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Transient expression of the cholecystokinin gene in male germ cells and accumulation of the peptide in the acrosomal granule: Possible role of cholecystokinin in fertilization

(spermatogenesis/neuropeptides/sperm/egg activation)

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ABSTRACT **Expression of the gene encoding the neurotransmitter/neuromodulator cholecystokinin (CCK) was demonstrated in testis of several different species. Two testicular CCK mRNA transcripts of different sizes were detected, and studies on the ontogeny of CCK gene expression indicated that the gene was expressed in male germ cells. *In situ* hybridization revealed CCK mRNA-expressing cells in the peripheral parts of the seminiferous tubules. Biochemical identification showed that the majority of prepro-CCK products in the testis represented pro-CCK. Immunofluorescence studies revealed CCK-like peptides primarily in spermatocytes and spermatids of mouse, rat, and monkey. Immuno electron microscopy of monkey testis demonstrated CCK immunoreactivity in the acrosomal granule of spermatids. Hence, an interesting possibility is that CCK peptides can be released during the acrosome reaction and thus may be of importance in the fertilization process.**

Cholecystokinin (CCK) was first purified by Jorpes and Mutt (1, 2) as a 33-amino acid peptide (CCK-33) from pig gut. Several different forms of CCK have been described in central and peripheral tissues (3–6). The brain contains CCK-8 and several CCK desoctapeptides but no detectable CCK-33. In the gut, however, intact CCK-33, CCK-39, and CCK-58 have been found together with the forms also seen in the brain (3–6). CCK-8 has been detected in many regions of the vertebrate nervous system (7–14) and fulfills several of the criteria of a neurotransmitter/neuromodulator (for review, see ref. 15), although the overall role of CCK in the brain is poorly understood.

Molecular cloning of cDNAs for both pig and rat CCK mRNA has established that all identified CCK peptides are derived by proteolytic processing from a 114- or 115 (rat)-amino acid precursor that is identical in brain and gut (16, 17). The various molecular forms of CCK in the brain and gut are therefore generated by tissue-specific posttranslational events. In accordance with this an ≈0.8-kilobase (kb) CCK mRNA has been demonstrated in rat brain and gut (16, 17).

In this study the distribution of CCK mRNA and CCK peptides was examined in several tissues, including the male reproductive system. CCK immunoreactivity was detected in spermatogenic cells, and its occurrence in the acrosome of male germ cells indicates a possible role of CCK in the fertilization process.

MATERIALS AND METHODS

RNA Preparations and Blot Analysis. Total and poly(A)$^+$ RNAs (18) were prepared from the indicated tissues, electrophoresed in 1% agarose gels containing 0.7% formaldehyde, and transferred to nitrocellulose filters (19, 20). The filters were hybridized to a 540-base-pair rat CCK cDNA insert (16) labeled with [α-^{32}P]dCTP by nick-translation to a specific activity of ≈5 × 10^8 cpm/μg. Filters containing RNA prepared from rat tissues were washed at high stringency (0.1 × SSC/0.1% SDS, 54°C; 1 × SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0), whereas filters with RNA prepared from other species were washed at reduced stringency (0.2 × SSC/0.1% SDS, 45°C). The filters were exposed to Kodak XAR films with DuPont intensifying screens.

***In Situ* Hybridization.** Eight-week-old BALB/c mice were anesthetized and perfused with 10% formalin containing picric acid (21). Cryostat sections (14 μm) were hybridized to a 44-mer oligonucleotide complementary to rat and human CCK mRNA and to mouse and pig CCK mRNA with one and two mismatches, respectively, as well as to several unrelated control oligonucleotides as previously described (22). The oligonucleotide probes were labeled at their 3′ ends with [α-[^{35}S]thio]dATP, by using terminal deoxynucleotidyltransferase (International Biotechnologies), to a specific activity of ≈7 × 10^8 cpm/μg. After hybridization and washing, the sections were first exposed to x-ray film for an initial assessment of the results. To allow analysis at a higher resolution NTB2 (Kodak) nuclear track emulsion was applied, and finally the sections were stained with cresyl violet.

