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Calcium regulates motility and protein phosphorylation by changing cAMP and ATP concentrations in boar sperm *in vitro*

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ABSTRACT

Considering the importance of calcium (Ca^{2+}) in regulating sperm capacitation, hyperactivation and acrosome reaction, little is known about the molecular mechanism of action of this ion in this process. In the present study, assessment of the molecular mechanism from the perspective of energy metabolism occurred. Sperm motility variables were determined using computer-assisted sperm analysis (CASA) and the phosphorylation of PKA substrates, tyrosine residues and AMP-activated protein kinase (AMPK) were analyzed by Western blot. Moreover, intracellular sperm-specific glyceraldehyde 3-phosphatedehydrogenase (GAPDH) activity, 3′-5′-cyclic adenosine monophosphate (cAMP) and adenosine 5′-triphosphate (ATP) concentrations were assessed in boar sperm treated with Ca^{2+}. Results of the present study indicated that, under greater extracellular Ca^{2+} concentrations (≥3.0 mM), sperm motility and protein phosphorylation were inhibited. Interestingly, these changes were correlated with that of GAPDH activity, AMPK phosphorylation, cAMP and ATP concentrations. The negative effects of Ca^{2+} on these intracellular processes were attenuated by addition of the calmodulin (CaM) inhibitor W7 and the inhibitor of calmodulin-dependent protein kinase (CaMK), KN-93. In the presence of greater extracellular Ca^{2+}, however, the phosphorylation pathway was suppressed by H-89. Taken together, these results suggested that Ca^{2+} had a dual role in regulating boar sperm motility and protein phosphorylation due to the changes of cAMP and ATP concentrations, in response to cAMP-mediated signal transduction and the Ca^{2+} signaling cascade. The present study provided some novel insights into the molecular mechanism underlying the effects of Ca^{2+} on boar sperm as well as the involvement of energy metabolism in this mechanism.

1. Introduction

To acquire fertilization competence, mammalian sperm need to undergo a series of biochemical and physiological modifications in the female reproductive tract in a process known as capacitation (Austin, 1951; Chang, 1951). From a molecular perspective, this process requires activation of a cyclic AMP signal transduction and Ca^{2+} signaling processes and the increase in tyrosine phosphorylation (Visconti et al., 1995; Salicioni et al., 2007). The cAMP/PKA pathway and Ca^{2+} signaling are essential for the regulation of sperm motility in many mammals (Marín-Briggiler et al., 2005; Lasko et al., 2012). In particular, Ca^{2+} is reported to be an important factor in regulating sperm motility (Bhoumik et al., 2014). There is some evidence that Ca^{2+} stimulates the soluble adenylyl cyclase Adcy10 (Cooper

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et al., 1995; Jaiswal and Conti, 2003; Sunahara et al., 1996) which catalyzes cAMP from ATP (Tate et al., 2010) and initiates cAMP-mediated signal transduction (Visconti et al., 1995; Visconti et al., 1999). Although it is well-established that Ca^{2+} is an important factor involved in most processes leading to fertilization (Breitbart, 2002; Ren and Xia, 2010), little is known about the molecular basis of how capacitation and subsequent sperm cell hyper-activation are induced by an increase of Ca^{2+} concentration.

Previous research reported that a certain amount of Ca^{2+} promoted protein phosphorylation and sperm motility. In contrast, however, relatively greater concentrations of Ca^{2+} inhibited protein phosphorylation and sperm motility in many species such as mice (Baker et al., 2004; Navarrete et al., 2015; Si and Olds-Clarke, 2000), cattle (Ho et al., 2002), and horses (Gonzalez-Fernandez et al., 2012). The inhibitory mechanism of greater concentrations of extracellular Ca^{2+} on protein phosphorylation and sperm motility remains to be elucidated. Even so, several studies have demonstrated that these signaling pathways and subsequent initiation and maintenance of hyper-activation of sperm were dependent on ATP and energy metabolism in the cell (Ford, 2006; Miki et al., 2004).

In all cell types, ATP concentrations are regulated by a dynamic balance between two primary processes: glycolysis and oxidative phosphorylation (Takei et al., 2014). In sperm, ATP synthesized by either of these processes is involved in the maintenance of progressive motility and also are necessary for the changes in sperm motility pattern known as hyper-activation. In other cell types, the cellular energy sensor AMPK is regulated by the ratio of AMP/ATP (Gowans and Hardie, 2014; Hardie, 2011). It has been suggested that the changes of AMPK activity might be involved in regulation of sperm motility, which is essential for sperm fertilization (de Llera et al., 2012). Other research indicated that relatively greater concentration of Ca^{2+} suppressed tyrosine phosphorylation by decreasing the availability of intracellular ATP (Baker et al., 2004).

