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Na^+/Ca^{2+} exchanger contributes to asterosap-induced elevation of intracellular Ca^{2+} concentration in starfish spermatozoa

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Summary

Asterosap, a group of equally active isoforms of sperm-activating peptides from the egg jelly of the starfish *Asterias amurensis*, functions as a chemotactic factor for sperm. It transiently increases the intracellular cGMP level of sperm, which in turn induces a transient elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Using a fluorescent Ca^{2+}-sensitive dye, Fluo-4 AM, we measured the changes in sperm $[Ca^{2+}]_i$ in response to asterosap. KB-R7943 (KB), a selective inhibitor of Na^+/Ca^{2+} exchanger (NCX), significantly inhibited the asterosap-induced transient elevation of $[Ca^{2+}]_i$, suggesting that asterosap influences $[Ca^{2+}]_i$ through activation of a K^+-dependent NCX (NCKX). An NCKX activity of starfish sperm also shows K^+ dependency like other NCKXs. Therefore, we cloned an NCKX from the starfish testes and predicted that it codes for a 616 amino acid protein that is a member of the NCKX family. Pharmacological evidence suggests that this exchanger participates in the asterosap-induced Ca^{2+} entry into sperm.

Keywords: Asterosap, Intracellular Ca^{2+}, NCX, NCKX, Starfish

Introduction

Extracellular Ca^{2+} is generally accepted as an important factor inducing changes in sperm essential for fertilization, such as chemotactic behaviour and the acrosome reaction. In the echinoderms, peptides released from the jelly coat of eggs play a pivotal role as a signal for such changes. For example, resact is a chemoattractant for sperm of the sea urchin *Arbacia punctulata* and binds to the receptor guanylyl cyclase on the plasma membrane of sperm flagella (Ward *et al.*, 1985). The stimulation of sperm by resact evokes changes in intracellular concentrations of cGMP, cAMP and Ca^{2+}, and in the membrane potential (for review see Darszon *et al.*, 2005). In the starfish *Asterias amurensis*, asterosap serves as a sperm attractant (Bohmer *et al.*, 2005), and as a factor for triggering the acrosome reaction in concert with two other jelly components: acrosome reaction-inducing substance (ARIS, a proteoglycan-like molecule; Koyota *et al.*, 1997; Gunaratne *et al.*, 2003) and Co-ARIS (a group of sulfated steroidal saponins; Nishiyama *et al.*, 1987) (Hoshi *et al.*, 1994). Asterosap is a group of equally active isoforms of sperm-activating peptide (Nishigaki *et al.*, 1996) that transiently increases the intracellular concentration of cGMP ($[cGMP]_i$), the intracellular pH (pH_i) and the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) via the activation of asterosap receptor, guanylyl cyclase (Nishigaki *et al.*, 2000; Matsumoto *et al.*, 2003). However, the signalling pathway leading to such changes has not been elucidated.

Na^+/Ca^{2+} exchange across the plasma membrane is an important determinant of Ca^{2+} entry (Li *et al.*, 1994). The process is catalysed by the Na^+/Ca^{2+} exchanger complex that electrogenically exchanges Na^+ and Ca^{2+} across the plasma membrane in either the Ca^{2+}-efflux (forward) or Ca^{2+}-influx (reverse) mode, depending on the electrochemical gradients of the substrate ions.

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Detailed studies on the function and structure of the complex have revealed that it consists of two types of proteins: Na^+/Ca^{2+} exchanger (NCX), which catalyses the exchange of three sodium ions for one calcium ion (Blaustein & Lederer, 1999; Fujioka *et al.*, 2000), and K^+-dependent Na^+/Ca^{2+} exchanger (NCKX), which exchanges four sodium ions for one calcium and one potassium ion (Schnetkamp, 1995). Six subfamilies of NCKX (NCKX1–6) have been cloned from different animals. Among them NCKX1 was first cloned from bovine retinal rods (Reilander *et al.*, 1992), and then from those of dolphin (Cooper *et al.*, 1999), chicken (Prinsen *et al.*, 2000), rat (Poon *et al.*, 2000) and human (Tucker *et al.*, 1998). Subsequently, NCKX2 was demonstrated in rat brain (Tsoi *et al.*, 1998) and in retinal cone cells of chicken and human (Prinsen *et al.*, 2000), and NCKX3 (Kraev *et al.*, 2001) and NCKX4 (Li *et al.*, 2002) in rodents and human. More recently NCKX5 (Schnetkamp, 2004) and NCKX6 (Cai & Lytton, 2004) have also been cloned and characterized from human and mouse, respectively. NCKX1–2 play a significant role in regulation of $[Ca^{2+}]_i$ during phototransduction, while NCKX3–6 are expressed in a variety of tissues, indicating their broad roles in the homeostasis of $[Ca^{2+}]_i$. Recently a sea urchin homologue of NCKX (suNCKX) was cloned and found to play a major role in keeping sperm $[Ca^{2+}]_i$ low during swimming (Su & Vacquier, 2002).

