Volume 302, number 3, 284–286 FEBS 11048 May 1992

Selective inhibition of membrane fusion events in echinoderm gametes and embryos by halenaquinol sulfate

Susumu Ikegami[a], Noboru Kajiyama[a], Yoshihiro Ozaki[a], Yuki Myotoishi[a], Shigeyoshi Miyashiro[b], Seiji Takayama[b], Motomasa Kobayashi[c] and Isao Kitagawa[c]

[a]*Department of Applied Biochemistry, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 724, Japan,* [b]*Central Research Laboratories, Ajinomoto Co., 1-1 Suzuki-cho, Kawasaki, Kanagawa 210, Japan* and [c]*Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565, Japan*

Received 24 March 1992

Halenaquinol sulfate, a hydroquinone sulfate obtained from the sponge *Xestospongia sapra*, prevented cell membrane fusion events of echinaderm gametes but did not affect early embryonic development of fertilized eggs up to the gastrula stage. However, halenaquinol sulfate inhibited secretion of hatching enzyme, resulting in the formation of gastrulae that were surrounded by the fertilization envelope. Therefore, the use of halenaquinol sulfate offers a unique opportunity to analyze the role of secretory events in complex populations of cells without affecting other cellular functions.

Halenaquinol sulfate; Membrane fusion; Hatching; *Asterina pectinifera*

1. INTRODUCTION

Membrane fusion is an important cellular event but there are few, if any, specific inhibitors of this cellular event which are not accompanied by non-specific cytotoxicity. We searched for chemicals which prevented fertilization and hatching of the starfish *Asterina pectinifera*, events which both require membrane fusion and exocytosis, but did not prevent mitotic cell division of fertilized eggs and embryonic development up to the mesenchymal gastrula stage. Halenaquinol sulfate (HS, Fig. 1), obtained from the sponge, *Xestospongia sapra* [1–3], was one such compound and may be a useful tool for studying the role of secretory events in such complex cellular systems as the developing embryo.

2. MATERIALS AND METHODS

2.1. *Materials*

The starfish, *Asterina pectinifera*, and the sea urchin, *Hemicentrotus pulcherrimus*, were collected from various areas of Japan and were kept in artificial sea water (ASW) in laboratory aquaria. HS was obtained as described [1]. Hexaprenylhydroquinone sulfate from the sponge, *Dysidea* sp. [4], was a gift from Dr. N. Fusetani, University of Tokyo. The DNA-staining Hoechst dye, 33342, and calcium ionophore, A23187, were obtained from Calbiochem-Behring. ASW was Jamarin, obtained from Jamain Laboratory, Osaka. 1-Methyladenine was from Sigma.

Correspondence address: S. Ikegami, Department of Applied Biochemistry, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 724, Japan. Fax: (81) (824) 22-7046.

2.2. *Methods*

All the experiments were carried out at 20°C unless otherwise stated. The acrosomal reaction of starfish sperm was induced as described by Ikadai and Hoshi [5]. Starfish eggs were fertilized at 50 min after the start of 1-methyladenine incubation, an inducer of oocyte maturation [6]. Fusion of the plasma membrane of a starfish egg with the acrosome-reacted sperm was examined as described by Hinckley et al. [7] with slight modifications. Maturing starfish oocytes were dejellied by brief exposure to acidified sea water (pH 5.5), treated with Hoechst 33342 (18 μM) for 1 h, washed five times in ASW and then either untreated or treated with HS (0.1 mM) for 5 min. Then, they were fertilized with sperm which had been pre-incubated in ASW containing the egg jelly to undergo the acrosome reaction. Three minutes later they were fixed in 2% glutaraldehyde and viewed with a fluorescent microscope. Microinjection into fertilized starfish eggs was carried out according to the method described by Hiramoto [8].

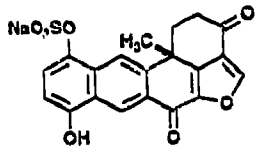
Starfish and sea urchin embryos were cultivated as described [9]. The hatching enzyme activities present in the exudate and cells of sea urchin embryos were determined as described [10].

3. RESULTS AND DISCUSSION

An essential initial step in fertilization is an intracellular membrane fusion event in the sperm known as the acrosome reaction [5]. HS inhibited the jelly coat-induced acrosomal reaction (ED_{50} 68 μM). HS also inhibited the acrosome reaction induced by the Ca^{2+} ionophore, A23187 (0.15 mM) to a comparable degree (ED_{50} 72 μM).

The second fusion event required for fertilization is fusion of the plasma membrane of the acrosome-reacted sperm with the plasma membrane of the egg. Sperm binding was observed in the HS-treated egg 25 s after insemination, as in the control egg. The sperm that had established cytoplasmic continuity with the control egg

 Published by Elsevier Science Publishers B.V.



halenaquinol sulfate

Fig. 1. Structure of halenaquinol sulfate.

became fluorescent [7] whereas the sperm bound to the HS-treated egg did not, showing that sperm-egg fusion but not the binding of sperm to egg plasma membrane was inhibited by HS.

