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The Effects of Ca^{2+} and Mg^{2+} on the Major Gelatinase Activities Present in the Sea Urchin Embryo

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We have examined the effects of Ca^{2+} and Mg^{2+} on the activities of the two most prominent gelatinases present in the developing sea urchin embryo. The EDTA-inhibited 41 kDa gelatinase was variously reactivated by concentrations of Mg^{2+} at or below 15 mM while the EDTA-inhibited 87 kDa gelatinase could not be reactivated by Mg^{2+}. Magnesium partially inhibited the activities of both gelatinases and Ca^{2+} was protective against these inhibitory effects. In competition studies Mg^{2+} was shown to compete with Ca^{2+} for binding to the gelatinase. These results demonstrate competition between Ca^{2+} and Mg^{2+} for binding to and regulating the activities of these gelatinases in a marine environment which contains 10 mM Ca^{2+} and 50 mM Mg^{2+}.

The matrix metalloproteinases (MMPs) utilize various components of the extracellular matrix (ECM) as substrates. Based on substrate specificities the MMPs are divided into five subclasses; (a) collagenases (b) gelatinases (c) elastases (d) stromelysins and (e) membrane-type MMPs (1,2). The ECM is known to control such cellular properties as shape, growth, migration and differentiation suggesting that normal growth and development require a regulated ECM composition (3). The cell surface location and substrate specificities of the MMPs suggest that they are good candidate molecules for roles in remodeling the ECM (4).

The sea urchin embryo contains two ECMs, the hyaline layer on the apical surface and the basal lamina on the basal surface of ectoderm cells. Species in both the hyaline layer and basal lamina cross-react with antisera prepared against vertebrate ECM components (5) and recently several collagen encoding genes have been shown to be expressed during sea urchin embryogenesis (6, 7). We and others have begun to identify and characterize gelatinase activities present in the sea urchin egg and embryo (8, 9, 10, 11, 12). We have purified a 41 kDa activity from the sea urchin egg (11) and partially purified an 87 kDa activity from gastrula stage embryos (12); both species cleave gelatin in a Ca^{2+}-dependent manner and the 87 kDa gelatinase also appears to require Zn^{2+} for activity.

Since sea water contains 50 mM Mg^{2+} in addition to 10 mM Ca^{2+} we have recently undertaken a study of the effects of Mg^{2+}, in the presence and absence of Ca^{2+}, on the activities of these gelatinases. The results presented here clearly demonstrate a role for Mg^{2+} in modulating the activities of the 41- and 87 kDa gelatinases.

METHODS

Growth of embryos. *Strongylocentrotus purpuratus* (*Sp*) were purchased from Seacology, Vancouver, Canada, and gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed three times in ice-cold Millipore-filtered-seawater (MFSW; 0.45 μm) and fertilized with a 100-fold numerical excess of sperm. Embryos were cultured with constant aeration, at 12°C, in cylindrical chambers containing paddles rotating at 40 rpm. Samples were harvested at the times indicated after fertilization (HPF).

Purification of the gelatinases. The 41 kDa gelatinase was purified as described previously (11). The 87 kDa gelatinase was partially purified as described previously (12).

Substrate gel zymography. Substrate gel zymography was performed essentially as described previously (13). Sodium dodecyl sulfate containing gels were prepared by copolymerizing acrylamide and gelatin at a final concentration of 0.1% (w/v). Samples of eggs, embryos, or the purified gelatinases were dispersed for 30 min at room temperature in Laemmli solubilizing solution from which both DTT and bromophenol blue had been omitted (14). Electrophoresis was performed at 10 mA and 4°C for 4.5 h. After electrophoresis, the gels were incubated for 60 min at room temperature in 50 mM Tris-HCl, pH 8.0, containing 2.5% (v/v) Triton X-100 followed by 16 h incubation at room temperature in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue R-250 using a standard protocol.

