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Asterosap-induced elevation in intracellular pH is indispensable for ARIS-induced sustained increase in intracellular Ca^{2+} and following acrosome reaction in starfish spermatozoa

Osamu Kawase[1], *Hiroyuki Minakata*[2], *Motonori Hoshi*[1] *and Midori Matsumoto*[1]
Department of Biosciences and Informatics, and Center for Life Science and Technology, Keio University, Yokohama, and SUNBOR, Osaka, Japan

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Summary

In the starfish, *Asterias amurensis*, the cooperation of three components of the egg jelly, namely ARIS (acrosome reaction-inducing substance), Co-ARIS and asterosap, is responsible for the induction of acrosome reaction. For the induction, ARIS alone is enough in high-Ca^{2+} or high-pH seawater, but, besides ARIS, the addition of either Co-ARIS or asterosap is required in normal seawater. Asterosap transiently increased both the intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$), while ARIS slightly elevated the basal level of $[Ca^{2+}]_i$. However, a sustained elevation of $[Ca^{2+}]_i$ and acrosome reaction occurred if sperm were simultaneously treated with ARIS and asterosap. EGTA inhibited the sustained $[Ca^{2+}]_i$ elevation and acrosome reaction. The sustained $[Ca^{2+}]_i$ elevation and acrosome reaction were highly susceptible to SKF96365 and Ni^{2+}, specific blockers of the store-operated Ca^{2+} channel (SOC). These results suggest that sustained $[Ca^{2+}]_i$ elevation, mediated by the SOC-like channel, is a prerequisite for the acrosome reaction. In high-pH seawater, ARIS alone induced a prominent $[Ca^{2+}]_i$ increase and acrosome reaction, which were similarly sensitive to SKF96365. The acrosome reaction was effectively induced by ARIS alone when pH_i was artificially increased to more than 7.7. Asterosap increased pH_i from 7.6 $\pm$ 0.1 to 7.7 $\pm$ 0.1. Furthermore, the sustained $[Ca^{2+}]_i$ elevation and acrosome reaction, induced by a combination of ARIS and asterosap, were drastically inhibited by a slight reduction in pH_i. Taking these results into account, we suggest that an asterosap-induced pH_i elevation is required for triggering the ARIS-induced sustained $[Ca^{2+}]_i$ elevation and consequent acrosome reaction.

Keywords: Acrosome reaction, Cooperative action, Intracellular Ca^{2+}, Intracellular pH, Starfish

Introduction

The acrosome reaction, namely the exocytosis of the acrosomal vesicle in the sperm head, is an essential event for fertilization in many species (Tilney, 1985). The acrosome reaction is initiated when a sperm interacts with an egg investment. In many marine invertebrates, including echinoderms, exocytosis is followed by the formation of an acrosomal process that projects from the anterior tip of the sperm head (Dan, 1952). In the starfish, *Asterias amurensis*, three components of the egg jelly, namely ARIS (acrosome reaction-inducing substance), Co-ARIS and asterosap, cooperatively trigger the acrosome reaction of the sperm (Hoshi *et al*., 1994). ARIS is a sulfated proteoglycan-like molecule of an extremely large molecular size (Ikadai & Hoshi, 1981*b*; Koyota *et al*., 1997), Co-ARIS is a group of sulfated steroidal saponins (Nishiyama *et al*., 1987) and asterosap is a group of equally active isoforms of sperm-activating peptide (Nishigaki *et al*., 1996). ARIS induces the acrosome reaction in cooperation with Co-ARIS or asterosap in normal seawater, whereas ARIS alone induces it in high-Ca^{2+} or high-pH seawater (Matsui *et al*., 1986*a*). Thus, ARIS is regarded as the major acrosome reaction-inducing molecule.

All correspondence to: Dr M. Matsumoto, Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi Kouhoku-ku Yokohama, 223-8522 Japan. Tel: +81 45 566 1774. Fax: +81 45 566 1448. e-mail: mmatsumo@bio.keio.ac.jp
[1] Department of Biosciences and Informatics, and Center for Life Science and Technology, Keio University, Yokohama, Japan.
[2] SUNBOR, Osaka, Japan.

An anti-asterosap antibody drastically reduces the capacity of the egg jelly to induce the acrosome reaction (Nishigaki *et al*., 1996). Furthermore, sperm did not undergo the acrosome reaction in response to the egg jelly if the asterosap-induced changes are blocked by the pretreatment of sperm only with asterosap (Kawase *et al*., 2004). Thus, it is clear that, besides ARIS, asterosap is essential for the egg-jelly-induced acrosome reaction.