The third fusion event is the elevation of the fertilization envelope, a structure formed by the exocytosis of cortical secretory granules [11]. Eggs were fertilized 50 min after the start of treatment with 1-methyladenine. The cortical reaction induced by the acrosome-reacted sperm was blocked by HS (ED_{50} 51 μM). Similarly, the cortical reaction induced by the treatment of the egg with the Ca^{2+} ionophore, A23187 (3.8 μM), was blocked by HS (ED_{50} 44 μM) as shown in Fig. 2. Since HS was barely permeable to the plasma membrane, HS was microinjected into an egg to give a final intracellular concentration of 70 μM and the egg was subsequently inseminated. The elevation of the fertilization envelope was prevented, showing that HS was capable of blocking the cortical reaction from inside the cell.

Although fusion of the cell membrane is required for the final 'pinching-off' process, the force exerted by the contractile ring during cytokinesis was expected to be large enough to overcome the blockade of the fusion process by HS. In fact, when HS was added to the suspension of fertilized eggs at a final concentration of 0.1 mM immediately following fertilization, they expelled two polar bodies normally and segregated completely to form two blastomeres of equal size. The embryo blastulated without delay after passing through a rapid cleavage period, and formed cilia on schedule. The rotating blastula, however, was unable to hatch

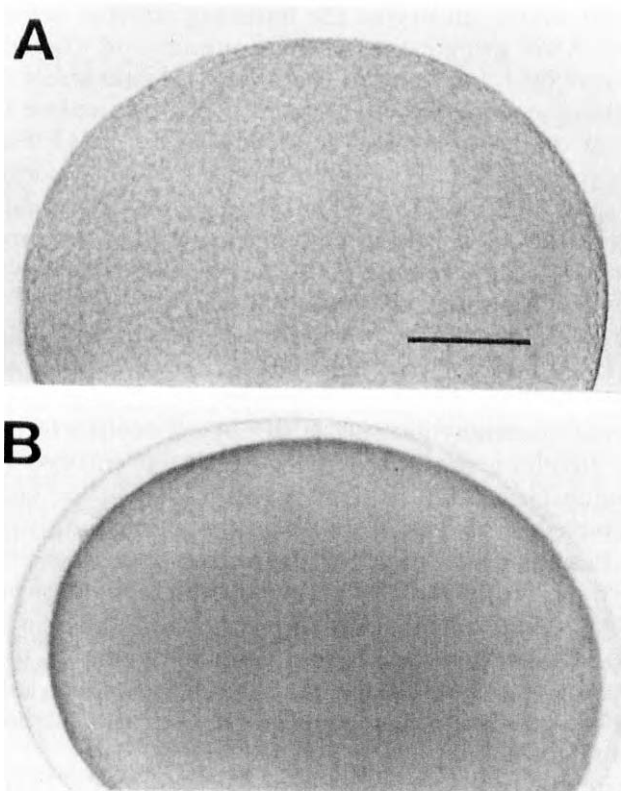


Fig. 2. (A) A starfish egg pre-incubated with HS (0.1 mM) for 15 min failed to elevate the fertilization envelope after addition of the Ca^{2+} ionophore, A 23187 (3.8 μM). (B) A control egg which elevated the fertilization membrane in the presence of the same concentration of the ionophore as used in A. Scale bar, 50 μm.

(Fig. 3A,B). The hatching enzyme of starfish embryos is quite labile; therefore, we examined the effect of HS on secretion of the hatching enzyme from the embryos of the sea urchin, *Hemicentrotus pulcherrimus*. When introduced to the culture of fertilized sea urchin eggs at 5 min after fertilization, HS (0.1 mM) did not affect cleavage, blastulation, cilia formation and gastrulation, but blocked hatching at the late blastula stage. HS (0.1 mM) did not affect the capability of hatching enzyme present in 100 ml of ASW conditioned by 1×10^6 hatch-