Biochemical Analysis of CCK Peptides. Testis and epididymis from adult rats were immersed in boiling water for 20 min, homogenized, and centrifuged at 10,000 × *g* for 30 min. The supernatant (the neutral water extract) was stored at −20°C. The pellet was reextracted in 0.5 M acetic acid, rehomogenized, and centrifuged, and the supernatant (the acid extract) was stored at −20°C. The extracts were assayed with sequence-specific CCK radioimmunoassays before and after incubation with trypsin and carboxypeptidase B (23). To measure CCK precursors extended beyond the glycine residue in the amidation site (Gly-Arg-Arg, residues 104–106 of rat prepro-CCK), tissue extracts and chromatographic fractions were incubated sequentially with trypsin and carboxypeptidase B. Tyrosine sulfation of CCK was examined by measurement with antiserum (no. 4698) requiring tyrosine O-sulfation of CCK peptides as described (9). The sequence specificity of the antisera used in this study is as follows: antiserum 1561 binds residues 86–90, antiserum 4698 binds residues 95–99, antiserum 2609 binds residues 99–103, and antiserum 3208 binds residues 99–104 of human prepro-CCK. Antiserum 4698 is dependent on O-sulfation of tyrosine-97, antiserum 2609 requires amidation of phenylalanine-103, and antiserum 3208 requires a free carboxyl group on glycine-104 of prepro-CCK. Hence, antiserum 2609 binds only bioactive

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Abbreviation: CCK, cholecystokinin.

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products of prepro-CCK. In order to further control the specificity of the measurements, a radioimmunoassay using antiserum 2604, which is specific for the CCK-related hormone gastrin, was also used.

Neutral and acid extracts were applied separately or together to Sephadex G-50 superfine columns before and after trypsin and carboxypeptidase B treatment. Elution was with 0.02 M barbital buffer (pH 8.4), and columns were calibrated with extracts of jejunal mucosa and cerebral cortex, with sulfated porcine tritriacontapeptide CCK-33 (a gift from V. Mutt, The Karolinska Institute), with sulfated and nonsulfated CCK octapeptides, and with glycine-extended CCK-8 (custom synthesized by Cambridge Research Biochemicals, Cambridge, U.K.). Void and total volumes of the columns were determined with ^{125}I-labeled albumin and ^{22}NaCl. Tryspin-treated extracts were also subjected to reverse-phase HPLC using a Nucleosil C_{18} column with a gradient elution [10–80% (vol/vol) B in A; solvent A, 0.1% trifluoroacetic acid in water; solvent B, is 0.045% trifluoroacetic acid in ethanol].

Immunohistochemistry. Animals were perfused with picric acid-containing formalin in phosphate buffer (21). After rinsing, 14-μm sections were cut on a cryostat and processed for indirect immunofluorescence histochemistry (24) using a rabbit CCK antiserum (ref. 25; dilution, 1:400). Antiserum preabsorbed with 1 μM CCK-8 served as control serum.

Immuno Electron Microscopy. Pieces of monkey testis were immersion-fixed in a mixture of formalin (10%) and glutaraldehyde (0.2%) in phosphate buffer. After cryoprotection with 30% sucrose, 50-μm frozen sections were cut on a Vibratome R and stained free-floating by the pre-embedding modification (26) of the peroxidase-antiperoxidase method (27). Endogenous peroxidase activity was blocked by incubating the sections in 0.5% H_2O_2 in methanol for 30 min, and 0.2% Triton X-100 was applied for 15 min to enhance antibody penetration. Following incubation with 10% normal rabbit or goat serum the sections were incubated for 12–16 hr with mouse monoclonal CCK antibody (ref. 28; hybridoma supernatant dilution, 1:2000) and appropriate secondary antibodies. Peroxidase activity was visualized with 0.03% diaminobenzidine and 0.004% H_2O_2 in phosphate-buffered saline. Sections were postfixed with aqueous 2% OsO_4, dehydrated through graded ethanol solutions, and embedded in Agar 100 resin. Semithin (10-μm) sections were cut from blocks and attached to glass slides by warming, and a capsule filled with Agar 100 was inverted on the sections. Ultrathin sections were cut with an OMU3 ultramicrotome (Reichert–Jung) and collected on 100-mesh copper grids, contrasted with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

RESULTS

Tissue Distribution of CCK mRNA. High levels of a 0.8-kb CCK mRNA were found in rat duodenum and brain (Fig. 1*a*). In addition, the testis also contained a 0.8-kb CCK mRNA. The level of CCK mRNA in the testis was ≈10% of the level in the brain. The rat epididymis also contained CCK mRNA, at a level ≈30% of the level found in the testis (data not shown). No CCK mRNA was detected in the other tissues analyzed, including ovaries taken from rats in various stages of the estrous cycle.