Asterosap transiently increases the intracellular cGMP, pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) via the activation of asterosap receptor, guanylyl cyclase (Nishigaki *et al*., 2000; Matsumoto *et al*., 2003), but these changes are not sufficient for the induction of the acrosome reaction. The ARIS-induced intracellular change has not yet been clearly detected (Matsui *et al*., 1986*a*). On the other hand, the egg jelly induces a transient elevation of pH_i and uptake of $^{45}Ca^{2+}$ (Matsui *et al*., 1986*a*,*b*). The uptake of Ca^{2+} is considered to be essential for the induction of the acrosome reaction, because the acrosome reaction is induced by a Ca^{2+} ionophore and the egg-jelly-induced acrosome reaction is inhibited by Ca^{2+} channel blockers (Matsui *et al*., 1986*a*). The egg-jelly-induced elevation of pH_i seems to facilitate the acrosome reaction, because the sperm undergo the acrosome reaction in high-pH seawater containing ARIS or in high-Ca^{2+}, high-pH seawater, but not in high-Ca^{2+} seawater or high-pH seawater (Ikadai & Hoshi, 1981*a*; Matsui *et al*., 1986*a*).

In sea urchins, FSP (fucose sulfate polymer) in the egg jelly (Alves *et al*., 1998) induces the sequential activation of two Ca^{2+} channels of sperm (Guerrero & Darszon, 1989; Hirohashi & Vacquier, 2002*a*). After the binding of FSP with its receptor, REJ (receptor for egg jelly; Moy *et al*., 1996), the first channel opens transiently and the second channel causes a sustained increase in $[Ca^{2+}]_i$ and the acrosome reaction, but the linkage between these two channels is unclear (Guerrero & Darszon, 1989). The second channel may be a store-operated Ca^{2+} channel (SOC; González-Martínez *et al*., 2001; Hirohashi & Vacquier, 2003). It is suggested that the FSP-induced increase in pH_i is essential for the activation of the second channel (García-Soto & Darszon, 1985; Guerrero *et al*., 1998). Similarly, in mammals, ZP (a complex of glycoproteins in the zona pellucida) activates the SOC through the increase in pH_i and gives rise to the acrosome reaction (Arnoult *et al*., 1996*a*; O'Toole *et al*., 2000). These observations suggest the signal transduction system leading to the acrosome reaction is widely preserved.

In starfish, the acrosome reaction is induced by the cooperative action of multiple molecules, not by each of them. In this report, we show that the sustained $[Ca^{2+}]_i$ elevation occurs via the SOC-like channel when sperm is simultaneously treated with ARIS and asterosap. The sustained $[Ca^{2+}]_i$ elevation depends on the asterosap-induced increase in pH_i and is prerequisite for the acrosome reaction. This report demonstrates that the starfish shares the general intracellular mechanism underlying the acrosome reaction with other animals, even though starfish are the only group so far in which the cooperation of multiple egg-coat components is required for the acrosome reaction.

Materials and methods

Chemicals and the modified seawaters

SKF96365, 1-methyladenine, 9-aminoacridine and Pluronic F-127 were purchased from Sigma Chemical (St Louis, MO). Fluo-4 AM was purchased from Nacalai Tesque (Kyoto, Japan). The synthetic asterosap isoform, P15, was used throughout this study (Nishigaki *et al*., 1996). The egg jelly and ARIS were prepared by a previously reported method (Ushiyama *et al*., 1993). ARIS and P15 were dissolved in H_2O at 1 mg sugar/ml and 100 μM respectively and diluted 100-fold at use. The sugar concentration of ARIS was determined by a resorcinol–sulfuric acid method (Monsigny *et al*., 1988) using L-fucose as the standard. Artificial seawater (ASW, pH 8.2) and seawater of pH 7.6 consisted of 430 mM NaCl, 9 mM $CaCl_2$, 9 mM KCl, 23 mM $MgCl_2$ and 25 mM $MgSO_4$ in 10 mM EPPS (*N*-2-hydroxyethylpiperazine-*N*′-3-propane sulphonic acid) buffer. High-Ca^{2+} seawater was prepared by adding $CaCl_2$ into ASW up to 70 mM and Ca^{2+}-free seawater was prepared by omission of $CaCl_2$ from ASW. To prepare higher pH (pH 8.7, 9.0 and 9.5) or lower pH (pH 6.8 and 7.1) seawaters, 10 mM EPPS buffer in ASW was replaced with 25 mM glycine buffer or 10 mM PIPES (piperazine-*N*, *N*′-*bis*(2-ethane sulphonic acid)) buffer, respectively.

Animals and gametes

Starfish, *Asterias amurensis*, were collected from several locations in Japan and Tasmania, Australia. The animals from Tasmania are known to be the offspring of those invaded from Tokyo Bay (Byrne *et al*., 1997), and no significant differences were observed between the Japanese and Tasmanian animals except for the breeding season. The mature eggs were collected in ASW by treating the ovaries with 10 μM 1-methyladenine and sperm were obtained as 'dry' sperm by cutting the testes and kept on ice before use.

