Acrosome reaction-inducing substance triggers two different pathways of sperm intracellular signaling in newt fertilization

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ABSTRACT The acrosome reaction is induced in the sperm of *Cynops pyrrhogaster* immediately in response to a ligand protein called acrosome reaction-inducing substance (ARIS) in the egg jelly at fertilization, whereas a spontaneous acrosome reaction occurs time-dependently in correlation with the decline of sperm quality for fertilization. The ARIS-induced acrosome reaction was recently found to be mediated by TRPV4 in association with the NMDA type glutamate receptor, although the intracellular mediators for the acrosome reaction are largely unclear. In the present study, spontaneous acrosome reaction was significantly inhibited by Ni^{2+}, RN1734, and diltiazem, which blocks Cav3.2, TRPV4 or TRPM8, and the cyclic nucleotide-gated channel, respectively. In contrast, expression of Ca^{2+}-activated transmembrane and soluble adenylyl cyclases was detected in the sperm of *C. pyrrhogaster* by reverse transcription-polymerase chain reaction. Activator of transmembrane or soluble adenylyl cyclases (forskolin or HCO_3^-) independently promoted spontaneous acrosome reaction, while an inhibitor of each enzyme (MD12330A or KH7) inhibited it only in the sperm with high potential for spontaneous acrosome reaction. An inhibitor of protein kinase A (H89) inhibited spontaneous acrosome reaction in a manner independent of sperm potential for spontaneous acrosome reaction. Surprisingly, KH7 significantly inhibited ARIS-induced acrosome reaction, but its effect was seen in a small percentage of sperm. H89 had no effect on ARIS-induced acrosome reaction. These results suggest that *C. pyrrhogaster* sperm possess multiple intracellular pathways for acrosome reaction, involving Ca^{2+} permeable channels, adenylyl cyclases and PKA, and that two pathways having distinct dependencies on adenylyl cyclases may contribute to ARIS-induced acrosome reaction at fertilization.

KEY WORDS: *acrosome reaction, Ca²⁺ channel, adenylyl cyclase, PKA, sperm, urodele*

Introduction

Sperm acrosome reaction (AR) is a critical event for the success of fertilization in many animal species. It is mediated by intracellular Ca^{2+} and cyclic adenosine monophosphate (cAMP) (Buffone *et al.*, 2014; Vacquier *et al.*, 2014), both of which are increased in response to a variety of extracellular triggers depending on the species (SeGall and Lennarz, 1979; Ikadai and Hoshi, 1981; Cherr and Clark, 1985; Thomas and Meizel, 1989; Ueda *et al.*, 2002, 2003; Sasanami *et al.*, 2003, 2007; Okumura *et al.*, 2004). The wide variation of the triggers suggests that the signaling pathway for the AR has been modified multiple times during animal evolution.

The acrosome reaction of amphibian sperm is induced by a glycoprotein called acrosome reaction-inducing substance (ARIS) on the surface of egg jelly (Picheral, 1977; Campanella *et al.*, 1997; Sasaki *et al.*, 2002) or in the vitelline envelope (Ueda *et al.*, 2002;

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Barisone *et al.*, 2002). In the Japanese red-bellied newt, *Cynops pyrrhogaster*, ARIS is co-localized with a sperm motility-initiating substance on the egg jelly surface to construct a unique mechanism of acrosome reaction-associated initiation of sperm motility (Watanabe *et al.*, 2009; Watanabe *et al.*, 2010; Takayama-Watanabe *et al.*, 2014; Yokoe *et al.*, 2016). For the success of internal fertilization of the newt, it is critical for sperm to undergo AR on the egg jelly surface, since ectopic induction of AR severely decreases the fertilization efficiency (Takahashi *et al.*, 2006). On the other hand, sperm of this newt have the potential to undergo a spontaneous acrosome reaction (Kon *et al.*, 2017). This potential is enhanced during storage in the vas deferens and time-dependently manifested in the presence of Ca^{2+} and pH at levels similar to those in egg jelly. Although spontaneous AR is a risk for declining fertilizability of the newt sperm, it is known to occur in mammalian capacitated sperm (Visconti *et al.*, 1999), suggesting that spontaneous AR may be a fundamental feature of sperm in internally fertilizing vertebrates. In mice, sperm possess an another signaling pathway for the induction of AR that is triggered by a glycoprotein of the zona pellucida (Bleil and Wassarman, 1980). Although which pathway works at fertilization is controversial (Jin *et al.*, 2011), spontaneous AR may work as a substitute of species-specific signaling pathway for the induction of AR in evolution. However, the relationship between the signaling pathways for ligand-induced AR and spontaneous AR has not been evaluated in correlation with the evolution of the regulatory mechanism underlying the induction of AR.