Expression of CCK mRNA in Testis of Various Species. CCK mRNA was detected in the testis of the five species studied (Fig. 1*b*). In addition to the 0.8-kb CCK mRNA, a 1.5-kb CCK mRNA was also seen in the testis of all species. The relative abundance of the two testicular CCK mRNAs varied between different species. In rat and monkey the 0.8-kb CCK mRNA predominated, whereas both mRNAs were present at roughly equal levels in mouse and guinea pig testis. In the pig testis, on the other hand, the 1.5-kb mRNA was more abundant than the 0.8-kb mRNA.

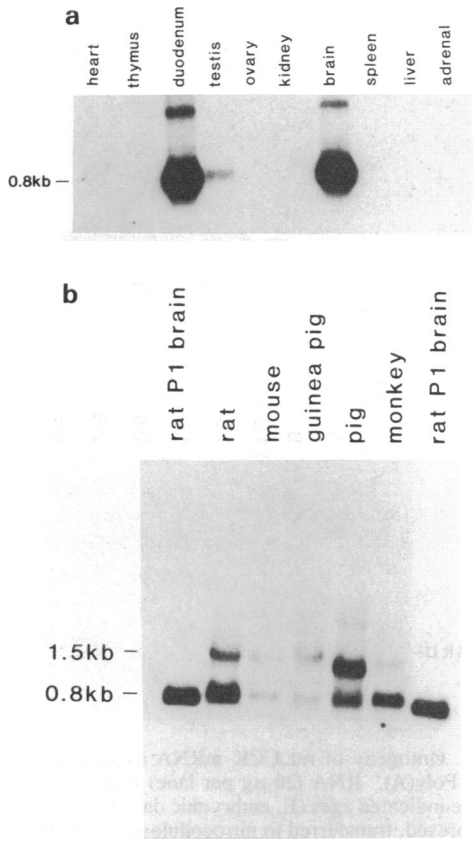

FIG. 1. Testicular expression of CCK mRNA. (*a*) Poly(A)$^+$ RNA (20 μg per lane) prepared from the indicated tissues of adult Sprague–Dawley rats was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde. The RNA was transferred to a nitrocellulose filter and hybridized to a nick-translated CCK cDNA insert. The filter was washed at high stringency and then exposed to x-ray film. (*b*) Poly(A)$^+$ RNA (20 μg) prepared from testis of the indicated species as well as from total brain of 1-day-old rats (5 μg) was fractionated by electrophoresis, transferred to nitrocellulose, and hybridized to the CCK cDNA probe. The filter was washed at reduced stringency and exposed to x-ray film. P1, postnatal day 1.

Ontogeny of CCK mRNA Expression in Rat Brain and Testis. CCK mRNA was already detectable in rat brain by embryonic day 16, and the level increased around birth (Fig. 2*a*). At 2 weeks of age, the level of CCK mRNA in the brain was similar to the level found in the adult rat brain. No testicular CCK mRNA was detected during the early postnatal development of rat testis (days 5–16, Fig. 2*b*). Expression of CCK mRNA in rat testis was first detected at day 19, and the peak level was observed at day 28.

Localization of CCK mRNA-Producing Cells in the Mouse Testis by *in Situ* Hybridization. In the seminiferous tubules of mouse testis, dense labeling was found overlaying the basal part of the tubules, where spermatogonia and spermatocytes as well as Sertoli cells are found (Fig. 3). Interestingly, some tubules lacked labeling altogether. No labeling of any cells in the mouse testis was seen when a similar-length oligonucleotide complementary to neuropeptide Y was used (data not shown).

