaM (10 $\mu\text{g/ml}$), EGTA (1 mM) and CaCl_2 (0.5 mM). The activation of proacrosin was started by elevating the pH from 3.0–7.1. At various time intervals, samples were removed and the hydrolysis of BAPNA during 10 min was determined. Control experiments showed that activation of proacrosin under these conditions (presence of BAPNA) was negligible during the enzymatic assay.

because very high backgrounds were observed (results not shown).

The next experiments were designed to obtain information on the interaction of CaM with the proacrosin-acrosin system in a more native state. As a first step, the effects of CaM and Ca^{2+} on the activation of proacrosin into acrosin were determined. Figure 2 shows that the presence of CaM and EGTA accelerated the activation of proacrosin at pH 7.1.

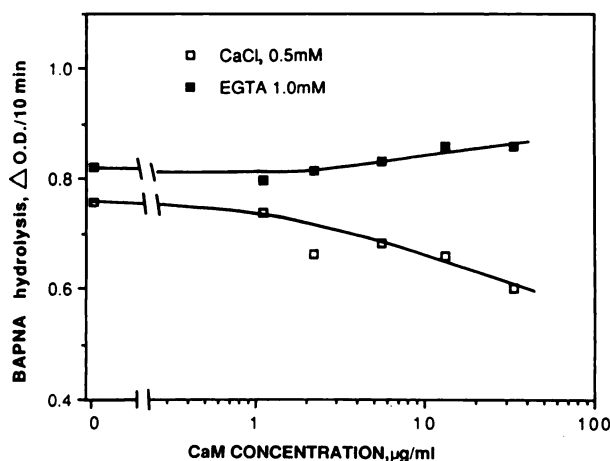


Fig. 3. Effect of increasing concentrations of CaM on the hydrolysis of BAPNA in the presence of 1 mM EGTA or 0.5 mM CaCl_2 . The concentration of purified acrosin in the assay was 10 $\mu\text{g/ml}$.

Indeed, the time required for half-activation was 55 min in the presence of EGTA alone versus 23 min in the presence of both EGTA and CaM. By contrast, when 0.5 mM Ca^{2+} was present with CaM, the rate of activation was decreased ($t_{1/2} = 73$ min). It should be noted that in this experiment, the activation was done at pH 7.1 (instead of 8.0) in order to respect physiological conditions. Similar results were also obtained when the activation was performed at pH 8.0, although the rate of activation was increased approximately six to sevenfold (results not shown).

Thereafter, we determined the effects of Ca^{2+} , EGTA, and increasing concentrations of CaM on the enzymatic activity of purified acrosin. Figure 3 shows that CaM at concentrations up to 33 $\mu\text{g/ml}$ (2 μM) had only a small influence on the hydrolysis of BAPNA in the presence of EGTA in the reaction mixture, where as 20% decrease was observed in the presence of Ca^{2+} and high concentration of CaM.

Because acrosin is a proteolytic enzyme, we wondered whether the absence of effects of CaM in the presence of EGTA on the acrosin activity might be explained by proteolysis of CaM. To verify this hypothesis, we studied the rate of hydrolysis of CaM by acrosin in the presence of both EGTA and various concentrations of Ca^{2+} . In the absence of Ca^{2+} (with or without 1 mM EGTA), CaM was hydrolysed rapidly (Fig. 4). After 2.5 min, no

