rab3-Peptide stimulates exocytosis of the ram sperm acrosome via interaction with cyclic AMP and phospholipase A_2 metabolites

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Abstract Acrosomal exocytosis triggered with A23187/Ca^{2+} was enhanced by rab3AL, a synthetic peptide corresponding to the effector domain of the small GTP-binding protein rab3. Exocytosis was further enhanced when spermatozoa were also exposed to dibutyryl-cAMP, but was prevented when H-89, a protein kinase A (PKA) inhibitor, was included. The action of rab3AL was not on, or upstream of, phospholipase A_2 (PLA_2). Inhibition of exocytosis by the PLA_2 inhibitor aristolochic acid was overcome by rab3AL when it was included together with lysophosphatidylcholine; this effect was prevented by H-89. These results suggest a functional coupling between rab3 protein, metabolites generated by PLA_2, and cAMP-activated PKA, in the final steps leading to membrane fusion during acrosomal exocytosis.

Key words: Exocytosis; rab3; Phospholipase A_2; Lysophosphatidylcholine; cAMP; Spermatozoa

1. Introduction

Mammalian spermatozoa undergo exocytosis of the acrosomal granule (the 'acrosome reaction') in response to oocyte-derived stimuli [1–3]; exocytosis can also take place after treatment with the ionophore A23187 and Ca^{2+} [4,5]. Treatment with these agonists, including A23187/Ca^{2+}, results in a rapid generation of diacylglycerol (DAG) due to the hydrolysis of polyphosphoinositides by phosphoinositidase C [3,6–8] and hydrolysis of phosphatidylcholine by a specific phospholipase C [3,8,9]. No evidence for the participation of the phospholipase D/phosphatidic acid phosphohydrolase pathway in the generation of DAG has been found [3]. DAG has an important role during exocytosis because it activates phospholipase A_2 (PLA_2) [10] which, in turn, generates fatty acids and lysophospholipids important for membrane fusion [11,12].

There is little information on the mechanisms actually involved in membrane fusion during acrosomal exocytosis. Recent work [13] has shown that cAMP and protein kinase A (PKA) may act downstream of Ca^{2+} entry, modulating the late steps of exocytosis. However, little attention has been paid to other molecular systems involved in the final steps of exocytosis in other cell systems [14], such as the ras-like small GTP-binding proteins rab3 [15], and related molecules, that regulate docking and fusion of secretory granules with the plasma membrane [16–19].

Synthetic peptides corresponding to a putative effector domain of rab3 proteins have been shown to stimulate exocytosis in permeabilized [20–23] and intact cells [24], and membrane fusion in a cell-free assay system [25]. We have therefore used a sperm model system, where cells are stimulated to undergo exocytosis with A23187/Ca^{2+}, to explore whether rab3 might be involved in the final steps of membrane fusion during acrosomal exocytosis. We present, for the first time, evidence suggesting that rab3 protein, together with PLA_2-derived metabolites and the cAMP/PKA signalling pathway, play an important role during the final steps of acrosomal exocytosis in mammalian spermatozoa.

2. Materials and methods

2.1. Materials

[1-^{14}C]Arachidonic acid (toluene solution; spec. act. 54–56 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., UK). The ionophore A23187 was purchased from Calbiochem (Nottingham, UK). Poly(vinyl)alcohol (type II) and polyvinylpyrrolidone were from Sigma (Poole, Dorset, UK). HEPES was from BDH (Poole, Dorset, UK). Organic solvents were from BDH or Fisons (Loughborough, Leics., UK). The following lipids were purchased from Sigma: 1,2-dioleoyl-*sn*-glycerol, 1,3-dioleoylglycerol, arachidonic acid, and lysophosphatidylcholine (lysoPC) (article L 4129). The PKA inhibitor H-89 was from Biomol (Affiniti Research Products, Nottingham, UK). Aristolochic acid was purchased from Aldrich (Milwaukee, MI, USA) or Sigma. All other chemicals were of reagent grade and were purchased from BDH or Sigma. The rab3AL peptide was kindly donated by Dr. G.J. Law (The Babraham Institute).

2.2. Preparation, labelling and treatment of spermatozoa

Throughout the experiments the standard saline incubation medium used consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM HEPES, adjusted to pH 7.55 at 20°C with NaOH [6]; a medium containing 222 mM sucrose in place of the NaCl was used for washing spermatozoa. Both media also contained 1 mg poly(vinyl) alcohol/ml and 1 mg polyvinylpyrrolidone/ml, and had an osmolality of 305 mOsm/kg.

Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium ($400\times g_{max}$ for 5 min, and $1000\times g_{max}$ for 10 min), as described [26].

Labelling was routinely carried out by incubating washed spermatozoa (about 1×10^8/ml) in 5–10 ml of saline medium containing 0.5 μCi [^{14}C]arachidonic acid/ml [11] for 60 min at 37°C. Before stimulation, spermatozoa were washed through sucrose medium ($400\times g_{max}$ for 5 min, and $1000\times g_{max}$ for 10 min) and resuspended in the saline medium containing 3 mM Ca^{2+}.

Exocytosis of the sperm acrosome was induced by treating cells with Ca^{2+} (3 mM) and the divalent cation ionophore A23187 (1 μM) in saline medium at 37°C, and was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples [4].

2.3. Lipid analyses

Reactions were stopped by the addition of chloroform/methanol (1:2, v/v) and lipids were then extracted according to Bligh and

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Abbreviations: DAG, diacylglycerol; db-cAMP, dibutyryl-cAMP; PKA, protein kinase A; lysoPC, lysophosphatidylcholine; PLA_2, phospholipase A_2

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Dyer [27]. Lipids were separated by thin-layer chromatography on silica gel 60-coated glass plates (0.25 mm thickness) (E. Merck, Darmstadt, Germany). Neutral lipids were separated by developing the plates twice using as solvent *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v). Lipids were visualized by staining in an iodine tank, identified by comparison with arachidonic acid, 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoylglycerol standards run on the same plate, scraped off, and the radioactivity in each determined by liquid scintillation counting

2.4. Statistics

Results are means ± S.E.M. Significance of results was examined after data transformation (arcsin $\sqrt{(x \div 100)}$ for percentages of exocytosis, and $\log_{10}$ for other variables), using Student's *t*-test or analysis of variance (ANOVA). Values of $P < 0.05$ were regarded as statistically significant.

3. Results

Treatment of ram spermatozoa with A23187 (1 μM) and Ca^{2+} (3 mM) resulted in a time-dependent increase in the number of cells undergoing acrosomal exocytosis (Fig. 1); maximal percentages of exocytosis (~90%) were seen 30 min after the beginning of treatment (not shown). Exocytosis did not take place when cells were not exposed to A23187 (Fig. 1A,B) or were treated with A23187 in the absence of Ca^{2+} (not shown).

We tested on exocytosis triggered with A23187/Ca^{2+} the effect of rab3AL peptide (VSALGIDFKVKTIYRN), a two-amino-acid substituted derivative of the effector domain of rab3 (VSTVGIDFKVKTIYRN), which was previously found to be more potent than the authentic peptide [21,22,24]. Addition of 25 or 50 μM rab3AL, together with A23187/Ca^{2+}, caused a slight reduction in the number of cells undergoing exocytosis (Fig. 1A). At 100 μM, rab3AL inhibited exocytosis at 5 and 10 min (2-factor ANOVA, treatment: $P < 0.001$; time: $P < 0.0001$), but it was stimulatory at 15 min (Student's *t*-test, $P = 0.003$). Exposure of sperm cells to rab3AL alone (100 μM), in the presence of Ca^{2+}, did not affect cell integrity (i.e. did not result in exocytosis) (Fig. 1A,B) and did not alter sperm viability, as revealed by a lack of change in sperm motility (not shown). On the other hand, when rab3AL was added 2.5 min after spermatozoa were stimulated with A23187, there was a concentration-dependent increase in the number or cells undergoing exocytosis (2-factor ANOVA, treament: $P < 0.007$; time: $P < 0.0001$) (Fig. 1B). The concentrations of rab3AL enhancing exocytosis in spermatozoa are similar to those found to stimulate exocytosis in other cells [21,22,24]. It is not entirely clear why rab3AL showed a slight inhibitory activity when added at 0 min, and a stimulatory effect when added at 2.5 min. One simple explanation is that the ionophore A23187 may bind to rab3AL when they are mixed together before addition of sperm suspension; this would decrease its activity, as seen when bovine serum albumin is a component of incubation media. This would not take place if rab3AL is added once spermatozoa have been exposed to the ionophore. Another possibility is that, after A23187 treatment, sperm membranes become more permeable to the rab3-peptide allowing its entry into the cells.