In this paper we present the evidence that the asterosap-induced transient elevation of $[Ca^{2+}]_i$ is mediated by sfNCKX, an NCKX named after cloning of the cDNA from starfish testes.

Materials and methods

Animal

Starfish, *Asterias amurensis*, were collected from several locations in Japan and in Tasmania. Those from Tasmania are the offspring of invaders most probably from Tokyo Bay and show no significant differences from the Japanese animals (Byrne *et al.*, 1997). Sperm were obtained in the form of 'dry sperm' by cutting the testes, and kept on ice until use.

Materials

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 sulfonic acid, pH 8.2). Low Ca^{2+} ASW was also treated as ASW but had 1 mM $CaCl_2$. K^+-divalent cation free seawater (KFSW) contained (in mM): 480 NaCl and 10 EPPS, pH adjusted to 8.2 with NaOH. Li^+/K^+ was buffer prepared as (in mM): 480 LiCl, 10 KCl, 1 EGTA and 20 EPPS, pH 8.2. Li^+ buffer was prepared as Li^+/K^+ buffer but omitting KCl. Pluronic F-127 and nifedipine were from Sigma (St Louis, MO), and Fluo-4 AM from Nacalai Tesque (Kyoto, Japan). Nitrendipine from Research Biochemical (Natick, MA), 2-(2-(4-nitrobenzyloxy)-phenyl)-isothiourea methanosulfonate (KB-R7943 mesylate, KB) from Tocris (Bristol, UK) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from Sigma were dissolved in dimethylsulfoxide (DMSO) for the stocks. The remaining reagents used were of the highest available quality. The synthetic asterosap isoform, P15 (Nishigaki *et al.*, 1996), was dissolved in ASW at a concentration of 1 μM before use.

$[Ca^{2+}]_i$ measurement

Dry sperm 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 incubated for 2 h at 16 °C. The sperm were washed once to remove excess Fluo-4 AM (and free Fluo-4 that might exist in the medium) 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. In the K^+-dependency experiments, sperm were loaded into KFSW following the same protocol as described above. For $[Ca^{2+}]_i$ measurements, 20 μl of the loaded sperm 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 RF-540 (Shimadzu Science, Tokyo, Japan) with excitation at 494 nm and emission at 516 nm.

Cloning of the full-length K^+-dependent Na^+/Ca^{2+} exchanger in starfish, sfNCKX

We screened a starfish testes cDNA library with a PCR-amplified cDNA fragment employing a pair of degenerate primers [5′-GTNGCNGGNGCNACNTT(C/T) ATG-3′ and 5′-GCN(C/G)(T/A)NCCNAC(T/G/A) ATNGTNCC-3′]. We designed primers from a conserved region sequence of suNCKX (Su & Vacquier, 2002). The PCR was performed under the following conditions: 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 53 °C for 30 s and 74 °C for 1 min, and 74 °C for 10 min. The resulting fragment was subcloned into pGEM-T Easy vector (Promega) according to the manufacturer's instructions. The inserted DNA fragment was fully sequenced on both strands using an automated DNA sequencer (3100 Genetic Analyzer, Applied Biosystems). To obtain the full-length cDNA we used the 5′/3′-RACE system from Clontech according to the manufacturer's instructions. The

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resulting fragments were combined by subcloning into pGEM-T vector and sequenced as indicated above.

