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Regulation of the starfish sperm acrosome reaction by cGMP, pH, cAMP and Ca^{2+}

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ABSTRACT In the starfish, *Asterias amurensis,* three components in the jelly coat of eggs, namely acrosome reaction-inducing substance (ARIS), Co-ARIS and asterosap, act in concert on homologous spermatozoa to induce the acrosome reaction (AR). Molecular recognition between the sperm surface molecules and the egg jelly molecules must underlie signal transduction events triggering the AR. Asterosap is a sperm-activating molecule, which stimulates rapid synthesis of intracellular cGMP, pH and Ca^{2+}. This transient elevation of Ca^{2+} level is caused by a K^+-dependent Na^+/Ca^{2+} exchanger, and the increase of intracellular pH is sufficient for ARIS to induce the AR. The concerted action of ARIS and asterosap could induce elevate intracellular cAMP levels in starfish sperm and the sustained increase in [Ca^{2+}], which is essential for the AR. The signaling pathway induced by these factors seems to be synergistically regulated to trigger the AR in starfish sperm.

KEY WORDS: *invertebrate fertilization, cyclic nucleotides, signal transduction, ion channel, spermatozoa*

Induction of the acrosome reaction

In marine invertebrate sperm, the acrosome reaction (AR), which makes the sperm capable of fertilization, involves the exocytosis of the acrosome vesicle and the polymerization of actin to form the acrosome process (Dan, 1952; Tilney, 1985). The AR is initiated when the sperm interacts with the egg jelly layer. Induction of the AR can be species-specific, indicating that molecular recognition between the sperm surface molecules and the egg jelly molecules must underlie the signal transduction events triggering the AR.

Requirements for the starfish AR

In the starfish *Asterias amurensis*, three components of egg jelly: ARIS (AR-inducing substance), co-ARIS and asterosap, cooperatively trigger the AR (Hoshi *et al*., 1994). ARIS is a sulfated proteoglycan-like molecule with an extremely large molecular mass (Ikadai & Hoshi, 1981*a,b*; Koyota *et al*., 1997; Gunaratne *et al*., 2003, Fig. 1). A pronase digest of ARIS (P-ARIS) retains the biological activity to induce the AR. It was subsequently discovered that this activity is associated with a sugar chain of ARIS. The minimum polysaccharide functional unit is called fragment 1 (Fr-1) and it contains 10 repeats of the pentasaccharide sequence Xyl-Gal-sulfatedFuc-sulfatedFuc-Fuc-. Cleavage of this sugar chain by either mild periodate treatment, or desulfation causes the complete inactivation of the AR inducing activity.

Co-ARIS is a group of sulfated steroidal saponins (Nishiyama *et al*., 1987). Asterosap is a group of equally active isoforms of sperm-activating peptides (Nishigaki *et al*., 1996). In normal seawater, ARIS induces the AR in cooperation with Co-ARIS or asterosap, whereas ARIS by itself induces the AR only in seawater with high Ca^{2+} (70 mM), or elevated-pH (pH 9.5) (Matsui *et al*., 1986*a*). Thus, ARIS is regarded as the major AR-inducing molecule. However, when the asterosap-induced changes are blocked by the pretreatment of sperm with asterosap alone, no AR is induced following treatment of the sperm with whole egg jelly containing all three components. Thus, it is clear that ARIS and asterosap are indispensable for the egg-jelly-induced AR.

Previous research has shown that egg jelly induces both a transient increase in intracellular pH ([pH]i) and the uptake of Ca^{2+}

Abbreviations used in this paper: AR, acrosome reaction; ARIS, acrosome reaction inducing substance; FSP, fucose sulfate polymer; pHi, intracellular pH.

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from the seawater (Matsui *et al.*, 1986*a*,*b*). The egg jelly-induced increase in pHi appears to facilitate the AR because sperm undergo the AR when treated with ARIS in seawater (pH 8.2) with a higher than normal pH (Ikadai & Hoshi, 1981*a*). Furthermore, the AR is induced by Ca^{2+} ionophores and the egg jelly-induced AR is inhibited by Ca^{2+} channel blockers such as dihydropyridines and verapamil. The uptake of Ca^{2+} through specific ion channels is thus essential for AR induction.

