Cholecystokinin receptors regulate sperm protein tyrosine phosphorylation via uptake of HCO_3^-

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Abstract

Cholecystokinin (CCK), a peptide hormone and a neurotransmitter, was detected in mature sperm two decades ago. However, the exact role of CCK and the types of CCK receptors (now termed CCK1 and CCK2) in sperm have not been identified. Here, we find that CCK1 and CCK2 receptors are immunolocalized to the acrosomal region of mature sperm. The antagonist of CCK1 or CCK2 receptor strongly activated the soluble adenylyl cyclase/cAMP/protein kinase A signaling pathway that drives sperm capacitation-associated protein tyrosine phosphorylation in dose- and time-dependent manners. But these actions of stimulation were abolished when sperm were incubated in the medium in the absence of HCO_3^- . Further investigation demonstrated that the inhibitor of CCK1 or CCK2 receptor could accelerate the uptake of HCO_3^- and significantly elevate the intracellular pH of sperm. Interestingly, the synthetic octapeptide of CCK (CCK8) showed the same action and mechanism as antagonists of CCK receptors. Moreover, CCK8 and the antagonist of CCK1 or CCK2 receptor were also able to accelerate human sperm capacitation-associated protein tyrosine phosphorylation by stimulating the influx of HCO_3^- . Thus, the present results suggest that CCK and its receptors may regulate sperm capacitation-associated protein tyrosine phosphorylation by modulating the uptake of HCO_3^- .

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Introduction

During the process of mammalian fertilization, sperm must undergo a series of biochemical and physiological changes after maturation in the epididymis before they are able to fertilize eggs. This time-dependent acquisition of fertilizing competence has been defined as capacitation (Chang 1951, 1955, Austin 1952). Sperm capacitation normally occurs in the female genital tract. However, it has also been accomplished *in vitro* by using chemically defined media. In most cases, these media contain appropriate concentrations of electrolytes, metabolic energy sources and serum albumin (Visconti *et al.* 1998, Naz & Rajesh 2004).

Capacitation involves an increase in membrane fluidity, cholesterol efflux, modifications in the distribution of surface protein; changes in enzymatic activities; modulation of intracellular constituents such as cAMP, Ca^{2+} and HCO_3^- ; and protein tyrosine phosphorylation (Brewis *et al.* 2005). In many mammalian species, protein tyrosine phosphorylation is considered an indicator of sperm capacitation and is associated with hyperactivated

motility, zona pellucida binding and acrosome reaction (Visconti *et al.* 1995a,b, Visconti & Kopf 1998, Zeng & Tulsiani 2003, Baker *et al.* 2004). Now, it is widely accepted that sperm protein tyrosine phosphorylation is regulated by the soluble adenylyl cyclase (sAC)/cAMP/protein kinase A (PKA) signaling pathway (Visconti *et al.* 2002). During capacitation, the entry of bicarbonate and calcium from the medium into the sperm cell activates sAC, resulting in elevated cAMP levels, subsequent PKA activation and protein tyrosine phosphorylation. However, little is known about hormonal control of this signaling pathway in sperm.

Cholecystokinin (CCK) is a small peptide hormone as well as an important neurotransmitter (Chandra & Liddle 2007). It is widely and abundantly expressed in the central system and in digestive organs (Crawley & Corwin 1994, Mutt 1994) and plays a critical role in digestion, feeding, cardiovascular function, respiratory function, neurotoxicity and seizures, cancer cell proliferation, analgesia, sleep, memory, anxiety and dopamine-mediated exploratory and rewarded behaviors (Crawley & Corwin 1994). CCK has also been found in

spermatogenic cells in the testis and epididymis (Persson *et al.* 1988, 1989, Peltö-Huikko *et al.* 1989) although its exact role remains elusive. CCK exerts physiologic functions through its specific membrane-spanning receptors. CCK receptors can be subdivided pharmacologically into type A and B receptors (now termed as CCK1 and CCK2 respectively according to the guidelines of the International Union of Pharmacology (IUPHAR) Committee on Receptor Nomenclature and Drug Classification) (Vanhoutte *et al.* 1996). The contribution of each receptor to CCK-stimulated action is varied. Some pharmacological studies using CCK receptor antagonists have indicated that the CCK2 is involved in anxiety, while CCK1 has been implicated in satiety and behavior (Hughes *et al.* 1990, Miyasaka *et al.* 1994, 2002, Wank 1995). The gene expression and/or protein distribution of CCK1 and CCK2 have been reported (Wank 1995, Bourassa *et al.* 1999). CCK1 is enriched in the pancreas and specific brain regions, whereas CCK2 is widely distributed throughout the CNS (Honda *et al.* 1993, Wank 1995, Matsusue *et al.* 1999). However, the expression of CCK receptors in mature sperm has not been reported. Interestingly, our previous studies have shown that tripeptidyl peptidase II (TPPII), a CCK-inactivating serine peptidase (Rose *et al.* 1996), could regulate sperm function (Zhou *et al.* 2013). Thus, we speculated that CCK and its receptors might play the specific roles in sperm function. We undertook the present study to examine the roles of CCK and its receptors in mature sperm and to elucidate the underlying mechanisms.