Sperm of *C. pyrrhogaster* possess multiple types of Ca^{2+} permeable channels including T-type and L-type voltage-dependent Ca^{2+} channels (VDCC; Cav3.2, 1.1, and 1.2), transient receptor potential vanilloid 4 (TRPV4), TRP melastatin 8 (TRPM8) and N-methyl D-aspartate-type glutamate receptor (NMDAR) (Watanabe and Takayama-Watanabe, 2014; Endo *et al.*, 2019). TRPV4 mediates the induction of AR by ARIS in association to NMDAR (Endo *et al.*, 2019), although it is largely unknown which channel participates in
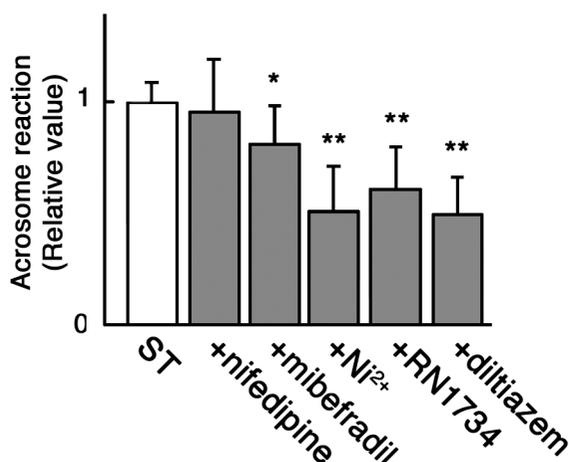

Fig. 1. Effects of blockers of Ca^{2+} permeable channels on spontaneous AR. *Sperm were incubated in Steinberg's salt solution (ST) containing a blocker of Ca^{2+} permeable channel for 1 h and fixed by glutaraldehyde. The acrosome reaction was evaluated by the absence of acrosome in the tip of the sperm head using a dark field microscope. Percentages of the acrosome reacted sperm were expressed by relative values against a mean percentage of them in ST. Asterisks indicate significant differences against ST (** P<0.01).*

TABLE 1

CNGB3 CDNA DETECTED BY THE RNASEQ FROM SPERMATOGENIC TESTES OF *C. PYRRHOGASTER*

genes	accession	species	bit score	E-value	identical (%)
comp24190	gi|345326098	*Ornithorhynchus anatinus*	137.5	3.75E-33	45.7
	gi|397501025	*Pan paniscus*	131	2.09E-30	44.8
	gi|114620818	*Pan troglodytes*	129	7.87E-30	42.5

spontaneous AR. Whereas, cyclic nucleotide-gated (CNG) channel is sometimes critical for AR in other species (Darszon *et al.*, 2008). Although its involvement in the induction of AR is not examined in the newt sperm, an analogue of cAMP can induce the AR (Kon *et al.*, 2017). In the present study, to consider the possible evolutional correlation between intracellular signaling pathways of spontaneous and ligand-induced ARs, we characterized Ca^{2+} permeable channels, and downstream mediators with focusing to adenylyl cyclases and cyclic AMP-dependent protein kinase (PKA), in the spontaneous AR and ARIS-induced AR of *C. pyrrhogaster* sperm.