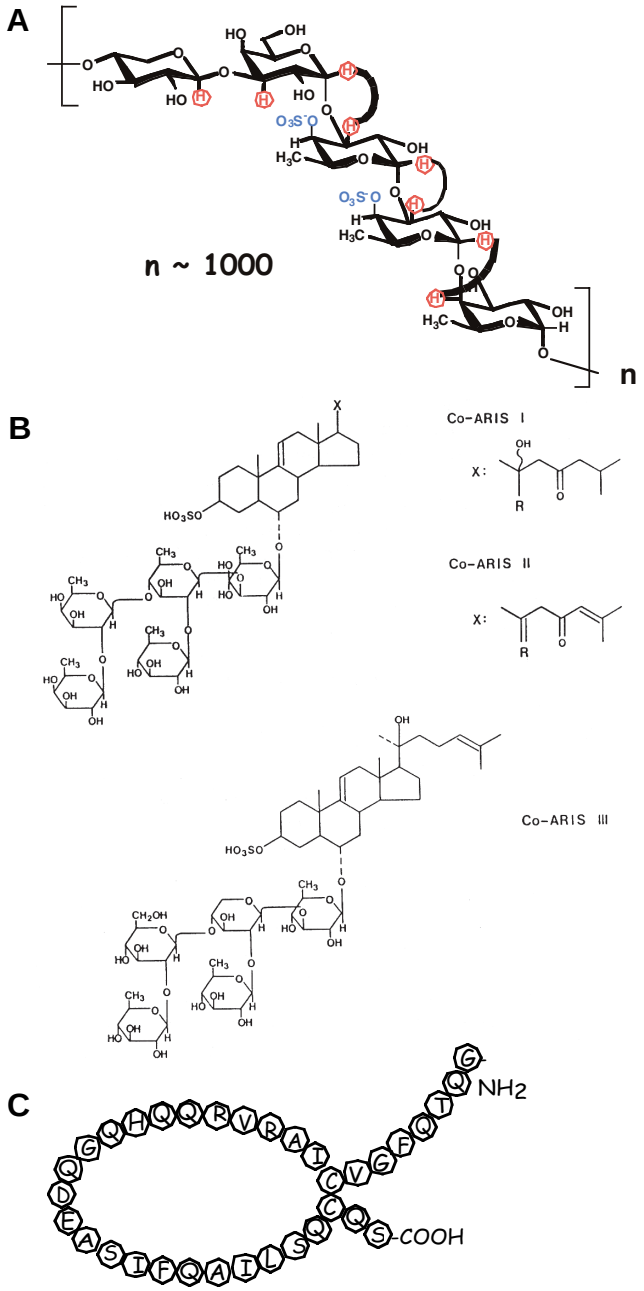

Fig. 1. Egg jelly components involved in acrosome reaction induction in starfish sperm. (A) *Acrosome reaction inducing substance (ARIS) is a sulfated proteoglycan-like molecule with an extremely large molecular size.* **(B)** *Co-ARIS is a group of sulfated steroidal saponins.* **(C)** *Asterosap is a group of equally active isoforms of sperm-activating peptides.*

In sea urchin sperm, the purified fucose sulfate polymer (FSP) of egg jelly is the inducer of the AR. The binding of FSP to sperm regulates the opening of two distinct Ca^{2+} channels and increases [pH]i (Guerrero & Darszon 1989, Darszon *et al.*, 2006). Sea urchin egg jelly also contains sialic acid-rich glycans (sialoglycans), which markedly potentiate AR induction by FSP, thus sea urchins have at least two pathways involved in triggering the sperm AR in the same manner as observed in starfish sperm (Hirohashi and Vacquier, 2002).