To investigate if the stimulatory effect of rab3AL is related to the activation of pathways underlying exocytosis in spermatozoa, we examined generation of arachidonic acid as an indicator of PLA_2 activation [11]. Ram spermatozoa were labelled with [^{14}C]arachidonic acid, washed, resuspended in saline medium and stimulated with A23187/Ca^{2+} in the abscence or presence of rab3AL. Stimulation with A23187/Ca^{2+} resulted in a considerable increase in arachidonic acid release. When cells were stimulated with A23187/Ca^{2+}, and rab3AL (100 μM) was included 2.5 min after the beginning of treatment, the rise in arachidonic acid was not different from that seen with A23187/Ca^{2+} alone (Table 1). Since PLA_2 is stimulated by messengers generated by upstream events, such as the DAG formed by phosphoinositidase C and phospholipase C actions [10], these results also suggest that rab3AL does not affect events upstream of PLA_2.

To test whether the effect of rab3AL on sperm acrosomal exocytosis is related to the activation of the cAMP/PKA messenger system, we first examined whether dibutyryl-cAMP (db-cAMP) modulated the effect of rab3AL. Fig. 2 shows that when spermatozoa were stimulated with A23187/Ca^{2+}, inclusion of db-cAMP (1 mM) resulted in an enhancement of exocytosis, similar to that observed with inclusion of rab3AL; the exposure of sperm cells to db-cAMP alone, in the

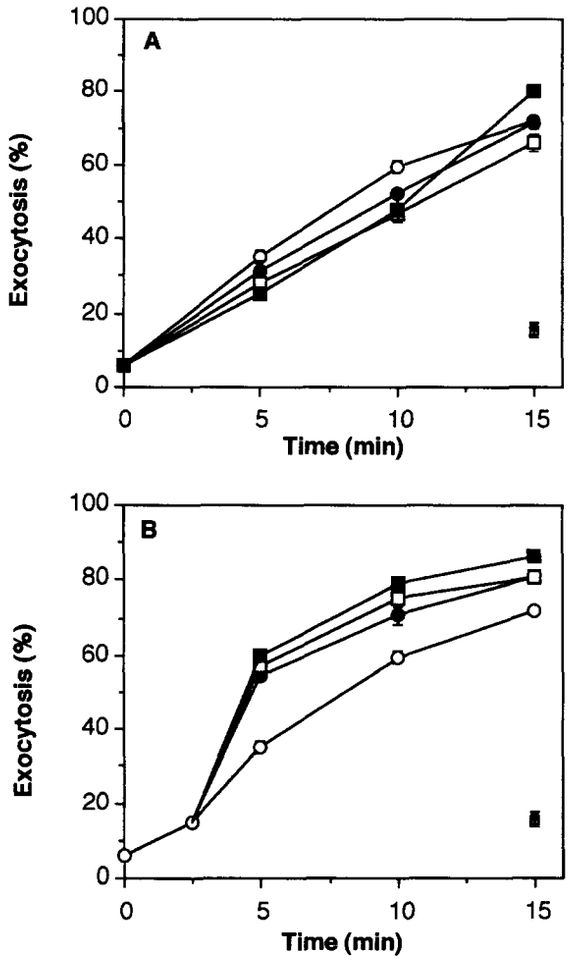

Fig. 1. Effect of rab3AL on A23187/Ca^{2+}-induced acrosomal exocytosis. Ram spermatozoa in saline medium with 3 mM Ca^{2+} were stimulated with A23187 (1 μM) for various times in the absence or presence of rab3AL. Sperm sub-samples were fixed with a glutaraldehyde fixative and examined by phase contrast microscopy. (A) rab3AL added at 0 min, together with A23187 (n=3). (B) rab3AL added 2.5 min after A23187 (n=3). (⊞) Control (Ca^{2+} alone); (○) A23187/Ca^{2+}; (●) A23187/Ca^{2+} plus rab3AL 25 μM; (□) A23187/Ca^{2+} plus rab3AL 50 μM; (■) A23187/Ca^{2+} plus rab3AL 100 μM, (×) rab3AL 100 alone μM.

presence of Ca^{2+}, did not affect cell motility or integrity (data not shown). When spermatozoa stimulated with A23187/Ca^{2+} were exposed to both db-cAMP and rab3AL, the levels of exocytosis were higher (Student's t-test, $P < 0.01$) than those seen when either db-cAMP or rab3AL were added (Fig. 2), suggesting a coupling of processes activated by the cAMP/PKA and rab3 systems (see below).