Results

Ca²⁺ permeable channels in spontaneous acrosome reaction

In order to examine the participation of Ca^{2+} permeable channels of *C. pyrrhogaster* sperm in spontaneous AR, sperm were treated with a channel blocker for 1h in Steinberg's salt solution (ST) that was modified to have a Ca^{2+} concentration and pH equivalent to those in egg jelly (Ukita *et al.*, 1999). Spontaneous AR that occurred in 24±0.82% of the sperm in ST was significantly suppressed by a blocker of T type VDCC (mibefradil), a selective blocker of Cav3.2 (Ni^{2+}), or by a blocker of TRPV4 and TRPM8 (RN1734) (Endo *et al.*, 2019) (Fig. 1). A blocker of L-type VDCC (nifedipine) showed no effect. Spontaneous AR is recently shown to be inhibited by a blocker of NMDAR (MK801) (Endo *et al.*, 2019). These results suggest that spontaneous AR occurs through the gating of T-type VDCC, TRPV4 (or TRPM8), and NMDAR. T type VDCC possibly present in the sperm of *C. pyrrhogaster* is Cav3.2 (Watanabe and Takayama-Watanabe, 2014). In addition, we examined the involvement of CNG channel in the spontaneous AR. RNAseq from spermatogenic testes included a contig sequence with high homology to the CNGB3 subunit of CNG channel (Table 1). The *CNGB3* gene was abundantly expressed in the spermatogenic testes compared to the nonspermatogenic testes (Supplementary Fig. S1), suggesting the possible presence of the CNG channel in sperm. Spontaneous AR was inhibited by a blocker of the CNG channel (diltiazem) (Fig. 1). This result suggests that the CNG channel also mediates the spontaneous AR.

Adenylyl cyclases in spontaneous acrosome reaction

RNAseq of the spermatogenic testes included contigs with high homology to transmembrane adenylyl cyclases, AC-3, -6, -9 (Table 2), and soluble AC, AC-10. RT-PCR analysis showed the abundant expression of *AC-3* gene in spermatogenic testes and the equivalent expression of the gene of the other three adenylyl cyclases between spermatogenic and nonspermatogenic testes (Fig. 2). Acrosome reacted sperm that were 25±6.3% of total sperm in ST were increased by forskolin and HCO_3^- that are activators of the transmembrane and soluble adenylyl cyclases, respectively (Chen *et al.*, 2000; Wertheimer *et al.*, 2013) (Fig. 3A). In addition to the acrosome reacted sperm, sperm having a granular vesicle at the tip of sperm head were often observed, which suggests that spontane-
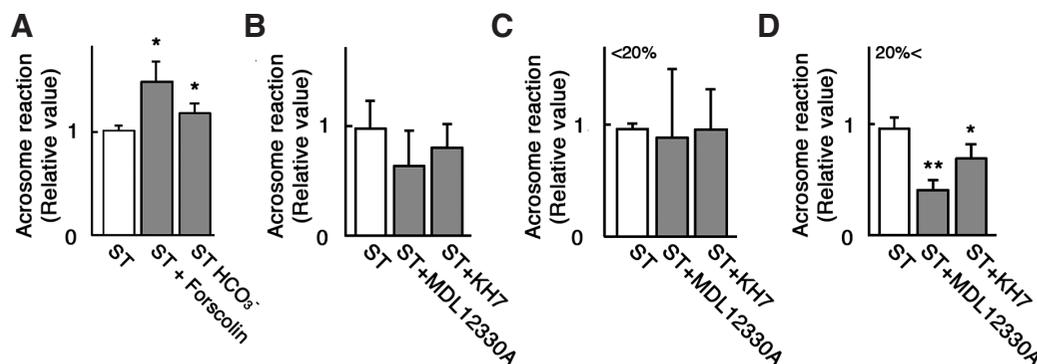
TABLE 2