In this review we describe the regulation of AR in starfish by ARIS and asterosap through changes in intracellular levels of guanosine 3-,5-cyclic phosphate (cGMP), pH, adenosine cyclic 3-,5-phosphate (cAMP) and Ca^{2+}.

Asterosap is a sperm-activating molecule

Asterosap is isolated as a sperm-activating molecule because it stimulates the motility of the sperm in seawater at the abnormally low pH of 6.6. The stimulation of motility in sperm of the sea urchin *Arbacia punctulata* at pH 6.6 occurs when the sperm binds the 14 amino acid, egg released peptide, resact. Resact is a chemoattractant for sperm and it evokes elevations of intracellular Ca^{2+} ($[Ca^{2+}]i$) in the flagellum (Garbers & Kopf, 1989; Kaupp *et al.,* 2008). As in the case of sea urchin, in starfish, asterosap is also a chemotactic agent for sperm (Kaupp *et al.,* 2003, Shiba *et al.*, 2006). The increase in $[Ca^{2+}]i$ elicits a turn in the sperm trajectory followed by a period of straight swimming called "turn-and run" (Boehmer *et al.,* 2005).

Asterosap stimulates rapid synthesis of intracellular cGMP and Ca^{2+}

Using rapid mixing techniques such as the stopped-flow method, asterosap was found to evoke a rapid and transient increase in the intracellular cGMP levels ([cGMP]i) in starfish sperm, followed by a transient cGMP-stimulated increase in $[Ca^{2+}]i$ (Matsumoto *et al.,* 2003). At a high concentration of asterosap, [cGMP]i increased up to 60-fold within 100–200 ms. After the increase, [cGMP]i decayed for 2–3 sec. Molecular biological methods showed that the asterosap receptor is a guanylyl cyclase located in the sperm flagellar plasma membrane. Sperm responses are exquisitely sensitive to picomolar concentrations of asterosap, suggesting that the asterosap has a chemosensory function.

When sperm are pretreated with asterosap, guanylyl cyclase is irreversibly inactivated by dephosphorylation (Matsui *et al.*, 1986c). Also, the sperm cease to respond to further additions of asterosap. However, in the presence of 3-isobutyl-1-methylxanthine (IBMX) or zaprinast, inhibitors of phosphodiesterases (PDEs), the sperm retain their capacity to undergo the AR on interaction with egg jelly or purified ARIS (Kawase *et al.,* 2004). IBMX and zaprinast suppress the intracellular catabolism of cGMP, but not the catabolism of cAMP. These results show that guanylyl cyclase and cGMP-specific PDEs are involved in the regulation of the AR.

Asterosap, transiently hyperpolarizes the sperm membrane potential (Vm). This phenomenon is inhibited by increasing external the K^+ concentration (9 mM KCl in normal seawater and 90 mM KCl in high K^+ seawater), which transiently depolarized the Vm (Nishigaki *et al.*, 2000). These changes in Vm are completely

inhibited when the K^+ concentration is increased (to 90 mM). Therefore, the transient hyperpolarization is attributed to the opening of a K^+ channel. IBMX sustaines the asterosap-induced hyperpolarization. Moreover, $[Ca^{2+}]i$ rapidly increases at high asterosap concentrations (1 µm) (Matsumoto *et al.*, 2003). At lower concentrations, the waveform of the asterosap-induced Ca^{2+} signals depends on the asterosap concentration. In the presence of 11 mM EGTA, which can completely chelate Ca^{2+} in seawater, the asterosap-stimulated increase in $[Ca^{2+}]i$ is completely inhibited. These findings demonstrate an increase of $[Ca^{2+}]i$ due to influx of this ion from seawater.

cGMP increases Ca^{2+} in starfish sperm

To confirm the cyclic nucleotide-induced elevations in $[Ca^{2+}]i$, we used novel [6,7-bis(ethoxycarbonylmethoxy)coumarin-4-yl]methyl-substituted forms of cAMP (BECMCM-caged cAMP) and cGMP (BECMCM-caged cGMP). With a flash of UV light, the caged compounds are changed into their active forms (Matsumoto *et al.,* 2003). A transient increase in $[Ca^{2+}]i$ was observed after release of the caged cGMP. In contrast, the intracellular cAMP ([cAMP]i) did not change significantly and the Ca^{2+} response evoked by the photolysis of caged cAMP was significantly smaller than that evoked using caged cGMP. Thus, a unifying principle emerges, i.e., chemosensory transduction in marine invertebrate sperm uses cGMP as the primary messenger.

