Roles for Ca^{2+} , Mg^{2+} and NaCl in modulating the self-association reaction of hyalin, a major protein component of the sea-urchin extraembryonic hyaline layer

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The self-association reaction of hyalin, a major protein component of the sea-urchin extraembryonic hyaline layer, was examined. Concentrations of Ca^{2+} below 1 mM had little effect on the hyalin gelation reaction, but higher concentrations of the cation induced protein aggregation. Quantitative aggregate formation required a Ca^{2+} concentration in excess of 10 mM. This reaction was modulated by both NaCl and Mg^{2+} . The effectiveness of Ca^{2+} in inducing hyalin gelation was markedly enhanced in the presence of 500 mM-NaCl, the concentration found in sea water. Similarly, 20 mM- Mg^{2+} also enhanced Ca^{2+} -induced hyalin gelation. Neither NaCl nor Mg^{2+} alone induced hyalin gelation. Concentrations of Ca^{2+} as low as 1 mM effectively protected hyalin from tryptic digestion both in the presence and in the absence of 500 mM-NaCl. The latter result suggested that, although higher concentrations of Ca^{2+} were required to induce the hyalin gelation reaction, lower concentrations of the cation could mediate a protein–protein interaction in an NaCl-independent fashion. These results identify the parameters that modulate hyalin self-association, a reaction that is essential for hyaline-layer assembly around the developing sea-urchin embryo.

INTRODUCTION

Throughout embryonic and larval development the sea-urchin is surrounded by a proteinaceous layer known as the hyaline layer (Cameron & Holland, 1985). Immediately following fertilization the hyaline layer is assembled from components released from granules that reside in the cortex of the unfertilized egg. The structural integrity of the hyaline layer is dependent upon Ca^{2+} ions, and formation of the layer can be envisaged as a Ca^{2+} -mediated protein–protein interaction. Although the composition of the layer has been studied in some detail (Hall & Vacquier, 1982; McCarthy & Spiegel, 1983), relatively little is known about the topological arrangement within the layer or the parameters that influence its formation. However, several lines of evidence suggest that the hyaline layer is a structure of defined composition and architecture: (a) the hyaline layer appears by scanning electron microscopy to be composed of two layers, one of which changes in appearance during embryonic development (Cameron & Holland, 1985); (b) the hyaline layer is a multicomponent structure consisting of at least five distinct polypeptides (McCarthy & Spiegel, 1983); (c) the composition of the hyaline layer is invariant between members of a given species and is distinct from the nearby fertilization membrane, the components of which are also derived from the cortical granules (Hall & Vacquier, 1982; McCarthy & Spiegel, 1983). These observations suggest that formation of the hyaline layer involves a selective interaction between at least five distinct polypeptides.

The glycoprotein hyalin has been implicated in the hyaline-layer assembly reaction partly because it is a major component of the layer and also because of its property of self-association into an insoluble gel in the presence of high concentrations of Ca^{2+} (Stephens & Kane, 1970; Kane, 1973; Gray *et al.*, 1986). Concentrations of Ca^{2+} in excess of 20 mM have routinely been

used to induce hyalin aggregation into an insoluble gel, thereby facilitating purification of the protein. This property is unique to hyalin and is not shared by the other polypeptide components of the hyaline layer. Recently, an additional role for Ca^{2+} in stabilizing hyalin conformation against the disruptive effects of high concentrations of NaCl has been reported (Robinson *et al.*, 1988). This stabilizing effect of Ca^{2+} occurs with concentrations of the cation as low as 1 mM. Clearly, the dual role for Ca^{2+} in stabilizing hyalin structure and facilitating protein–protein interactions are important for assembly of the hyaline layer around the developing embryo.

In an effort to determine the parameters that influence hyaline layer formation, I have undertaken a study of the effects of Ca^{2+} , Mg^{2+} and NaCl on the hyalin self-association reaction. The results reported here clearly demonstrate roles for both Mg^{2+} and NaCl in potentiating the effectiveness of Ca^{2+} in inducing the hyalin gelation reaction. In addition, concentrations of Ca^{2+} that stabilize hyalin secondary structure also mediate a hyalin–hyalin interaction in an NaCl-independent manner. A model is proposed to account for these effects of Ca^{2+} , Mg^{2+} and NaCl on hyalin self-association during hyaline-layer assembly.