Identification of CCK Peptides in Rat Testis and Epididymis. Low levels of α-carboxyamidated CCK peptides were observed in rat testis and epididymis (Table 1). The amounts of α-carboxyamidated CCK peptides were <1% of the amounts found in extracts from the duodenal mucosa or cerebral cortex. The majority of the CCK peptides in both testis and

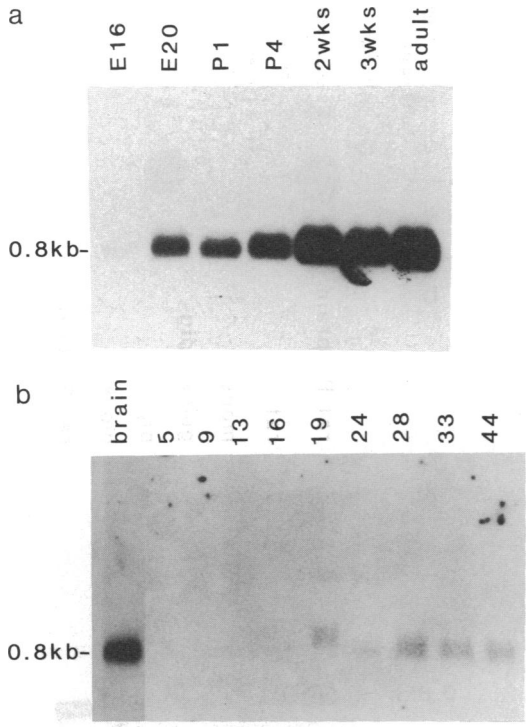

FIG. 2. Ontogeny of rat CCK mRNA expression in brain and testis. (*a*) Poly(A)$^+$ RNA (20 μg per lane) prepared from total rat brain at the indicated ages (E, embryonic day; P, postnatal day) was electrophoresed, transferred to nitrocellulose, and hybridized to the rat CCK cDNA probe. (*b*) Total RNA (40 μg per lane, except the 24-day sample, which contained 20 μg) prepared from the testis of rats at the indicated days of age was analyzed. Lane at left contained 20 μg of poly(A)$^+$ RNA from adult rat brain. Autoradiograms were exposed for 1 day (*a* and left lane in *b*) or 1 week (rest of *b*).

epididymis were nonsulfated, but substantial amounts of glycine-extended CCK intermediates and larger molecular

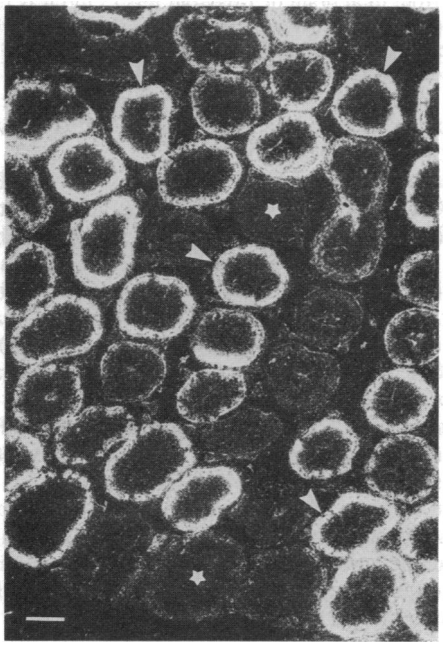

FIG. 3. Emulsion autoradiograph of sections through mouse testis after *in situ* hybridization. Hybridization probe was an ^{35}S-labeled oligonucleotide complementary to CCK mRNA. Dense labeling can be seen over the basal one-third of most seminiferous tubules (arrowheads). Note the absence of labeling in some tubules (stars). (Bar = 50 μm.)

Table 1. CCK and its precursors in adult rat tissues

Tissue	Concentration, pmol/g of tissue		
	Amidated CCK	Glycine-extended intermediates	Pro-CCK
Testis*	0.2 ± 0.1	1.8 ± 0.7	7.2 ± 2.6
Epididymis*	0.5 ± 0.1	2.6 ± 0.9	2.2 ± 0.8
Duodenal mucosa†	58.1 ± 12.6	7.9 ± 2.2	1.1 ± 0.4
Cerebral cortex†	120.4 ± 16.9	25.1 ± 4.7	1.5 ± 0.7

Concentrations of CCK peptides (mean ± SEM, n = 6) in neutral and acid extracts were measured as described (9, 23).
*Less than 25% of CCK peptides are O-sulfated on tyrosine.
†More than 98% of CCK peptides are O-sulfated on tyrosine.

forms containing the intact CCK-8 sequence were found. In the testis, these precursor forms were 10–40 times more abundant than the α-carboxyamidated peptides, and the amounts of pro-CCK peptides were 7 times higher in the testis than in brain and gut. In the epididymis, glycine-extended intermediates predominated. The various CCK peptides present in the testis and epididymis (Table 1) were characterized by gel filtration and reverse-phase HPLC monitored by the sequence-specific radioimmunoassays (data not shown).