ADENYLYL CYCLASE CDNAS DETECTED BY THE RNASEQ FROM SPERMATOGENIC TESTES OF *C. PYRRHOGASTER*

genes	accession	species	bit score	E-value	identical (%)
AC3					
comp52271	gil156717224	*Xenopus (Silurana) tropicalis*	1936	0	96.2
	gil348534829	*Oreochromis niloticus*	1810	0	91.7
	gil363732440	*Gallus gallus*	1806	0	92.1
AC6					
comp54794	gil2734867	*Takifugu rubripes*	815.5	0	87.4
	gil444515388	*Tupaia chinensis*	799.7	0	86.8
	gil351697635	*Heterocephalus glaber*	798.9	0	86.6
AC9					
comp57215	gil449278870	*Columba livia*	2024	0	85.0
	gil432111555	*Myotis davidii*	1945	0	83.4
	gil440901440	*Bos grunniens mutus*	1940	0	83.6
AC10					
comp55328	gil169261973	*Squalus acanthias*	185.3	4.19E-51	80.9
	gil465976920	*Chelonia myda*	177.9	5.25E-48	74.8
	gil444435659	*Bos taurus*	161.8	2.63E-44	71.9

ous AR was not completed in those sperm. The effect of HCO_3^- on sperm soluble adenylyl cyclase was further confirmed by the suppression of spontaneous AR by cotreating with HCO_3^- and KH7, an inhibitor of soluble adenylyl cyclase (Supplementary Fig. S2). These results suggest that cAMP produced by those transmembrane and soluble adenylyl cyclases can mediate spontaneous AR. However, spontaneous AR was not significantly suppressed by MDL12330A, an inhibitor of transmembrane adenylyl cyclases, and KH7 (Fig. 3B). Potential of spontaneous AR is different among sperm population depending on the duration of sperm storage in the vas deferens (Kon *et al.*, 2017). Thus, we independently analyzed the data of the sperm population that exhibited spontaneous AR at more than 20% (30±2.9%) in ST for 1h and those at less than 20% (19±0.91%). Both MDL12330A and KH7 significantly suppressed spontaneous AR in the sperm population with high potential (>20% in 1 h incubation), while they did not suppress the spontaneous AR in the population with low potential (<20% in 1 h incubation).

Protein Kinase A in spontaneous acrosome reaction

Involvement of PKA was examined using a specific PKA inhibitor (H89). When sperm were incubated in ST containing H89 for 1 h, spontaneous AR was significantly inhibited regardless of the sperm's potential for spontaneous AR (Fig. 4).
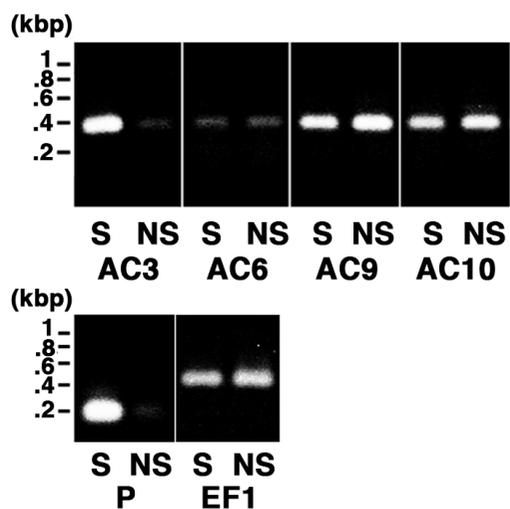

Fig. 2. Expression of mRNA for adenylyl cyclases in the spermatogenic and non-spermatogenic testis. *One-μg of total RNA purified from spermatogenic* **(S)** *and non-spermatogenic* **(NS)** *testes was reverse-transcribed, and polymerase chain reaction was performed using DNA primers specific for AC-3, -6, -9, and -10. Expressions of* protamine **(P)** *and* elongation factor1 **(EF1)** *mRNA were examined as controls.*

CNG channel, adenylyl cyclases, and PKA in ARIS-induced acrosome reaction

Acrosome reaction-inducing substance induces AR immediately in sperm being suspended in egg jelly extract (JE) including ARIS (Hiyoshi *et al.*, 2007). The ARIS-induced AR is mediated by TRPV4 and NMDAR (Endo *et al.*, 2019) but not by VDCCs (Takayama-Watanabe *et al.*, 2015). To examine the involvement of CNG channel in ARIS-induced AR, sperm were incubated in JE containing diltiazem. This blocker did not inhibit the induction of AR in JE (Fig. 5A). Next, the involvement of transmembrane and soluble adenylyl cyclases was examined in ARIS-induced AR. The acrosome reaction was significantly inhibited by KH7 in JE, although many sperm underwent AR (Fig. 5B). MDL 12330A also tended to decrease the induction of AR in JE although the significant difference was not observed. Finally, the involvement of PKA in the ARIS-induced AR was examined using H89. That inhibitor showed no effect on the induction of AR in JE (Fig. 5C).