Transient elevation of Ca^{2+} is caused by a K^+-dependent Na^+/Ca^{2+} exchanger

Asterosap transiently increases [cGMP]i of sperm, which in turn induces a transient increase in $[Ca^{2+}]i$. Using the fluorescent Ca^{2+}-sensitive dye Fluo-4 AM, we measured the changes in $[Ca^{2+}]i$ of the sperm in response to asterosap. KB-R7943 (KB), a selective inhibitor of Na^+/Ca^{2+} exchanger (NCX), significantly inhibited the asterosap-induced transient increase in $[Ca^{2+}]i$, suggesting that asterosap influences $[Ca^{2+}]i$ through the activation of a K^+-dependent NCX (NCKX) (Islam *et al.,* 2006a). The NCKX activity in starfish sperm also shows K^+ dependency similar to other NCKXs. Voltage-gated Ca^{2+} channels and the store-operated channels do not affect this system. An NCKX cDNA from the starfish testes predicts that it codes for a 616-amino-acid protein that is a member of the NCKX family. Pharmacological evidence suggests that NCX participates in asterosap-induced Ca^{2+} entry into the sperm. Therefore, NCX may contribute to the transient elevation of $[Ca^{2+}]i$ induced by asterosap.

Sustained increase in Ca^{2+} induced by ARIS and asterosap

For the induction of the AR, ARIS alone is sufficient in seawater with high $[Ca^{2+}]$ (70 mM) or high pH seawater (pH 9.5) but in normal seawater, the addition of either Co-ARIS or asterosap is also required (Matsui *et al.,* 1986a). Asterosap transiently increases both the pHi and $[Ca^{2+}]i$, while ARIS slightly elevates the basal level of $[Ca^{2+}]i$. However, if sperm are simultaneously treated with ARIS and asterosap, a sustained increase in $[Ca^{2+}]i$ and consequent AR occurs (Kawase *et al.,* 2005). EGTA inhibits the sustained increase in $[Ca^{2+}]i$ and AR. The sustained increase in $[Ca^{2+}]i$ and AR induction are highly susceptible to SKF96365 and Ni^{2+}, specific blockers of store-operated Ca^{2+} channels (SOC). Thus, the sustained increase in $[Ca^{2+}]i$, mediated by the SOC-like channel, seems be a required for triggering the AR.

The asterosap-induced increase in pHi is sufficient for ARIS to induce the AR

In seawater at high pH (pH 9.5) ARIS alone induces a prominent $[Ca^{2+}]i$ increase and the AR. When the change in pHi was measured using 9-amino-acidine, the pHi in normal seawater (pH 8.2) is 7.6 ± 0.1. But the AR is also induced by ARIS alone when the pHi is artificially increased to more than 7.7 (Kawase *et al.,* 2005). Furthermore, the sustained increase in $[Ca^{2+}]i$ and AR induction by a combination of ARIS and asterosap, were both drastically inhibited by a slight reduction ($\Delta pH = 0.1$) in [pH]i. The asterosap-induced increase in [pH]i is required for triggering the ARIS-induced sustained increase in $[Ca^{2+}]i$ that triggers the AR.