Electrophysiological measurements and acrosome reaction assay

The measurement of $[Ca^{2+}]_i$ was performed by using Fluo-4 AM as previously reported (Kawase *et al*., 2004). The level of pH_i was monitored by using 9-aminoacridine as previously reported (Matsui *et al*., 1986*b*) with several modifications. Dry sperm (5 μl)

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was diluted with 2 ml of each seawater containing 0.17 μM 9-aminoacridine in a round cuvette and the fluorescence intensity was monitored with a Shimadzu Spectrofluorophotometer RF-540 (Shimadzu Science, Tokyo, Japan) at 382 nm excitation and 454 nm emission. To chelate the external Ca^{2+}, 2 M EGTA in 6 M NaOH was added into the sperm suspension at a final concentration of 11 mM. For the acrosome reaction assay, 100 μl of sample was fixed by adding 20 μl of 5% formaldehyde in ASW and stained with 3 μl of 0.5% erythrosine in 70% ethanol. The acrosome reaction was assayed under a Nomarski microscope by counting the number of sperm that extruded the acrosomal process. More than 200 sperm were scored for each experiment.

Results

Sustained $[Ca^{2+}]_i$ elevation and the acrosome reaction are induced by the cooperative action of ARIS and asterosap P15

The $[Ca^{2+}]_i$ change was monitored in Fluo-4-loaded sperm. ARIS slightly elevated the basal concentration of $[Ca^{2+}]_i$ (Fig. 1, top trace). The ARIS-induced changes in the sperm were clearly detected for the first time in this study. P15 transiently increased the level of $[Ca^{2+}]_i$ and elevated its basal level (Fig. 1, middle trace). Although neither ARIS-induced nor P15-induced change in $[Ca^{2+}]_i$ triggered the acrosome reaction, the mixture of ARIS and P15 induced a sustained $[Ca^{2+}]_i$ elevation and the acrosome reaction (Fig. 1, bottom trace). This result obviously shows that the signal transduction system leading to the acrosome reaction is sufficiently activated only by the combination of ARIS and P15.

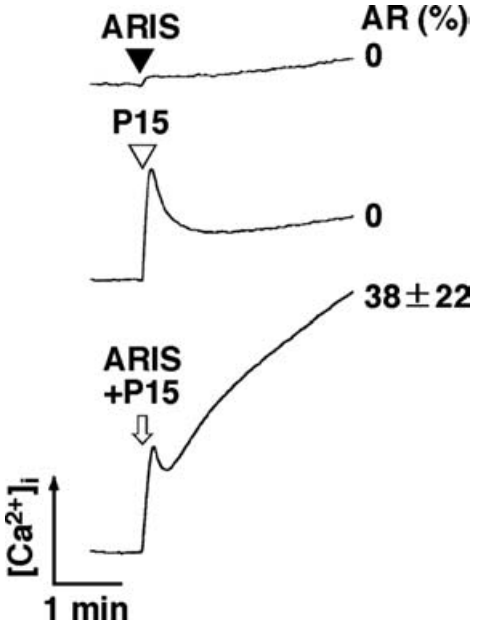

Figure 1 Sustained increase in $[Ca^{2+}]_i$ induced by the mixture of ARIS and asterosap (P15). The effects of ARIS (black arrowhead), P15 (white arrowhead) and ARIS plus P15 (white arrow) on the $[Ca^{2+}]_i$ level were monitored with Fluo-4-loaded sperm. The fluorescence intensity was recorded at 516 nm; excitation was at 494 nm. ARIS was used at 10 μg sugar/ml and P15 at 1 μM. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

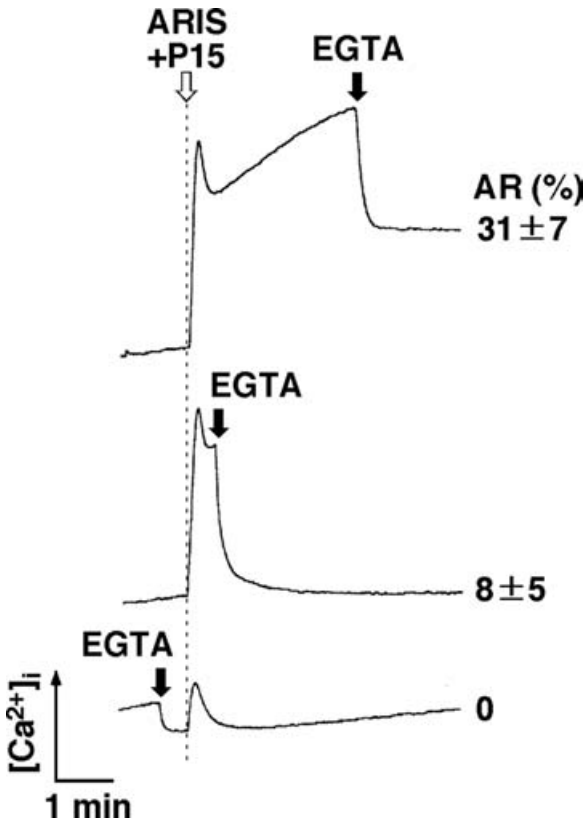

Figure 2 EGTA blocked the sustained $[Ca^{2+}]_i$ elevation induced by ARIS plus P15 and consequent acrosome reaction. EGTA was added to the suspension of Fluo-4-loaded sperm (black arrows) 3 min after (top trace), 30 s after (middle trace) or 30 s before the addition of ARIS plus P15 (dotted line with white arrow). ARIS was used at 10 μg sugar/ml and P15 at 1 μM. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