EXPERIMENTAL

Preparation of hyalin

Strongylocentrotus droebachiensis were collected from an intertidal habitat off the east coast of Newfoundland and gametes were obtained by intracoelomic injection of 0.5 M-KCl. Eggs were washed three times in ice-cold Millipore (0.45 μm pore size)-filtered sea water and resuspended to 10% (v/v) in Millipore-filtered sea water. The eggs were dejuellied by titration to pH 5.5 with HCl, left for 2 min and the pH was returned to 8.0 by the addition of 1 M-Tris base. After extensive washing in

Millipore-filtered sea water the eggs were resuspended to 10% (v/v) and their vitelline layer was disrupted by treatment with 10 mM-dithiothreitol as described previously (Epel *et al.*, 1970). Following the dithiothreitol treatment the eggs were extensively washed in Millipore-filtered sea water containing 10 mM-Tris/HCl buffer, pH 8.0, resuspended to 10% (v/v) and fertilized by the addition of a few drops of sperm, diluted 1:100 in Millipore-filtered sea water. The eggs were kept suspended for 3 min and then allowed to settle for 2 h. The supernatant was removed by aspiration and the clumped eggs were washed in Millipore-filtered sea water. The eggs were resuspended to 10% (v/v) in ice-cold Ca^{2+} - and Mg^{2+} -free sea water (500 mM-NaCl, 10 mM-KCl, 2 mM- NaHCO_3 and 3 mM- Na_2SO_4) and kept in suspension for 15 min by gentle rotation. EDTA was added to 2 mM and the rotation continued for an additional 15 min. The eggs were allowed to settle, the supernatant was removed and the proteinase inhibitor benzamidine hydrochloride was added to a final concentration of 5 mM. After centrifugation at 20000 *g* for 30 min at 4 °C the supernatant was removed and CaCl_2 was added to a final concentration of 100 mM. Hyalin was allowed to form a precipitate overnight at 4 °C, collected by centrifugation at 20000 *g* for 30 min at 4 °C and resuspended in 20 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-EDTA and 5 mM-benzamidine hydrochloride. The solution was centrifuged at 20000 *g* for 30 min at 4 °C, and the supernatant was again made 100 mM in CaCl_2 and left overnight at 4 °C. This cycle of Ca^{2+} precipitation/solubilization was repeated one more time, and the solubilized hyalin was kept on ice until used. Before use 1 ml samples of hyalin preparations were extensively dialysed against 2 × 4 litres of 20 mM-Tris/HCl buffer, pH 8.0, at 4 °C.

Purified hyalin migrated as a single species of apparent M_r 350000 when analysed by electrophoresis in a 3–12% polyacrylamide gradient gel in the presence of SDS (Laemmli, 1970).

Assay of hyalin gelation reaction

Samples (75 μg) of hyalin in 20 mM-Tris/HCl buffer, pH 8.0, were incubated at room temperature with various combinations of CaCl_2 , MgCl_2 and NaCl at the final concentrations indicated. After 20 min at room temperature the samples were centrifuged at 16000 *g* for 30 min in an Eppendorf centrifuge (model 5415), and the pellet and supernatant fractions were assayed for protein with bovine serum albumin as a standard (Lowry *et al.*, 1951).

Tryptic digestions of hyalin

Samples (20 μg) of hyalin in 20 mM-Tris/HCl buffer, pH 8.0, containing various concentrations of CaCl_2 were incubated at room temperature for 20 min. Trypsin (L-tosylphenylalanylchloromethane-treated; Sigma Chemical Co.) was then added to give a hyalin/trypsin ratio of 100:1 (w/w) and the incubation was continued. At the indicated times after trypsin addition portions of the reaction mixture were withdrawn and each was added to an equal volume of ice-cold 20% (w/v) trichloroacetic acid. After 30 min on ice the precipitates were isolated by centrifugation, resuspended in solubilizing solution containing 0.1 M-dithiothreitol, 0.4 M-Tris base, 2% (w/v) SDS, 30% (v/v) glycerol and 0.1% (w/v) Bromophenol Blue and analysed by electrophoresis

in a 3–12% polyacrylamide gradient gel (Laemmli, 1970). The gel was stained with AgNO_3 (Goldman *et al.*, 1981).