To obtain additional evidence for the involvement of the cAMP/PKA system, we then examined the effects of H-89, a PKA specific inhibitor [28] (Fig. 3). When spermatozoa were treated with A23187/Ca^{2+} and H-89 (0.3 μM), there was a reduction in the number of cells undergoing exocytosis in relation to the response seen with A23187/Ca^{2+} alone. Furthermore, the stimulatory effect of rab3AL was clearly reduced by H-89. The effect of H-89 was not on the viability of cells, because exposure to H-89 alone did not alter sperm motility or integrity (data not shown). It could be argued that, although H-89 inhibited the stimulatory effect of rab3AL, these results do not allow for a clear distinction between the inhibition exerted by H-89 on exocytosis stimulated by A23187 and the inhibition of rab3AL-stimulated exocytosis. We have, therefore, carried out an additional series of experiments concentrating on events downstream of PLA_2.

Recent work [13] has suggested that, in the final steps of membrane fusion, the cAMP/PKA messenger system could modulate events downstream of PLA_2, acting together with metabolites generated by PLA_2 and, perhaps, proteins of the rab3/rabphilin system. We have therefore tested whether lysoPC, a metabolite generated by PLA_2, affects the ability of rab3AL to enhance exocytosis. To concentrate on events downstream of PLA_2, spermatozoa were treated with the PLA_2 inhibitor aristolochic acid [29,30], a reagent found to inhibit acrosomal exocytosis [13]; test reagents were then included to examine whether they would restore exocytosis.

Spermatozoa treated with A23187/Ca^{2+} in the presence of aristolochic acid (400 μM) did not undergo exocytosis (Fig. 4A); aristolochic acid by itself affected neither cell integrity nor motility (data not shown). When spermatozoa were treated with A23187/Ca^{2+} and aristolochic acid, inclusion of rab3AL (100 μM) did not restore the ability of sperm cells to undergo exocytosis. However, when spermatozoa were exposed to a sub-optimal concentration of lysoPC (2.5 μg/ml) [11] plus rab3AL, spermatozoa did respond, with a higher proportion of cells undergoing exocytosis than that seen when only lysoPC was included (2-factor ANOVA, treatment: $P < 0.0001$; time: $P < 0.0001$) (Fig. 4A). Previous work has shown that this sub-optimal concentration of lysoPC contributed to restoring exocytosis when spermatozoa exposed to A23187 and aristolochic acid were treated with db-cAMP [13]. Therefore, we examined whether the effect of lysoPC plus rab3AL described above was coupled to the cAMP/PKA system by including the PKA inhibitor H-89. As seen in Fig. 4B, inclusion of H-89 reduced the occurrence of exocytosis stimulated with rab3AL and lysoPC, thus strengthening the idea that the rab3 protein system is functionally coupled to the cAMP/PKA signalling system. Exposure of cells to all reagents in the absence of A23187 revealed that no acrosome loss took place and, hence, that cell integrity was not affected (Fig. 4B); under these conditions, cell motility was also not altered (data not shown).

Table 1
Effect of rab3AL on the A23187/Ca^{2+}-induced release of arachidonic acid

Treatments	[^{14}C]Arachidonic acid after 5 min (cpm/10^8 spermatozoa)
Control (Ca^{2+} alone)	378 ± 22
A23187/Ca^{2+}	1392 ± 97[a]
A23187/Ca^{2+}+rab3AL	1284 ± 36[a]
rab3AL alone	450 ± 50

Ram spermatozoa were labelled with 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37°C, washed, resuspended in saline medium with Ca^{2+} (3 mM), treated with A23187 (1 μM) and, 2.5 min later, rab3AL (100 μM) or its solvent as control. After 5 min, lipids were extracted, separated and quantified. Results are means ± S.E.M. of duplicate assays carried out on two occasions.
[a]When compared with control: $P < 0.01$.