External Ca^{2+} is required for sustained $[Ca^{2+}]_i$ elevation the acrosome reaction

EGTA, a Ca^{2+} chelator, was added into the sperm suspension to reduce the extracellular Ca^{2+} at various times before or after the addition of ARIS and P15. If EGTA was added after the sustained $[Ca^{2+}]_i$ elevation was accomplished (3 min after the addition of ARIS and P15), the $[Ca^{2+}]_i$ decreased and its elevation stopped, but the acrosome reaction had already occurred prior to the addition of EGTA (Fig. 2, top trace). However, if

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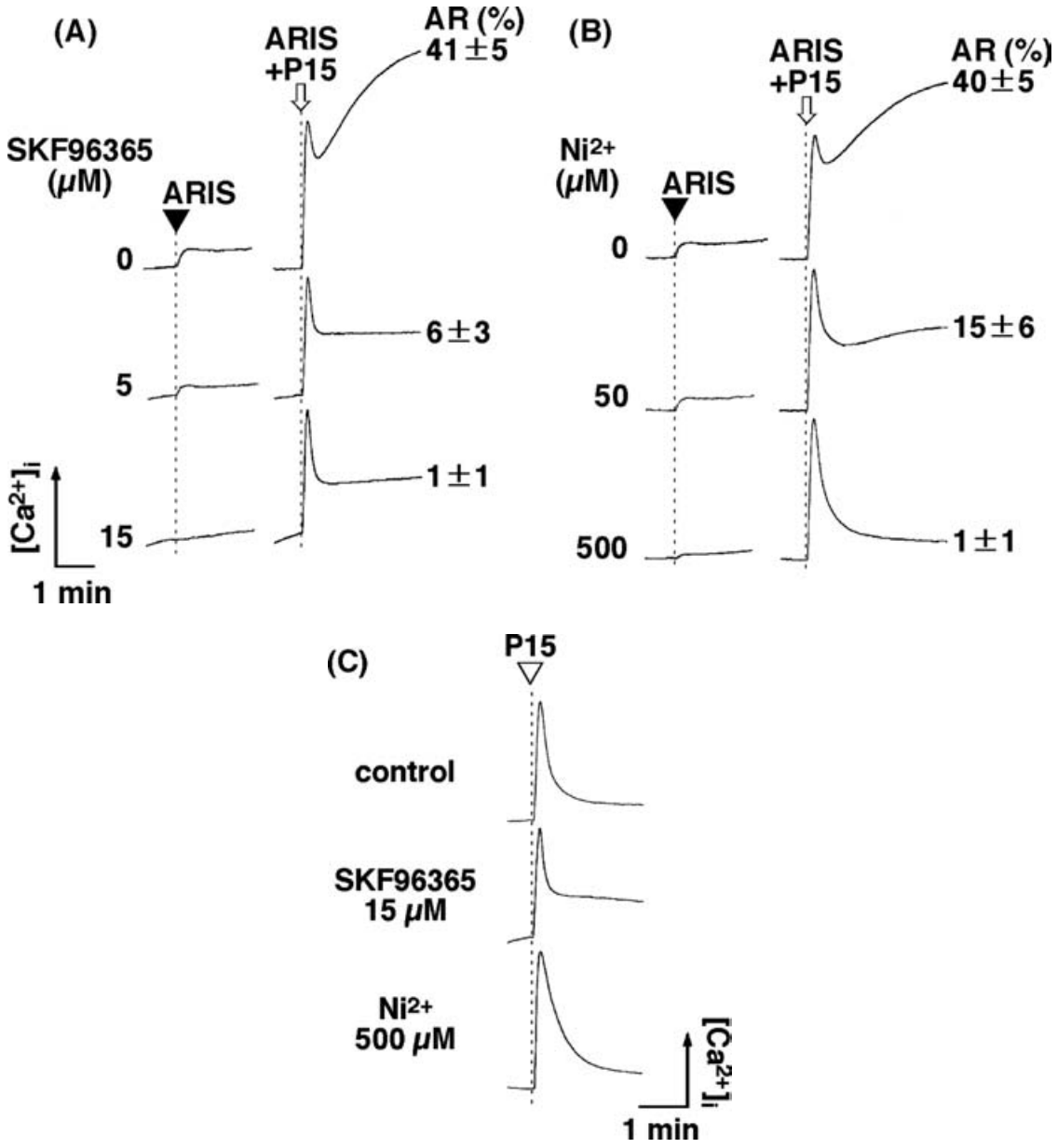