ARIS and asterosap both elevate intracellular cAMP in starfish sperm

In sea urchins, the sperm-activating peptide speract increases [cAMP]i, but in starfish sperm, asterosap does not significantly increase [cAMP]i (36 pmol/10^8 cells, Matsumoto *et al.,* 2003). By using a cAMP enzyme-immunoassay, when sperm were stimu-

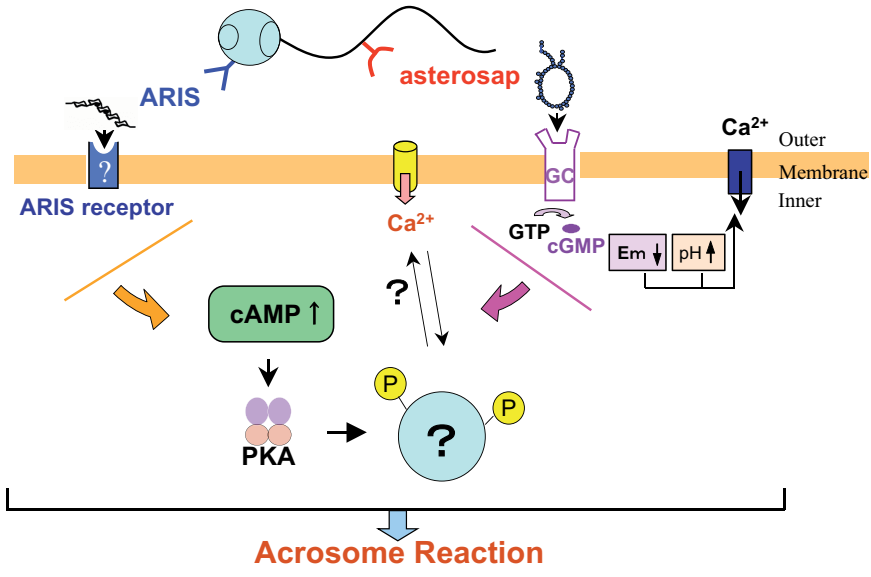

Fig. 2. Concerted regulation of AR in starfish by ARIS and asterosap. *ARIS is a key molecule for triggering AR and asterosap is a sperm-activating peptide. ARIS induces the AR in cooperation with Co-ARIS or asterosap in normal seawater. Asterosap stimulates a rapid synthesis of intracellular [cGMP]i, [pH]i and $[Ca^{2+}]i$, and the elevation of [cGMP]i stimulated $[Ca^{2+}]i$, which is caused by K^+-dependent Na^+/Ca^{2+} exchanger. ARIS and asterosap can induce the sustained increase in $[Ca^{2+}]i$ and co-elevate [cAMP]i levels.*

lated with egg jelly, or with ARIS plus asterosap, [cAMP]i was elevated to 54 pmol/10^8 cells and stayed for more than 30 s after both the treatments. However, ARIS alone could slightly increase [cAMP]i (41 pmol/10^8 cells, Islam *et al.,* 2006b).

The [pH]i increase is required for triggering the ARIS-induced AR in starfish sperm. To determine the pH dependency of the increase in [cAMP]i in ARIS-treated sperm, the sperm were treated at different external pH values, and [cAMP]i then determined. The rate of cAMP elevation also depends on the external pH. At pH 9.5, [cAMP]i was 48 pmol/10^8 cells; this value is similar to that observed for egg-jelly stimulated sperm in normal seawater of pH 8.2. Therefore, the synergistic effects of the asterosap and ARIS are linked to [pH]i and [cAMP]i.

Protein kinase activation is involved in AR induction

In a cAMP enzyme immunoassay, ARIS also increased [cAMP]i in the presence of seawater with high pH (pH 9.5, Islam *et al.,* 2006b). Pretreatment of spermatozoa with two specific, cell-permeable PKA inhibitors, H89 and KT5720, prevented the induction of the AR in a concentration-dependent manner. Therefore, the PKA activity should participate in the induction of the AR with ARIS and asterosap. To investigate this, we have cloned a gene encoding a regulatory subunit of PKA that had been identified in starfish sperm. In Fig. 2, we summarized the concerted regulation of the AR in starfish sperm by ARIS and asterosap.

