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# ASTEROSAP, AN EGG JELLY PEPTIDE, ELEVATE INTRACELLULAR $Ca^{2+}$ AND ACTIVATE THE MOTILITY OF SPERMATOZOA

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## ABSTRACT

Components from the outer envelopes of the egg that influence the flagellar beating and acrosome reaction of spermatozoa are regulated by ion flux across the plasma membrane. Asterosap, a sperm-activating peptide from the starfish egg jelly layer, causes a transient increase in intracellular cyclic GMP (cGMP) through the activation of the asterosap receptor, a guanylyl cyclase (GC), and causes an increase in intracellular $Ca^{2+}$. Here we describe the pathway of asterosap-induced $Ca^{2+}$ elevation using different $Ca^{2+}$ channel antagonists. Fluo-4 AM, a cell permeable $Ca^{2+}$ sensitive dye was used to determine the channel caused by the asterosap-induced $Ca^{2+}$ elevation in spermatozoa. Different L-type $Ca^{2+}$ channel antagonists, a non specific $Ca^{2+}$ channel antagonist (nickel chloride), and a store-operated $Ca^{2+}$ channel (SOC) antagonist do not show any significant response on asterosap-induced $Ca^{2+}$ elevation, whereas KB-R7943, a selective inhibitor against $Na^{+}/Ca^{2+}$ exchanger (NCX) inhibited effectively. We also analyzed the flagellar movement of spermatozoa in artificial seawater (ASW) containing the asterosap at 100 nM $ml^{-1}$. We found that spermatozoa swam vigorously with more symmetrical flagellar movement in asterosap than in ASW and KB-R7943 significantly inhibited the flagellar movement.

**Key words** : Asterosap, Flagellar motility, Intracellular $Ca^{2+}$ elevation, Spermatozoa, Starfish

## INTRODUCTION

In marine invertebrates, peptides released from eggs play a central role in the activation of spermatozoa and chemotactic behavior. Extra-cellular $Ca^{2+}$ is generally accepted as an important factor in the introduction of chemotactic behavior in animal species. Resact is a chemo-attractant for the spermatozoa of sea urchin (*Arbacia punctulata*), which binds to the receptor, guanylyl cyclase (GC), on the spermatozoa (Yoshida *et al.*, 1994). The stimulation of spermatozoa by resact evokes changes in intracellular cyclic GMP (cGMP), cyclic AMP (cAMP), $Ca^{2+}$ concentration, and membrane potential (for review see ref. Darszon *et al.*, 2001). It was believed that resact increases $Ca^{2+}$ elevation through the cAMP-gated $Ca^{2+}$ channel; however, a recent study has shown that the $Ca^{2+}$ response

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evoked by the photolysis was significantly smaller using caged cAMP as compared to caged cGMP (Matsumoto *et al.*, 2003). Both resact and cyclic nucleotide causes a turn or brief tumbling in the swimming path of spermatozoa. In the ascidians *Ciona savigni* and *C. intestinalis*, the sperm-activating and -attracting factor (SAAF) induces extra-cellular $Ca^{2+}$ for activating the motility of spermatozoa and eliciting a chemotactic behavior in the activated spermatozoa towards the egg. The modulators of the store-operated $Ca^{2+}$ channels (SOC) do not inhibit the chemotactic movement of the spermatozoa, even though they inhibited the activation of spermatozoa operated by the voltage-dependent $Ca^{2+}$ channels (VDCC). The increase in the intracellular $Ca^{2+}$ concentration in the SOC induces asymmetrical flagellar movements to establish the chemotactic behavior in spermatozoa (Yoshida *et al.*, 2003).

In the starfish, *Asterias amurensis,* the outermost egg coat is a thick gelatinous layer and composed of three components namely acrosome reaction-inducing substance (ARIS), Co-ARIS and asterosap, cooperatively trigger the acrosome reaction (AR) of spermatozoa (Hoshi *et al.*, 1994). ARIS is a sulphated glycoprotein with an extremely large molecular size and is one of the major factors involved in triggering the AR (Gunaratne *et al.*, 2003), Co-ARIS is a family of sulphated steroidal saponins (Nishiyama *et al.*, 1987) and asterosap is a group of equally active isoforms of sperm-activating peptides (Nishigaki *et al.*, 1996). Twelve sperm-activating peptides were purified and structurally identified from the starfish *Asterias amurensis,* and they were collectively named asterosaps (Nishigaki *et al.*, 1996). Asterosap binds to 130 kDa membrane protein that is likely to be a GC (Nishigaki *et al.*, 2000).

