Soluble adenylate cyclase generated cAMP acts via protein kinase-A and not Epac 1/2 to direct capacitation-associated protein tyrosine phosphorylation in stallion sperm

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Capacitation is a complex process that encompasses the molecular changes sperm must undergo to successfully fertilize an oocyte. Our laboratory recently defined for the first time *in vitro* incubation conditions that yielded significant time-dependent increases in protein tyrosine phosphorylation, coupled with significant rates of progesterone-induced acrosomal exocytosis, suggesting that sperm are undergoing changes consistent with capacitation. Using pharmacological inhibitors and activators, our objectives were to investigate the roles of soluble adenylate cyclase (sAC), cAMP-dependent protein kinase-A (PKA) and the recently identified cAMP-dependent guanine nucleotide exchange factors, Epac 1 and 2, in capacitation-associated protein tyrosine phosphorylation in stallion sperm. Semen was collected with an artificial vagina from six adult stallions of proven fertility. Seminal plasma was removed by centrifugation at 37 °C and sperm were resuspended at 10×10^6 sperm/mL in modified Whitten's medium (MW: 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1.0 mM pyruvic acid, 25 mM $NaHCO_3$ and 7 mg/mL BSA; pH 7.25) and incubated at 37 °C in a humidified atmosphere for 0, 2, 4, and 6 h. In all experiments, mouse sperm were used as a positive control; cauda epididymal sperm were isolated using the swim-out method into 37 °C MW (pH 7.35). Sperm were centrifuged, resuspended at 2×10^6 sperm per 300 μL MW, and incubated in a 37 °C water bath for 0, 30, and 60 min. Immunoblot analysis using anti-phosphotyrosine antibodies was used to determine the effects of treatment on protein tyrosine phosphorylation. The sAC specific inhibitor KH7 prevented the time-dependent increase in protein tyrosine phosphorylation in both stallion (60 μM) and mouse sperm (30 μM). Because PKA is a downstream target of cAMP, we tested two different PKA inhibitors: (1) H89, which inactivates the active catalytic subunit of PKA; and (2) PKI, which binds to the regulatory subunits and prevents disassociation of the holoenzyme. In stallion sperm, incubation in the presence of H89 caused a dose-dependent increase in tyrosine phosphorylation, whereas PKI (60 μM) effectively inhibited tyrosine phosphorylation. In mouse sperm, as expected, both H89 (30 μM) and PKI (40 μM) inhibited tyrosine phosphorylation. Next, we wanted to determine whether Epac 1/2 are present in equine sperm, and their plausible role in promoting protein tyrosine phosphorylation. Using indirect immunofluorescence, we localized Epac 1 to the acrosome and Epac 2 to the midpiece in both stallion and mouse sperm. However, incubation of stallion or mouse sperm in the presence of 8-pCPT-2′-O-Me-cAMP (100 μM), an Epac-specific cAMP analogue that does not activate PKA, had no effect in the pattern or levels of time-dependent protein tyrosine phosphorylation. Collectively, these experiments demonstrated for the first time the requirement for sAC generated cAMP, and that cAMP is acting through PKA and not Epac 1/2, to direct these capacitation-associated phosphorylation events. We are presently investigating the potential role of Epac 1/2 in other capacitation-associated events in stallion sperm.

Keywords: Stallion; Sperm; Capacitation; Protein kinase-A; Epac

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Luteinizing hormone-induced release by kisspeptide in primary cultures of equine pituitary cells

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Kisspeptins, in association with their receptor GPR54, are believed to have a ''gatekeeper''-like effect on reproductive function by mediating GnRH release at the level of the hypothalamus. Recently we demonstrated that in the horse, as in many other species, IV administration of the decapeptide KiSS-10 increased serum concentrations of both LH and FSH. Although the main role of kisspeptide occurred at the hypothalamus, some reports indicated a secondary effect at the level of the pituitary gland. The objective of this series of experiments was to determine if KiSS-10 was able to release LH by a direct effect at the level of the pituitary. Primary cultures of gelding (age, 2 to 5 y) pituitary cells were conducted in October ($n = 2$) and January ($n = 2$). In October, cultures (6×10^5 cells/well, $n = 3$ wells/treatment) were treated for 60 min with 100 nM KiSS-10 (AnaSpec, Inc., San Jose, CA, USA), or control (no treatment), or 10 nM GnRH (Bachem, Fine Chemicals,