Figure 3 SKF96365 and Ni^{2+} inhibited the sustained $[Ca^{2+}]_i$ elevation induced by ARIS plus P15. In the presence of 0–15 μM SKF96365 (A) or 0–500 μM Ni^{2+} (B), Fluo-4-loaded sperm were treated with ARIS (dotted line with black arrowhead) or ARIS plus P15 (dotted line with white arrow). In the presence of 15 μM SKF96365 or 500 μM Ni^{2+}, the sperm were treated with P15 (dotted line with white arrowhead, C). ARIS was used at 10 μg sugar/ml and P15 at 1 μM. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

EGTA was added 30 s after the addition of ARIS and P15, both the sustained $[Ca^{2+}]_i$ elevation and acrosome reaction were markedly inhibited (Fig. 2, middle trace). The acrosome reaction was not induced despite the initial $[Ca^{2+}]_i$ spike. If EGTA was added 30 s before the addition of ARIS and P15, the whole change in $[Ca^{2+}]_i$ and the acrosome reaction were drastically inhibited (Fig. 2, bottom trace). The small $[Ca^{2+}]_i$ spike could be attributed to the remaining external Ca^{2+}, because it did not appear in Ca^{2+}-free seawater (data not shown). These results suggest that the sustained $[Ca^{2+}]_i$ elevation and ARIS- or P15-induced changes depend on extracellular Ca^{2+} and that the sustained $[Ca^{2+}]_i$ elevation is essential for the acrosome reaction.

Sustained $[Ca^{2+}]_i$ elevation induced by ARIS plus P15 is highly susceptible to SOC blockers

We investigated whether the SOC-like channel was involved in the sustained $[Ca^{2+}]_i$ elevation induced by ARIS and P15 by using the SOC blockers SKF96365

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and Ni^{2+} (Leung & Kwan, 1999; González-Martínez *et al.*, 2001). The sustained $[Ca^{2+}]_i$ elevation and acrosome reaction were remarkably inhibited by 5 μM SKF96365 (IC_{50} values around 2.2 μM), although the ARIS-induced $[Ca^{2+}]_i$ elevation was not blocked unless the concentration of SKF96365 was higher (IC_{50} values around 8.2 μM; Fig. 3A). Similarly, the sustained $[Ca^{2+}]_i$ elevation and acrosome reaction were inhibited by 50 μM Ni^{2+} (IC_{50} values around 36 μM), whereas the ARIS-induced signal was suppressed at much higher concentrations (IC_{50} values around 400 μM; Fig. 3B). Neither 15 μM SKF96365 nor 500 μM Ni^{2+} affected P15-induced changes in $[Ca^{2+}]_i$ (Fig. 3C). Cyclopiazonic acid (CPA) is known as a drug reducing the Ca^{2+} concentration of the intracellular Ca^{2+} store and then activating the SOC. The co-treatment of sperm with CPA and ARIS induces the acrosome reaction (data not shown). Because the sustained $[Ca^{2+}]_i$ elevation and the acrosome reaction are highly sensitive to the blockers, they seem to be mediated via the SOC-like channel.

ARIS induces a prominent $[Ca^{2+}]_i$ increase in high-pH or high-Ca^{2+} seawater

ARIS induces the acrosome reaction in high-pH or high-Ca^{2+} seawater, although these seawaters themselves do not (Matsui *et al.*, 1986*a*). A prominent increase in $[Ca^{2+}]_i$ and the acrosome reaction were shown when sperm were treated with ARIS alone in high-pH (pH 9.5) or high-Ca^{2+} seawater (Fig. 4). However, in each condition the patterns of Ca^{2+} signal were different: The speed of $[Ca^{2+}]_i$ elevation was slow in high-pH seawater but rapid in high-Ca^{2+} seawater, suggesting that the mechanisms involved in the $[Ca^{2+}]_i$ elevation are different in high-pH and high-Ca^{2+} seawater.

ARIS-induced prominent $[Ca^{2+}]_i$ elevation in high-pH seawater is highly susceptible to SKF96365

If the Ca^{2+} channel activated by the combination of ARIS and P15 in ASW is similarly activated by ARIS in high-pH or in high-Ca^{2+} seawater, the ARIS-induced prominent Ca^{2+} increases in these modified seawaters should be highly sensitive to SKF96365. The ARIS-induced prominent $[Ca^{2+}]_i$ elevation in high-pH seawater (pH 9.5) and the acrosome reaction were drastically blocked by 5 μM SKF96365 (IC_{50} values around 3.1 μM; Fig. 5A), while 15 μM SKF96365 was required to suppress those in high-Ca^{2+} seawater (IC_{50} values around 7.3 μM; Fig. 5B). The ARIS-induced prominent $[Ca^{2+}]_i$ increase in high-pH seawater was highly susceptible to SKF96365 as was the sustained $[Ca^{2+}]_i$ increase induced by ARIS and P15 in ASW (Figs. 3A, 5A). Thus, it is suggested that the pH_i increase and ARIS action cooperatively activate the SOC-like channel to trigger the acrosome reaction. On the other hand, ARIS-induced $[Ca^{2+}]_i$ elevations both in ASW and in high-Ca^{2+} seawater were inhibited by SKF96365 in a similar dose-dependent manner (Figs. 3A, 5B). ARIS-induced $[Ca^{2+}]_i$ influx seems to be enhanced by a high concentration of extracellular Ca^{2+}. This conclusion agrees with the previous result