Quantitative gelatinase assay. This assay was based on quantitating the gelatinase-dependent cleavage of gelatin into trichloroace-

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Abbreviations: EDTA, Ethylenediaminetetracetic acid; MFSW, Millipore filtered sea water; ECM, Extracellular matrix; MMP, Matrix metalloproteinase; TCA, Trichloroacetic acid.

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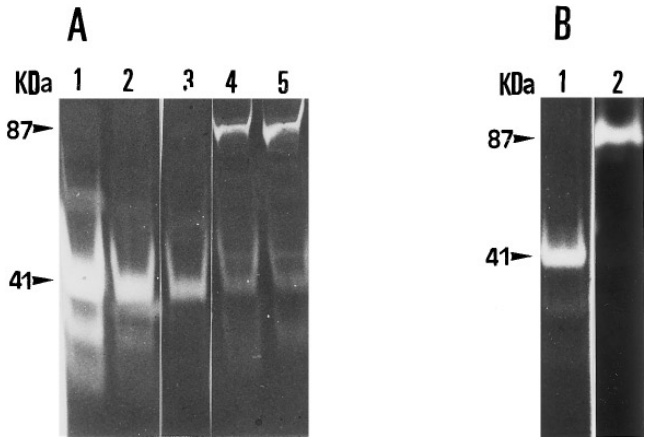

FIG. 1. Gelatin substrate gel zymography of eggs and various stage embryos (A) or the purified gelatinases (B). Aliquots (15 μg) of eggs and various stage embryos were analysed by gelatin substrate gel zymography as described in Methods. Lanes 1, 2, 3, 4, and 5 represent unfertilized eggs, 1, 21.5, 53 and 70.5 hour old embryos, respectively. (B) Aliquots (1 μg) of the purified 41 kDa gelatinase (lane 1) or (3 μg) of the partially purified 87 kDa gelatinase (lane 2) were analyzed by gelatin substrate gel zymography as described in Methods.

tic acid (TCA) soluble peptides. One unit of gelatinase activity was defined as the amount of gelatinase required to cleave 0.1 μg of gelatin/min into TCA soluble peptides at 37°C. Typically, 1- to 5 μL aliquots of gelatinase were added to a reaction volume of 100 μL containing the following: 0.1% (w/v) gelatin, 50 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. After incubation at 37°C, an equal volume of ice-cold 20% (w/v) TCA was added and the pellet was harvested by centrifugation and assayed for protein (15). In all assays, a reaction mixture consisting of the gelatinase, gelatin, Tris-HCl, and $CaCl_2$ was added to an equal volume of ice-cold 20% (w/v) TCA at zero time. The resultant pellet was used to establish a zero time value for protein content. Percentage cleavage was determined using the formula

$$\frac{\text{OD zero time} - \text{OD after incubation}}{\text{OD zero time}} \times 100$$

The number of units of gelatinase activity were calculated from the value of percentage cleavage. In all assays, the time points were within the linear range of gelatinase activity versus time and gelatinase concentrations were within the linear range of gelatinase activity versus gelatinase concentration.

Competition assays. In the competition assay, 5 μg aliquots of the purified 41 kDa on the partially purified 87 kDa gelatinase were dot-blotted onto nitrocellulose and the filters incubated for 60 min at room temperature in 10 mM Imidazole-HCl, pH 6.8 and 60 mM

