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Participation of membrane adenylyl cyclase in heparin-induced capacitation in cryopreserved bovine spermatozoa

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Summary

The aim of this work was to study the participation of membrane adenylyl cyclase in heparin-induced capacitation in cryopreserved bovine spermatozoa. Sperm suspensions were incubated in Tyrode's albumin lactate pyruvate medium in the presence of heparin (10 IU ml^{-1}) or forskolin (1–75 μM), a well-known membrane adenylyl cyclase activator. The participation of membrane adenylyl cyclase was confirmed using a specific inhibitor, 2′,5′-dideoxyadenosine (6–25 μM). Spermatozoa capacitated with forskolin (25 μM) were incubated with bovine follicular fluid to evaluate their ability to undergo acrosome reaction. Capacitation percentages were determined by the fluorescence technique with chlortetracycline, and true acrosome reaction was determined by trypan blue and differential interferential contrast. The forskolin concentrations employed had no effect on progressive motility or sperm viability. Capacitation values induced by 25-μM forskolin treatment (27.80 ± 2.59%) were significantly higher respect to the control (4.80 ± 1.30%). The inhibitor 2′,5′-dideoxyadenosine prevented forskolin-induced capacitation and significantly diminished capacitation induced by heparin. Follicular fluid induced physiological acrosome reaction in spermatozoa previously capacitated with 25-μM forskolin ($P < 0.05$). Forskolin acts as a capacitation inducer and involves the participation of membrane adenylyl cyclase as part of the intracellular mechanisms that lead to capacitation in cryopreserved bovine spermatozoa.

Introduction

Mammalian spermatozoa require a preparation period denominated capacitation to acquire the ability to fertilise mature oocytes. During this process, changes in plasma membrane fluidity, O_2 uptake and metabolism, intracellular ionic concentration and the activity of several enzymes, take place. Capacitation is followed by an exocytotic event termed acrosome reaction that is an absolute requisite for fertilisation (Yanagimachi, 1994). The molecular mechanism that underlies sperm capacitation has not been completely clarified but the process is regulated by a number of intracellular signals involving protein kinase A (PKA) (Leclerc *et al.*, 1996; O'Flaherty *et al.*, 2006; Menzel *et al.*, 2007; Rodriguez & Beconi, 2009; Rotman *et al.*, 2010; Breininger *et al.*, 2010), protein kinase C (PKC) (Thundathil *et al.*, 2002; O'Flaherty *et al.*, 2006; Rotman *et al.*, 2010) and protein tyrosine kinase (PTK) (Leclerc *et al.*, 1997; O'Flaherty *et al.*, 2006; Rodriguez & Beconi, 2009).

Adenylyl cyclases (ACs) are the main enzymes responsible for the intracellular production of cAMP. In particular, membrane-associated AC (mAC) isoforms can be regulated by activated cell surface receptors and associated G proteins, leading to modulation of cAMP generation. This results in a temporary change in cAMP-mediated signalling, for instance through PKA, to bring about a specific cellular response; usually cAMP levels later return to normal through the effects of both (i) diffusion of stimulus from the cell surface and (ii) continued cAMP breakdown catalysed by cyclic nucleotide phosphodiesterases (PDEs) (Baxendale & Fraser, 2003).

Changes in cAMP concentrations have been linked to important processes regulating the fertilising potential spermatozoa, namely maturation, motility, capacitation

and gamete fusion (de Lamirande *et al.*, 1997). Previous studies in spermatozoa, however, have produced inconsistent data regarding the nature of ACs responsible. Although several investigators (e.g. Stein *et al.*, 1986; Fraser & Duncan, 1993; Fraser & Adeoya-Osiguwa, 1999) detected the modulation of sperm AC activity in response to various agents known to affect somatic mACs (e.g. forskolin, guanosine nucleotides, specific bacterial toxins and purified G proteins), many others did not (Hanski & Garty, 1983; Hildebrandt *et al.*, 1985). The subsequent identification and characterisation of a highly active Mn^{+2}-dependent soluble AC (sAC), regulated by bicarbonate but not receptor/G protein modifiers (Buck *et al.*, 1999), led to the proposal that this is the isoform regulating cAMP in mammalian spermatozoa.

