Evidence for Involvement of Metalloendoproteases in a Step in Sea Urchin Gamete Fusion

Judith L. Roe, Horacio A. Farach, Jr., Warren J. Strittmatter,* and William J. Lennarz

Department of Biochemistry and Molecular Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030; and *Department of Neurology, Baylor College of Medicine, Houston, Texas 77030

Abstract. Membrane fusion events are required in three steps in sea urchin fertilization: the acrosome reaction in sperm, fusion of the plasma membrane of acrosome-reacted sperm with the plasma membrane of the egg, and exocytosis of the contents of the egg cortical granules. We recently reported the involvement of a Zn^{2+}-dependent metalloendoprotease in the acrosome reaction (Farach, H. C., D. I. Mundy, W. J. Strittmatter, and W. J. Lennarz. 1987. *J. Biol. Chem.* 262: 5483–5487). In the current study, we investigated the possible involvement of metalloendoproteases in the two other fusion events of fertilization. The use of inhibitors of metalloendoproteases provided evidence that at least one of the fusion events subsequent to the acrosome reaction requires such enzymes. These inhibitors did not block the binding of sperm to egg or the process of cortical granule exocytosis. However, sperm–egg fusion, assayed by the ability of the bound sperm to establish cytoplasmic continuity with the egg, was inhibited by metalloendoprotease substrate. Thus, in addition to the acrosome reaction, an event in the gamete fusion process requires a metalloendoprotease.

At least three plasma membrane fusion events are required for fertilization in the sea urchin *Strongylocentrotus purpuratus* (Kay and Shapiro, 1985; Schuel, 1985; Trimmer and Vacquier, 1986; Rossignol et al., 1984). The first membrane fusion event, depicted in Fig. 1, is the acrosome reaction (*I*), which involves Ca^{2+}-dependent fusion of the membrane of the acrosomal vesicle with the sperm plasma membrane. We recently reported evidence that a Zn^{2+}-dependent metalloendoprotease in the sperm is involved in this process and that sperm contain metalloendoprotease activity (Farach et al., 1987). In the second fusion event (*II*), which occurs after the sperm undergoes the acrosome reaction and binds to an egg receptor, the sperm plasma membrane fuses with the plasma membrane of the egg. The third fusion event (*III*) is an exocytotic event involving the cortical granule membrane and the egg plasma membrane.

Insight into the possible role of proteins in the fusion process has been provided by a number of studies. First, the fusion of certain viral envelopes with the host cell membrane was shown to be mediated by viral coat proteins (White et al., 1983). Second, studies on the cell-cell fusion of myoblasts, as well as on exocytosis in mast cells and adrenal chromaffin cells, showed that certain proteases may be directly involved in membrane fusion (Couch and Strittmatter, 1983, 1984; Mundy and Strittmatter, 1985). Third, we recently established that the acrosome reaction in sperm is inhibited by inhibitors of metalloendoprotease and that metalloendoprotease activity is present in these cells (Farach et al., 1987).

In the current report we studied the effect of both inhibitors and substrates of metalloendoproteases on the fertilization process. Because the membrane fusion events can be assayed independently in this system, we were able to investigate the requirement for metalloendoprotease activity in both cortical granule exocytosis and gamete fusion. The results of these studies, coupled with our earlier observations on the acrosome reaction (Farach et al., 1987), implicate one or more metalloendoproteases in both the acrosome reaction and an event in the gamete fusion process.

Materials and Methods

Gamete Collection and Handling

Gametes were collected from *Strongylocentrotus purpuratus* as previously described (SeGall and Lennarz, 1979).