Sequence analysis

The complete nucleotide and deduced amino acid sequences were analysed using commercial software (GENETYX-MAC v.11.2, Software Development, Japan). Homologues of sfNCKX were constructed at Genome Net server (Bio-informatics Center, Kyoto University, Japan; http//www.genome.ad.jp). Glycosylation sites were detected using the Scan Prosite web site http://kr.expasy.org/cgi-bin/scanprosite. The signal sequence was predicted using the web site http://psort.nibb.ac.jp/cgi-bin/runpsort.pl. GenBank accession numbers for NCKX sequences are: sea urchin (suNCKX), AY077699; chicken rod (cNCKX1), AF177984; rat brain (rNCKX2), AF021923; partial human (hNCKX3), AF169257; human (hNCX1), M91368; guinea pig (gNCX1), U04955; bovine (bNCX1), L06438; mouse (mNCX1), U70033; and human (hNCX2), AB029010. The GenBank accession number of the sfNCKX is AB188343.

Southern blot analysis

Genomic DNA from *A. amurensis* testes was isolated, digested with restriction enzymes, separated by 0.7% agarose gel electrophoresis and then transferred to a nitrocellulose membrane (Pall Biodyne B, Pensacola, USA). The membrane was prehybridized for 2 h at 65 °C in 6× SSC, 0.5% SDS and 5× Denhardt's solution, 0.12 mg/ml salmon sperm DNA and 4 mM EDTA (pH 7). The full-length sfNCKX was labeled with [α-^{32}P]dCTP by random priming (Amersham Biosciences). The probe was added to the hybridization mixture and the incubation was continued for 18 h at 65 °C. After terminating hybridization the membranes were washed once (1 h) with 2× SSC, 0.1% SDS and once (1 h) with 0.2× SSC, 0.1% SDS at 65 °C. After washing, the blots were analysed using a BAS 5000 Bio-Image Analyzer (Fuji Photo Film).

Northern blot analysis

The RNA (7.5 µg of total RNA) from the testes and ovaries of *A. amurensis* was denatured and separated by electrophoresis on a 1% agarose gel containing formaldehyde. The separated RNA was transferred onto a nitrocellulose membrane (Pall Biodyne B, Pensacola, USA). The Northern blot hybridization was carried out as follows: hybridization – 4× SSC, 0.12 mg/ml salmon sperm DNA, 0.2% SDS, 5× Denhardt's solution, 50% formaldehyde at 42 °C for 16 h; washing – 2× SSC–0.1% SDS at 50 °C for 10 min, 0.2× SSC–0.1% SDS at 50 °C for 1 h, repeated once. The 3′ region of the cDNAs was labelled with [α-^{32}P]dCTP (3000 Ci/mmol) using a random prime labelling system (Amersham Biosciences) for hybridizing probes. After washing, the blots were analysed using a BAS 5000 Bio-Image Analyzer (Fuji Photo Film).

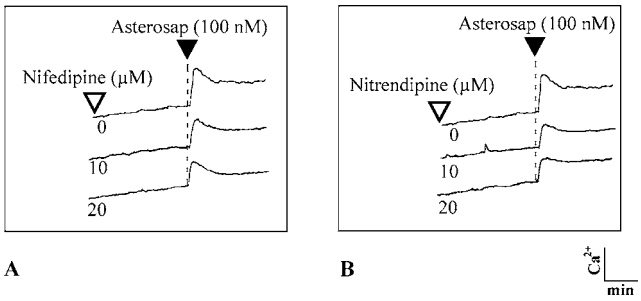

Figure 1 Effect of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ elevation induced by asterosap. Fluo-4 loaded sperm were diluted in a cuvette containing 1.5 ml of ASW. At the time shown by the white arrowhead, different concentrations of Ca^{2+} channel blockers (*A*, nifedipine; *B*, nitrendipine) were added. Following a 3 min incubation of loaded sperm in ASW containing different concentrations of the blockers, asterosap (shown by the black arrowhead) was added. Experiments were repeated three times.

Results

Ca^{2+} channel inhibitors do not have significant effects on asterosap-induced changes in $[Ca^{2+}]_i$