However, more recently it has been shown that ligands involved in promoting sperm fertilising potential, such as adenosine, fertilisation promoting peptide (FPP) and calcitonin, can act via cell surface G protein-linked receptors to differentially regulate cAMP in uncapacitated and capacitated spermatozoa (Fraser & Adeoya-Osiguwa, 1999; Adeoya-Osiguwa & Fraser, 2002, 2003). As a result, initial stimulation of cAMP appears to accelerate capacitation, while the subsequent inhibition of cAMP in capacitated cells reduces spontaneous acrosome loss. This suggests that these specialised events are regulated by the modulation of AC activity and cAMP levels; although the precise mechanisms are unclear, changes in specific protein tyrosine phosphorylation appear to be involved (Adeoya-Osiguwa & Fraser, 2000).

Additional evidence that mammalian spermatozoa have mACs comes from studies showing that AC activity in permeabilised cells can be stimulated by forskolin, with responses to forskolin being inhibited by dideoxyadenosine, an inhibitor that acts on the P site of mAC. Forskolin, which binds directly to and activates mACs, also accelerated capacitation and acrosome reactions in intact, live spermatozoa, the predicted outcome of constant stimulation of mAC/cAMP. The inclusion of dideoxyadenosine, a specific mAC inhibitor, inhibited responses in the agonists. Thus, there was biochemical and physiological evidence for the presence of mAC, but these approaches did not provide information on the presence and location of specific isoforms (Fraser *et al.*, 2005).

Taking into account that mAC is involved in sperm capacitation and regulates sperm motility in several species, and that PKA participate in capacitation induced by heparin in cryopreserved bovine spermatozoa, the aim of this work was to study the participation of mAC in heparin-induced capacitation in cryopreserved bovine spermatozoa.

Material and methods

Semen freezing

Semen was collected by means of an artificial vagina from five pedigree Holstein bulls (4–5 years old) of proven fertility. The bulls belong to a controlled programme of artificial insemination and were maintained under uniform conditions during the period of research. Progressive motility was greater than 70% and the percentage of abnormal spermatozoa was less than 20%. Two ejaculates from each bull were obtained once a week during 12 weeks; they were diluted in a buffer containing Tris (0.20 mM), citrate (0.06 mM), glycine (0.12 mM), fructose (0.06 mM), 20% egg yolk and 7% glycerol at a 2 : 1 ratio. Final concentration was within 3.0–4.5 $\times$ 10^7 sperm/ml. A slow cooling curve at 5 °C (1 °C per min) was performed, and the semen was then equilibrated at 5 °C for further 90 min. It was frozen at −76 °C on dry ice, and pellets were preserved at −196 °C in liquid nitrogen (O'Flaherty *et al.*, 1999).

Evaluation of progressive motility and sperm viability

Progressive motility was evaluated by light microscopy (400 $\times$ magnification) with a thermal stage (37 °C) three times by the same observer after each treatment (45 min). The percentage of live spermatozoa was determined by the supravital eosin/nigrosin technique (Pintado *et al.*, 2000). At least 200 spermatozoa were counted in each sample.

Determination of sperm concentration

Assessment of sperm concentration was conducted in a Neubauer chamber.

Preparation of the sperm suspension for capacitation

Samples of frozen semen were thawed for 10 min in Tyrode's albumin lactate pyruvate (TALP) medium, pH 7.4 at 36 °C, without calcium or bovine serum albumin (BSA, Parrish *et al.*, 1988) in a 1 : 3 ratio. Samples were centrifuged twice by at 600 $\times$ ***g*** for 5 min to separate the seminal plasma and the freezing buffer. The pellets were resuspended to a final concentration of 1.5 $\times$ 10^7 spermatozoa/ml, in TALP with the addition of $CaCl_2$ (2 mM) and BSA (6 mg ml^{-1}) for all the experiments (completed TALP, Rodriguez *et al.*, 2005). Sperm suspensions corresponding to each experience were incubated for 45 min at 38 °C under 5% CO_2 in humidified air in the presence or absence of a capacitation inducer (Fukui *et al.*, 1990 and O'Flaherty *et al.*, 1997). Sperm concentration and progressive motility were evaluated before and after capacitation.

