

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 MgCl₂, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1.0 mM pyruvic acid, 25 mM NaHCO₃ 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,