Materials and methods

Animals

Mature C57 male mice (10–12 weeks) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). They were housed in the animal housing at our institute before manipulation. Food and water were freely available throughout the experiments. All protocols were conducted according to the approval of the Institute Animal Care Committee of Shanghai Institute of Biochemistry and Cell Biology (Permit Number: SYXK2007-0017).

Detection of CCK and its receptor proteins on the sperm

Western blot analysis of CCK and its receptor proteins in spermatozoa was conducted according to a previously described protocol (Zhou *et al.* 2008, 2013). Briefly, total protein extracts obtained from spermatozoa of cauda epididymis were resolved by electrophoresis on 12% SDS-polyacrylamide gels, transferred into PVDF (0.45 µm) membranes and probed with rabbit polyclonal antibodies (anti-CCK8 antibody (Sigma: C2581): 1:5000; anti-CCK1 antibody (Sigma: SAB4503488): 1:5000; anti-CCK2 antibody (Abcam, Cambridge, UK: ab183124): 1:2000). The bound IgG was detected with goat anti-rabbit HRP (dilution: 1:10 000) (Calbiochem, Nottingham, UK) and

developed using ECL Plus (Amersham). Protein was assayed by probing the blots with monoclonal antibodies against α -tubulin (Sigma: T6074; 1:20 000).

Indirect immunofluorescence

Immunofluorescence was indirectly detected as described previously (Zhou *et al.* 2008, 2013). Sperm were washed out from the epididymal cauda and fixed in 4% paraformaldehyde for 30 min at room temperature, and the 1:100 diluted rabbit polyclonal antibodies to CCK8 (Sigma: C2581), CCK1 (Sigma: SAB4503488) and CCK2 (Abcam: ab183124) were applied. Then, FITC-labeled goat anti-rabbit IgG (Sigma: F9887) were used as the secondary antibodies (dilution: 1:200). All the images were taken using a BX51 fluorescence microscope (Olympus).

Culture media and solvents

Enriched Krebs-Ringer bicarbonate (EKR) medium was used throughout the study for mouse sperm preparation and capacitation, and the preparation was adopted from previously published reports (Zeng & Tulsiani 2003). The final composition of the medium was 120 mM NaCl; 4.8 mM KCl; 1.0 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 5 mM glucose, 21 mM sodium lactate, 0.25 mM sodium pyruvate, 25 mM NaHCO_3 and 3 mg/ml BSA. All the chemicals were purchased from Sigma and were of the highest purity available. A tenfold concentrated solution of all the ingredients was first prepared without CaCl_2 , BSA and NaHCO_3 , sterilized by passage through a 0.22-µm filter and stored at -20°C in single-use aliquots. Working media were prepared by adding CaCl_2 , NaHCO_3 and BSA and gassing the medium with a mixture of 5% CO_2 and 95% air overnight at pH 7.2–7.4. As described in a previous report (Visconti *et al.* 1995a), in some experiments, medium without NaHCO_3 was derived by adding 25 mM NaCl instead of 25 mM NaHCO_3 . In some experiments, Ca^{2+} - and BSA-free media were used and the Ca^{2+} and BSA were added back to above final concentrations if necessary.

DMSO was used as solvent for CR1409 (Phoenix, CA, USA), PD135158 (Tocris, MO, USA), H89 (Sigma) and KH7 (Sigma). The maximal concentrations of CR1409 and PD135158 stock solution are 100 mM respectively according to the instructions. All the concentration of DMSO solvent is equal 0.1% among all samples in each experiment, except that the concentration of DMSO is 0.25% when CR1409 and PD135158 were used at the dose of 250 µM. We have found that the very low final concentration of nonaqueous solvents (0.1, 0.25 and 0.5% DMSO) present in these experiments have no detectable effect on sperm function.

Preparation of sperm

The cauda epididymis was excised and freed from the fat pad, blood vessels and connective tissue. The tissue was then transferred to a dish containing 1 ml EKR medium pre-warmed to 37°C and cut in several places with iridectomy scissors to release the spermatozoa into the medium. After 5 min, the sperm suspension was transferred to a 5 ml centrifuge tube. The final concentration of sperm was adjusted

to $3\text{--}4 \times 10^6$ cells/ml in appropriate medium. After incubation for various time periods and following treatment with different molecules or antagonists (CCK8 (Sigma: C2175); CR 1409 (Phoenix); PD135158 (Tocris), the sperm were concentrated by centrifugation at 6000 *g* for 2 min at room temperature, washed in PBS three times, resuspended in Laemmli's sample buffer without mercaptoethanol and boiled for 5 min. After centrifuging at 6000 *g* for 2 min, the supernatant was collected and 2-mercaptoethanol was added to attain a final concentration of 5%. The sperm extract was either used immediately or stored at -70°C until analysis.