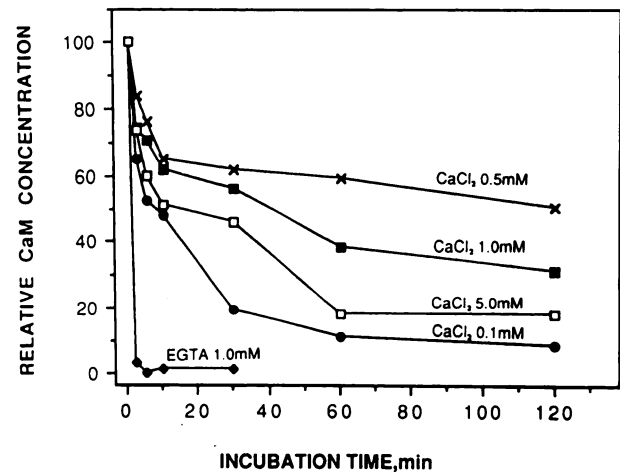


Fig. 4. Hydrolysis of CaM by ram acrosin. Pure CaM (90 $\mu\text{g/ml}$ final concentration) and pure acrosin (10 $\mu\text{g/ml}$ final concentration) were incubated together in 1 mM EGTA or various concentrations (0.1–5 mM) of CaCl_2 . At each indicated time, a sample was removed and mixed with twofold concentrated electrophoresis sample buffer (Laemmli, 1970). CaM relative concentration was determined as described in Materials and Methods.

detectable CaM or fragments of CaM were found. The presence of Ca^{2+} at concentrations of 0.1–5.0 mM afforded considerable protection against CaM hydrolysis, the optimal effect being observed at 0.5 mM. At higher Ca^{2+} concentrations (1 and 5 mM), the protective effect was slightly attenuated.

In order to determine the specificity of the proteolytic attack on CaM, we compared the peptides generated by the action of acrosin and trypsin in the presence of EGTA or Ca^{2+} (Fig. 5). Trypsin appeared to be more active than acrosin on a weight basis on CaM hydrolysis. The major peptides generated with the two enzymes had similar molecular weights. However, a short-lived high molecular weight fragment not seen with trypsin was generated with acrosin. This fragment was particularly evident in the presence of Ca^{2+} . Furthermore, the peptide patterns differed slightly in low molecular weight bands whether EGTA or Ca^{2+} was present.

Discussion

Our results show that CaM interacts physically with various forms of proacrosin and acrosin that have been denatured during SDS gel electrophoresis and ^{125}I -CaM overlay procedure (Fig. 1). Although our preparation of proacrosin was not entirely pure, the identity of the 45 and 43 kDa CaM binding proteins as proacrosin forms was confirmed by Western blot analysis with antiacrosin antibodies. Furthermore, these 45 and 43 kDa proteins were major Coomassie blue stained proteins which were transformed during proacrosin activation into two proteins, one which corresponded exactly to the electrophoretic mobility of acrosin purified by affinity chromatography.

The *in vitro* interaction of CaM with the proacrosin-acrosin system would remain an interesting curiosity if it did not affect some functions of acrosin in its native state. However, in the native state and in conditions of physiological pH, acrosin rapidly degrades calmodulin. Therefore, studies with calmodulin affinity columns or with gel filtration columns would be meaningless. For this reason, we decided to determine the effect of calmodulin on the activity of proacrosin, since this proenzyme form should have little proteolytic activity toward calmodulin. The modulation of proacrosin autoconversion into acrosin by CaM in the absence of Ca^{2+} suggests that this phenomenon could be physiologically significant, since the physical interaction of proacrosin and CaM was also higher in absence of