Asterosaps that are diffusible peptides evoke a transient increase in the cGMP concentration in spermatozoa, followed by a cGMP-stimulated increase in the intracellular $Ca^{2+}$ concentration. The mechanism of asterosap produces signaling in starfish which is similar to that of resact in sea urchin. The stimulation of spermatozoa with asterosap also evokes changes in the intracellular cGMP, $Ca^{2+}$ concentration, and membrane potential. However, in starfish, the cAMP levels do not change significantly, and the $Ca^{2+}$ response is not evoked by the photolysis of caged cAMP; however the $Ca^{2+}$ elevation is evoked by the photolysis of caged cGMP (Matsumoto *et al.*, 2003). The cGMP induces $Ca^{2+}$ fluctuations, which control the swimming trajectory, and a $Ca^{2+}$ pulse evokes a turn or bends in the trajectory of spermatozoa, followed by a period of straight swimming (Nishigaki *et al.*, 1996). It has been reported that *Asterias* spermatozoa respond to asterosap with tumbling episodes and swimming in narrow circles when the hydrolysis of cyclic nucleotides is prevented by preincubation of spermatozoa with IBMX (Matsumoto *et al.*, 2003).

In this study, we report an asterosap-induced $Ca^{2+}$ signaling. The data supports the hypothesis that asterosap causes a transient intracellular $Ca^{2+}$ elevation probably by a $Na^{+}/Ca^{2+}$ exchanger (NCX) in spermatozoa and controls the movement of spermatozoa.

## MATERIALS AND METHODS

### *Animals and dry spermatozoa*

Starfish, *A. amurensis*, were collected from several locations of Japan and of Tasmania. Spermatozoa were obtained in the form of "dry spermatozoa" by cutting the testes and were stored in a refrigerator until use.

### *Materials*

Pluronic F-127 and Fluo-4 AM were purchased from Sigma (St. Louis MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Verapamil was purchased from Wako Chem. Co. (Osaka, Japan), 2-(2-(4-nitrobenzyloxy)-phenyl)-isothiurea methanosul-phonate (KB-R7943 mesylate, KB) from Tocris (Bristol, UK), and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from Sigma and were dissolved in DMSO. The remaining reagents used were of the highest available quality. The synthetic asterosap isoform, P15 (Nishigaki *et al.*, 1996), was used at a concentration of 1 μM. Artificial seawater (ASW) contained 430 mM NaCl, 9 mM $CaCl_2$, 9 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, and 10 mM EPPS (*N*-2-hydroxyethyl-piperazine-*N*'-3-propane sulphonic acid, pH 8.2). Low $Ca^{2+}$ ASW was also treated as ASW but had 1 mM $CaCl_2$.

### *Intracellular Ca2+ measurement*

Dry spermatozoa were diluted 10-fold in low $Ca^{2+}$ ASW and incubated with 10 μM Fluo-4 AM plus 0.1 mM EDTA (ethylenediamine-*N*, *N*, *N*', *N*'-tetraacetic acid), and 0.5% Pluronic F-127, and then incubated for 2 h at 16°C. Free Fluo-4 was washed with low $Ca^{2+}$ ASW by centrifugation (1000 g for 5 min at 4°C), resuspended in the original volume of low $Ca^{2+}$ ASW and kept on ice in the dark until use. For the measurement of intracellular $Ca^{2+}$, 20 μl loaded spermatozoa were diluted in 1.5 ml of ASW in a round cuvette at 16°C under constant stirring. The fluorescence intensity was recorded on a spectrofluorophotometer (Shimadzu Science, Tokyo, Japan) with an excitation of 494 nm and emission of 516 nm.