Future perspectives

The treatment of mammalian sperm with albumin is essential for capacitation, which in turn, is essential for the AR (Toyota *et al.,* 1971). The AR is also induced by the zona pellucida in concert with some components of female genital fluid and increases [cAMP]i and [Ca^{2+}]i (Nolan *et al.,* 2004). In sea urchins, two pathways with FSP and polysialic acid are involved in the induction of the pHi increase in sperm. The polysialic acid present on the surface of the egg plays a role in triggering the sperm AR (Hirohashi and Vacquier, 2002). Furthermore, we showed that the ARIS and asterosap signaling pathway seems to be synergistically regulated to trigger the AR in starfish sperm. Since the AR in sperm of different animals could be regulated by many different factors, the analysis of the AR induction mechanism in a wide variety of animals is essential for our further understanding of fertilization.

References

BOHMER, M., VAN, Q., WEYAND, I., HAGEN, V., BEYERMANN, M., MATSUMOTO, M., HOSHI, M., HILDEBRAND, E., & KAUPP, B. (2005). Ca^{2+} spikes in the flagellum control chemotactic behavior of sperm. *EMBO J.* 24: 2741–52.

DAN, J.C. (1952). Studies of the acrosome. I. Reaction to egg water and other stimuli. *Biol. Bull.* 103: 54–66.

DARSZON, A., ACEVEDO, J. J., GALINDO, B.E., HERNÁNDEZ-GONZÁLEZ, E.O., NISHIGAKI, T., TREVIÑO, C. L., WOOD, C. AND BELTRÁN, C. (2006) Sperm channel diversity and functional multiplicity. *Reproduction* 131: 977-988.

GARBERS, D.L. & KOPF, G.S. (1980). The regulation of spermatozoa by calcium and cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* 13: 251–306.

GUERRERO, A. & DARSZON, A (1989) Evidence for the activation of two different Ca^{2+} channels during the egg jelly-induced AR of sea urchin sperm. *J. Biol. Chem.* 264: 19593–9

GUNARATNE, H.M., YAMAGAKI, T., MATSUMOTO, M., & HOSHI, M. (2003). Biochemical characterization of inner sugar chains of AR-inducing substance in jelly coat of starfish eggs. *Glycobiology* 13: 567–80.

HIROHASHI, N. & VACQUIER, V.D. (2002a). Egg sialoglycans increase intracellular pH and potentiate the AR of sea urchin sperm. *J. Biol. Chem.* 277: 8041–47.

HOSHI, M., NISHIGAKI, T., USHIYAMA, A., OKINAGA, T., CHIBA,K., & MATSUMOTO, M. (1994). Egg-jelly signal molecules for triggering the AR in starfish spermatozoa. *Int. J. Dev. Biol.* 38: 167–74.

IKADAI, H. & HOSHI, M. (1981a). Biochemical studies on the AR of the starfish, *Asterias amurensis*. I. Factors participating in the AR. *Dev. Growth Differ.* 23: 73–80.

IKADAI, H. & HOSHI, M. (1981*b*). Biochemical studies on the AR of the starfish, *Asterias amurensis*. II. Purification and characterization of AR inducing substance. *Dev. Growth Differ.* 23: 81–8.

ISLAM, M.S., KAWASE, O., HASE, S., MINAKATA, H., HOSHI, M., & MATSUMOTO M. (2006a) Na^+/Ca^{2+} exchanger contributes to asterosap-induced elevation of intracellular Ca2+ concentration in starfish spermatozoa. *Zygote* 14: 133–41.

ISLAM, M.S., KAWASE O., HASE S., HOSHI M., & MATSUMOTO, M. (2006b) PKA activation in concert with ARIS and asterosap induces the AR in starfish. *Zygote* 14: 329–40.

KAUPP, U.B., SOLZIN, J., HILDEBRAND, E., BROWN, J.E., HELBIG, A., HAGEN, V., BEYERMANN, M., PAMPALONI, F., & WEYAND, I. (2003). The signal flow and motor response controlling chemotaxis of sea urchin sperm. *Nat. Cell. Biol.* 5: 109–17.