The presence of extracellular Ca^{2+} is required for the activation of *A. amurensis* sperm by asterosap. It is known that store-operated Ca^{2+} channels (SOC) are not involved in the asterosap-induced elevation of $[Ca^{2+}]_i$ (Kawase *et al.*, 2005). We thus examined whether antagonists against voltage-dependent Ca^{2+} channels (VDCC) affected the asterosap-induced elevation of $[Ca^{2+}]_i$. Nifedipine and nitrendipine, potent and specific blockers of the L-type Ca^{2+} channel (Su & Vacquier, 2002; Rodriguez & Darszon, 2003), affected the asterosap-induced elevation of $[Ca^{2+}]_i$ only slightly, if at all, when the sperm had been incubated with these drugs for 3 min prior to the asterosap treatments (Fig. 1). The results indicate that the VDCC does not play an important role in the asterosap-induced elevation of $[Ca^{2+}]_i$. It is also reported that CCCP prevents mitochondrial Ca^{2+} uptake by collapsing the transmitochondrial membrane potential (Babcock *et al.*, 1997). However, pretreatments of sperm with 5 µM CCCP for 3 min did not reduce the asterosap-induced $[Ca^{2+}]_i$ increase (data not shown). Our observations suggest the presence of a distinct signalling pathway for the asterosap-induced $[Ca^{2+}]_i$ changes.

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Asterosap induces $[Ca^{2+}]_i$ elevation through an NCKX

An NCX is involved in the $[Ca^{2+}]_i$ changes induced by speract in sea urchin sperm (Schackmann & Chock, 1986). Speract signalling causes K^+ efflux through cGMP-dependent K^+ channels, resulting in a decrease in sperm membrane potential (E_m), namely hyperpolarization. The hyperpolarization activates Na^+/H^+ exchange, adenylyl cyclase and a hyperpolarization-activated and cyclic nucleotide-gated K^+ channel. These changes lead to increases in pH_i, $[cAMP]_i$ and Na^+ influx. Furthermore, hyperpolarization enhances Na^+/Ca^{2+} exchange to keep $[Ca^{2+}]_i$ low. Also, the increase in $[Na^+]_i$ and depolarization of the membrane potential lead to reversal of the NCX (Darszon *et al.*, 2005).

In *A. amurensis*, asterosap activates a K^+ channel of sperm plasma membrane and causes a transient hyperpolarization, which then activates Na^+/H^+ exchange and eventually leads to an increase in pH_i (Nishigaki *et al.*, 2000). Recently Matsumoto *et al.* (2003) reported that asterosap activates a cGMP pathway and thereby increases the $[Ca^{2+}]_i$. Thus it is of much interest to ask which exchanger is involved in the asterosap-induced increase in $[Ca^{2+}]_i$. Pretreatments of sperm with KB-R7943 (KB), a potent inhibitor of the reverse mode of the NCX (Iwamoto *et al.*, 1996; Watano *et al.*, 1996), inhibited the asterosap-induced transient increase in $[Ca^{2+}]_i$ only partially at 1 μM but significantly at 5 μM (Fig. 2). The results suggest that the asterosap-induced transient influx of Ca^{2+} is caused by the reverse mode of an NCKX.

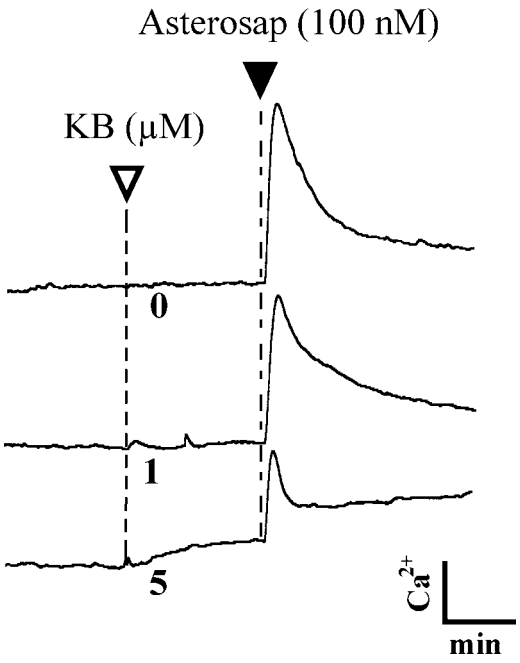

Figure 2 KB inhibits the asterosap-induced Ca^{2+} elevation by the NCKX. Fluo-4 loaded sperm were diluted in a cuvette containing 1.5 ml of ASW. At the time indicated by the white arrowhead, a different concentration of KB was added. Following a 2 min incubation, asterosap (indicated by the black arrowhead) was added. KB blocks the asterosap-induced Ca^{2+} elevation in a dose-dependent manner. Data shown in the figure are representative of at least six different batches of sperm.