### *Motility of spermatozoa*

To examine the chemotactic activity of the spermatozoa, stored semen was diluted about $10^3$-fold into 0.5% (w/v) polyvinylpyrrolidone in ASW and incubated for 1 min at room temperature for the induction of motility. The suspension of spermatozoa was immediately placed on a glass slide coated with BSA. All observations and recordings of movements were performed within 20 min after dilution and pictures of the movements were recorded at room temperature. Movements of the spermatozoa were observed under a phase-contrast microscope (Nikon, Optiphot) with an x20 objective lens (Olympus, BX51). The swimming trajectories were recorded after mixing with asterosap (100 nM) or/and spermatozoa incubated with 1 μM of KB for 3 min then added asterosap. A computer at 50 msec intervals for 1 msec, by using a high-speed charge-coupled device camera (HAS-200R, Ditect, Tokyo) and a video card (HAS-PCI, Ditect) were used to record the movements. The position of each spermatozoon was analyzed by using an image-analyzing program (Dip-motion 2D, Ditect).

## RESULTS

***Asterosap-induced $Ca^{2+}$ elevation in spermatozoa have a distinct pathway***

Extracellular $Ca^{2+}$ is required for the activation of *A. amurensis* spermatozoa by asterosap (Islam *et al.*, 2006). We examined the possibility that asterosap activates a transient $Ca^{2+}$ elevation using a signaling pathway. In order to unequivocally identify the pathway, we employed different voltage-dependent calcium channels (VDCC) antagonists to resolve the intracellular $Ca^{2+}$ elevation induced by the asterosap. Verapamil, specific blocker of the L-type $Ca^{2+}$ channel (Su and Vacquier, 2002; Rodriguez and Darszon, 2003), produced no effect on the $Ca^{2+}$ elevation in spermatozoa against asterosap, even if administered in 5 to 10 times higher doses than the suggested effective reference concentrations (Fig. 1A). Nifedipine and nitrendipine are also specific blockers of the L-type $Ca^{2+}$ channel and showed no significant inhibition on the elevation of intracellular $Ca^{2+}$ (Islam *et al.*, 2006). Furthermore, a nonspecific $Ca^{2+}$ channel antagonist, nickel chloride (Darszon *et al.*, 2001), which inhibits SOC at higher concentrations, also did not produce any effect on the $Ca^{2+}$ elevation in spermatozoa against asterosap (Fig. 1B). Kawase *et al.* (2005) also found that SOC are not involved in the asterosap-induced elevation of $[Ca^{2+}]_i$. The above findings indicate that VDCC and SOC are not involved in intracellular $Ca^{2+}$ induced by asterosap. The CCCP is thought to prevent mitochondrial $Ca^{2+}$ uptake by collapsing the transmitochondrial membrane potential (Babcock *et al.*, 1997). We used CCCP to explore the possible participation of mitochondrial source in the asterosap response. Incubation of spermatozoa for 4 min with 5 μM CCCP did not reduce the asterosap induced increase in intracellular $Ca^{2+}$ (Fig. 1C). Our observations indicate that the intracellular $Ca^{2+}$ changes induced by asterosap occurs in a diverse pathway.

In sea urchin, speract is involved in the intracellular $Ca^{2+}$ changes by a $Na^+/Ca^{2+}$ exchanger (NCX) (Schakmann and Chock, 1986). Functionally, speract binding to its receptor(s) activates guanylyl cyclase (Bentley *et al.*, 1988). Speract signaling then causes $K^+$ efflux through cGMP-dependent $K^+$ channels (Galindo *et al.*, 2000), causing a decrease in membrane potential (*E*m) of spermatozoa. Asterosap activates a $K^+$ channel of plasma membrane of spermatozoa and causes a transient hyperpolarization leads to increase the pH (Nishigaki *et al.*, 2000). In addition, asterosap activates a cGMP pathway and thereby increase the $[Ca^{2+}]_i$ (Matsumoto *et al.*, 2003). Accordingly, asterosap-induced $Ca^{2+}$ elevation in spermatozoa was interesting to investigate. Here we found an influx of $[Ca^{2+}]_i$ induced by asterosap, probably is a result of NCX (Fig. 2).