Western blot for tyrosine and PKA substrate phosphorylation

SDS-PAGE was carried out in 12% gel. The sperm extracts were electrophoretically transferred to PVDF membranes (0.45 μm) in all experiments. The blots were blocked with blocking buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.05% (v/v) Triton X-100, 0.25% (m/v) gelatin; pH 7.5) and probed with a monoclonal antibody against phosphotyrosine (clone 4G10, Millipore) or the anti-phospho-PKA substrate (anti-pPKAs; 1:5000; clone 100G7E, Cell Signaling). The reactive bands were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). To confirm equal protein loading, the blots were stripped and reprobed with anti- α -tubulin monoclonal antibody (Sigma: T6074; 1:20 000).

Immunofluorescence staining for PKA substrate phosphorylation

Sperm from epididymal cauda were collected and adjusted to $3\text{--}4 \times 10^6$ cells/ml in EKR medium. After treatment with CR 1409 (Phoenix) and PD135158 (Tocris) for five min, they were washed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. The 1:50 diluted rabbit anti-phospho-PKA substrate (anti-pPKAs; clone 100G7E, Cell Signaling) were applied. Then, FITC-labeled goat anti-rabbit IgG (Sigma: F9887) were used as the secondary antibodies (dilution: 1:200). All the images were taken using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of intracellular pH in sperm

Sperm were collected and loaded with 1.2 μM BCECF-AM for 15 min in the medium (37°C , under 5% CO_2). Afterward, the cells were pelleted and washed twice to remove free dye and adjusted to 1×10^7 cells per ml in incubation medium. When needed, CCK8 or CCK receptor antagonists were added and incubated for further appropriate time. The pH was determined as previously described (Fraire-Zamora & Gonzalez-Martinez 2004). Fluorescence was detected by an excitation ratio of 500:439 nm (emission, 550 nm) using a luminescence spectrometer (BioTek, VT, USA). Calibration was performed according to the previous method (Fraire-Zamora & Gonzalez-Martinez 2004).

Assays on human sperm

Human sperm capacitation medium (Biggers, Whitten and Whittingham (BWW)) had the following composition in mM:

94.8 NaCl, 4.8 KCl, 1.7 CaCl_2 , 1.2 KH_2PO_4 , 1.2 MgSO_4 , 0.27 Na-pyruvate, 13.21 Na-lactate, 5.5 glucose and 25 NaHCO_3 , with 3.5 mg/ml BSA (fraction V; Sigma, MO, USA). These experiments using human sperm were approved by an institutional human research committee, and informed consent was obtained from the participants. Human ejaculates were obtained by masturbation, and processing began within 1 h. The ejaculates had normal volume, sperm concentration and sperm motility according to World Health Organization criteria. Once washed by BWW solution, sperm were resuspended in the BWW medium and adjusted to 1×10^7 cells/ml in incubation medium. Samples were used for western blot analysis of tyrosine phosphorylation and pH determination as described above after they were treated by CCK8 (Sigma: C2175) or CCK receptor antagonists (CR 1409 (Phoenix); PD135158 (Tocris)).

Results

CCK and its receptors locate on the acrosome region of mature sperm

The indirect immunofluorescence assay indicated that the positive CCK signal was mainly located in the acrosome region of mouse sperm (Fig. 1A), consistent with the previous observation (Pelto-Huikko *et al.* 1989). However, a weak signal was also found on the principal piece of mouse sperm (Fig. 1A). This may be a non-specific signal because of several unexpected bands in the western blotting analysis by using CCK antibody (Fig. 1B). Western blot analysis and immunofluorescence assay all validated that the CCK1 receptor and CCK2 receptor were specifically located on the region of sperm acrosome (Fig. 1C, D, E and F). The negative control with the omission of the first antibody showed no immunoreactive signals and bands in the immunofluorescence assay and western blot analysis (Fig. 1G and H). It was observed that the size of the band for CCK1 receptor or CCK2 receptor (≈ 72 kDa) was larger than that of the deduced size (≈ 50 kDa).