Fertilization Bioassay

Eggs were washed twice with Millipore-filtered sea water (MFSW),[1] then diluted 1:100 in MFSW, and 0.5-ml aliquots were treated with the peptides or chelator for 1 min. The sperm suspension was then further diluted 1:1,000 in MFSW. A 10-μl aliquot was removed and diluted to 1 ml. In this dilution step the synthetic peptide or chelator was added for 30 s before the fertilization assay was begun. Subsequently, 10 μl of the final suspension of diluted sperm was mixed into the 0.5-ml suspension of eggs, incubated for 5 min, and then assessed by light microscopy for elevation of the fertilization envelope. At least 200 eggs were counted for each point. In this assay, the percentage of eggs fertilized must be linearly dependent on the concentration of sperm; therefore, the sperm concentration was routinely adjusted so that

1. *Abbreviation used in this paper*: MFSW, Millipore-filtered sea water.

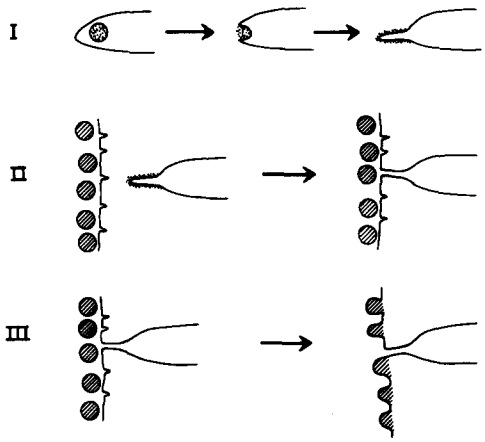

Figure 1. Scheme depicting three membrane fusion events in fertilization: *I*, acrosome reaction; *II*, sperm–egg plasma membrane fusion; and *III*, cortical granule exocytosis.

~55% fertilization occurred in control samples to be in the linear range of fertilization. After dilution, sperm were used as quickly as possible (within 30 s) to avoid sperm death. To measure fertilization using sperm preinduced to undergo the acrosome reaction, the jelly coat was removed from the eggs (pH 5.5, 2 min, and washing 2× with MFSW). These dejellied eggs were mixed with sperm pretreated with purified jelly coat (SeGall and Lennarz, 1979). The acrosome reaction was monitored by observation in the electron microscope and conditions were established such that 80–99% of the sperm were acrosome reacted within 30 s. In this assay, the eggs were exposed to the metalloendoprotease inhibitor or substrate before the pretreated sperm were added, thus eliminating any effect these compounds might have on the acrosome reaction. Pretreated sperm were added to the eggs within 30 s because sperm that have undergone the acrosome reaction die quickly (Kinsey et al., 1979). For the sperm binding assay and the sperm–egg fusion assay, sperm were diluted 1:1 with MFSW and then diluted to a concentration that gave 100% fertilization in controls (after induction with jelly coat).

Ionophore Activation of Eggs

Eggs were induced to undergo the cortical reaction by the addition of the ionophore A23187 (Sigma Chemical Co., St. Louis, MO) in DMSO (final concentration = 0.1%). After 5 min, the percentage of eggs with elevated fertilization envelopes was determined. Calcium ionophore A23187 was added to a concentration (typically 3–16 μM) that induced the cortical reaction in ~50% of the eggs. At least 200 eggs were counted for each experiment.

Sperm Binding Assay

Sperm binding was determined as described (Kinsey et al., 1980). Briefly, 25 s after sperm were added, samples were fixed in an equal volume of 2% glutaraldehyde in MFSW (10 mM Tris, pH 8.35) and washed twice in 4 ml MFSW (10 mM Tris, pH 8.35); the number of sperm remaining bound to the circumference of the egg was then counted. The number of fertilized eggs was determined by fixing the samples in an equal volume of 6% glutaraldehyde in MFSW (10 mM Tris, pH 8.35) 5 min after addition of sperm and determining the percentage of eggs with fertilization envelopes.

Sperm–Egg Fusion Assay

The assay was performed essentially as described by Hinckley et al. (1986). A 10% suspension of acid-dejellied eggs was incubated in 10 μg/ml Hoechst dye 33342 (Calbiochem-Behring Corp., San Diego, CA) in MFSW at 14°C for 30–60 min, washed twice in MFSW, and brought to a 1% suspension in MFSW. Sperm preinduced to undergo the acrosome reaction were added to a 0.5-ml aliquot of eggs to give 100% fertilization in the control sample. The samples were fixed in an equal volume of 2% glutaraldehyde in MFSW (10 mM Tris, pH 8.35). Samples were washed twice in 4 ml MFSW (10 mM Tris, pH 8.35) after eggs had settled and were viewed on a Nikon Diaphot microscope equipped for epifluorescence. Fluorescent images were visualized using a UV-1A filter and were recorded on Kodak Tri-X film.