KB-R7943 mesylate was originally identified as a NCX inhibitor (Iwamoto *et al.*, 1996). Here we also used KB-R7943, to check the participation of NCX. We found that an influx of $Ca^{2+}$ was partially inhibited by the treatment with 1μM when it was pre-incubated for two minutes (Fig. 2). When we tested the effects of higher concentrations of KB-R7943, a significant inhibition of the exchanger occurred in the transient waveform of the $Ca^{2+}$ signals in a dose-dependent manner (Fig. 2).

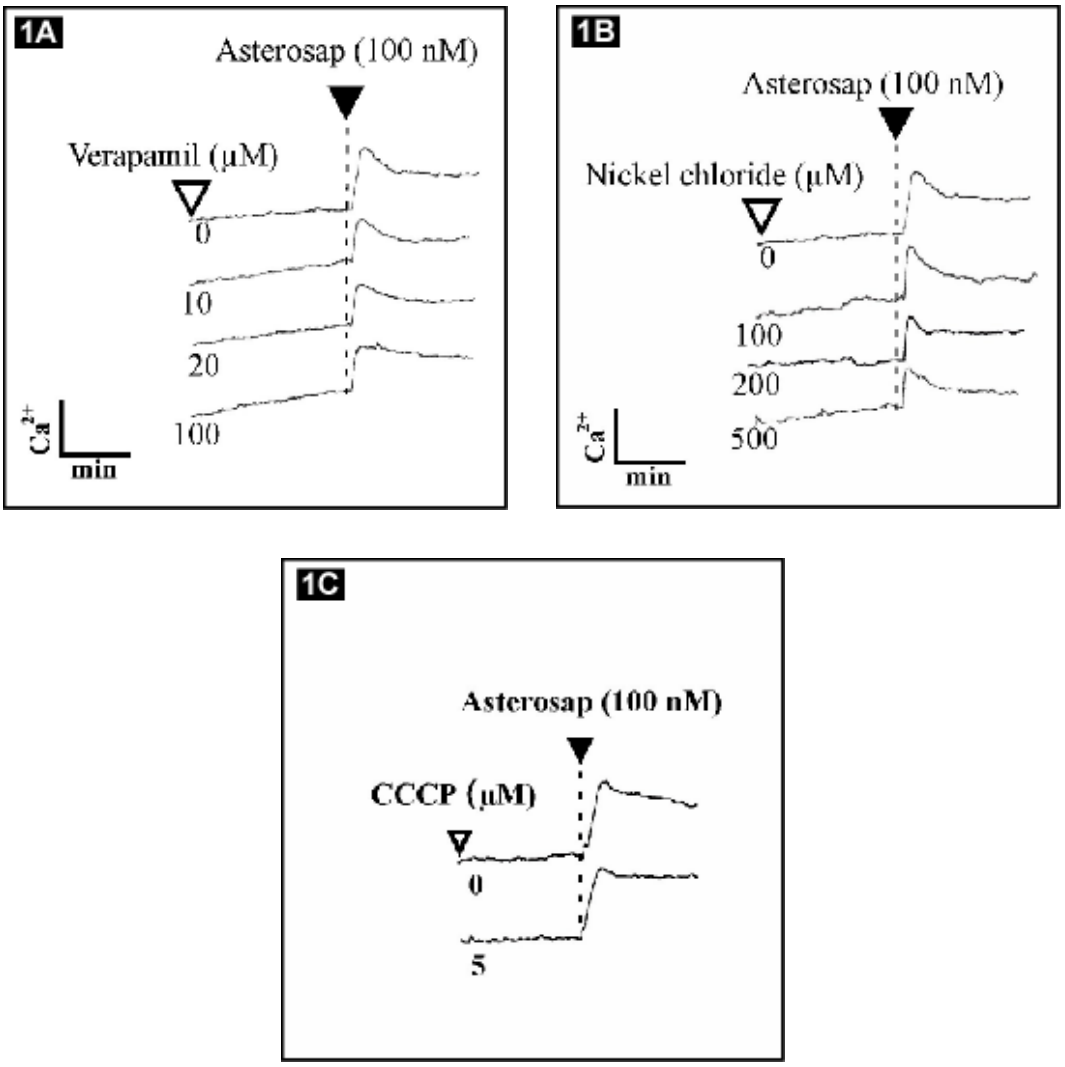


Fig. 1. Effects of $Ca^{2+}$ channel blockers on intracellular $Ca^{2+}$ elevation induced by asterosap. For the $Ca^{2+}$ measurements, Fluo-4 loaded spermatozoa (described in *Materials and methods*) were diluted in cuvette containing 1.5 ml of ASW. At that time shown by the white arrowhead, different concentrations of $Ca^{2+}$ channel blockers (A, verapamil and B, nickel chloride) were added. Following 3 min incubation of loaded spermatozoa in ASW containing different concentrations (final, shown on each trace) of the blockers, asterosap (shown by black arrowhead) was added. CCCP was also incubated with Fluo-4 loaded spermatozoa for 3 min and at the point (black arrowhead) asterosap was added (C). The trace is a representative of at least four experiments.

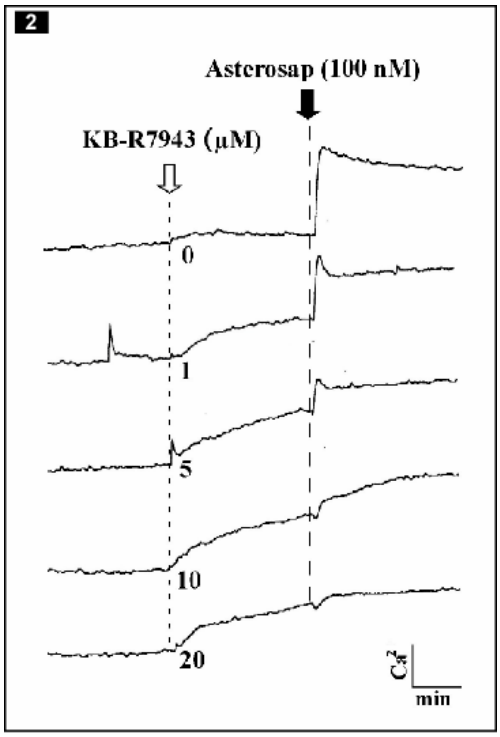


Fig. 2. Inhibition of asterosap-induced $Ca^{2+}$ elevation by KB-R7943. Spermatozoa were loaded with Fluo-4 as mentioned in the *Materials and methods*. Loaded spermatozoa were diluted in cuvette containing 1.5 ml of ASW. Different concentrations of KB-R7943 were added as mentioned in the trace by the white arrow and then incubated for 2 min. At that time 100 nM (final) asterosap was added (indicated by the black arrow). 