CCK receptor antagonists can accelerate sperm capacitation-associated protein tyrosine phosphorylation

Since the function of CCK is mediated by its receptors, we considered that CCK1 and CCK2 receptors might play an important role in sperm function. Thus, we used CCK1 and CCK2 antagonists to test their effect on mature sperm function. We found that CCK1 or CCK2 receptor antagonist could accelerate protein tyrosine phosphorylation in a dose- and time-dependent manner (Fig. 2A and B). The tyrosine phosphorylation reached the highest levels when the sperm were incubated for 60 min with 100 μM CCK1 and 50 μM CCK2 receptor antagonists. Unless otherwise noted, we used these two concentrations of the two antagonists and the incubation time of 60 min for the subsequent experiments in this study. These data suggested that CCK1 or CCK2 receptor

Figure 1 Characteristics of CCK and its receptor proteins in mouse sperm. (A, C, E and G) Immunofluorescence staining of CCK (A), CCK1 receptor (C) and CCK2 receptor (E) proteins on spermatozoa. Mouse cauda sperm were collected and probed with anti-CCK-8, CCK1 and CCK2 polyclonal antibodies. Control sperm were incubated only with (FITC)-labeled goat anti-rabbit IgG (G). Sperm DNA was stained with propidium iodide (PI) and can be seen in red. Bars: 10 μ m. A representative of three independent experiments is shown. (B, D, F and H) western blot analysis of CCK (B), CCK1 receptor (D) and CCK2 receptor (F) proteins in cauda epididymal sperm of mouse. The negative control (H) was conducted only by using goat anti-rabbit antibody conjugated with iollian HRP. The blots were probed with monoclonal antibodies against α -tubulin to assess protein loading. The western blot is a representative of three independent experiments.

antagonist could significantly accelerate the sperm capacitation-associated tyrosine phosphorylation.

CCK receptor antagonists can accelerate PKA substrate phosphorylation

The serine/threonine phosphorylation of PKA substrate proteins is a critical event on the upstream of sperm capacitation-associated protein tyrosine phosphorylation (Battistone *et al.* 2013). To examine whether CCK receptor antagonists have the influence on PKA substrate phosphorylation, we determined the change of PKA substrate phosphorylation in sperm treated with CCK receptor antagonists. The western blot analysis demonstrated that CCK1 or CCK2 receptor antagonist could increase PKA substrate phosphorylation (Fig. 3A). The indirect immunofluorescence staining further showed

that the signals of PKA substrate phosphorylation were enhanced by CCK1 or CCK2 receptor antagonist (Fig. 3B). These data indicated that CCK1 or CCK2 receptor antagonist could significantly promote PKA substrate phosphorylation.

Activation of sperm sAC/cAMP/PKA pathway by CCK receptor antagonists depends on the extracellular HCO_3^-

It is widely accepted that sperm capacitation-associated protein tyrosine phosphorylation is regulated by the sAC/cAMP/ PKA pathway. The presence of BSA , Ca^{2+}

Figure 2 Effects of CCK receptor antagonists on sperm capacitation-associated protein tyrosine phosphorylation. (A) Dose-dependent effects of CCK receptor antagonists on sperm protein tyrosine phosphorylation. Sperm were incubated with CCK receptor antagonists CR 1409 (CCK1-in: 0, 5, 10, 50, 100 and 250 μ M) and PD135158 (CCK2-in: 0, 5, 10, 50, 100 and 250 μ M) for 1 h. Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of five independent experiments. (B) Time-dependent effects of CCK receptor antagonists on sperm protein tyrosine phosphorylation. Spermatozoa were incubated with CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 5, 30, 60 and 90 min. Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of five independent experiments.

Figure 3 Effects of CCK receptor antagonists on sperm PKA substrate phosphorylation. (A) Spermatozoa were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μM) and PD135158 (CCK2-in, 50 μM) for 5 and 30 min respectively. PKA substrate phosphorylation (α-pPKAs) was then assessed by western blot analysis. α-Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Spermatozoa were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μM) and PD135158 (CCK2-in, 50 μM) for 5 min. PKA substrate phosphorylation (α-pPKAs) was then assessed by indirect immunofluorescence analysis. Control sperm were incubated only with (FITC)-labeled goat anti-rabbit IgG (negative). Sperm DNA was stained with propidium iodide (PI) and can be seen in red. Bars: 10 μm. A representative of three independent experiments is shown.

and HCO_3^- in the medium is essential for the activation of this pathway (Visconti *et al.* 1995a). To test whether the effects of CCK receptor antagonists on tyrosine phosphorylation are related to the sAC/cAMP/PKA pathway, we used a highly selective blocker (H89) of PKA and a specific inhibitor (KH7) of sAC. The results showed that H89 and KH7 all suppressed the increase of protein tyrosine phosphorylation stimulated by CCK receptor antagonists (Fig. 4A and B). These findings suggested that the CCK receptor antagonist-induced activation of tyrosine phosphorylation was located upstream of PKA and sAC action. Furthermore, we examined the effect of CCK receptor antagonists on tyrosine phosphorylation of sperm incubated in media devoid of BSA, HCO_3^- and Ca^{2+} . When the sperm were incubated in the absence of BSA or HCO_3^- for 1 h, the acceleration of tyrosine phosphorylation induced by CCK receptor antagonists disappeared (Fig. 5A and B). This demonstrated that the effect of CCK receptor antagonists on tyrosine phosphorylation was dependent on the presence of BSA and HCO_3^- in the medium. As illustrated in Fig. 5C, CCK receptor antagonists could accelerate tyrosine phosphorylation in the absence of extracellular Ca^{2+} . To chelate the traces of Ca^{2+} in Ca^{2+} -free medium, EGTA at a final concentration of 25 μM was used. But this treatment could not abolish CCK receptor antagonist-induced change of sperm tyrosine phosphorylation (Fig. 5C). Moreover, BAPTA-AM (25 μM), an intracellular Ca^{2+} chelator, could not still block the CCK receptor antagonist-induced increase of sperm tyrosine phosphorylation (Fig. 5C). These results revealed that the acceleration of

tyrosine phosphorylation by CCK receptor antagonists was independent on extracellular or intracellular Ca^{2+} of sperm.