Considering the close relationship between tyrosine phosphorylation and ATP, it was hypothesized in the present study that Ca^{2+}, cAMP and AMPK activity contributes to the regulation of sperm motility and protein phosphorylation and that these regulations might be mediated by intracellular ATP. In the present study, therefore, the mechanism of Ca^{2+} was evaluated in regulation of boar sperm motility and protein phosphorylation as related to energy metabolism. Moreover, changes of GAPDH activity, cAMP concentrations and ATP concentrations, and AMPK activity in sperm treated with different concentrations of Ca^{2+} were assessed to comprehensively analyze these intracellular mechanisms. This study, therefore, contributed to a more comprehensive understanding of complex mechanisms underlying the regulation of Ca^{2+} in boar sperm physiological functions.

2. Materials and methods

2.1. Materials

Polyvinylidene fluoride (PVDF) membrane and anti-phospho-AMPK antibody (Thr172) were purchased from Millipore (Billerica, MA). Phospho-PKA Substrate (RRXS*/T*; 100G7E) Rabbit mAb, anti-phosphotyrosine antibody, anti-GSK3β antibody, anti-rabbit IgG HRP-conjugated secondary antibody and anti-mouse IgG HRP-conjugated secondary antibody were purchased from Life Technologies Corporation (Camarillo, CA). Alexa 555-conjugated anti-rabbit antibody, Alexa488-conjugated anti-mouse antibody, peanut agglutinin (PNA) and propidiumiodide (PI) were from Molecular Probes (Invitrogen). The chemiluminescence detection kit (ECL) was from GE healthcare. Other chemical products were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

2.2. Culture media and experimental design

The basal medium was modified Whitten (MW) medium and the non-capacitating (N-Cap) medium consisted of 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 137 mM NaCl, 5.55 mM glucose and 2.0 mM sodium pyruvate. The capacitating (Cap) medium was similar to N-Cap medium but contained 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$ and 0.4% BSA (4.8 mM KCl, 1.2 mM KH_2PO_4, 95 mM NaCl, 5.55 mM glucose, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.4% BSA, and 2.0 mM sodium pyruvate) as described by Tardif et al. (Tardif et al., 2001; Tardif, 2002). For other medium, 25 mM $NaHCO_3$ and 0.4% BSA were added without 2.0 mM $CaCl_2$ and was defined as the Cap-Ca^{2+} medium. The pH was maintained at 7.2–7.4 with NaCl to an osmolarity of 290–310 mOsm/kg until the beginning of the experiment. (Gonzalez-Fernandez et al., 2013).

To study the effect of Ca^{2+} on sperm *in vitro*, sperm were incubated with a final concentration of Ca^{2+} (0.5, 1.0, 2.0, 3.0, 4.0 mM) in N-Cap medium or Cap-Ca^{2+} medium, respectively. When necessary, some regulators associated with signaling pathways were added in the incubation medium.

2.3. Sperm collection and incubation

Semen was routinely collected using the manual method from eight mature (2–3 year-old) sexually mature Duroc boars that were managed under similar conditions (King and Macpherson, 1973). Boars were selected solely for greater sperm quality and proven fertility (over 75% pregnancy rates). The sperm samples were subsequently transported to the laboratory within 20 min. The semen were counted and collected by centrifugation at 800*g* for 5 min at room temperature and sperm pellets were re-suspended with N-Cap media. The sperm suspension was diluted 1:4 (v/v) in designated media to a final concentration of 1×10^6 sperm/mL. If addition of regulators (inhibitors or accelerators) were needed, the regulators were supplemented with the designated treatment before sperm addition. According to the previously used *in vitro* CO_2 culture method (Zhen et al., 2016), the sperm were incubated in a different medium for 2.5 h (Tardif, 2002; Zhen et al., 2016) and the medium was shaken every 10 min to prevent the precipitation formed by CO_2 and Ca^{2+}. Meanwhile, sperm were also incubated in a 37 °C water bath for 2.5 h as a precaution so as to ensure that there was no difference between these two different methods.