sfNCKX activity is dependent on K^+

We used Fluo-4 for fluorescent calcium imaging to examine the transport function of sfNCKX. When we studied K^+-dependent Ca^{2+} influx, the medium was changed from one containing 480 mM sodium to one containing 480 mM lithium, solution in the presence or absence of potassium (Tsoi *et al.*, 1998; Cooper *et al.*, 1999; Prinsen *et al.*, 2000; Poon *et al.*, 2000; Kraev *et al.*, 2001). This manoeuvre will reverse the sodium gradient and remove sodium competition at the outwardly facing calcium binding sites, and it should therefore favour calcium entry, employing the reverse mode of the exchanger. If sfNCKX function requires the co-transport of potassium with calcium, however, there should be no chance of an increase in $[Ca^{2+}]_i$ until potassium is present in the medium. To examine the $[Ca^{2+}]_i$ changes, loaded sperm in KFSW were suspended in Li^+ or Li^+/K^+ buffer. Addition of 10 mM $CaCl_2$ to Li^+ or Li^+/K^+ buffer resulted in a greater Ca^{2+} increase in the presence of K^+. We investigated the idea that sfNCKX is K^+-dependent and that Ca^{2+} can be induced through the reverse mode of NCX (Fig. 3*A*).

KB is a potent inhibitor of NCX. Fig. 3*A* shows that we found sfNCKX causes an increase in $[Ca^{2+}]_i$ in the presence of K^+. Accordingly, we used KB to monitor the dependence of sfNCKX on K^+. A final concentration of 10 μM KB was required to inhibit the K^+-dependent Ca^{2+} influx (Fig. 3*B*). The above findings ratify the sfNCKX activity in starfish sperm.

Cloning of sfNCKX

We isolated an NCKX clone from the testes cDNA library of *A. amurensis*. The full-length cDNA of 3169 bp showed a 1848 bp open reading frame encoding a 616 amino acid protein with a calculated molecular mass of 67.7 kDa. It displays a significant sequence similarity to the known NCKXs (Tsoi *et al.*, 1998; Prinsen *et al.*, 2000; Kraev *et al.*, 2001; Su & Vacquier, 2002), and was thus designated sfNCKX (Fig. 4). It contains 12 transmembrane domains (TM1–12), and a potential cleavage site for signal peptidase is present on TM1, at the position analogous to the known sites of NCKXs (Kraev *et al.*, 2001; Li *et al.*, 2002; Schnetkamp, 2004; Cai & Lytton, 2004). It shares a few more features

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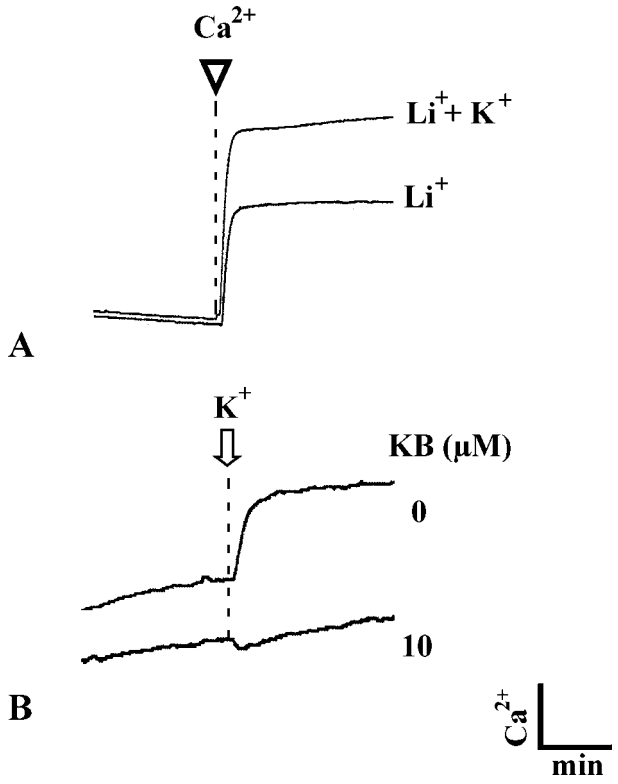