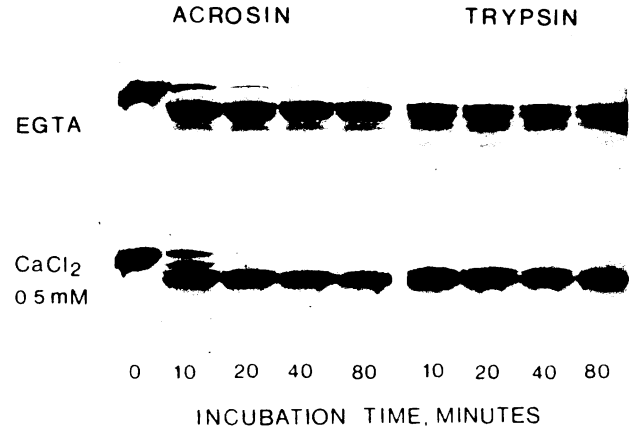


Fig. 5. Comparison of the proteolytic fragments of calmodulin obtained either with acrosin or trypsin in the presence of 1 mM EGTA or 0.5 mM CaCl_2 . To obtain fragments of comparable staining intensities, the concentration of acrosin in the presence of EGTA or CaCl_2 were fixed respectively at 2 and 6.3 μg and those of trypsin in the presence of EGTA or CaCl_2 at 100 and 500 ng per 0.2 ml. Calmodulin concentration was the same (45 $\mu\text{g}/0.2$ ml) in all incubations. The proteins were separated by SDS gel electrophoresis according to Swank and Munkres (1971) to achieve a good resolution of the low molecular weight components.

Ca^{2+} (Fig. 1). However, the influence of CaM on the rate of activation of proacrosin was small (2–3 fold increase) compared with the previously reported *in vitro* influence of glycosaminoglycans (Parrish et al, 1980) and of phospholipid vesicles (Parrish et al, 1978) on the conversion of boar proacrosin into acrosin. A concentration of 4 μg per ml of heparin or of 0.4 mM phospholipid increased boar proacrosin activation rate by more than a 1000-fold and a 100-fold, respectively.

It is also possible that CaM could influence other physicochemical properties of proacrosin not determined here. These properties include zona pellucida binding/fucose binding (Topfer-Peterson, 1988) and digestion of zona pellucida proteins (Polakoski and McRorie, 1973).

The most dramatic effect observed in this study was the rapid and extensive hydrolysis of CaM by acrosin in absence of Ca^{2+} . This result, which is similar to the one previously observed with trypsin (Walsh and Stevens, 1977), suggests that under certain conditions *in vivo* acrosin could attenuate the action of CaM. It is not surprising that no effects of CaM are observed on acrosin activity *in vitro* (Fig. 3) even though acrosin is a CaM-binding protein.

It must be remembered that acrosin in the overlay procedure (Fig. 1) was in a denatured form with no enzymatic activity. This result suggests that the CaM-binding domain of acrosin is different from the active site of the enzyme and does not need complex secondary and tertiary structure. Furthermore, the CaM-acrosin interaction probably has no physiological meaning unless acrosin is complexed to an inhibitor. This possibility should not be excluded since the presence of such inhibitors has been shown previously in mammalian sperm acrosome (Zaneveld et al, 1973).

Although we have demonstrated that CaM is able to interact physically *in vitro* with the proacrosin-acrosin system, its *in vivo* influence on the acrosin regulated functions remains difficult to predict solely on the basis of this demonstration. In order to elaborate a coherent model, one must consider the precise ultrastructural localization of CaM and acrosin, the timing of Ca^{2+} influx as well as the timing of proacrosin activation, and the presence of other Ca^{2+} dependent and Ca^{2+} independent CaM binding proteins. The main area of uncertainty remains the *in vivo* timing and mechanism of proacrosin activation. We are not certain that a single mechanism could explain this activation, since the proacrosin-acrosin system has been attributed many roles, namely in the acrosome reaction, in the binding of sperm to zona pellucida, in the digestion of zona pellucida proteins, and in other processes (Polakoski and Siegel, 1986). The different localization of proacrosin-acrosin in the outer and inner acrosomal membranes (Johnson et al, 1983) could also account for these different actions and mechanisms of activation. Therefore, we think that any model on the role of the CaM and proacrosin association would be presently premature. However, the demonstration by immunological methods at the electron microscopy level that some CaM is found in the tip of the acrosome of ram ejaculated spermatozoa (Weinman et al, 1986) suggests that this association is physiologically significant. Finally, the CaM-proacrosin association could also be involved in sperm maturation since CaM concentration is much higher in the acrosome of ovine immature spermatozoa (spermatids and caput epididymis spermatozoa) than in the acrosome of ejaculated spermatozoa (Weinman et al, 1986).

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