Torrance, CA, USA) to serve as a positive control. Media was then collected for detection of equine LH via RIA. There was a nine-fold increase in LH in GnRH vs control cells (mean ± S.E.M., 997.5 ± 114.8 vs 169.0 ± 59.2 ng/mL, $P < 0.006$), but there was no significant difference in the control vs KiSS-10 (182.4 ± 32.9 ng/mL) cells. To determine if cell density or KiSS-10 dose affected the response to KiSS-10, cells in January were also plated at two concentrations (6×10^5 vs 1.2×10^6/well), and we used three doses of KiSS-10 (1×10^2, 1×10^3, and 1×10^4 nM; $n = 3$ wells/treatment). In the absence of a significant trend in response using the two cell densities, we inferred that increasing cell density did not have an effect on gonadotrope cell function nor LH release. Nonetheless, there was a two-fold increase in LH following GnRH vs control (78.1 ± 9.3 vs 41.5 ± 6.2 ng/mL, $P < 0.005$). Interestingly, KiSS-10 also elicited a two-fold increase in LH vs control cells, regardless of dose ($P < 0.02$ for all treatments). Both the reduced LH response to GnRH and the reduced mean LH concentration obtained from non-treated control cells in January were consistent with decreased GnRH receptor number and LH content in the equine pituitary at the depth of the non-breeding season. The direct pituitary response to KiSS-10 in January may be explained by increased pituitary sensitivity to KiSS-10, mediating the ''gatekeeper'' effect in seasonal transition. Assessing seasonal changes in equine pituitary GPR54 may yield a more precise evaluation of pituitary sensitivity to KiSS-10 and delineate the hierarchal role of kisspeptins.

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Keywords: Kisspeptide; LH; GnRH; Pituitary culture; Horse

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Evaluation of cryopreserved-thawed stallion sperm before and after density gradient centrifugation with silane-coated silica particles (EquiPure®)

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By enhancing the sperm population used for AI, fewer cells can be used and dead or damaged sperm, which may have deleterious effects on the normal sperm, can be removed. Specifically, we hypothesized that the removal of dead or damaged sperm will increase the quality of live sperm after thawing of frozen semen, as measured by mitochondrial membrane potential, motility, and morphology of live sperm.

Semen from five mature stallions (two ejaculates each) was cryopreserved (in 0.5-mL straws) using a standard protocol, and stored in liquid nitrogen at −196 °C. For each ejaculate, four straws were thawed (37 °C for 30 s), diluted in a modified Tyrode's medium containing 0.1% polyvinyl alcohol (TALP-PVA), layered over an 80% EquiPure® gradient, and centrifuged at 200 × *g* for 35 min (EquiPure® sample). After centrifugation, sperm were collected from the bottom of the tube, resuspended in TALP-PVA, and washed once. Four additional straws were similarly thawed, diluted with TALP-PVA, and washed once to remove freezing extender (untreated sample). Sperm motility was determined by computerized semen analysis (CASA) and concentrations of both sperm preparations were adjusted to 5×10^6 sperm/mL. Aliquots of both preparations were analyzed for changes in membrane structure by staining with YoPro-I and propidium iodide (DNA stains with differential permeability across the plasma membrane). Mitochondrial function was assessed by staining with JC-1, a potentiometric probe which can be used to quantify mitochondrial membrane potential in equine sperm. Following incubation with YoPro-I, propidium iodide, and JC-1, samples were assessed with flow cytometry. Sperm morphology was assessed with differential interference contrast (DIC) microscopy (1000×) by an observer blinded to treatment group.

The separation of frozen-thawed equine sperm by centrifugation through the EquiPure® gradient increased ($P < 0.001$) total (80 ± 2.4% vs 41.7 ± 2.4%) and progressive sperm motility (69.5 ± 2.9% vs 31.5 ± 2.9%), as well as the percentage of morphologically normal sperm (45 ± 3.9% vs 27.7 ± 3.9%) compared to the control. In addition, the proportion of sperm in the treated sample with high mitochondrial membrane potential increased (81.6 ± 1.8% vs 42.1 ± 1.8%), as