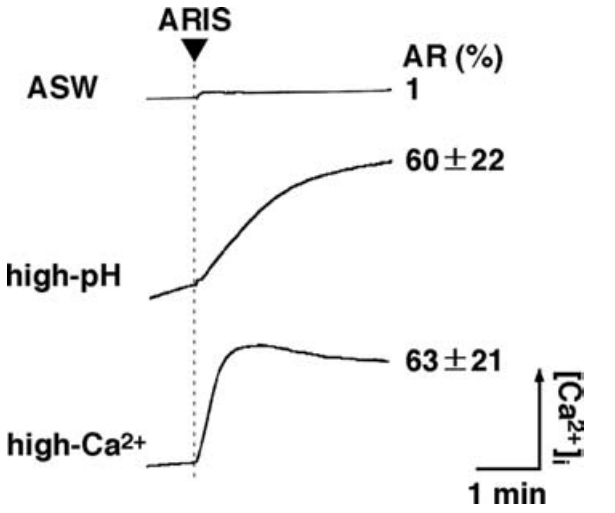

Figure 4 ARIS induced a prominent $[Ca^{2+}]_i$ increase in high-pH or high-Ca^{2+} seawater. Fluo-4-loaded sperm were treated with ARIS (dotted line with black arrowhead) in high-pH (pH 9.5, middle trace) or high-Ca^{2+} seawater (bottom trace). ARIS was used at 10 μg sugar/ml. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

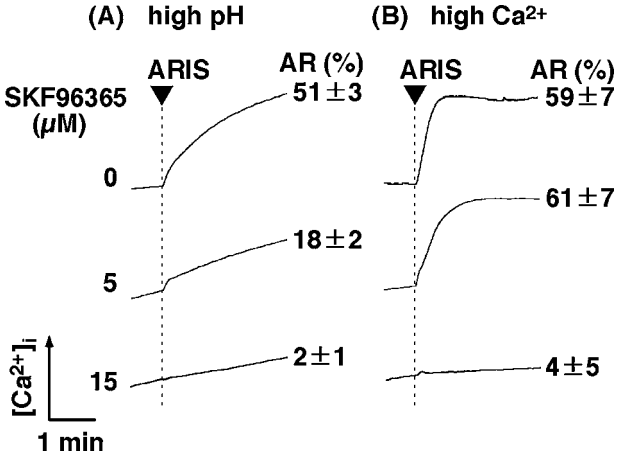

Figure 5 SKF96365 inhibited the ARIS-induced prominent $[Ca^{2+}]_i$ increase in high-pH seawater. In the presence of 0–15 μM SKF96365, Fluo-4-loaded sperm were treated with ARIS (dotted line with black arrowhead) in high-pH (pH 9.5, A) or high-Ca^{2+} seawater (B). ARIS was used at 10 μg sugar/ml. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

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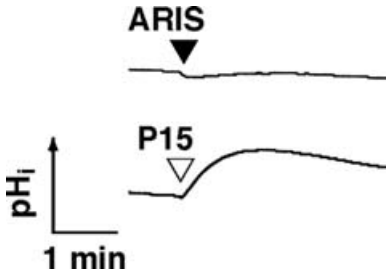

Figure 6 P15-induced elevation of pH_i. ARIS (black arrowhead) or P15 (white arrowhead) was added to the sperm suspension containing 9-aminoacridine. The fluorescence intensity was recorded at 454 nm; excitation was at 382 nm. ARIS was used at 10 μg sugar/ml and P15 at 1 μM. The pH_i was 7.6 ± 0.1 in ASW and increased up to 7.7 ± 0.1 ($n = 4$, $\Delta pH = 0.1$) after the addition of P15, but not ARIS.