KAUPP, U.B., KASHIKAR, N.D., WEYAND, I. (2008). Mechanisms of Sperm Chemotaxis *Annual Review of Physiology,* 70 (in press)

KAWASE, O., MINAKATA, H., HOSHI, M., & MATSUMOTO, M. (2005). Asterosap-induced elevation in intracellular pH is indispensable for ARIS-induced sustained increase in intracellular Ca^{2+} and following AR in starfish spermatozoa. *Zygote* 13: 63–71.

KAWASE, O., UENO, S., MINAKATA, H., HOSHI, M., & MATSUMOTO, M. (2004). Guanylyl cyclase and cGMP specific phosphodiesterase participate in the AR of starfish sperm. *Zygote* 12: 345–55.

KOYOTA, S., WIMALASIRI, K.M.S., & HOSHI, M. (1997). Structure of the main saccharide chain in AR-inducing substance of starfish, *Asterias amurensis*, *J. Biol. Chem.* 272: 10372–6.

MATSUI, T., NISHIYAMA, I., HINO, A., & HOSHI, M. (1986*a*). Induction of the AR in starfish. *Dev. Growth Differ.* 28: 339–48.

MATSUI, T., NISHIYAMA, I., HINO, A., & HOSHI, M. (1986b). AR-inducing substance purified from the egg jelly inhibits the jelly-induced AR in starfish: an apparent contradiction. *Dev. Growth Differ.* 28: 349–57.

MATSUI, T., NISHIYAMA, I., HINO, A., & HOSHI, M. (1986*c*). Intracellular pH changes of starfish sperm upon the AR. *Dev. Growth Differ.* 28: 359–68.

MATSUMOTO, M., SOLZIN, Z., HELBIG, A., HAGEN, V., UENO, S., KAWASE, O., MARUYAMA, Y., OGISO, M., GODDE, M., MINAKATA, H., KAUPP, U.B., HOSHI, M., & WEYAND, I. (2003). A sperm-activating peptide controls a cGMP signaling pathway in starfish sperm. *Dev. Biol.* 260: 314– 24.

NISHIGAKI, T.,CHIBA,K., MIKI,W., & HOSHI, M. (1996). Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. *Zygote* 4: 237–45.

NISHIGAKI, T., CHIBA, K., & HOSHI, M. (2000). A 130-kDa membrane protein of sperm flagella is the receptor for asterosaps, sperm-activating peptides of starfish *Asterias amurensis*. *Dev. Biol.* 219: 154–62.

NISHIYAMA, I., MATSUI, T., FUJIMOTO, Y., IKEKAWA, N., & HOSHI, M. (1987). Correlation between the molecular structure and biological activity of Co-ARIS, a cofactor for AR-inducing substance. *Dev. Growth Differ.* 29: 171–76.

NOLAN, M.A., BABCOCK, D.F., WENNEMUTH, G., BROWN, W., BURTON, K.A., & MCKNIGHT G.C. (2004) Sperm-specific protein kinase A calytytic subunit Calpha2 orchestrates cAMP signaling for male fertility. *Proc. Natl. Acad. Sci. USA* 101: 13483–8

SHIBA,K., TAGATA,T., OHMURA,J., MOGAMI, Y., MATSUMOTO, HOSHI M. and BABA, S.A. (2006). Peptide-induced hyperactivation-like vigorous flagellar movement in starfish sperm *Zygote* 14: 23–3.

TOYODA, Y., YOKOYAMA, M. & HOSHI, T. (1971) Studies on the fertilization of mouse eggs in vitro I In vitro fertilizatrion of eggs by fresh epididymal sperm. *Jpn. J. Anim. Reprod.* 16: 147–51

TILNEY, L.G. (1985). The AR. In *Biology of Fertilization*, vol. 2 (ed. C. B. Metz & A. Monroy), pp. 157– 213. Orlando, FL: Academic Press.