Fig. 3. **Effect of adenylyl cyclase activators and inhibitors on spontaneous acrosome reaction.** **(A)** *Sperm were incubated in Steinberg's salt solution (ST) containing an activator of transmembrane adenylyl cyclase (***forskolin***) or soluble adenylyl cyclase (***HCO_3^-***). The acrosome reaction was evaluated by the absence of acrosome in the tip of the sperm head using a dark field microscope. Percentages of the* acrosome reacted sperm were expressed by relative values against a mean percentage of them in ST. **(B)** *Sperm were incubated in ST containing an inhibitor of transmembrane adenylyl cyclase (***MDL12330A***) or soluble adenylyl cyclase (KH7).* **(C,D)** *The sperm populations that underwent spontaneous AR at <20%* **(C)** *or at 20%<* **(D)** *within 1h were independently examined. Asterisks indicate significant differences against ST (*$*P<0.05$, $**P<0.01$*).*

Discussion

Acrosome reaction is generally induced by the increase of intracellular Ca^{2+} and sperm of an identical species sometimes possess multiple intracellular signaling pathways for AR (Shur, 1999; Visconti *et al.*, 1999; Darszon *et al.*, 2006). Results of the present study suggest that multiple signaling pathways are also present for AR in the sperm of *C. pyrrhogaster*. Based on the results and the previous studies, they are characterized by the different dependencies on Ca^{2+} permeable channels, adenylyl cyclases, and PKA. Possible pathways for ARs are proposed in Fig. 6. Cav3.2 and TRPV4 are initial triggers for spontaneous AR. Those channels seem to independently gate in sperm since the spontaneous AR was not completely inhibited by blocking neither channels (Fig. 1). CNG channel is gated in the downstream of Cav3.2 and TRPV4 since Ca^{2+} influxed through these channels should activate Ca^{2+}-activated adenylyl cyclases, ie. AC3 and AC10 (Choi *et al.*, 1992; Jaiswal and Conti, 2003). Both types of adenylyl cyclases are suggested to be present in the sperm (Fig. 2). Actually, cAMP level of *C. pyrrhogaster* sperm is raised in ST (Kon *et al.*, 2017). NMDAR also mediates spontaneous AR by the Ca^{2+} influx through either Cav3.2 or TRPV4 (or TRPM8) (Fig. 1), which causes depolarization of sperm membrane allowing the cation transport through the NMDAR (Aidley and Stanfield, 1996). This contrasts the fact that NMDAR works in association with not Cav3.2 but TRPV4 in the ARIS-induced AR (Takayama-Watanabe *et al.*, 2015; Endo *et al.*, 2019). ARIS signal appears to selectively gate TRPV4 during the fertilization process.