Figure 3 Dependence of sfNCKX activity on K^+. (*A*) K^+-dependent Ca^{2+} influx of sfNCKX. Loaded sperm in KFSW were added to Li^+/K^+ or Li^+ buffer and left to equilibrate for 2 min. An addition of 10 mM Ca^{2+} (white arrowhead) indicates the K^+ dependence of sfNCKX. Note that if this gene is present in starfish sperm, Ca^{2+} will move into the cells by reversing the Na^+ movement in the presence of K^+. (*B*) KB blocks K^+-dependent Ca^{2+} influx. Loaded sperm were diluted in Li^+ buffer and 10 mM $CaCl_2$ added to it. Following a 2 min incubation in buffer containing different concentrations of KB (in μM), KCl (10 mM) was added (white arrow). Experiments were repeated three times.

with them – a cytoplasmic amino-terminus, conserved N-linked glycosylation sites, a long cytoplasmic loop between TM6 and TM7, and a short cytoplasmic carboxyl-terminus (Tsoi *et al.*, 1998; Prinsen *et al.*, 2000; Kraev *et al.*, 2001; Su & Vacquier 2002) – suggesting further similarities to other NCKXs. There are 10 protein kinase C (PKC) phosphorylation sites: two in the cytoplasmic amino-terminus, one in the inside loop between TM2 and TM3, and the rest in the cytoplasmic loop between TM6 and TM7. The highest level of sequence identity among different NCKXs occurs between TM3 and TM4 and between TM8 and TM10 (Fig. 4). A BLAST search has revealed that 46% of the amino acid sequence is identical to, and 59% of the sequence is similar to, that of the suNCKX.

Although suNCKX contains an unusual His-rich region in its intracellular loop, sfNCKX does not contain such a His-rich region. The relationships between NCKX and NCX sequences of different animals are shown in Fig. 5. The analysis reveals that sfNCKX groups with NCKX and is separated from NCX.

To confirm the copy number of sfNCKX in the *A. amurensis* genome, we carried out Southern blot analysis (Fig. 6*A*). This sfNCKX cDNA sequence contains two *Bgl*II sites and there are no *Eco*RI, *Sal*I or *Xho*I sites. The samples digested with *Eco*RI (lane 1), *Sal*I (lane 2) and *Xho*I (lane 3) show one band of hybridization, and that with *Bgl*II (lane 4) shows two bands. Simple patterns of hybridizing fragments suggest that sfNCKX is a unique and single-copy gene in the *A. amurensis* genome.

The transcripts of the tissue distribution of sfNCKX are shown in Fig. 6*B*. Approximately 2.6 kb was found to be abundantly expressed in the testes but not in the ovaries, which corresponds to the size of the cloned cDNA.

Discussion

The main finding of this study is that an NCKX, most probably by the reverse mode of sfNCKX, plays a pivotal role in the asterosap-induced transient increase in $[Ca^{2+}]_i$ in *A. amurensis* sperm. An NCKX mechanism is found in a number of species and in a variety of tissues. This exchanger can run in either direction; however, the exchanger usually operates to pump Ca^{2+} out of the cell (forward mode) in most systems. In previous studies on the K^+ dependence of NCKX expressed in cultured cells (Tsoi *et al.*, 1998; Kraev *et al.*, 2001), the dependence of the reverse mode NCX on external K^+ was observed. In Fig. 3, we investigated whether Ca^{2+} influx can be induced through the reverse mode of NCX in starfish sperm and its dependence on external K^+. However, we summarize our results sequentially here. First, using nifedipine and nitrendipine, our data suggested that VDCC does not play an important role in the asterosap-induced transient elevation of $[Ca^{2+}]_i$. In addition, SOC-like channels are not involved (Kawase *et al.*, 2005). Secondly, by using KB, we showed that an NCKX is involved in this process. Thirdly, we cloned, named and characterized an NCKX gene, sfNCKX.

The NCX electrogenically exchanges three Na^+ for one Ca^{2+} and can function to cause Ca^{2+} accumulation (reverse mode) or Ca^{2+} extrusion (forward mode) depending on the concentrations of each ion on either side of the membrane and on the membrane potential (Blaustein & Lederer, 1999). Speract signalling causes an increase in $[Na^+]_i$; it is possible that this process, along with membrane depolarization, favours the influx of Ca^{2+} via the NCX during the stimulus (Darszon *et al.*, 2005). It has been hypothesized that NCKX contributes to asterosap-induced Ca^{2+}