Determination of capacitation

The chlortetracycline (CTC) fluorescent technique was used to detect changes in the plasma membrane of the bovine spermatozoon (Fraser *et al.*, 1995). Three patterns were observed: F (fluorescent), intact noncapacitated spermatozoa displaying fluorescence throughout their surface; C (capacitated), intact capacitated spermatozoa that lost fluorescence in the postacrosomal region; acrosome-reacted (AR), spermatozoa with a reacted acrosome that lost fluorescence in the postacrosomal and acrosomal regions, expressing fluorescence only in the midpiece. Chlortetracycline (500 μl) was added to an equal volume of the medium containing the spermatozoa. Glutaraldehyde was then added to the mixture reaching a final concentration of 0.1%. Slides were examined at 400 × magnification under epifluorescence excitation at 410 nm using a Carl Zeiss Jena Jenamed 2 epifluorescence microscope. The percentage of capacitated spermatozoa determined at zero time was subtracted from the values obtained in control and capacitated samples to rule out cells damaged during freezing–thawing.

Determination of true acrosome reaction

An aliquot of sperm suspensions of the different treatments was incubated with the same volume of trypan blue (0.25%), during 15 min at 37 °C. To remove the excess of stain, it was centrifuged 10 min at 600 ***g*** and resuspended in PBS with 5% of formaldehyde. Reacted acrosomes were evaluated in live and dead spermatozoa (O'Flaherty *et al.*, 1999) by optic microscopy of differential interferential contrast (DIC). The percentage of reacted spermatozoa determined at zero time was subtracted from the values obtained in control and acrosome-reacted samples to rule out cells damaged during freezing–thawing.

Experiment 1: capacitation induced by forskolin (FSK)

To study the participation of mAC in cryopreserved bovine sperm *in vitro* capacitation, sperm suspensions were incubated during 45 min at 38 °C, in the presence of heparin or different concentrations (1–75 μM) of forskolin (FSK), a well-known activator of mAC (Baxendale & Fraser, 2003). In these samples, the percentage of capacitated spermatozoa was determined by CTC.

Experiment 2: acrosome reaction (AR) induced by follicular fluid in spermatozoa capacitated with forskolin (FSK)

To determine whether spermatozoa capacitated with FSK were able to undergo AR, spermatozoa capacitated with 75 μM FSK or 10 IU ml^{-1} of heparin were incubated during 15 min in the presence of 30% of bovine follicular fluid (McNutt & Killian, 1991), as an acrosome reaction inducer. The percentages of acrosome-reacted live spermatozoa were determined by trypan blue/DIC.

Experiment 3: participation of membrane adenylyl cyclase (mAC) during capacitation induced by heparin or forskolin (FSK)

To discern the possible involvement of mAC during capacitation induced by heparin or FSK, spermatozoa were incubated in the presence of heparin (10 IU ml^{-1}) or FSK (75 μM) and different concentrations (6–25 μM) of 2′,5′-dideoxyadenosine (Baxendale & Fraser, 2003), a mAC inhibitor. The percentages of capacitated spermatozoa were determined by CTC.

Statistical analysis

Percentages of progressive motility, viability, capacitated spermatozoa and acrosome-reacted spermatozoa are given as means ± SD. For the analysis of treatments in the different experiences, the analysis of variances was performed (ANOVA) and the Bonferroni test was used as a post-ANOVA analysis, in the cases where differences were significant. A value of $P < 0.05$ was considered as statistically significant.

Results

The addition of FSK at concentration ranging from 1 to 75 μM or 2′,5′-dideoxyadenosine at the concentrations used as mAC inhibitor, failed to modify progressive motility or sperm viability (data not shown).

The addition of 25 μM FSK to cryopreserved bovine sperm suspensions reached similar percentages of capacitated spermatozoa to the ones obtained with heparin. Capacitation percentages were significantly higher respect to the control (Fig. 1).

To confirm the physiological capacitation induced by 25 μM FSK, the AR was induced by bovine follicular fluid. Spermatozoa previously capacitated with FSK, responded to follicular fluid, reaching percentages of AR significantly higher than control group and similar to the ones obtained with heparin (Fig. 2).