Results

Inhibitors of Metalloendoproteases Block Fertilization at a Step after the Acrosome Reaction

Previously, we have shown that the membrane fusion required for the sperm acrosome reaction was blocked by inhibitors of metalloendoproteases and that a metalloendoprotease was present in sperm homogenates (Farach et al., 1987). In this study, we initially determined whether or not the membrane fusion events in fertilization subsequent to the acrosome reaction were similarly blocked by these compounds. Using a sensitive fertilization assay (see Materials and Methods) where fertilization is linearly dependent on the amount of sperm added, we found that the fluorogenic protease substrate succinyl-Ala-Ala-Phe-4-aminomethylcoumarin (Succ-Ala-Ala-Phe-AMC), which has been used to assay metalloendoproteases in a variety of cells including sea urchin sperm (Mumford et al., 1980; Mundy and Strittmatter, 1985; Farach et al., 1987), inhibited the overall process of fertilization (ID_{50} = 0.45 mM; Fig. 2 *A*). Another metalloendoprotease substrate, carbobenzoxy-Gly-Phe-NH_2 (CBZ-Gly-Phe-NH_2), also inhibited fertilization (ID_{50} = 1.37 mM).

Both of these substrates could affect the overall fertilization process merely by their inhibitory action on the acrosome reaction or by blocking a subsequent step in this multistep process. Therefore, we examined the effect of these substrates on the individual steps in fertilization. We previously reported (Farach et al., 1987) that the first fusion event in fertilization, the acrosome reaction in sperm, was inhibited by the peptide Succ-Ala-Ala-Phe-AMC. Therefore, to study the effects of this protease substrate on subsequent events (i.e., gamete binding, sperm and egg plasma membrane fusion, and the cortical granule reaction) we assayed fertilization using sperm that had been induced to undergo the acrosome reaction before the introduction of peptide substrate. We found (Fig. 2 *B*) that Succ-Ala-Ala-Phe-AMC in-

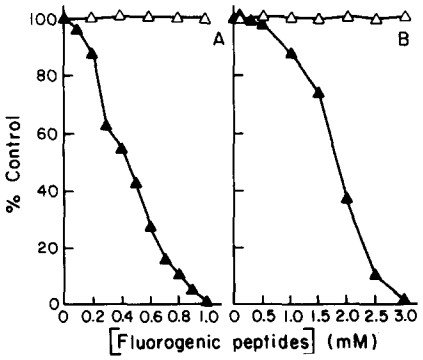

Figure 2. (*A*) Effects of fluorogenic protease substrates on the overall process of fertilization. Eggs and sperm were incubated with increasing concentrations of either Succ-Ala-Ala-Ala-AMC (△) or Succ-Ala-Ala-Phe-AMC (▲). (*B*) Effects of fluorogenic protease substrates on fertilization using sperm preinduced to undergo the acrosome reaction. Eggs were incubated with increasing concentrations of the two fluorogenic peptides.

hibited fertilization (ID_{50} = 1.8 mM). Succ-Ala-Ala-Ala-AMC, which is not a metalloendoprotease substrate, did not inhibit the overall process of fertilization (Fig. 2 *A*) or fertilization using acrosome-reacted sperm (Fig. 2 *B*). The dipeptide substrate CBZ-Gly-Phe-NH_2 also inhibited fertilization by preinduced sperm (ID_{50} = 2.2 mM), whereas CBZ-Gly-Gly-NH_2, which is not a substrate, had no effect. These observations establish that the metalloendoprotease substrates inhibited not only the sperm acrosome reaction (Farach et al., 1987), but also some subsequent step in fertilization.