CCK receptor antagonists can elevate intracellular pH by uptake of HCO_3^-

The presence of extracellular free HCO_3^- in the medium is essential for sperm protein tyrosine phosphorylation. Media lacking HCO_3^- and buffered to various pHs (pH 5–9) and alkalinization of intracellular pH with NH_4Cl all did not mimic this HCO_3^- effect on protein tyrosine phosphorylation (Schackmann & Chock 1986, Visconti *et al.* 1995a). Our above observation indicated that CCK receptor antagonists accelerated protein tyrosine phosphorylation only when sperm were incubated in a presence of HCO_3^- medium. We also found that the antagonist of CCK1 or CCK2 receptor have no influence on pH of medium (data not shown). We hypothesized that intracellular HCO_3^- in sperm might be elevated by receptor antagonists. To test this hypothesis, we investigated the effect of receptor antagonists on intracellular HCO_3^- in sperm by examining the change of intracellular pH according to the previous report (Xu *et al.* 2007). The two antagonists were respectively added to the sperm suspension and incubated for 1 h. The result showed that both antagonists significantly increased the sperm intracellular pH in the dose-dependent manners (Fig. 6A). Moreover, as shown in Fig. 6B, the antagonist-induced increase in pH level was fast. The free cytosolic pH in the sperm was continuously elevated since the CCK receptor antagonists were added into the

Figure 4 Activation of sperm sAC/cAMP/PKA pathway by CCK receptor antagonists. (A) Spermatozoa were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 60 min in the absence or presence of 20 μ M the protein kinase A (PKA) inhibitor H89. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Spermatozoa were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 60 min in the absence or presence of 50 μ M KH7. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments.

HCO_3^- containing medium. These data suggested that CCK receptor antagonists can stimulate uptake of HCO_3^- in sperm.

CCK8 can activate sAC/cAMP/ PKA pathway by inducing the influx of HCO_3^-

Since CCK is the ligand of CCK1 and CCK2 receptors, we investigated its action by using chemically synthetic polypeptide CCK8. We found that CCK8 could accelerate protein tyrosine phosphorylation in the dose-dependent manner (Fig. 7A) and promote the PKA substrate phosphorylation (Fig. 7B). The increase of protein tyrosine phosphorylation was abolished by the blocker (H89) of PKA and the inhibitor (KH7) of sAC (Fig. 7C and D). Moreover, the CCK8-stimulating acceleration of tyrosine phosphorylation was dependent on the presence of BSA and HCO_3^- , but not Ca^{2+} in the medium (Fig. 7E, F and G). Further investigation indicated that CCK8 could trigger the rapid increase of pH in the sperm (Fig. 7H). These results suggested that CCK8 can regulate the activation of sAC/cAMP/ PKA pathway by inducing the influx of HCO_3^- in the sperm.

CCK8 exerts its action by inhibiting CCK1 and CCK2 receptors

Generally, the inhibitors have the biphasic dose-response. Although CCK8 showed the same action on sperm function as the inhibitors of CCK receptors, the activation or inhibition of CCK1 and CCK2 receptors by

CCK8 and their inhibitors needs to be addressed. To answer this question, we further investigated the effects of antibodies against CCK1 and CCK2 receptors on sperm function. The results indicated that the antibody against CCK1 or CCK2 receptor could accelerate the sperm protein tyrosine phosphorylation (Fig. 8A) and elevate the level of intracellular pH in sperm (Fig. 8B). Moreover, the high (100 μ M CCK1-in; 50 μ M CCK2-in) and low (0.25 μ M CCK1-in and CCK2-in) doses of inhibitors, as well as the antibodies against CCK receptors showed similar superposition effect on sperm with CCK8 (Fig. 8). These data demonstrated that CCK8

Figure 5 Activation of sperm tyrosine phosphorylation by CCK receptor antagonists depends on the extracellular BSA and HCO_3^- . (A) Sperm were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 60 min in the absence or presence of 3 mg/ml BSA. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Sperm were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 60 min in the absence or presence of 25 mM HCO_3^- . Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (C) In the Ca^{2+} -free medium, Sperm were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 60 min in the absence or presence of EGTA (25 μ M) or BAPTA-AM (25 μ M). Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments.