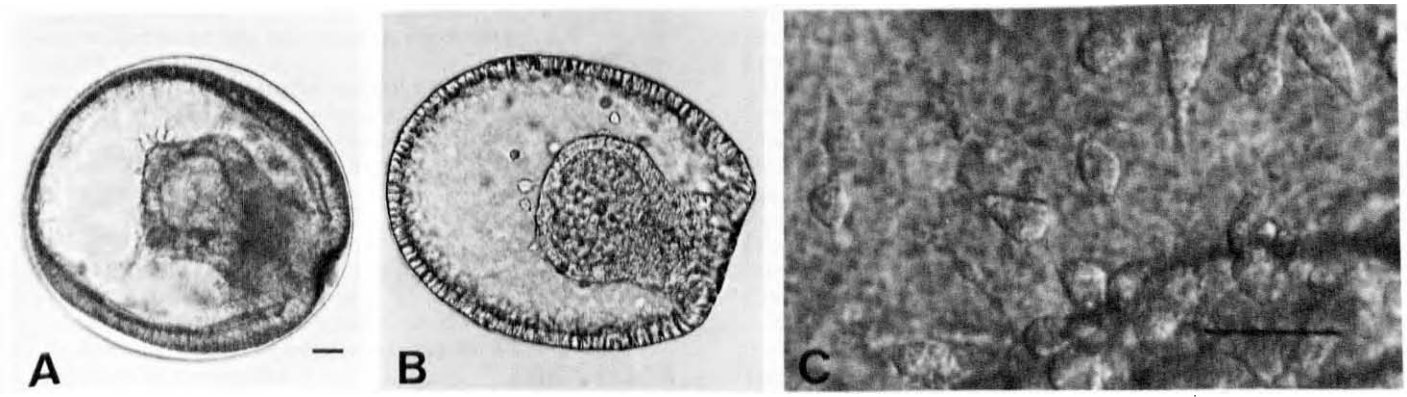


Fig. 3. (A) A starfish embryo incubated in ASW containing HS (0.1 mM) for 24 h from the time of fertilization. The embryo was unable to hatch. (B) An embryo incubated for the same period as in A but in the absence of HS. (C) A 24-h embryo treated with HS (0.1 mM) from the time of hatching (16 h after fertilization). Nomarski optics. Scale bar, 20 μm.

ing sea urchin embryos: the hatching enzyme activities in the ASW preparation in the presence and absence of HS were 60.1 ± 1.7 and 61.3 ± 1.7 mU, respectively. The hatching enzyme activity present in the particulate fraction of the homogenate [10] of a HS (0.1 mM)-treated embryo was 14.3 nU and that of a control embryo of the same age was 15.0 nU. These results demonstrate that HS affected neither enzymatic activity nor synthesis of hatching enzyme but that HS specifically prevented secretion of the hatching enzyme.

Starfish embryos cultured in the continuous presence of HS (0.1 mM) from the time of fertilization gastrulated inside the fertilization envelope and subsequently formed mesenchymal cells (Fig. 3A). Since the fertilization envelope prevented the gastrulated embryo from growing larger, HS (0.1 mM) was added to the culture of starfish embryos from the time immediately after hatching. It was found that the archenteron invaginated on time, followed by the filopodial extension of mesenchymal cells from the tip of the archenteron (Fig. 3C). However, mesenchymal cells were unable to migrate into the blastocoel cavity, possibly through inhibition of secretion of some components of the extracellular matrix.

Embryos cultured in *p*-nitrophenylsulfate (0.25 mM), halenaquinol [1] (0.25 mM) or hexaprenylhydroquinone sulfate [4] (0.25 mM) became normal bipinnaria, suggesting that the action of HS is rather specific. Although the mechanism of action of HS to block membrane fusion remains to be clarified, the use of HS offers a unique opportunity to analyze the role of secretory events in complex morphogenetic changes occurring during early embryonic development of higher animals.

Acknowledgements: We thank the Ministry of Education, Science and Culture, Japan, and the Fisheries Agency, Japan, for support.

REFERENCES

[1] Kobayashi, M., Shimizu, N., Kyogoku, Y. and Kitagawa, I. (1985) Chem. Pharm. Bull. 33, 1305–1308.
[2] Kobayashi, M., Shimizu, N., Kitagawa, I., Kyogoku, Y., Harada, N. and Udu, H. (1985) Tetrahedron Lett. 26, 3833–3836.
[3] Harada, N., Udu, H., Kobayashi, M., Shimizu, N. and Kitagawa, I. (1989) J. Am. Chem. Soc. 111, 5668–5674.
[4] Fusetani, N., Sugano, M., Matsunaga, S., Hashimoto, K., Shikama, H., Ohta, A. and Nagano, H. (1987) Experientia 43, 1233–1234.
[5] Ikadai, H. and Hoshi, M. (1981) Dev. Growth Diff. 23, 73–80.
[6] Kanatani, H. (1973) Int. Rev. Cytol. 35, 253–298.
[7] Hinsley, R.E., Wright, B.D. and Lynn, J.W. (1986) Dev. Biol. 118, 148–154.
[8] Hiramoto, Y. (1974) Exp. Cell Res. 87, 403–406.
[9] Tsuchimori, N., Miyashiro, S., Shibai, H. and Ikegami, S. (1988) Development 103, 343–351.
[10] Nakatsuka, M. (1985) Dev. Growth Diff. 27, 653–661.
[11] Roe, J.L., Farach Jr., H.A., Strittmatter, W.J. and Lennarz, W.J. (1988) J. Cell Biol. 107, 539–544.