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Figure 4 Alignment of NCKX proteins obtained from starfish, sea urchin, chicken, rat and human. The shaded regions indicate identical sequences, or at least three similar residues in all sequences. Locations of the putative transmembrane segments (TM1–TM12) are over-lined. An asterisk indicates potential sites of protein kinase C phosphorylation. Other potential sites for signal peptidase cleavage (SPC ↓) and four *N*-linked glycosylation (•) are also shown. Dashes indicate gaps introduced to maximize the alignment.

accumulation, but it has been difficult to test this hypothesis rigorously given the lack of specific inhibitors. The use of KB, a selective inhibitor of reverse NCX, allowed us to evaluate the role of this exchanger in the $[Ca^{2+}]_i$ changes caused by asterosap. KB exerts a preferential effect on reverse-mode NCX activity

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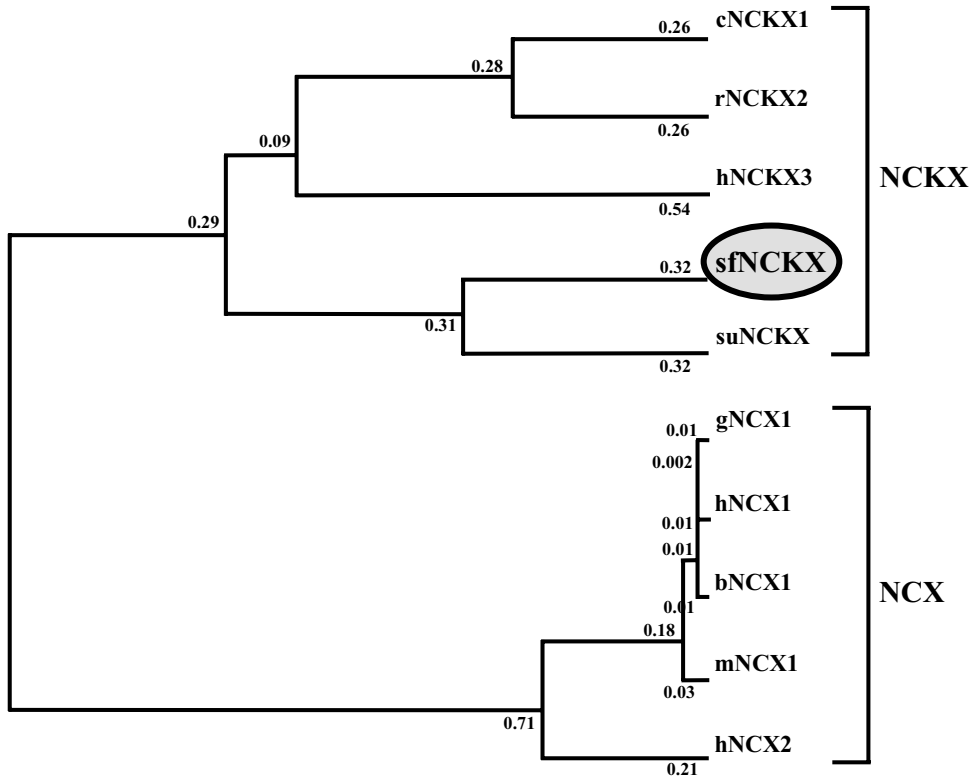

Figure 5 Phylogeny of sfNCKX. A rootless phylogenetic tree was constructed by the neighbour-joining method. Bootstrap percentage values (1000 replicates) are shown over the corresponding nodes. All branch lengths are proportional to the distances between sequences. Protein IDs in GenBank are given in Materials and Methods. b, c, g, h, m, r, sf and su denote bovine, chicken, guinea pig, human, mouse, rat, starfish and sea urchin, respectively.