1μM KB-R7943 inhibited the asterosap-induced $Ca^{2+}$ elevation slightly but when the concentration was increased, inhibition rate was also increased. Data shown in the figure are representative of at least six different batches of spermatozoa.

***Asterosap activate the motility of spermatozoa***

We recorded the trajectories and changes of spermatozoa while they were swimming in the asterosap containing ASW. Shiba *et al*. (2006) reported that starfish (*Aphelasterias japonica*) spermatozoa swam along circular paths with the head oscillating synchronously. Our studies on *A. amurensis* found that spermatozoa in ASW also swam along a circular path but beat frequency increased those in asterosap (Fig. 3A, B; Table 1). KB-R7943 is a potent inhibitor of the flagellar motility and one micromolar KB-R7943 partially blocks the motility in sea urchin spermatozoa (Su and Vacquier, 2002). Here we examined the effect of KB-R7943 on asterosap-induced activation of spermatozoa. The circular trajectory patterns of motility induced by asterosap (Fig. 3B) was partially inhibited by 1 μM of KB-R7943 (Fig. 3D), whereas no significant difference was found in the trajectory

patterns of the spermatozoa between ASW (Fig. 3A) and 1 μM KB (Fig. 3C). An inhibition by KB-R7943 states that flagellar motility is sensitive to the level of intracellular $Ca^{2+}$ and we presume that asterosap-induced $Ca^{2+}$ elevation may plays a role on motility. The results of the swimming trajectories were summarized in Table 1. Movements of the heads were measured from the video recorded files. Significant differences of the spermatozoa trajectories occurred when asterosap administered into 1 μM KB containing suspension.