The activated adenylyl cyclases caused spontaneous AR only in the limited percentage of the sperm having high potential of spontaneous AR, while those having low potential did it independently of the adenylyl cyclases (Fig. 3). This indicates that sperm of *C. pyrrhogaster* possess two distinct pathways for AR regarding to the dependency on the adenylyl cyclases. Since the potential of spontaneous AR is increased when sperm are stored in the vas deferens for longer time-period (Kon *et al.*, 2017), adenylyl cyclase-dependent pathway is thought to become active by a certain change of sperm physiology concerning a decline of sperm quality for fertilization and leading to the sensing to increasing cAMP. Unexpectedly, both
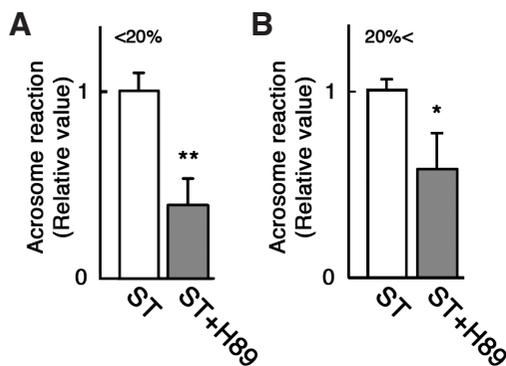
Fig. 4. Effect of a PKA inhibitor on the spontaneous acrosome reaction (AR). *Sperm populations that underwent spontaneous AR at <20% (A) or at >20% (B) within 1h were independently incubated in Steinberg's salt solution (ST) containing H89 for 1 h, and then fixed by glutaraldehyde. The acrosome reaction was evaluated by the absence of acrosome in the tip of the sperm head using a dark field microscope. Asterisks indicate significant differences against ST (*P<0.05, **P<0.01).*
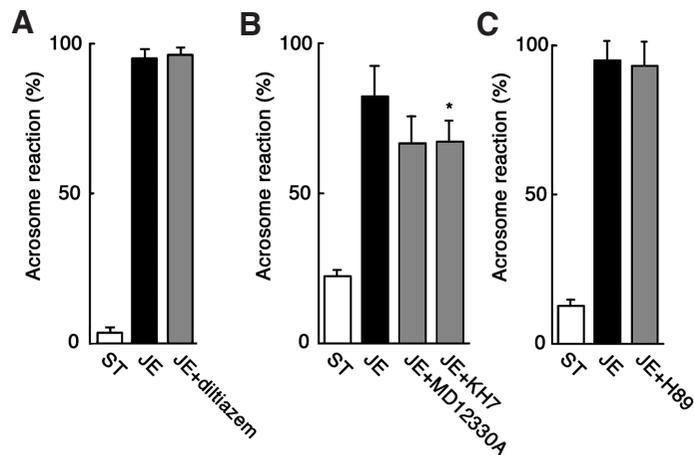
Fig. 5. Effects of inhibitors of cyclic nucleotide-gated (CNG) channel, adenylyl cyclases and PKA on ARIS-induced acrosome reaction. *Sperm were pretreated with an inhibitor of CNG channel (diltiazem) (A), transmembrane or soluble adenylyl cyclases (MD12330A or KH7) (B), or PKA (H89) (C), and then incubated in JE containing ARIS and the same inhibitor for 5min. Acrosome reaction was evaluated by the absence of acrosome in the tip of the sperm head using a dark field microscope. Asterisk indicates a significant difference against JE (P<0.05).*

adenylyl cyclase-independent and -dependent pathways are also present in the downstream of ARIS (Fig. 5). It is quite unique that two distinct pathways are activated by an identical ligand, ARIS, for the AR. Since transmembrane adenylyl cyclase involves in the adenylyl cyclase-dependent pathway, ARIS may trigger that pathway through a G-protein, which is a possible mediator for the AR in mammalian sperm (Ward *et al.*, 1992; Etkovitz *et al.*, 2009). The adenylyl cyclase-dependent pathway in the ARIS signal is activated in the quite limited number of sperm and may also correlate with the sperm quality as it does in spontaneous AR. Supposingly, the adenylyl cyclase-dependent pathway compensates for the malfunction of adenylyl cyclase-independent one and assures the immediate response to ARIS that is crucial for sperm to participate in fertilization. The adenylyl cyclase-dependent pathways in the ARIS-induced AR and the spontaneous AR are different in the dependency on PKA (Fig. 5). This means that two of the four different pathways in the downstream of Ca^{2+} permeable channels are selectively activated in the sperm to cause AR during the fertilization of *C. pyrrhgoaster*.