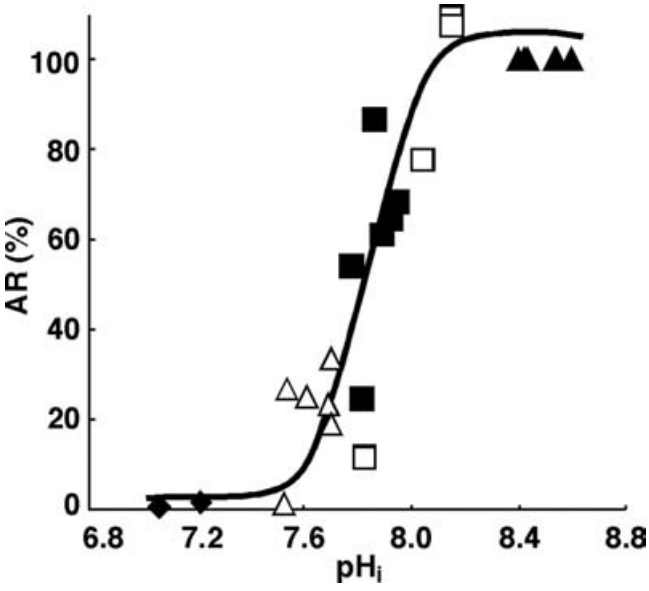

Figure 7 Ratio of ARIS-induced acrosome reaction increased steeply if the pH_i was elevated to 7.7 or higher. The pH_i was measured using 9-aminoacridine when sperm were diluted with seawater at pH 7.6 ($n = 2$, black rhombus), 8.2 ($n = 6$, white triangle), 8.7 ($n = 6$, black square), 9.0 ($n = 4$, white square) or 9.5 ($n = 6$, black triangle). The ratios of the ARIS-induced acrosome reaction were counted in these conditions and the correlation between the pH_i levels and these ratios was plotted. ARIS was used at 10 μg sugar/ml. Each ratio of the acrosome reaction was normalized to that in seawater with pH 9.5 as 100%.

showing that the elevation of pH_i is not required for the acrosome reaction induced by ARIS in high-Ca^{2+} seawater (Matsui *et al*., 1986*b*).

P15-induced elevation of pH_i is enough to facilitate ARIS-induced acrosome reaction

The change in pH_i was measured using 9-aminoacridine. The pH_i was 7.6 ± 0.1 in ASW and it increased

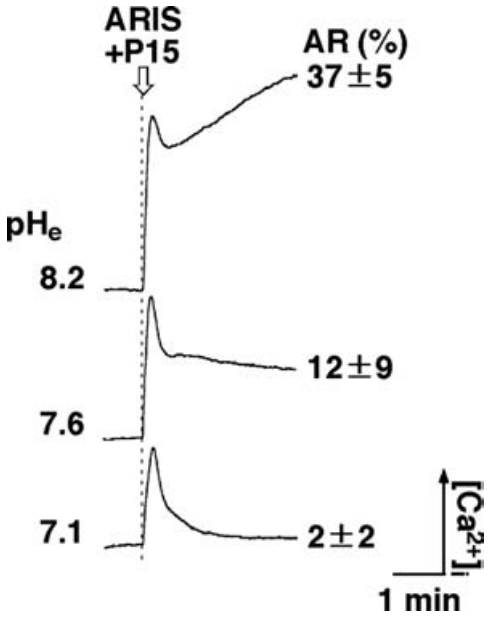

Figure 8 Slight decrease in pH_i reduced the capacity of sperm to undergo the sustained $[Ca^{2+}]_i$ increase and subsequent acrosome reaction in response to ARIS plus P15. Fluo-4-loaded sperm were treated with ARIS and P15 (dotted line with white arrow) in seawater at pH 7.1, 7.6 or 8.2 (ASW). ARIS was used at 10 μg sugar/ml and P15 at 1 μM. The ratio of the acrosome reaction was expressed at the end of each trace as the mean ± SE of three independent experiments.

up to 7.7 ± 0.1 ($n = 4$, $\Delta pH = 0.1$) by adding P15, but not ARIS (Fig. 6).

To determine whether the P15-induced elevation of pH_i is sufficient for ARIS to induce the acrosome reaction, we investigated the correlation between the level of pH_i and the ratio of acrosome reaction induced by ARIS alone (Fig. 7). The dry sperm was diluted in seawaters at different pH (7.6–9.5) and tested for ARIS-induced acrosome reaction. The ratio steeply increased if the pH_i was elevated more than 7.7, indicating that ARIS action and P15-induced increase of pH_i (up to 7.7 ± 0.1) cooperatively induce the acrosome reaction.

Slight decrease in pH_i reduces the sperm capacity to undergo the acrosome reaction in response to ARIS plus P15

The sustained $[Ca^{2+}]_i$ elevation and acrosome reaction induced by ARIS and P15 were significantly inhibited in low-pH seawater (pH 7.1 or 7.6; Fig. 8). In the seawater at pH 7.6, the pH_i was 7.4 ± 0.2 ($n = 7$) and elevated 0.1 ($n = 4$) by P15. The slight reduction in resting pH_i level may be enough to prevent P15 from facilitating the ARIS-induced acrosome reaction.

Taking all results into account, we conclude that the P15-induced increase in pH_i is required for the ARIS-induced sustained elevation in $[Ca^{2+}]_i$, which in turn leads to the acrosome reaction.