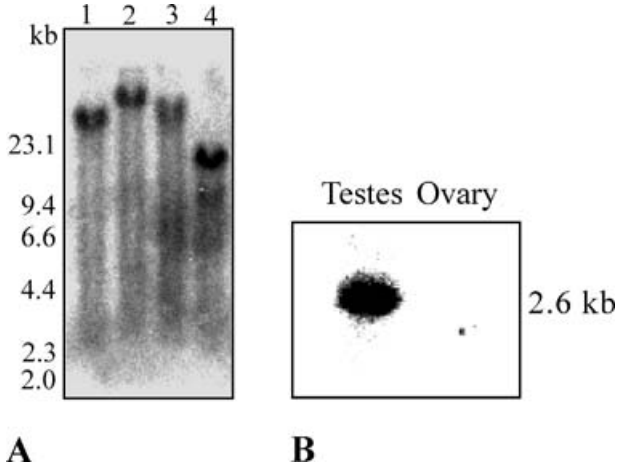

Figure 6 (*A*) Southern blot analysis of the sfNCKX gene. Samples of the *A. amurensis* DNA (10 μg/lane) were digested with *Eco*RI (lane 1), *Sal*I (lane 2), *Xho*I (lane 3) or *Bgl*II (lane 4). Size markers in kilobases are indicated on left. (*B*) Tissue distribution of sfNCKX transcripts. RNA (7.5 μg of the total RNA) was isolated from testes and ovaries, and analysed by Northern blotting at higher stringency from starfish NCKX.

(Iwamoto *et al.*, 1996; Watano *et al.*, 1996). It inhibits $[Ca^{2+}]_i$ accumulation into rat cardiomyocytes and some other NCX1-expressing cells with an IC_{50} of 1.2–2.4 μmol/l (Iwamoto *et al.*, 1996). We found a significant inhibition of the transient increase in $[Ca^{2+}]_i$ in a concentration-dependent manner when sperm were pretreated with KB for 2 min (Fig. 2). KB at 5 μM significantly inhibited the $[Ca^{2+}]_i$ increase. Therefore, we assume that KB can also inhibit an NCKX complex as we found in starfish sperm. Su & Vacquier (2002) reported that higher concentrations of KB (>10 μM) alter the resting sperm $[Ca^{2+}]_i$. KB at <5 μmol/l has been used as a fairly selective blocker for reverse-mode NCX activity in isolated cardiomyocytes of guinea pig (Watano *et al.*, 1999). In other cell types, such as bovine adrenal chromaffin cells (Pintado *et al.*, 2000), KB has recently been reported to inhibit neuronal nicotinic acetylcholine receptors (IC_{50} 0.3–6.5 μmol/l).

Although sperm-specific ion channels also regulate cation flux in the mammalian sperm flagellum, the regulation mechanism remains unknown. The sperm activating peptide-activated signalling pathways are known in sea urchin and starfish with some points of similarity. For example, resact, speract and asterosap activate the cGMP-signalling pathway and thereby increase the $[Ca^{2+}]_i$. In the sea urchin *Strongylocentrotus purpuratus*, speract also transiently increases $[cGMP]_i$ causing a transitory hyperpolarization, which is mediated by cGMP-modulated K^+-selective channels

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that activate the Na^+/H^+ exchanger and then stimulate other ion transporters (Darszon *et al.*, 2005). Our study indicates that the transient $[Ca^{2+}]_i$ elevation in sperm occurs in response to asterosap, and is suggested to be caused by the function of NCKX (Fig. 2).

We have identified the sfNCKX molecule expressed in starfish testes, which shares a significant sequence similarity with other NCKX proteins. There are 12 TM segments, with an extracellular loop between TM1 and TM2, with conserved *N*-linked glycosylation sites, and a large cytoplasmic loop between TM6 and TM7, which is typical of other NCKX proteins (Fig. 4). This is notable because these regions are believed to play a central role in the formation of an ion-binding domain (Schwarz & Benzer, 1997; Su & Vacquier, 2002). The long central loop, which is presumed to be cytoplasmic based on a comparison between NCKX1 and NCX1, contains consensus sequences for several protein kinases. As the amino acid sequence of suNCKX contains a His-rich region in the intracellular loop, it has been thought that this region plays an important role in maintaining the pH_i in sperm (Su & Vacquier, 2002). However, as the sfNCKX sequence does not have such a His-rich region, it is not certain whether this region is essential for sperm activation.

Taking the data presented in this paper together with our previous findings (Nishigaki *et al.*, 1996, 2000; Matsumoto *et al.*, 2003; Kawase *et al.*, 2005; Bohmer *et al.*, 2005), we conclude that the main signalling pathway for asterosap to trigger chemotactic behaviour and/or the acrosome reaction in sperm is initiated by asterosap binding to, and activation of, the asterosap receptor (guanylyl cyclase), which is followed by a transient increase in $[cGMP]_i$, activation of an NCKX, most probably the reverse mode of sfNCKX, and a transient increase in $[Ca^{2+}]_i$, in that order. Further details of the signalling pathway are currently being investigated.

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