Because metalloendoproteases require Zn^{2+} for activity, the chelator 1,10-phenanthroline should inhibit processes requiring metalloendoprotease activity. Earlier we showed that this chelator inhibited the acrosome reaction in sperm (Farach et al., 1987). We therefore tested the effect of 1,10-phenanthroline on fertilization using sperm preinduced to undergo the acrosome reaction. As shown in Fig. 3, this chelator inhibited fertilization (using preinduced sperm) in a dose-dependent fashion (ID_{50} = 0.34 mM). Ca^{2+} ions were far in excess (10 mM) of the chelator in this experiment; therefore, inhibition of fertilization by 1,10-phenathroline was not merely the result of Ca^{2+} chelation. Furthermore, the observed inhibition was not the result of irreversible perturbation of the egg membrane because pretreatment of eggs with 1,10-phenanthroline (2.2 mM) followed by washing to remove the chelator had no inhibitory effect on subsequent fertilization.

Another metal chelator, EDTA, also prevented the overall process of fertilization. As shown in Fig. 4, this inhibition of fertilization was reversed in a dose-dependent manner by the reintroduction of Zn^{2+}, consistent with results obtained by Clapper et al. (1985) for the acrosome reaction. Thus, both the acrosome reaction and a subsequent event inhibited by the chelator were restored by reintroduction of Zn^{2+}. Inhibition of fertilization at higher concentrations of Zn^{2+} may be due to inhibition of the acrosome reaction, an unexplained phenomenon that was observed earlier by Clapper et al. (1985).

Fertilization Envelope Elevation and Cortical Granule Exocytosis Is Not Blocked by Metalloendoprotease Inhibitors and Substrates

The previous experiments implicated a metalloendoprotease in events subsequent to the acrosome reaction. To distinguish between the possibilities that a metalloendoprotease was involved in gamete binding, sperm–egg membrane fusion, or cortical granule exocytosis, we studied the effect of 1,10-phenanthroline on the induction of the cortical reaction in eggs. The cortical granule reaction was initiated by directly introducing Ca^{2+} via the ionophore A23187. As shown in Fig. 5, at a concentration (1.1 mM) that blocks the acrosome reaction in sperm and blocks fertilization using preinduced sperm, 1,10-phenanthroline did not inhibit the cortical reaction. Jackson et al. (1985) also reported that 1,10-phenanthroline has no effect on the in vitro cortical reaction induced by calcium in isolated egg cortices. We have confirmed this finding in isolated egg cortices using the metalloendoprotease peptide substrate CBZ-Gly-Phe-NH_2, rather than chelators (Macek, C. B., W. J. Strittmatter, and W. J. Lennarz, unpublished observations). Thus, a metalloendoprotease does not appear to be involved in cortical granule exocytosis. Because the endpoint of the fertilization assay is formation of the fertilization envelope resulting from cortical granule exocytosis, these data also show that the effect on fertilization of the metalloendoprotease inhibitors is not simply due to an inhibition of fertilization envelope formation.

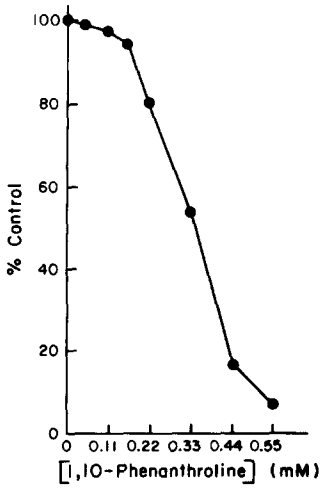

Figure 3. Inhibition of fertilization by 1,10-phenanthroline using preinduced sperm. Eggs were incubated with increasing concentrations of 1,10-phenanthroline and then mixed with sperm which had been preinduced with jelly coat to undergo the acrosome reaction.