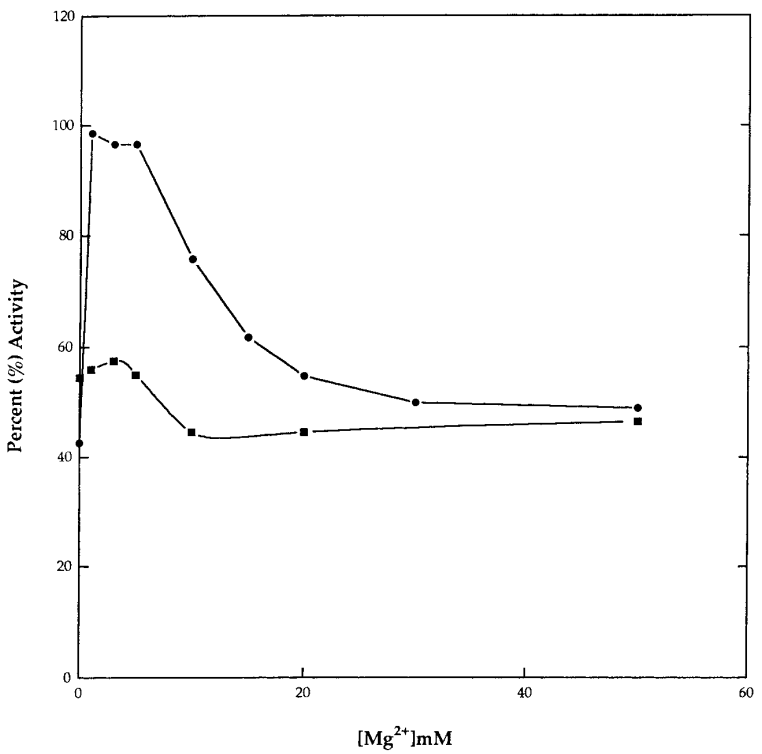

FIG. 2. The effect of Mg^{2+} on the EDTA-inhibited gelatinases. Aliquots of the gelatinases were made 5 mM in EDTA, incubated at room temperature for 20 min and diluted 50-fold into quantitative gelatinase assay mixes containing various concentrations of Mg^{2+} and no Ca^{2+}. Percent hydrolysis was measured after incubation at 37°C for 18 hr. All values were normalized with respect to aliquots of the gelatinases which were reactivated in the presence of 10 mM Ca^{2+} with no Mg^{2+} present. The 41 kDa gelatinase activity ●—● and the 87 kDa gelatinase activity ■—■ are displayed.

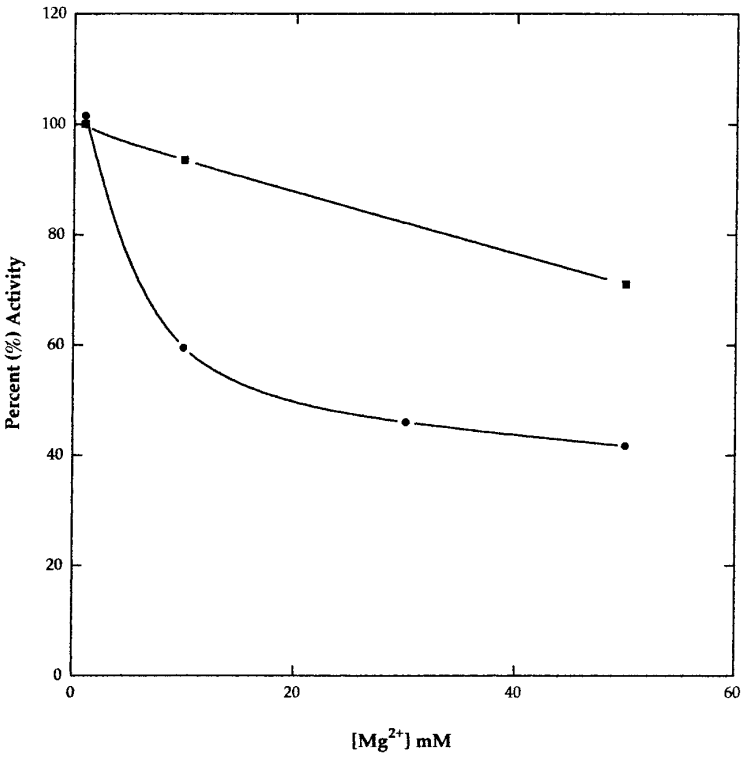

FIG. 3. The effect of Mg^{2+} on the 41 kDa gelatinase activity in the presence or absence of Ca^{2+}. Aliquots of the purified 41 kDa gelatinase were assayed in the standard assay mix containing various concentrations of Mg^{2+} in the absence ● — ● or the presence ■ — ■ of 10 mM Ca^{2+}. All values were normalized with respect to an aliquot of the gelatinase assayed in the presence of 10 mM Ca^{2+} with no Mg^{2+} present.