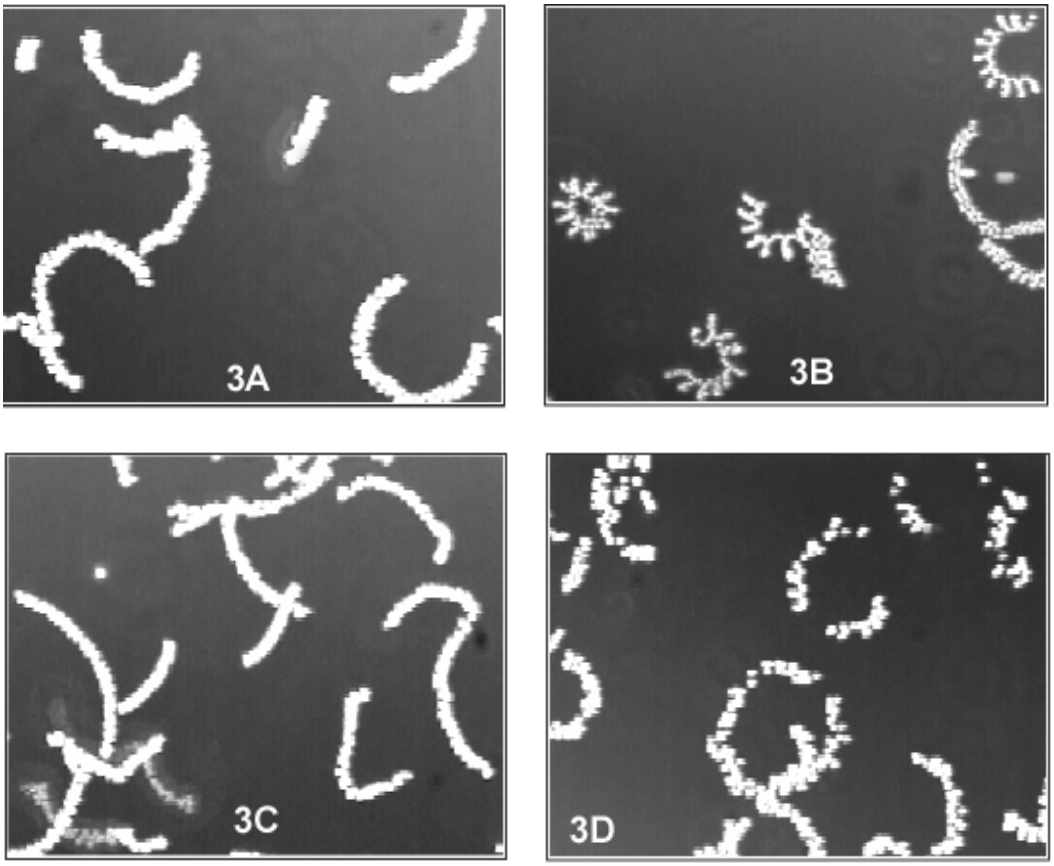


Fig. 3. Asterosap-induced changes in the swimming of *A. amurensis* spermatozoa. ASW containing spermatozoa were incubated for 3 min with or without 1 μM KB-R7943 and then asterosap was added (100 nM). Photographs were taken immediate after mixing with asterosap to the cell suspension. In A-D, the trajectories of spermatozoa were shown when they were suspended in ASW (A), ASW containing 100 nM asterosap (B), ASW containing 1μM KB-R7943 (C), and ASW containing 1μM KB-R7943 plus asterosap (100 nM) (D). ASW containing spermatozoa (A) and ASW plus KB-R7943 containing spermatozoa (C) are showing almost similar trajectories. In B, asterosap induces a circular and vigorous trajectory patterns of motility, and in D, KB-R7943 partially inhibited the trajectories. A summary of the trajectories are shown in Table 1.