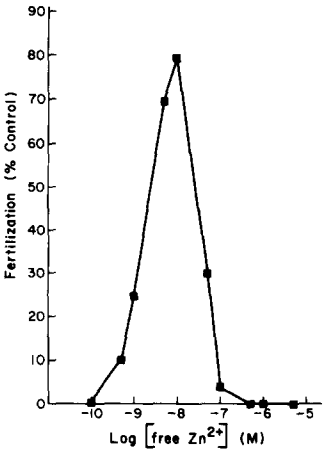

Figure 4. Effect of Zn^{2+} on fertilization inhibited by EDTA. Sperm and eggs were treated with 1 mM EDTA and increasing concentrations of $ZnCl_2$ to reach the final free Zn^{2+} concentrations shown. Free Zn^{2+} calculations were made using a program described by Fabiato and Fabiato (1979). Fertilization was assayed as described in Materials and Methods.

Metalloendoprotease Substrates Block Gamete Fusion and Not Gamete Binding

Having excluded the cortical granule exocytotic reaction as

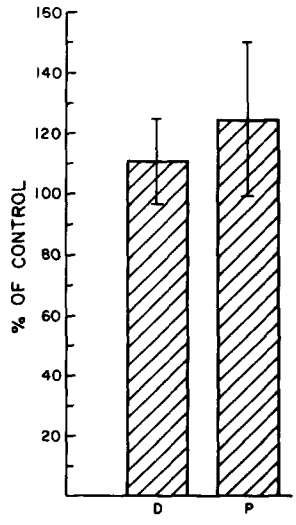

Figure 5. Effect of 1,10-phenanthroline on ionophore-induced activation of eggs. Eggs were preincubated for 15 min at 14°C with 0.1% DMSO (*D*) or 1.1 mM 1,10-phenathroline (*P*) in 0.1% DMSO and induced to undergo the cortical reaction by the addition of the ionophore A23187. Data are expressed as percentage of control (no pretreatment of eggs) and each bar represents the mean ± SEM of four experiments.

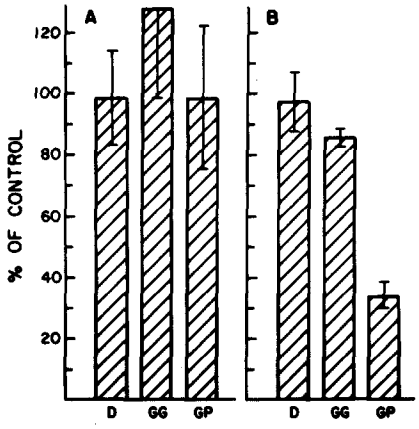

Figure 6. Effect of dipeptides on sperm binding and fertilization. Acid-dejellied eggs were pretreated with 1% DMSO (*D*), 2 mM CBZ-Gly-Gly-NH_2 (*GG*), or 2 mM CBZ-Gly-Phe-NH_2 (*GP*) in 1% DMSO followed by addition of sperm preinduced to undergo the acrosome reaction. The number of sperm bound per egg (*A*) and the percent of fertilized eggs (*B*) are shown. The data are expressed as percentage of control (no pretreatment of eggs) and each bar represents the mean ± SEM of four experiments.

a site of action of metalloendoprotease, we investigated gamete binding and the subsequent fusion event involving sperm and egg plasma membranes. Because binding is a prerequisite for fusion (Ruiz-Bravo and Lennarz, 1987), it was important to exclude the possibility that an inhibitor blocks fertilization by simply blocking sperm–egg binding. It has been previously established (Kinsey et al., 1980) that the optimum time for measuring sperm binding in *S. purpuratus* is 25 s postfertilization, when the number of sperm bound is maximal just before elevation of the fertilization envelope. As seen in Fig. 6, although fertilization using preinduced sperm was inhibited by 66% by CBZ-Gly-Phe-NH_2 (Fig. 6 *B*), the number of sperm bound per egg was unchanged by this peptide (Fig. 6 *A*). The control dipeptide, CBZ-Gly-Gly-NH_2, was not inhibitory in either assay. We conclude from these observations that the dipeptide CBZ-Gly-Phe-NH_2 inhibited gamete fusion, not merely sperm–egg binding. These results also show that the inhibition of fertilization by metalloendoprotease inhibitors observed in experiments using acrosome-reacted sperm is not due to inhibition of the acrosome reaction in the small percentage of sperm that were not prereacted because in this case a decrease in sperm binding would have been observed.