KCl. The filters were then incubated for 15 min at room temperature in Imidazole-KCl buffer containing 50 μ Ci $^{45}CaCl_2$, 10 mM $CaCl_2$ and 0, 1, 10 or 30 mM $MgCl_2$ followed by washing for 5 min at room temperature in 1 mM Tris-HCl, pH 7.5. The filters were air-dried, sectioned and the dots counted in 10 mL aliquots of Scinti Verse E (Fisher) in a Beckman model LS9000 liquid scintillation counter. Corrections for background binding were made by subtracting the counts bound to dots which did not contain protein.

RESULTS AND DISCUSSION

We have previously shown that the sea urchin exhibits a dynamic pattern of gelatinase activities during embryogenesis (11). This data is reproduced in Fig. 1A; the egg (lane 1) possesses a 41 kDa activity which predominates during early development. However, by the gastrula stage (lane 4) the 87 kDa activity has become quantitatively the most important. Clearly, these species are the most abundant in the sea urchin egg and embryo. The gelatin substrate gel zymograms for the purified 41 kDa and the partially purified 87 kDa preparations used in this study are presented in Fig. 1B, lanes 1 and 2, respectively.

Our previous studies have shown a Ca^{2+} requirement for activity of both the 41- and 87 kDa species (11, 12). In the results reported here we have extended this study to the effects of Mg^{2+} in the presence and absence of Ca^{2+}. Differing effects of Mg^{2+} were noted for the EDTA-inhibited 41- and 87 kDa gelatinases (Fig. 2). Concentrations of Mg^{2+} up to about 15 mM could variously reactivate the 41 kDa gelatinase; maximal reactivation occurred at concentrations of 5 mM or less and decreased substantially above this concentration. By comparison, Mg^{2+} was largely ineffective at reactivating the EDTA-inhibited 87 kDa gelatinase. The contrasting effects of Mg^{2+} probably reflect differences in the nature of the catalytic sites of the 41- and 87 kDa gelatinases. However, at the high concentrations of Mg^{2+} present in sea water this cation was ineffective at reactivating either gelatinase. The effect of Mg^{2+} on the uninhibited gelatinases was also examined. In the absence of Ca^{2+} increasing concentrations of Mg^{2+} resulted in partial inhibition of the 41 kDa gelatinase (Fig. 3); at a Mg^{2+} concentration of 50 mM approximately 40% of the gelatinase activity remained uninhibited. This result is reminiscent of that seen in Fig. 2. In the presence of 10 mM Ca^{2+} the inhibitory effect of Mg^{2+} greatly diminished. Clearly, Ca^{2+} effectively protects the gelatinase against the inhibitory effect of Mg^{2+}. However, at the concentrations of Ca^{2+} and Mg^{2+} present in sea water, 10- and 50 mM respectively, the 41 kDa gelatinase activity is about 70% of the uninhibited level. Similar results were seen for the 87 kDa gelatinase (Fig. 4).