The specific inhibitor of the enzyme mAC, 2′,5′- dideoxyadenosine, was used to confirm mAC participation in FSK-induced capacitation in our experimental model (FSK 25 μM) and in bovine spermatozoa capacitated with heparin, a well-known capacitation inducer. Capacitation induced by FSK, was entirely blocked by 25 μM 2′,5′-dideoxyadenosine ($P < 0.05$) (Fig. 3). The addition

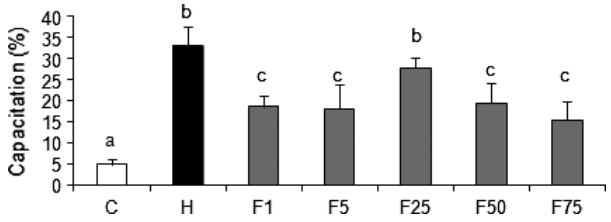

Fig. 1 Effect of forskolin (FSK) on capacitation of cryopreserved bovine spermatozoa. C, control; H, heparin; FSK, forskolin (μM). Samples were incubated for 45 min at 38 °C in complete Tyrode's albumin lactate pyruvate medium. Different letters indicate significant differences ($P < 0.05$), $n = 5$.

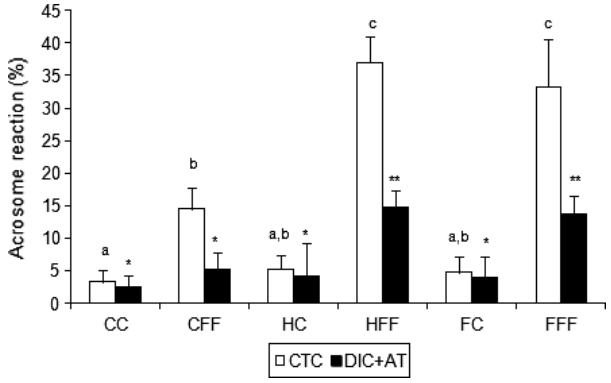

Fig. 2 Effect of follicular fluid (30%) on acrosome reaction of spermatozoa previously capacitated with heparin or forskolin (FSK) (25 μM). CC, control; CFF, control + follicular fluid (FF) (30%); HC, control (capacitated with heparin); HFF, heparin + FF (30%); FC, control (capacitated with 25 μM FSK); FFF, 25 μM FSK + FF (30%). Samples were incubated at 38 °C in complete Tyrode's albumin lactate pyruvate medium, for 45 min (with or without heparin or FSK) and 15 min (with or without FF). Different letters or *, ** indicate significant differences ($P < 0.05$), $n = 5$. Differential interferential contrast-AT: optic microscopy of differential interferential contrast and trypan blue.

of 2′,5′-dideoxyadenosine (12 μM), significantly diminished capacitation induced by heparin, reaching control values (Fig. 4) and confirming the participation of mAC in heparin-induced capacitation.

Discussion

The molecular basis of sperm capacitation is still poorly understood, although a calcium uptake, an increase in cAMP concentration, a rise in intracellular pH, an efflux of cholesterol from the sperm plasma membrane (Yanagimachi, 1994; Visconti *et al.*, 1998), and tyrosine phosphorylation of specific proteins have been demonstrated to occur during this process (Aitken *et al.*, 1995; Visconti *et al.*, 1995; Leclerc *et al.*, 1996).

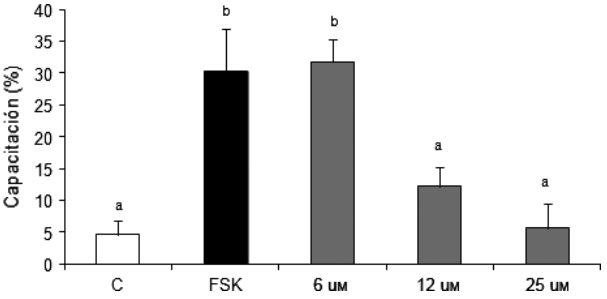

Fig. 3 Effect of membrane adenylyl cyclase inhibitor, 2′,5′-dideoxyadenosine, on sperm capacitation induced by forskolin. C, control; FSK, forskolin; different concentrations. Samples were incubated for 45 min at 38 °C in complete Tyrode's albumin lactate pyruvate medium. Different letters indicate significant differences ($P < 0.05$), $n = 5$.