The results of the present study suggest that ARIS limits the intracellular signaling pathways in the sperm of *C. pyrrhogaster* through determining the Ca^{2+} permeable channels and the dependency on PKA. On the other hand, co-presence of the multiple pathways for AR may give a potential to substitute one pathway for another and develop a new signaling pathway in the evolution of reproductive system. In *C. pyrrhogaster*, Cav3.2 does not work in the induction of AR at fertilization, whereas Ni^{2+}-sensitive cation channel such as Cav3.2 is critical for the AR induced by an egg envelope component in the toad *Bufo arenarum* (Krapf *et al.*, 2009). As Ni^{2+} inhibits the spontaneous AR in *C. pyrrhogaster* sperm (Fig.1), it is suggested that a signaling pathway mediated by Ni^{2+}-sensitive cation channel is conserved for the AR in the sperm of both species. However, that pathway works at fertilization only in *B. arenarum*. In the fertilization of *C. pyrrhogaster*, Cav3.2 mediates the regulation of sperm motility (Takahashi *et al.*, 2013; Watanabe and Takayama-Watanabe, 2014). Likewise, CatSper 1 participates in both acrosome reaction
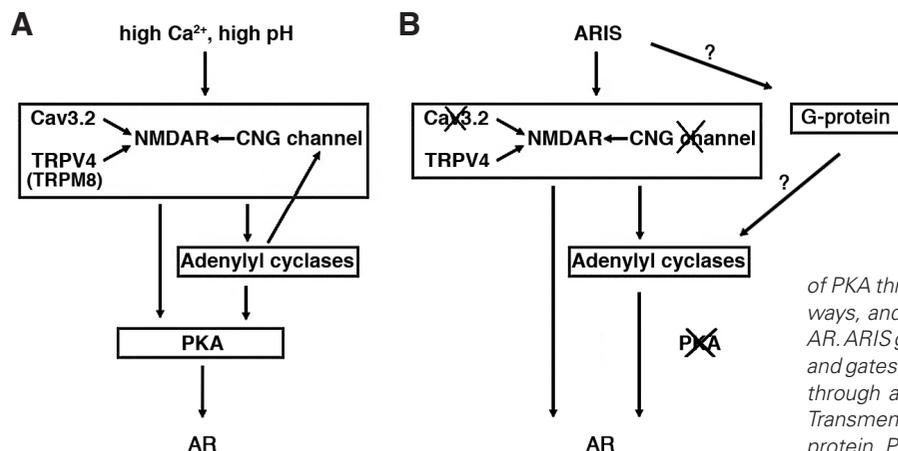
Fig. 6. Signaling pathways of the sperm for spontaneous and ARIS-induced acrosome reaction (AR) in *C. pyrrhogaster*. (A) *Pathways for spontaneous AR. High concentration of Ca²⁺ and pH that are equivalent to those in egg jelly gate Cav3.2 or TRPV4 (or TRPM8), cause depolarization of sperm membrane, and gate NMDAR. Influx of Ca²⁺ through those channels activates adenylyl cyclases and the increased cAMP gates cyclic nucleotide-gated (CNG) channel. Ca²⁺ further influxed leads to the activation of PKA through adenylyl cyclase-dependent or -independent pathways, and then AR was induced.* **(B)** *Pathways for ARIS-induced AR. ARIS gates TRPV4, causes depolarization of sperm membrane, and gates NMDAR. Ca²⁺ influxed through the channels induces AR through adenylyl cyclase-dependent or -independent pathways. Transmembrane adenylyl cyclase may be activated through a G-protein. PKA was not needed in the ARIS-induced AR.*

and motility in human (Tamburrino *et al.*, 2014), although CatSper cation channel is not present in amphibian sperm (Cai and Clapham, 2008). These facts suggest that some Ca²⁺ permeable channels for the AR are interchangeable with those for the regulation of motility. Such interchangeability of an intracellular signaling mediator is supposed to be a key to develop a new combination of the mediators, resulting in the evolution of the signaling pathway for the AR. Further comparative study about the usages of intracellular mediators will reveal how the multiple pathways for AR in the sperm correlate with the establishment of a variety of signaling pathways working in the fertilization of extant animal species.

Materials and Methods

Gametes

The experimental protocol was approved by the Committee for Animal Experiments of Yamagata University (No. 29148). Animals were treated under the Guidelines for the Proper Conduct of Animal Experiments in Japan. Mature eggs were obtained from the uterus, the most posterior region of the oviduct after females were daily injected with 300 IU human chorionic gonadotropin three times. Sperm were collected from the vas deferens.