Table 1. Effects of KB-R7943 on asterosap-induced trajectories of spermatozoa

| Parameters* | Trajectory (μm/sec) |
|---|---|
| ASW | 403.76 (±7.90) |
| KB-R7943 | 413.39 (±11.35) |
| Asterosap | 489.40 (±10.07) |
| Asterosap + KB-R7943 | 372.91 (±7.56) |

* Conditions were stated in Fig. 3. Trajectories were measured from individual spermatozoon. Data are expressed as mean ± SD (n = 4-10)

## DISCUSSION

The main finding of this study ratifies that asterosap, a sperm-activating peptide of *A. amurensis*, induces to increase intracellular $Ca^{2+}$ of spermatozoa that is mediated by NCX and activates flagellar motility. The NCX mechanism is found in a variety of tissues; however, in most systems, the exchanger operates to pump $Ca^{2+}$ out of the cell. Reverse mode of NCX has been described in mammalian smooth muscles, such as cardiac myocytes, canine and ferret red blood cells, pancreatic B cells, barnacle muscle cells, and squid axons (Rasgado-Flores *et al.*, 1991; Blaustein and Lederer, 1999;). In this study, we demonstrated a NCX of starfish spermatozoa that evokes the transient $Ca^{2+}$ elevation induced by asterosap (Fig. 2). In addition, participation of this exchanger approves no effect of different $Ca^{2+}$ blockers on the asterosap induced activation of spermatozoa. KB-R7943 is a potent inhibitor of NCX and inhibits this exchanger and inhibits the motility of spermatozoa. These data indicate that the NCX might play an important role in the regulation of the intracellular $Ca^{2+}$ level in starfish spermatozoa and activate the motility of spermatozoa.

In our previous findings, we have identified the NCX molecule that is expressed in starfish testes and shares a significant sequence similarity with this protein of other animals (Islam *et al.*, 2006). Therefore, this clone could have an NCX activity, which might play an important role in the asterosap induced-intracellular $Ca^{2+}$ level in starfish.

Sea urchin spermatozoa have an abundance of membrane bound GC that is activated by the egg peptide speract (Garbers, 1989; Darszon *et al.*, 2001). Cyclic GMP in turn regulates a $K^+$ channel that hyperpolarizes the spermatozoa membrane (Darszon *et al.*, 2001) and thereby increases intracellular $Ca^{2+}$. In starfish, we also found a cGMP regulated pathway and assume that in response to asterosap the transient $Ca^{2+}$ elevation occurs in spermatozoa which induces the function of NCX. This exchanger could also be physically associated with the sperm cGMP-regulated channel and GC, all of which are localized in the spermatozoa membrane (Nishigaki *et al.*, 2000; Islam *et al.*, 2006). Sperm-specific ion channels also regulate cation flux in mammalian spermatozoa flagella, although the mechanism of regulation remains unknown at this time. Motility of herring spermatozoa initiate when they bind a factor released from eggs. This initiation depends on a NCX that brings $Ca^{2+}$ into the spermatozoa in exchange for $Na^+$ efflux (Vines *et al.*, 2002).

During chemotaxis, the swimming trajectories of spermatozoa were controlled by $Ca^{2+}$ spikes in the flagellum (Bohmer *et al*., 2005). There have been a couple of proposals for a model of the spermatozoa chemotaxis mechanism based on the response to chemical concentration gradients. It has been verified that the gradient of attractant plays an important role in attracting abalone spermatozoa to an egg (Riffell *et al*., 2002, 2004). Cook *et al*. (1994) found that a diffusional gradient of speract spanning the picomolar to micromolar response range is established over a spherical radius of the order of 1 mm around the egg. In our findings, swimming trajectories of spermatozoa represents upon an approaching to the asterosap. This findings indicate that alteration of the flagellar movement is likely to be induced by asterosap through changes in $[Ca^{2+}]_i$, pHi or $[cAMP]_i$, which are occurred via the cGMP-signaling pathway (Matsumoto *et al*., 2003; Nishigaki *et al*., 2004).

Intracellular $Ca^{2+}$ level changes dynamic in various steps during fertilization and it possibly plays a key role in some phenomena subsequentially. In the present study, we report that the asterosap-induced $Ca^{2+}$ elevation is dependent on the NCX. It is possible that these changes in the intracellular $Ca^{2+}$ level are caused via the $Ca^{2+}$ channels or the $Ca^{2+}$ exchangers in spermatozoa and shows vigorous flagellar movement in the ASW containing asterosap. Thus, the vigorous flagellar movement of spermatozoa allows performing the acrosome reaction as effectively as possible.

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