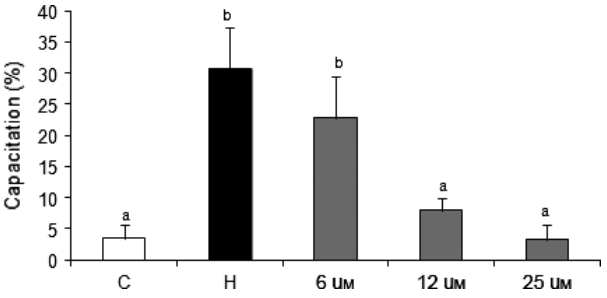

Fig. 4 Effect of membrane adenylyl cyclase inhibitor, 2′,5′-dideoxyadenosine, on sperm capacitation induced by heparin. C, control; H, heparin; different concentrations. Samples were incubated for 45 min at 38 °C in complete Tyrode's albumin lactate pyruvate medium. Different letters indicate significant differences ($P < 0.05$), $n = 5$.

There are controversial reports regarding the nature of the AC responsible for regulating the fertilising potential of sperm (Breininger *et al.*, 2010). Although several authors have detected the presence and modulation of sperm AC activity in response to various agents known to affect somatic transmembrane AC (Baxendale & Fraser, 2003; many others have not (Hildebrandt *et al.*, 1985). The transmembrane AC activity is regulated by G proteins in response to extracellular ligands (Taussig & Gilman, 1995). In contrast, soluble AC is associated with various intracellular organelles (Zippin *et al.*, 2003) and is regulated by bicarbonate and calcium (Litvin *et al.*, 2003) but does not respond to forskolin (Baxendale & Fraser, 2003).

The most commonly used compound to induce *in vitro* capacitation in bovine spermatozoa is heparin, a glycosaminoglycan (Parrish *et al.*, 1995). It interacts with spermatozoa in plasma membrane through binding bovine seminal proteins (BSP) (Chandonnet *et al.*, 1990), and it acts through specific ligand in the plasma

membrane by activating intracellular pathways that increase cAMP. However, the molecular events underlying these processes are poorly understood.

It was reported that FSK can bind directly and activate mACs, accelerating capacitation and acrosome reaction in intact, live spermatozoa (Fraser *et al.*, 2005). Our results demonstrate that FSK acts as a capacitation inducer in cryopreserved bovine spermatozoa, reaching similar values than heparin-capacitated spermatozoa. Follicular fluid (30%; McNutt & Killian, 1991) only induces physiological acrosome reaction in spermatozoa previously capacitated (Garde *et al.*, 1997). We have observed that spermatozoa capacitated with FSK reached similar acrosome reaction levels (trypan blue/DIC) than spermatozoa capacitated with heparin; confirming that the activation of mAC plays a pivotal role in the mechanisms that lead to capacitation in cryopreserved bovine spermatozoa.

It has been demonstrated that the inclusion of 2′, 5′-dideoxyadenosine, a specific mAC inhibitor, prevented the response to FSK in many biological systems (Baxendale & Fraser, 2003). In cryopreserved bovine spermatozoa, the addition of the mAC inhibitor prevented not only FSK-induced capacitation but also the physiological *in vitro* capacitation induced by heparin. As changes in the concentrations of cAMP have been linked to heparin-induced capacitation (Parrish *et al.*, 1994; Uguz *et al.*, 1994), our results would be confirming the participation of the membrane adenylyl cyclase in the intracellular mechanisms triggered by heparin during capacitation in bovine spermatozoa.

Strong evidence indicates that capacitation is associated with or controlled by different signal transduction elements, such as PKA (Visconti *et al.*, 1997; Lefièvre *et al.*, 2002) PKC (Thundathil *et al.*, 2002), PTK (Leclerc *et al.*, 1996; Aitken *et al.*, 1998), and components of the extracellular signal-regulated kinase family of mitogen-activated protein kinase pathway (de Lamirande & Gagnon, 2002; Thundathil *et al.*, 2003). It is well known that sperm capacitation is associated with a number of biochemical events, most notably an increase in protein tyrosine phosphorylation (Visconti *et al.*, 1998; Breitbart, 2003). Heparin could be acting as a ligand that triggers, among other mechanisms, the activation of mAC producing the cAMP that participates in the regulation of the protein tyrosine kinases involved in bovine sperm capacitation.

In conclusion, our results demonstrate the participation of membrane adenylyl cyclase in heparin-induced capacitation and contribute to elucidate the involvement of mAC and cAMP in the mechanisms that lead to physiological capacitation in cryopreserved bovine spermatozoa.

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