Egg jelly extract

Egg jelly extract was prepared according to Kon *et al.,* (2017). Briefly, mature eggs were shaken in Steinberg's salt solution (58.2 mM NaCl, 0.67 mM KCl, 6 mM Ca(NO₃)₂, 0.83 mM MgSO₄, 10 mM Tris-HCl: pH 8.5) for 1 h. The supernatant was collected and then centrifuged at 13,500 rpm at 4°C for 30 min. The supernatant was again collected as the JE and stored at -25°C until experimental use.

Chemicals

Chemicals were purchased from Sigma-Aldrich (Tokyo). Mibefradil (a blocker of T type VDCC; Bezprozvanny and Tsien, 1995; Takahashi *et al.*, 2013), NiCl₂ (a selective blocker of Cav3.2; Lee *et al.*, 1999; Takayama-Watanabe *et al.*, 2015), nifedipine (a blocker of L type VDCC; Furukawa *et al.*, 2009; Takayama-Watanabe *et al.*, 2015), RN1734 (a TRPV4 channel blocker; Vincent *et al.*, 2009; Endo *et al.*, 2019), diltiazem (a blocker of CNG channel; Kolesnikov *et al.*, 1990), mdl12330A (an inhibitor of transmembrane adenylyl cyclases; O'brien *et al.*, 2011), or KH7 (an inhibitor of soluble adenylyl cyclase; Bitterman *et al.*, 2013) was used at a final concentration of 100 μM (mibefradil, NiCl₂, and RN1734), 500 μM (MK801), 50 μM (diltiazem), 10 μM (mdl12330A), or 5 μM (KH7). Forskolin (an activator of transmembrane adenylyl cyclase; O'brien *et al.*, 2011), or NaHCO₃ (an activator of soluble adenylyl cyclase; Buffone *et al.*, 2014) was used at 10 μM (Forskolin), or 1 mM (NaHCO₃). H89 (an inhibitor of PKA; O'brien *et al.*, 2011) was used at 10 μM.

Acrosome reaction

Sperm were pretreated with one of the channel blockers or enzyme inhibitors in the reconstructed vas deferens solution (20 mM NaCl, 6 mM Na₂SO₄, 1 mM KCl, 0.1 mM Ca(NO₃)₂, 0.06 mM MgSO₄, 10 mM HEPES– NaOH: pH 6.9) for 3 min. For the induction of AR by ARIS, sperm were incubated in JE containing the same blocker or inhibitor for 5 min. They were then fixed in 2.5% glutaraldehyde, and the absence of acrosome in the tip of the sperm head was evaluated in more than 100 sperm using a dark field microscope (CX41; Olympus Co., Tokyo Japan). For spontaneous AR, sperm were incubated in ST containing a blocker or an inhibitor for 1 h according to Kon *et al.*, (2017). Pretreatment with a chemical was not performed in examining the effect of activators of adenylyl cyclases. Experiments were independently performed at least three times.

Statistics

Significant differences were evaluated by Welch's *t*-test.

Expression of CNG channel and adenylyl cyclases

RNAseq data from spermatogenic testes (Watanabe and Takayama-Watanabe, 2014) were surveyed by the BLAST protocol to find a contig with high homology to the CNGB3 subunit of CNG channel and adenylyl cyclases. Reverse-transcription PCR was performed in the spermatogenic and nonspermatogenic testes using specific DNA primers for mRNAs of the *CNGB3* (Forward: GGAATCGTCGCATGCAGATGTG, REVERSE: CATTCAGAAAGGCTCATCGCATG), AC3 (Forward: TAACACCAGACTC-CAGCACAAAT, Reverse: TCAAGATGTCACGGGTATCCTCC), AC6 (Forward: CAAGGTGGCTCTCAAGTACATGA, Reverse: TGTTCGCTTC-CAGCTCTACGTAG), AC9 (Forward: GACTACAGCAGCATTGAGAA-GAT, Reverse: TTGGATTCGACACTCCACCCCAG), or AC10 (Forward: GCAAAGCTTAATATCAGACAGCC, Reverse: AAACGTATCAATGTAGT-GTGCC) that were designed from the contig base sequences. Expressions of *protamine* and *elongation factor 1* mRNA were examined as the representatives of spermatid-specific and ubiquitous genes (Yokoe *et al.*, 2014).

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