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Discussion

The sustained increase in $[Ca^{2+}]_i$ is essential for the acrosome reaction of sperm in mammals (Arnoult *et al*., 1996*a*; O'Toole *et al*., 2000) and sea urchins (González-Martínez *et al*., 2001). In mammals and sea urchins, two distinct Ca^{2+} channels are required for the induction of acrosome reaction (Guerrero & Darszon, 1989; Arnoult *et al*., 1996*b*). The second channel, which is presumably the SOC, is somehow activated by the increase in $[Ca^{2+}]_i$ through the first channel and causes the sustained $[Ca^{2+}]_i$ elevation (Guerrero & Darszon, 1989; Arnoult *et al*., 1996b; O'Toole *et al*., 2000; Hirohashi & Vacquier, 2002*a*). Similarly, we demonstrated that a sustained $[Ca^{2+}]_i$ elevation was induced by the cooperation of ARIS and P15 and essential for the acrosome reaction. The sustained $[Ca^{2+}]_i$ elevation was highly susceptible to SKF96365 (IC_{50} values around 2.2 μM). Because it is widely accepted that SKF96365 inhibits the SOC with IC_{50} values from 1 to 4 μM (Leung & Kwan, 1999; Hirohashi & Vacquier, 2003), the sustained $[Ca^{2+}]_i$ elevation seems to be mediated by SOC-like channel also in starfish sperm. The assumption was supported by its high sensitivity to Ni^{2+}. On the other hand, ARIS slightly elevated the basal level of $[Ca^{2+}]_i$ and P15 caused the $[Ca^{2+}]_i$ spike. We do not know whether the ARIS-induced, the P15-induced $[Ca^{2+}]_i$ change or both are essential for the acrosome reaction. To answer this question, two events must be inhibited and/or estimated separately, which is not practical at present.

In sea urchins, the FSP-induced increase in pH_i is essential for the sustained $[Ca^{2+}]_i$ elevation and consequent acrosome reaction (García-Soto & Darszon, 1985; Guerrero *et al*., 1998). An inositol triphosphate (IP_3) receptor-like protein purified from sea urchin sperm attaches with IP_3 strongly in the high-pH condition (Zapata *et al*., 1997). In mammals, the increase in pH_i is accompanied by the ZP-induced acrosome reaction and facilitates the Ca^{2+} influx, although the relation between pH_i and $[Ca^{2+}]_i$ is not clear (Arnoult *et al*., 1996*a*). In starfish, the pH_i elevation is suggested to promote the acrosome reaction induced by ARIS alone (Matsui *et al*., 1986*a*). We found that the P15-induced increase in pH_i was sufficient to facilitate the ARIS-induced sustained $[Ca^{2+}]_i$ elevation and consequent acrosome reaction. The acrosome reaction was induced by ARIS alone in high-Ca^{2+} seawater, but it was sheer artifact lacking the elevation of pH_i and the activation of SOC-like channel. Therefore, the signal transduction leading to the acrosome reaction is similar in all animals so far studied including starfish, though the starfish is the only one so far in which multiple inducers are known.

Only a single molecule, FSP in sea urchin or ZP3 in mouse, is taken up as the major acrosome-reaction-inducing molecule and the above-mentioned mechanism has been proposed (Darszon *et al*., 2001). Both FSP-induced and ZP3-induced signals are branched and complex, but not separable. In the case of starfish, the roles of ARIS and asterosap may be clearly separated. Thus, ARIS and asterosap would be suitable tools to investigate the detailed steps leading to the acrosome reaction. The black box at present would be elucidated by using the starfish, namely how the sustained $[Ca^{2+}]_i$ elevation is mediated by the pH_i.

In the physiological environment for fertilization, several molecules are probably involved in the induction of the acrosome reaction. Although ARIS and astersoap are indispensable for the egg-jelly-induced acrosome reaction, the ratio of acrosome reaction induced by the egg jelly is quite high compared with that by ARIS and asterosap. It suggests that Co-ARIS or undetermined factors are involved in the physiological induction (Nishigaki *et al*., 1996). In a sea urchin, *Hemicentrotus pulcherrimus*, speract promotes the induction of acrosome reaction by acting as a cofactor of FSP (Yamaguchi *et al*., 1987, 1989). In another sea urchin, *Strongylocentrotus purpuratus*, sialoglycan and speract in the egg jelly potentiate the FSP-induced acrosome reaction in low-pH seawater (Hirohashi & Vacquier, 2002*c*) and sialoglycan reduces the concentration of FSP required for the induction in normal seawater (Hirohashi & Vacquier, 2002*b*). Both sialoglycan and speract seem to facilitate the acrosome reaction through the elevation of pH_i. In mammals, ANP (atrial natriuretic peptide) and progesterone in the follicular fluid initiate the acrosome reaction (Osman *et al*., 1989; Zamir *et al*., 1995; Rotem *et al*., 1998). The pretreatment of sperm with progesterone promotes the ZP-induced acrosome reaction (Roldan *et al*., 1994). Furthermore, the human reproductive tract has a nitric oxide (NO)-generating system (Rosselli *et al*., 1998) and exogenous NO induces the acrosome reaction (Revelli *et al*., 2001). All these data suggest that, even though the acrosome reaction is induced *in vitro* by a single molecule such as FSP or ZP3, multiple molecules act on sperm for the induction of the acrosome reaction. Thus, the analysis of their cooperation is an important issue in understanding what happens in nature.

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