The results of the preceding studies excluded sperm–egg binding and the cortical reaction as the site of inhibition by metalloendoprotease inhibitors and substrates and implied that the site of action was gamete membrane fusion. To directly test this, we used a recently described assay that allows direct visualization of the single sperm that fuses with the egg within seconds of gamete interaction (Hinckley et al., 1986). Eggs incubated before fertilization with the DNA-binding dye Hoechst 33342 (and then washed to remove excess dye) exhibit one brightly fluorescent sperm upon fertilization. Only the sperm that has established cytoplasmic continuity with the egg becomes fluorescent (Hinckley et al., 1986). We used this assay to confirm the hypothesis that sperm–egg fusion had been inhibited by the dipeptide CBZ-Gly-Phe-NH_2. The results are shown in Fig. 7. As shown in Fig. 7 *A*, 25 s after fertilization the fluorescent fertilizing sperm could be seen below the elevating fertilization enve-

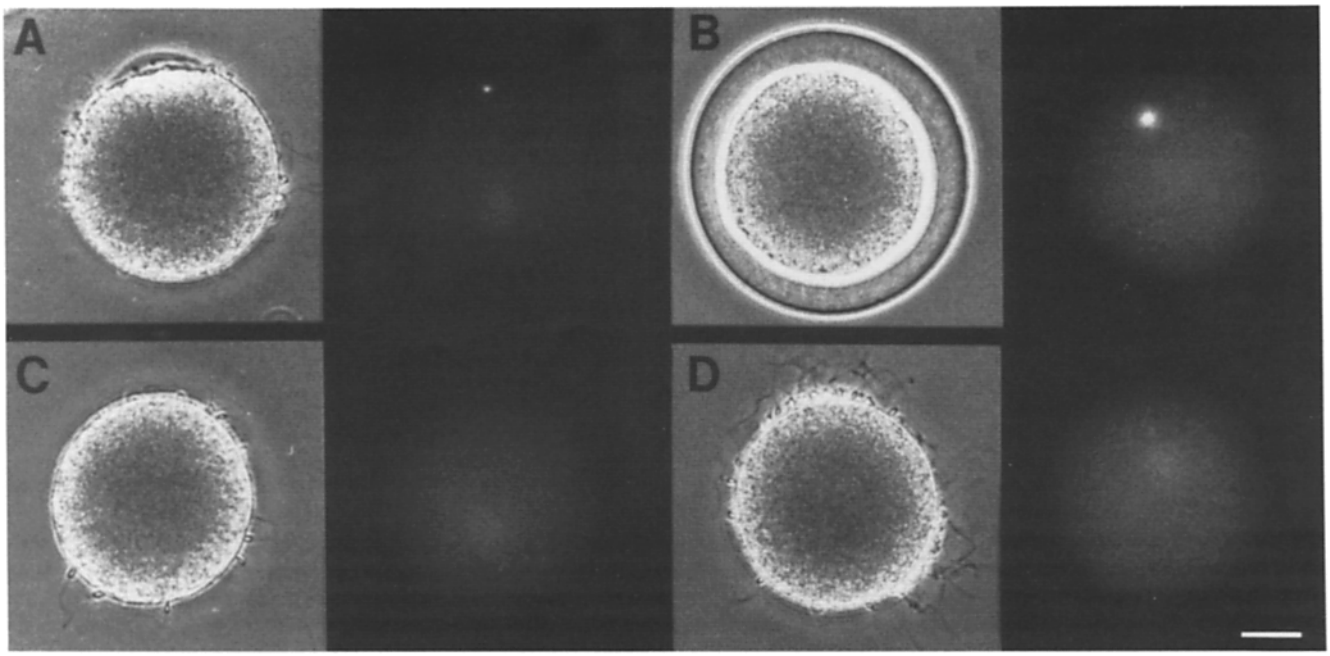

Figure 7. Effect of dipeptides on sperm–egg fusion. Eggs preloaded with Hoechst dye 33342 were fertilized with sperm preinduced to undergo the acrosome reaction. Eggs were either untreated (*A* and *B*) or preincubated in 2 mM CBZ-Gly-Phe-NH_2 (*C* and *D*). Samples were fixed in 2% glutaraldehyde 25 s (*A* and *C*) or 3 min (*B* and *D*) after addition of sperm. Phase-contrast micrographs are shown on the left and the corresponding fluorescent image on the right of each panel. The pronucleus of the egg is easily seen as a large diffuse ring, whereas the fertilizing sperm appears as a bright spot. Note the absence of a fluorescent sperm, even after 3 min (when control eggs are clearly seen fertilized) in eggs treated with CBZ-Gly-Phe-NH_2. Bar, 25 μm.

Table I. Metalloendoprotease Inhibitors Block Sperm–Egg Fusion

Treatment	Percent fertilized*	Percent inhibition of fertilization	Percent of fertilized eggs positive for sperm fusion‡	Percent of unfertilized eggs positive for sperm fusion§
Control	94.7 ± 2.5	–	73.0 ± 5.3	0
CBZ-Gly-Gly-NH_2	95.1 ± 1.1	0	71.0 ± 2.1	0.4 ± 0.2
CBZ-Gly-Phe-NH_2	22.1 ± 9.9	76.9 ± 9.6	73.5 ± 6.8	0.6 ± 0.6

Eggs were dejellied and loaded with Hoechst dye 33342 as described in Materials and Methods. Eggs were pretreated with 4 mM CBZ-Gly-Gly-NH_2 or 4 mM CBZ-Gly-Phe-NH_2 or untreated (control) and fertilized with sperm preinduced to undergo the acrosome reaction. Eggs were fixed after 3 min, washed, and viewed under phase optics to observe fertilization envelope elevation and fluorescence optics using a UV-1A filter to observe the presence of a fluorescent sperm, indicating sperm–egg fusion.