RESULTS AND DISCUSSION

In this paper I report on the effects of Ca^{2+} , Mg^{2+} and NaCl on the hyalin gelation reaction. The choice of conditions for study was mandated by the fact that open sea water contains 10 mM- Ca^{2+} , 50 mM- Mg^{2+} and 500 mM-NaCl. In addition, previous work has shown that an NaCl concentration of 500 mM induces a change in hyalin secondary structure and that this effect can be reversed by concentrations of Ca^{2+} as low as 1 mM (Robinson *et al.*, 1988). It was therefore of interest both to determine the conditions that facilitate hyalin gelation and to compare these conditions with those that stabilize hyalin structure.

The effects of Ca^{2+} , Mg^{2+} and NaCl on the hyalin gelation reaction were monitored by using a simple centrifugation assay. This assay involved removal of the hyalin gel, formed under a given set of conditions, by centrifugation at 16000 *g* for 30 min at room temperature. Gel formation was quantified by determining the amounts of protein in the pellet and supernatant fractions. As can be seen in Table 1, in the presence of

Table 1. Quantification of hyalin gelation

Samples (75 μg) of hyalin were incubated in the presence of various concentrations of CaCl_2 , MgCl_2 and NaCl as described in the Experimental section. After centrifugation at 16000 *g* for 30 min the pellet fractions were each resuspended in 100 μl of 1 M-NaOH. The supernatant fractions were added to equal volumes of ice-cold 20% (w/v) trichloroacetic acid and left on ice for 30 min. The precipitates were harvested by centrifugation and each was resuspended in 100 μl of 1 M-NaOH. Protein concentrations in the pellet and supernatant fractions were determined with bovine serum albumin as a standard (Lowry *et al.*, 1951). The results are means $\pm$ S.E.M. ($n = 3$).

Salt concentration (mM)			Percentage of hyalin present in:	
[CaCl_2]	[MgCl_2]	[NaCl]	Pellet fraction	Supernatant fraction
0	0	0	10.2 $\pm$ 0.8	89.8 $\pm$ 0.8
0	0	500	10.4 $\pm$ 1.2	89.6 $\pm$ 1.2
0.3	0	500	12.5 $\pm$ 0.8	87.5 $\pm$ 0.8
1.0	0	500	15.8 $\pm$ 1.0	84.2 $\pm$ 1.0
3.0	0	500	20.3 $\pm$ 0.5	79.7 $\pm$ 0.5
10.0	0	500	77.2 $\pm$ 2.2	22.8 $\pm$ 2.2
0	20	500	13.3 $\pm$ 1.2	86.7 $\pm$ 1.2
1.0	0	500	15.8 $\pm$ 1.0	84.2 $\pm$ 1.0
1.0	20	500	50.0 $\pm$ 2.1	50.0 $\pm$ 2.1
3.0	0	500	20.3 $\pm$ 0.5	79.7 $\pm$ 0.5
3.0	20	500	58.7 $\pm$ 2.3	41.3 $\pm$ 2.3
10.0	0	500	77.2 $\pm$ 2.2	22.8 $\pm$ 2.2
10.0	20	500	85.1 $\pm$ 3.1	14.9 $\pm$ 3.1
1.0	0	0	11.2 $\pm$ 1.4	88.8 $\pm$ 1.4
1.0	0	500	15.8 $\pm$ 1.0	84.2 $\pm$ 1.0
3.0	0	0	15.1 $\pm$ 1.6	84.9 $\pm$ 1.6
3.0	0	500	20.3 $\pm$ 0.5	79.7 $\pm$ 0.5
10.0	0	0	17.1 $\pm$ 1.9	82.9 $\pm$ 1.9
10.0	0	500	77.2 $\pm$ 2.2	22.8 $\pm$ 2.2

500 mM-NaCl increasing concentrations of Ca^{2+} resulted in a quantitative shift of hyalin into a high- M_r aggregate. Although Ca^{2+} concentrations between 0.3 and 1 mM had little effect on aggregate formation, higher concentrations of the cation resulted in a substantial fraction of hyalin aggregating. In a control experiment NaCl alone had no effect of hyalin gelation.

Since hyaline-layer formation *in vivo* occurs in the presence of Mg^{2+} , the effect of Mg^{2+} ions on the Ca^{2+} -mediated gelation reaction was examined. At 20 mM Mg^{2+} significantly increased hyalin aggregation in the presence of Ca^{2+} (Table 1). However, Mg^{2+} alone was ineffective at inducing this reaction and therefore acted only to potentiate the effect of Ca^{2+} .

The effect of NaCl on the Ca^{2+} -mediated gelation reaction was also examined. Although NaCl alone had no effect on this reaction, marked differences were found in the effectiveness of Ca^{2+} in inducing hyalin gelation in the presence and in the absence of 500 mM-NaCl (Table 1). In the absence of NaCl, concentrations of Ca^{2+} as high as 10 mM did not induce quantitative hyalin gelation.