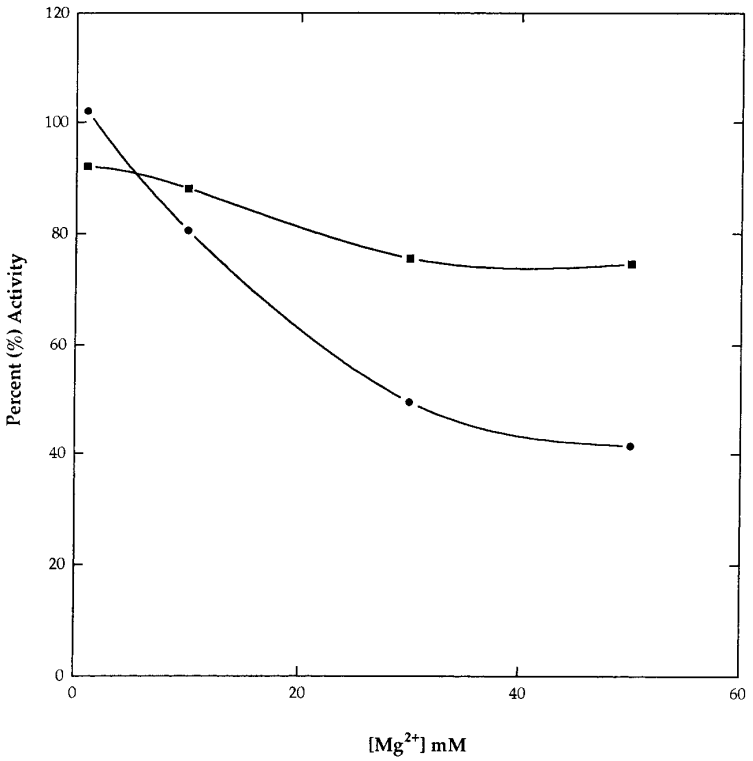

FIG. 4. The effect of Mg^{2+} on the 87 kDa gelatinase activity. Aliquots of the partially purified 87 kDa gelatinase were assayed in the standard assay mix containing various concentrations of Mg^{2+} in the absence ● — ● or the presence ■ — ■ of 10 mM Ca^{2+}. All values were normalized with respect to an aliquot of the gelatinase assayed in the presence of 10 mM Ca^{2+} with no Mg^{2+} present.

To further examine the interactions of Ca^{2+} and Mg^{2+} with the gelatinase activities we performed competition studies in which relative Ca^{2+} binding was measured in the presence of increasing concentrations of Mg^{2+} (Table 1). Both gelatinases bound decreasing amounts of Ca^{2+} as the Mg^{2+} concentration increased suggesting that Ca^{2+} and Mg^{2+} can compete for the same binding sites on the gelatinases. The ionic radii and hydration energies of Ca^{2+} and Mg^{2+} are sufficiently different to preclude these ions from binding to the same high affinity sites (16, 17). In mammals, extracellular Ca^{2+}-binding proteins are exposed to Ca^{2+} concentrations as high as 3 mM (18) and consequently can bind Ca^{2+} at multiple, weak binding sites which could not function intracellularly where Ca^{2+} concentrations vary from 10^{-7} to 10^{-5}M (19). At the high concentrations of Ca^{2+} and Mg^{2+} present in sea water, cell-surface located proteins, such as the gelatinases, can bind these cations at many weak binding sites some or all of which may be able to accommodate either ion. We have previously shown that the Ca^{2+} reactivation of the EDTA-inhibited 87 kDa gelatinase occurs with an apparent kd of approximately 0.1 mM (12) while the Ca^{2+}-dependent reactivation of the 41 kDa gelatinase occurs with an apparent kd of 3.7 mM (manuscript in preparation). These results suggest that both gelatinases require low affinity Ca^{2+} binding for activity. These weak Ca^{2+} binding sites can probably also accommodate Mg^{2+}; however, Mg^{2+} occupancy of at least some of these sites leads to inhibition as seen in the results reported here. The contrasting effects of Ca^{2+} and Mg^{2+} on the gelatinases could result in the regulation of these activities at the cell surface; small changes in the concentrations of either cation could result in changes in gelatinase activities. Such regulatory roles for Ca^{2+} and Mg^{2+} would be dependent upon the existence of microenvironments in which the concentrations of these cations differ from those in the bulk phase

TABLE 1

Competition between Ca^{2+} and Mg^{2+} for Binding to the Gelatinases

	Percentage of Ca^{2+} remaining bound	
$[Mg^{2+}]$ mM	41 kDa	87 kDa
0	100	100
1	54.6	65.8
10	35.7	45.6
30	26.2	27.7

environment. Localized environments could be created by cell-surface proteins which bind large numbers of Ca^{2+} and/or Mg^{2+} with low affinities. Interactions between these Ca^{2+} and/or Mg^{2+} storage proteins and other molecules such as the 41- and 87 kDa gelatinases may regulate the activities of the latter. Further experimentation will be required to explore this possibility.

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