Figure 6 CCK receptor antagonists stimulate sperm tyrosine phosphorylation by increasing intracellular pH via the influx of HCO_3^- . (A) Spermatozoa were treated with the CCK receptor antagonists CR 1409 (CCK1-in, 0, 5, 10, 50 and 100 μM) and PD135158 (CCK2-in, 0, 5, 10, 50 and 100 μM) for 60 min, and then the intracellular pH (pHi) level in the sperm was examined. Results are expressed as the means $\pm$ s.e.m. ($n=5$). * $P<0.05$, ** $P<0.01$, as compared with the corresponding controls (0) (unpaired t -test). (B) Effects of CCK receptor antagonists CR 1409 (CCK1-in, 100 μM) and PD135158 (CCK2-in, 50 μM) on sperm intracellular pH (pHi). The arrow indicates the time points at which the antagonists were added. A representative of four experiments is presented. Control (DMSO) is the basal level.

and CCK receptor antagonists increased protein tyrosine phosphorylation and influx of HCO_3^- by inhibiting CCK1 and CCK2 receptors.

CCK8 and CCK receptor antagonists can activate sAC/cAMP/ PKA pathway by inducing the influx of HCO_3^- in human sperm

To investigate whether CCK8 and its receptor antagonists play functional roles in human sperm, we treated human sperm with CCK8 and CCK receptor antagonists and then determined the levels of protein tyrosine and PKA substrate phosphorylation and pH in human sperm. The results demonstrated that CCK8 and CCK1 or CCK2 receptor antagonist could stimulate the acceleration of protein tyrosine and PKA substrate phosphorylation (Fig. 9A and B) and the increase of intracellular pH in human sperm (Fig. 9C). These data suggested that

CCK8 and CCK receptor antagonists could activate sAC/cAMP/PKA pathway by inducing the influx of HCO_3^- in human sperm.

Discussion

This study provides the first characterization of CCK1 and CCK2 receptors and their role in the mature sperm of mice and humans. Based on the localization of CCK in the spermatogenic cells, together with the presence of CCK receptors on oocytes, previous researchers raised the possibility that CCK may be of importance in the fertilization process by its release from sperm and then action on the oocyte (Moriarty *et al.* 1988, Persson *et al.* 1988, Peltö-Huikko *et al.* 1989). Our present results show that CCK and its receptors all locate on the acrosome region of mature sperm, and they are involved in the regulation of sperm capacitation-associated tyrosine phosphorylation. These results suggest that CCK and its receptors can regulate specific functions in mature sperm.

CCK1 and CCK2 receptors are all heavily glycosylated. Up to now, scarcely any reports showed that the native size of CCK1 or CCK2 receptor is identical to the predicted size. The deduced sequence of the rat CCK1 receptor corresponds to a 429-amino-acid protein with a calculated molecular mass of 48 kDa, but the purified CCK1 receptor from rat pancreas have a molecular mass of 85–95 kDa (Wank *et al.* 1992). The rat and canine CCK2 receptors are 452 and 453 amino acids long respectively, but their molecular masses are identified to be 74–78 kDa (Svoboda *et al.* 1982, Noble *et al.* 1999). The molecular mass of CCK2 receptor in human tissue is approximate 80 kDa (Kulaksiz *et al.* 2000). By sequence analysis at <http://www.uniprot.org/uniprot/O08786> for CCK1 receptor and <http://www.uniprot.org/uniprot/P56481> for CCK2 receptor. We found that mouse CCK receptor sequence contained three sites (CCK1 receptor at position 10, 24 and 190; and CCK2 receptor at position 7, 30 and 36) for N-linked glycosylation. These sites are the same as the glycosylation sites of other species CCK receptor, so it is reasonable that the molecular mass of mouse CCK1 or CCK2 receptor was all larger than that of the deduced size.

The specificity and toxicity should be considered with great caution when some inhibitors are used to evaluate the function of proteins or channels. Sperm are specialized cells and have a highly condensed nucleus. Generally, somatic cells are more sensitive than sperm to the same stimulation, so the different concentrations of inhibitors are required to modulate somatic cell and sperm functions. The IC_{50} values of H89 (PKA inhibitor) is 80 nM, but its working concentration for mouse sperm was 10–100 μM (Visconti *et al.* 1995b). Even for sperm, the dosage of a certain inhibitor is different between the species. The IC_{50} of the SFK (Src family kinase) inhibitor SK1606 in human sperm was five times lower than the

one observed in mouse sperm (Krapf *et al.* 2010, Battistone *et al.* 2013). Our present results showed that 5 μM CCK1 or CCK2 receptor antagonist could stimulate the increase of tyrosine phosphorylation (Fig. 2A). We further found that 0.1 μM CCK1 or CCK2 receptor antagonist could also moderately exert these actions (Supplementary Figure 1, see section on supplementary data given at the end of this article). However, CCK1 or CCK2 receptor antagonist at the dosage of 5 μM could moderately but not significantly increase the level of intracellular pH (Fig. 6A). Thus, we investigated the effects of CCK receptor antagonists on sperm functions at the relatively higher concentrations within the effectual doses.