* Percent of eggs with elevated fertilization envelope. The data are expressed as mean of three experiments ± SEM (at least 100 eggs were counted for each point).

‡ Number of fertilized eggs showing a fluorescent sperm divided by the total number of fertilized eggs.

§ Number of unfertilized eggs showing a fluorescent sperm divided by total number of unfertilized eggs.

lope in control eggs; other sperm were still bound but were not fluorescent. At 3 min after fertilization, the fertilization envelope had fully elevated and the fertilizing sperm could be seen clearly (Fig. 7 *B*). The control dipeptide CBZ-Gly-Gly-NH_2 did not inhibit the appearance of a fluorescent sperm (data not shown). In the presence of CBZ-Gly-Phe-NH_2, however, at both 25 s (Fig. 7 *C*) and 3 min (Fig. 7 *D*) after fertilization, no fluorescent sperm were seen on the inhibited eggs, although nonfluorescent sperm remained bound to the egg. Thus, sperm–egg fusion was inhibited by this metalloendoprotease substrate.

A quantitative analysis of eggs examined using this assay is summarized in Table I. In these assays, the amount of sperm was adjusted so that 100% of the control eggs were fertilized. Thus, we could directly observe inhibition of fertilization in the samples treated with inhibitors. The concentration of inhibitor used was adjusted to give <100% inhibition to exclude nonspecific effects of the inhibitors. As shown in the control, 95% of the eggs were fertilized; in 70% of these, a fluorescent sperm, indicating fusion of sperm and egg, could be detected. In 30% of the fertilized eggs, no fluorescent sperm was seen, presumably due to incomplete loading of the eggs with the dye or the positioning of the fluorescent sperm out of the field of focus. In eggs treated with CBZ-Gly-Gly-NH_2, not a substrate for metalloendoproteases, no inhibition of fertilization was observed and the percent of fertilized eggs with a fluorescent sperm was identical to that in the control. In the presence of the metalloendoprotease inhibitor, CBZ-Gly-Phe-NH_2, fertilization was inhibited by 76.9% and, most importantly, 0% of the unfertilized eggs showed the presence of a fluorescent sperm, indicating that sperm–egg fusion was inhibited by the peptide substrate.