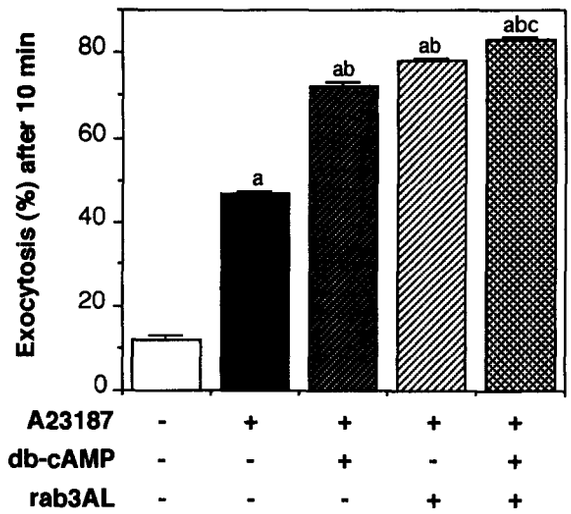

Fig. 2. Effect of db-cAMP and rab3AL on A23187/Ca^{2+}-induced acrosomal exocytosis. Ram spermatozoa in saline medium with 3 mM Ca^{2+} were stimulated with A23187 (1 μM) for various times in the absence or presence of db-cAMP (1 mM) or rab3AL (100 μM); rab3AL was added 2.5 min after A23187. Sperm samples were fixed with a glutaraldehyde fixative and examined by phase contrast microscopy ($n = 3$). a, different from control, $P < 0.001$; b, different from A23187/Ca^{2+}, $P < 0.001$; c, different from A23187/Ca^{2+} plus db-cAMP or rab3AL, $P < 0.01$.

4. Discussion

The results of this study suggest that rab3 protein has an important role in the sequence ending in membrane fusion during exocytosis of the sperm acrosome. The rab3 protein appears to be functionally coupled to both the cAMP/PKA messenger system and to metabolites generated by PLA_2.

In regulated secretory cells, rab3 proteins are leading candidates for the control of exocytosis. [16]. Among over 30 rab proteins, rab3 represents a small subfamily (rab3a, rab3b, rab3c, rab3d) expressed in a variety of cells: rab3a mainly in neurons and chromaffin cells; rab3b in pituitary cells, platelets, and mast cells; rab3c in neurons; and rab3d in adipocytes and mast cells [16,19,31]. It has been postulated that rab3 exercises a positive regulatory role in exocytosis because rab3AL, a synthetic peptide corresponding to the rab3 effector domain, stimulates secretion in a variety of cells [20–24]. Our results agree with this idea, because exposure of spermatozoa to rab3AL enhanced exocytosis stimulated with A23187/Ca^{2+}. However, since the putative effector domains of the different

rab3 isoforms are very similar (cf. [31,32]), the response to rab3AL does not allow us to implicate a specific member of the rab3 subfamily (cf. [31]).

It has been argued that some rab proteins do not necessarily act as positive regulators of membrane fusion because overexpression of wild type rab3a actually led to inhibition of exocytosis in chromaffin cells [33]. Thus, some rab3 isoforms may prevent exocytosis (i.e. act as negative regulators) until a signal is received and inhibition is relieved [19]; this has led to the separation of rab proteins into inhibitory rab proteins (I-rab) (e.g. rab3a) and facilitatory rab proteins (F-rab) (e.g. rab3b and rab3d) [18,34,35]. Nevertheless, an explanation of how rab3AL would affect rab3a intracellular function and cause stimulation of exocytosis has been offered [23].

It has also been argued that some of the effects seen with the synthetic peptide rab3AL may be related to actions on other cell processes (e.g. formation of inositol 1,4,5-trisphosphate [36]), or to the net positive charge of the peptide [37]. Our results, however, are most likely due to a stimulation of the final steps of fusion, rather than to alternative actions. Moreover, the results we obtained were also highly specific; i.e. not related to a simple perturbation of membranes. These conclusions are based on two sets of evidence. Firstly, it was observed that rab3AL was not able to influence PLA_2 activity. This indicates that the peptide acts neither on this enzyme nor on upstream events that regulate PLA_2 function [10]; if rab3AL were modulating events upstream of PLA_2, changes in the activity of the enzyme would have been observed. Furthermore, rab3AL helped to restore exocytosis in spermatozoa treated with a PLA_2 inhibitor. Secondly, responses to rab3AL were seen only under specific conditions and they were inhibited or enhanced by modulators of other messengers systems. Interestingly, we found that rab3AL could stimulate non-permeabilized sperm cells, in agreement with results found in other cell types [24], and the finding that exocytosis in non-permeabilized mouse spermatozoa could be triggered by either mastoparan or GTPγS [38,39].