The protein tyrosine phosphorylation is one of the most important events of sperm capacitation (Visconti & Kopf 1998, Roberts *et al.* 2003, Zeng & Tulsiani 2003, Baker *et al.* 2004). Our study indicated that the antagonists of CCK receptors and CCK8 could accelerate the capacitation-associated tyrosine phosphorylation

of sperm. Moreover, this regulation was found to be dependent on sAC and PKA, as well as extracellular BSA and HCO_3^- , consistent with the activation of the sAC/cAMP/PKA pathway.

Both the CCK1 and CCK2 receptors belong to the family of seven transmembrane domain G-protein coupled receptors which activate the signal transduction cascade of phospholipase C, with the formation of the second messengers, inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol, leading to the release of intracellular Ca^{2+} (Wank 1995, Dunlop 1998). More and more evidence indicated that CCK and native or recombinant CCK1 and CCK2 receptors were coupled to the mobilization of intracellular Ca^{2+} (Matozaki *et al.* 1988, 1990, Lee *et al.* 1993, Dunlop *et al.* 1996, 1997, Shimazoe *et al.* 2008). The antagonist of CCK1 or CCK2 receptor could significantly affect physiological functions of different cell types by modulating intracellular Ca^{2+} concentration (Muller *et al.* 1997, Dunlop *et al.* 1998, Simasko *et al.* 2002, Yang *et al.* 2007). Ca^{2+} is essential for the activation of the sAC/cAMP/PKA pathway in sperm. The time-dependent increase in sperm capacitation-associated tyrosine phosphorylation is

Figure 7 CCK8 can regulate sAC/cAMP/PKA pathway by increasing intracellular pH via the influx of HCO_3^- . (A) Dose-dependent effect of CCK8 on sperm protein tyrosine phosphorylation. Sperm were incubated with CCK8 (0, 2.5, 10, 25, 50 and 100 μM) for 60 min. Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Spermatozoa were treated with the CCK8 (25 μM) for 5 and 30 min respectively. PKA substrate phosphorylation (α -pPKAs) was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (C) Spermatozoa were treated with the CCK8 (25 μM) for 60 min in the absence or presence of 20 μM of the protein kinase A (PKA) inhibitor H89. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (D) Spermatozoa were treated with the CCK8 (25 μM) for 60 min in the absence or presence of 50 μM KH7. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (E) Sperm were treated with the CCK8 (25 μM) for 60 min in the absence or presence of 3 mg/ml BSA. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (F) Sperm were treated with the CCK8 (25 μM) for 60 min in the absence or presence of 25 mM HCO_3^- . Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (G) Sperm were treated with the CCK8 (25 μM) for 60 min in the absence or presence of 1 mM Ca^{2+} . Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (H) Effect of CCK8 (25 μM) on sperm intracellular pH (pHi). The arrow indicates the time points at which the antagonists were added. A representative of four experiments is presented.

Figure 8 CCK8 exerts its action by inhibiting CCK1 and CCK2 receptors. (A) The effects of CCK8, inhibitors and antibodies of CCK1 and CCK2 receptors on sperm protein tyrosine phosphorylation. Sperm were incubated with CCK8 (25 μ M), CCK1 antibody (20 μ g/ml) or CCK2 antibody (20 μ g/ml), and CCK1 antibody (20 μ g/ml), CCK2 antibody (20 μ g/ml), CR 1409 (CCK1-in, 100 μ M), PD135158 (CCK2-in, 50 μ M), CR 1409 (CCK1-in, 0.25 μ M) and PD135158 (CCK2-in, 0.25 μ M) in the presence of CCK8 (25 μ M) for 60 min. Protein tyrosine phosphorylation was assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Sperm were incubated with CCK8 (25 μ M), CCK1 antibody (20 μ g/ml) or CCK2 antibody (20 μ g/ml), and CCK1 antibody (20 μ g/ml), CCK2 antibody (20 μ g/ml), CR 1409 (CCK1-in, 100 μ M), PD135158 (CCK2-in, 50 μ M), CR 1409 (CCK1-in, 0.25 μ M) and PD135158 (CCK2-in, 0.25 μ M) in the presence of CCK8 (25 μ M) for 60 min, and then the intracellular pH (pHi) level in the sperm was examined. Results are expressed as the means $\pm$ S.E.M. ($n=6$). * $P<0.05$, ** $P<0.01$, as compared with the corresponding controls (0) (unpaired t -test).

dependent on the increase of intracellular Ca^{2+} (Visconti *et al.* 1995a, Zeng & Tulsiani 2003). Surprisingly, our data showed that the antagonist of CCK1 or CCK2 receptor, as well as CCK8, still accelerated the capacitation-associated tyrosine phosphorylation when extracellular and intracellular Ca^{2+} in the sperm were abolished. Therefore, it is reasonable to conclude that the activation of sAC/cAMP/PKA-mediated phosphotyrosine pathway in sperm by CCK8 and its receptor antagonists were not coupled to the extracellular and intracellular Ca^{2+} mobilization.