Discussion

The studies reported here, together with earlier studies on sperm (Farach et al., 1987), demonstrate that substrates and inhibitors of metalloendoproteases prevent the membrane fusion events in both the sperm acrosome reaction and sperm–egg fusion, but have no effect on egg cortical granule exocytosis. Several observations indicate that the inhibitors and substrates specifically inhibit these fusion events as a result of interaction with metalloendoprotease(s). First, the ability of peptides to inhibit fusion correlates well with their ability to interact with metalloendoproteases. Thus, peptides that interact with these proteases (e.g., Succ-Ala-Ala-Phe-AMC and CBZ-Gly-Phe-NH_2) blocked the fusion events in fertilization, whereas peptides that do not interact with protease (e.g., Succ-Ala-Ala-Ala-AMC and CBZ-Gly-Gly-NH_2) had no effect on fusion. Second, the metal chelators 1,10-phenanthroline and EDTA inhibited both the acrosome reaction and fertilization. Inhibition of both processes was reversed by Zn^{2+}, which reconstitutes the overall process of fertilization, suggesting that the effect was not merely due to an irreversible perturbation of the egg membrane by the chelators. Third, preincubation of the egg with 1,10-phenanthroline followed by removal by washing had no effect on fertilization.

The observations that metalloendoprotease inhibitors and substrates blocked the calcium-dependent exocytosis in the acrosome reaction in sperm but had no effect on the calcium-dependent exocytosis of the cortical granule reaction in the egg, suggests different requirements for fusion in these two cell types. These compounds having no effect in the ionophore-induced cortical granule reactions in the egg is not due to failure to enter the egg because they also had no effect on the calcium-induced reaction (Decker and Lennarz, 1979) in preparations of the isolated egg cortex.

We previously demonstrated that fusion of myoblasts to form myotubules is inhibited by metalloendoprotease inhibitors (Couch and Strittmatter, 1983, 1984). In this system the fusion of myoblasts, a developmentally regulated, homotypic event, results in large multinucleated syncytia. In contrast, the fusion of a sperm and an egg involves a two-cell heterotypic fusion in which the sperm genome is inserted into the egg. In this system, unlike the myoblast fusion system, cell binding can be assayed independently from cell fusion. Also, fusion can be directly monitored by observing the transfer of fluorogenic dye from the egg to the single fertilizing sperm immediately after fusion. As reported here, metalloendoprotease inhibitors specifically inhibited gamete fusion and not gamete binding. In addition, sperm–egg fusion was unambiguously demonstrated to be zinc dependent by reconstitution with exogenous zinc after chelation of endogenous metals.

These results, coupled with earlier studies on sperm (Farach et al., 1987), implicate one or more metalloendoproteases in both the acrosome reaction and in gamete fusion. The metalloendoprotease implicated in these studies appears to be different from other proteases previously studied in *S. purpuratus* sperm (Green and Summers, 1980; Resing et al., 1985). We do not yet know whether the metalloendoprotease implicated in gamete fusion is the same enzyme

we previously characterized in sperm and implicated in the acrosome reaction (Farach et al., 1987). What also remains to be established is how these enzymes function in the membrane fusion events that are common to both of these biological processes. In this context, the similarities between the heterotypic cell fusion process studied in this report and the virus–host fusion process are of interest. Both involve receptors and result in the introduction of a "foreign" genome. Also, in the case of viral–host fusion it is clear that prior proteolytic cleavage and exposure of a fusogenic protein domain is required (White et al., 1983). The current studies strongly implicate proteolytic cleavage in sperm–egg fusion. However, it remains to be established if such cleavage results in concomitant generation of a fusogen. Alternatively, the metalloendoprotease may be involved in proteolysis of either the egg vitelline layer or sperm cell surface proteins, thereby allowing the bilayers of the two gametes to come into close apposition before fusion.

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