The question that arises is how would rab3 associate with secretory granule membranes and regulate fusion, and how is it functionally coupled to other messenger systems? It is now clear that rab3 interacts with a series of proteins. Among them, rabphilin3A, originally described as a synaptic vesicle protein and recently identified in hormone secreting cells from various species [40], has been found to interact with rab3a and rab3c in a GTP-dependent manner [41,42]. The widely expressed protein rabin3 interacts with rab3a and rab3d (but not with rab3c) [43], whereas SNAP-25, VAMP/synaptobrevin-2, and syntaxins have been found to exist as a complex with rab3a in brain presynaptic terminals [44]. In addition, rab3a may also interact with SNARE [44,45] or the cytosolic protein doublet REEP-1 and -2 [23]. The mode of interaction with most of these target proteins is still unclear and it is not well known, as indicated above, if inhibitory or facilitatory 'complexes' are formed with rab3. For example, both inhibitory [19] and facilitatory [17] models have been presented to explain how rab3a-GTP may associate with target proteins on granule membranes, how they interact with putative 'docking' proteins in the plasma membrane, and how rab3a would dissociate from the complex before [19] or after [15,17,46] fusion.

The fusion process may also be regulated by the cAMP/PKA signalling system. Recently, it has been found that some rab3 target proteins are phosphorylated by cAMP-dependent PKA [47,48], with rab3a not serving as a substrate for this kinase. Thus, our results showing that db-cAMP or H-89 stimulates or inhibits, respectively, the occurrence of exocytosis support the idea [13] that the cAMP/PKA messenger system could modulate membrane fusion in spermatozoa. Our results also indicate that rab3 action in spermatozoa could be coupled to the cAMP/PKA messenger system because (a) the stimulatory effect of rab3AL was further enhanced by db-cAMP, (b) the effect of rab3AL was reduced by the PKA inhibitor H-89, and (c) in sperm cells where PLA_2 was inhibited with aristolochic acid, the stimulatory effect of rab3AL was, again, reduced by H-89. It is important to emphasise that although the cAMP/PKA system may be important in the modulation of the final steps of fusion, some fusion-related events may not be controlled by this signalling system. This is suggested by the finding that H-89 did not cause a complete inhibition of exocytosis triggered by A23187/Ca^{2+}, or that enhanced by rab3AL. It is thus likely that some protein(s) involved in the late steps of fusion may be regulated by other protein kinases (e.g. rabphilin3A is also phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II [48,49]), or that some of these proteins may not be regulated by phosphorylation.

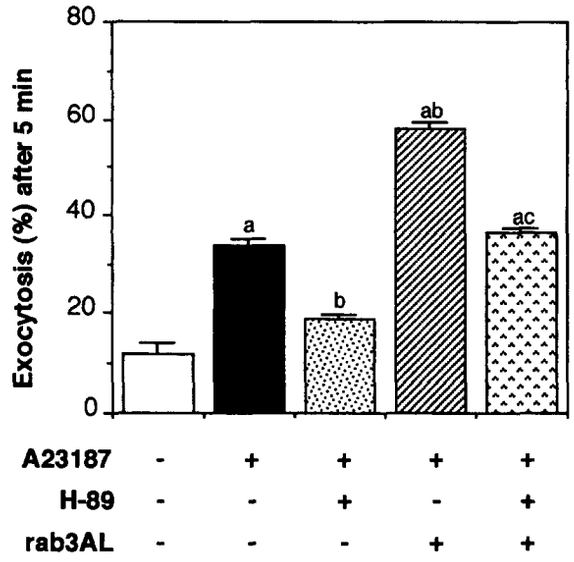

Fig. 3. Effect of rab3AL and H-89 on A23187/Ca^{2+}-induced acrosomal exocytosis. Ram spermatozoa in saline medium with 3 mM Ca^{2+} were incubated in the absence or presence of H-89 (0.3 μM) for 5 min, then stimulated, or not, with A23187 (1 μM), and after 2.5 min exposed to rab3AL (100 μM). Sperm samples were fixed with a glutaraldehyde fixative and examined by phase contrast microscopy ($n=3$). a, different from control, $P<0.001$; b, different from A23187/Ca^{2+}, $P<0.001$; c, different from A23187/Ca^{2+} plus rab3AL, $P<0.001$.