Emerging evidence indicated that CCK and its receptors could also be involved in regulating the transport of HCO_3^- (Sjoblom *et al.* 2013). Some evidence indicated that CCK was able to stimulate HCO_3^- secretion in a dose-dependent fashion, and this stimulatory effect of CCK was blocked completely by the CCK receptor antagonists (Yamazaki *et al.* 1996, Szalmay *et al.* 2001, Sjoblom *et al.* 2013). Here, we showed for the first time that CCK and its receptors could affect HCO_3^- uptake in sperm, namely CCK and its receptor antagonists could accelerate the influx of HCO_3^- in medium into sperm. HCO_3^- is another key factor for sperm protein tyrosine phosphorylation. Mammalian sperm are enriched in the atypical sAC, which is not regulated by G proteins but rather by HCO_3^- (Buck *et al.* 1999, Chen *et al.* 2000). sAC activation resulted in the stimulation of PKA and the enhancement of the tyrosine phosphorylation. Although it is well established that HCO_3^- is essential for sperm capacitation, including tyrosine phosphorylation, there is no consensus as to how intracellular levels of this anion were regulated

Figure 9 CCK8 and CCK receptor antagonists can activate sAC/cAMP/PKA pathway by increasing intracellular pH via the influx of HCO_3^- in human sperm. (A) Sperm were treated with CCK8 (25 μ M), CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 10 and 30 min in BWV medium. Protein tyrosine phosphorylation was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (B) Sperm were treated with CCK8 (25 μ M), CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) for 10 and 30 min in BWV medium. PKA substrate phosphorylation (α -pPKAs) was then assessed by western blot analysis. α -Tubulin was used as the loading control. The western blot is a representative of three independent experiments. (C) Effects of CCK8 (25 μ M), CCK receptor antagonists CR 1409 (CCK1-in, 100 μ M) and PD135158 (CCK2-in, 50 μ M) on human sperm intracellular pH (pHi). The arrow indicates the time points at which the antagonists were added. A representative of three independent experiments is presented.

(Florman *et al.* 2007). There is good evidence that the cystic fibrosis transmembrane conductance regulator (CFTR), initially described as a Cl^- channel, may be involved in the transport of sperm HCO_3^- (Xu *et al.* 2007). However, CFTR inhibitors were found to be unable to block the bicarbonate-dependent increase in tyrosine phosphorylation (Hernandez-Gonzalez *et al.* 2007). Some evidence has also suggested the involvement of the $\text{Na}^+/\text{HCO}_3^-$ co-transporter and the anion transporter SLC26A3/A6 in the importation of HCO_3^- into sperm (Parkkila *et al.* 1993, Demarco *et al.* 2003). Our present study demonstrated the involvement of CCK and its receptor in regulation of sperm capacitation-associated tyrosine phosphorylation via the transport of HCO_3^- in sperm; however, the detailed transport mechanism underlying the CCK receptor-regulated HCO_3^- uptake in sperm remains to be investigated.

An interesting finding from our present study was that CCK8 had the same action on sperm as the antagonists of CCK receptors. CCK dose-response studies on pancreatic acini typically revealed a biphasic dose-response relationship, namely stimulation at low CCK concentrations and inhibition at supramaximal concentrations (Wank 1995). In our present work, the CCK1 or CCK2 receptor antibody could accelerate the sperm protein tyrosine phosphorylation and elevate the level of intracellular HCO_3^- in sperm. The high and low dose of inhibitors also showed similar response. This suggests that CCK8 exerts the function by inhibiting its receptors in mature sperm. The endogenous CCK8 has been shown to be a substrate of TPPII, and TPPII is responsible for the major inactivation pathway of endogenous CCK8 (Rose *et al.* 1996). Our previous data demonstrated that TPPII protein was located in the region of mature sperm acrosome. TPPII antagonists could accelerate sperm capacitation-associated protein tyrosine phosphorylation. Furthermore, this effect of TPPII antagonists on tyrosine phosphorylation was also dependent on the presence of HCO_3^- in the medium (Zhou *et al.* 2013). This was in agreement with that CCK8 effect on sperm depends on HCO_3^- . This also implies the possibility that TPPII functions in association with CCK8 inactivation in sperm. However, further investigation is required to verify whether TPPII actually degrades CCK8 in sperm.

In conclusion, the present study has demonstrated for the first time the involvement of CCK and its receptors in the regulation of intracellular HCO_3^- levels, thereby eliciting the sAC/cAMP/PKA-mediated capacitation-associated protein tyrosine phosphorylation pathway. The present findings have revealed an intrinsic mechanism involving sperm CCK and its receptors in regulating sperm capacitation and thus fertilization.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0138>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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