Fig. 1. Effect of Ca^{2+} on the tryptic digestion of hyalin in the absence of NaCl

Samples (20 μg) of hyalin were incubated at room temperature in the presence or in the absence of CaCl_2 at a hyalin/trypsin ratio of 100:1. At 5 min, 35 min and 90 min intervals after the addition of trypsin, portions of the reaction mixture containing 5 μg of protein were added to an equal volume of ice-cold 20% (w/v) trichloroacetic acid. Digestion products were analysed by electrophoresis in a 3–12% polyacrylamide gradient gel. Lanes 1, 4 and 7 contain the products produced after 5 min of digestion, lanes 2, 5 and 8 contain the products produced after 35 min of digestion, and lanes 3, 6 and 9 contain the products produced after 90 min of digestion.

The addition of NaCl to a final concentration of 500 mM resulted in a marked enhancement of the effectiveness of Ca^{2+} in inducing hyalin gelation. In the presence of both 500 mM-NaCl and 10 mM- CaCl_2 almost quantitative hyalin gelation occurred.

The centrifugation assay used in the experiments described above is useful for monitoring hyalin-hyalin interactions that lead to the formation of large protein aggregates (gelation). However, smaller aggregates would not be detected in this assay. In an effort to detect protein-protein interactions that might be occurring without formation of large aggregates I determined the profile of tryptic peptides derived from hyalin as a function of Ca^{2+} concentration (Fig. 1). In the absence of Ca^{2+} , hyalin was quantitatively digested (lanes 1–3). However, the addition of Ca^{2+} to a concentration as low as 1 mM resulted in the protection of hyalin from proteolysis (lanes 4–6). Similarly, 20 mM- CaCl_2 also protected hyalin from proteolysis (lanes 7–9). These results suggested that hyalin-hyalin interactions were occurring even in the presence of low concentrations of Ca^{2+} that do not lead to quantitative formation of large aggregates. Similar results were obtained when the tryptic-digestion experiment was performed in the presence of 500 mM-NaCl (results not shown). The pattern of tryptic peptides derived from hyalin was unaltered by the presence of 500 mM-NaCl, and again a Ca^{2+} concentration of 1 mM effectively protected hyalin from proteolytic digestion.

The marked effect of 1 mM- Ca^{2+} in protecting hyalin against tryptic digestion both in the presence and in the absence of 500 mM-NaCl suggested that protein-protein interactions were occurring under these conditions. These interactions were distinct from those that lead to hyalin gel formation, since (1) they required 10-fold lower concentrations of Ca^{2+} and (2) they are independent of NaCl. These results are particularly interesting since it was previously shown that 500 mM-NaCl induced a measurable change in hyalin secondary structure and that this conformational change could be reversed by 1 mM- Ca^{2+} (Robinson *et al.*, 1988). The results reported here extend these earlier findings and demonstrate that, in addition to stabilizing hyalin secondary structure against the effects of NaCl, low concentrations of Ca^{2+} also facilitate protein-protein interactions.

The results reported here clearly show that the effectiveness of Ca^{2+} in inducing the hyalin gelation reaction is modulated by both Mg^{2+} ions and NaCl. However, NaCl is not required for all hyalin-hyalin interactions; low concentrations of Ca^{2+} mediate interactions in an NaCl-independent fashion. The following model is proposed to account for the effects of Ca^{2+} , Mg^{2+} and NaCl on hyalin self-association. Hyalin released from the cortical granules binds Ca^{2+} and is stabilized against any structural changes due to NaCl. This effect of Ca^{2+} requires a cation concentration of approx. 1 mM (Robinson *et al.*, 1988). In addition, Ca^{2+} binding induces a hyalin-hyalin interaction that, in the presence of higher concentrations of the cation, leads to the formation of small aggregates of the protein. The presence of Mg^{2+} potentiates the formation of small aggregates in response to Ca^{2+} . These aggregates can act as nucleation sites for the formation of larger aggregates (gelation), a reaction that is driven by the high ambient concentrations of NaCl present in sea water.

Clearly, the ionic composition of sea water plays an important role in mediating hyalin-hyalin interactions

and consequently hyaline-layer assembly. At present, no functional role in the assembly pathway can be assigned to the other polypeptide components of the hyaline layer.

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