Previous work has shown that metabolites generated by PLA_2 are important for membrane fusion during acrosomal exocytosis [11]. The present study has revealed that PLA_2-derived metabolites are functionally coupled to rab3 action: in sperm cells where PLA_2 was inhibited by aristolochic acid, the inclusion of lysoPC plus rab3AL caused higher levels of exocytosis than each of the compounds added alone. Thus, perturbation of membrane phospholipids by release of lysoPC may facilitate the action of the rab3 fusion complex. Finally, indication that the cAMP/PKA messenger system may be coupled both to PLA_2-derived metabolites and to rab3 comes

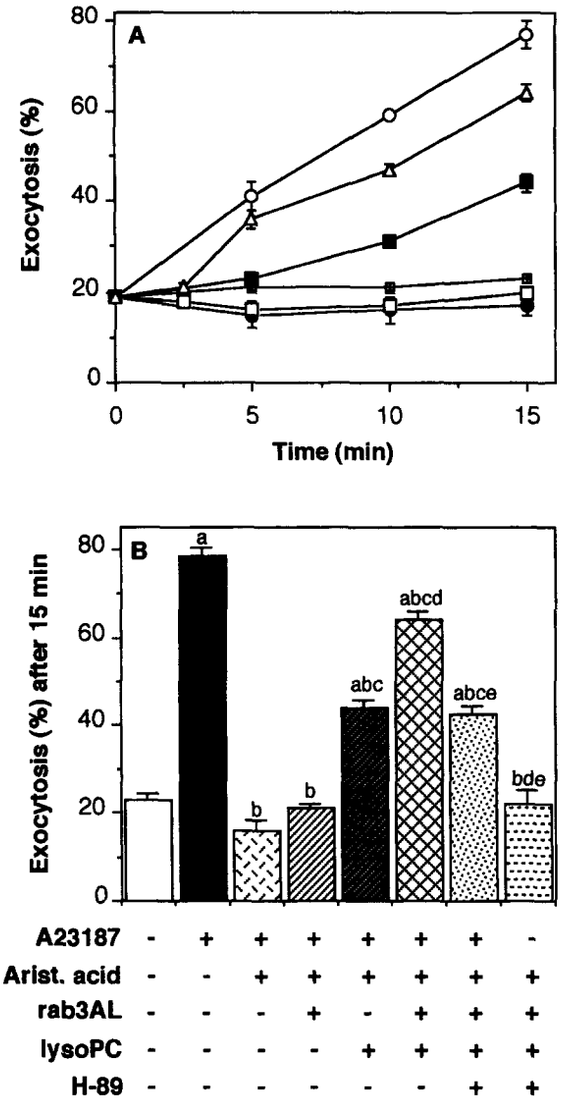

Fig. 4. Effect of aristolochic acid, rab3AL, lysoPC and/or H-89 on A23187/Ca^{2+}-induced acrosomal exocytosis. Ram spermatozoa in saline medium with 3 mM Ca^{2+} were exposed to aristolochic acid (400 μM), lysoPC (2.5 μg/ml), H-89 (0.3 μM), or solvent (as control) for 5 min, then exposed to A23187 (1 μM); rab3AL (100 μM) was then added, 2.5 min after A23187. Sperm sub-samples were fixed with a glutaraldehyde fixative and examined by phase contrast microscopy. (A) rab3AL and lysoPC overcome the inhibitory effect of aristolochic acid ($n=3$). (⊞) Control (Ca^{2+} alone); (○) A23187/Ca^{2+}; (●) A23187/Ca^{2+} plus aristolochic acid; (□) A23187/Ca^{2+} plus aristolochic acid and rab3AL; (■) A23187/Ca^{2+} plus aristolochic acid and lysoPC; (△) A23187/Ca^{2+} plus aristolochic acid, lysoPC and rab3AL. (B) H-89 reduces the rab3AL/lysoPC-induced stimulation of exocytosis ($n=3$). a, different from control, $P<0.001$; b, different from A23187/Ca^{2+}, $P<0.005$; c, different from A23187/Ca^{2+} plus aristolochic acid, $P<0.01$; d, different from A23187/Ca^{2+} plus aristolochic acid and lysoPC, $P<0.02$; e, different from A23187/Ca^{2+} plus aristolochic acid, rab3AL and lysoPC, $P<0.005$.

from the finding that H-89 reversed the stimulation seen with rab3AL plus lysoPC.

In conclusion, our results suggest that a rab3 fusion complex and the cAMP/PKA messenger system, in conjunction with PLA_2-derived metabolites such as lysoPC, may modulate the final steps of fusion during exocytosis of the sperm acrosome.

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