Immunohistochemical Localization of CCK Immunoreactivity in Testis of Different Species. In adult mouse, rat, and monkey testis, dot-like CCK-positive structures were observed in the seminiferous tubules, predominantly at a position corresponding to the localization of spermatocytes and spermatids (Fig. 4 *a*, *b*, *d*, and *e*). Different seminiferous tubule profiles showed varying numbers of CCK-positive structures with different intensities, and some tubule profiles seemed to lack CCK immunoreactivity (Fig. 4*a*). The most prominent immunoreactivity was seen in the monkey testis, where the positive structures exhibited somewhat different morphology, ranging from small round structures to large irregular structures, the latter located centrally in the tubules (Fig. 4*e*). In the rat testis, CCK-positive structures were less abundant and were irregularly distributed, forming small patches (Fig. 4*d*). Leydig cells in interstitial tissue, spermatogonia, and Sertoli cells present in the tubules close to the basement membrane were not immunoreactive. Preabsorption of the CCK antiserum with synthetic CCK-8 abolished the immunoreactivity (Fig. 4*c*).

Immuno Electron Microscopic Localization of CCK in Monkey Spermatogenic Cells. CCK immunoreactivity in the monkey testis was found in the acrosomal granule of spermatids (Fig. 5). No certain staining was observed in the acrosomal vesicle or the cytoplasm of spermatids or in spermatogonia. No labeling of acrosomal granules was seen after incubation with control serum (data not shown).

DISCUSSION

The present study demonstrates expression of CCK mRNA and peptides in the testis of several mammalian species as analyzed by RNA blot analysis, *in situ* hybridization, peptide characterization of tissue extracts, and immunohistochemistry. The results extend our recent detection of CCK immunoreactivity in the seminiferous tubules of the mouse testis (29) and document a transient expression of CCK mRNA in male germ cells, with accumulation of CCK peptide in the acrosomal granule of spermatids and possibly also in spermatozoa. The maximal expression of CCK mRNA in testis of prepubertal rat coincides with the occurrence of increasing numbers of spermatocytes in the seminiferous tubules (30, 31). Further evidence for this was obtained by *in situ* hybridization, which showed intense labeling over the basal part of the seminiferous tubules. *In situ* hybridization and immunohistochemical analyses revealed CCK mRNA and CCK

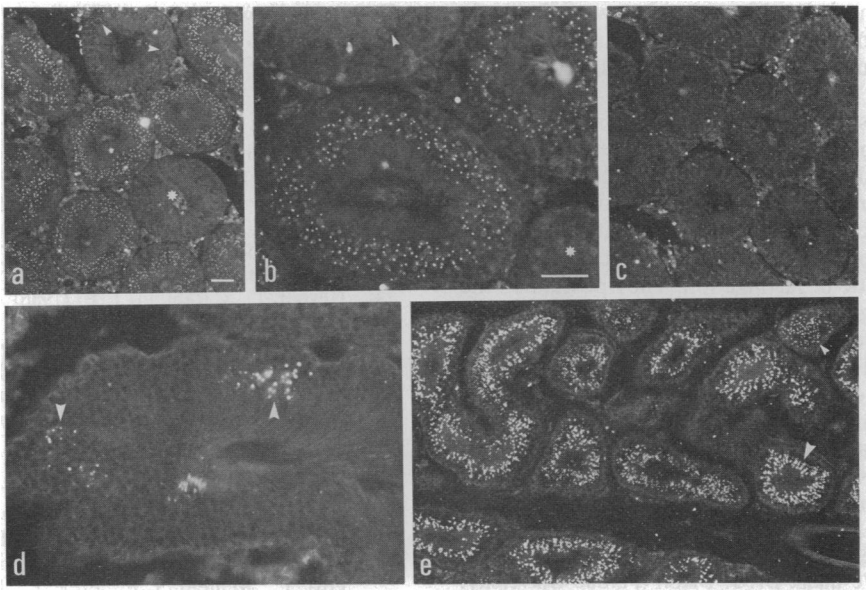

FIG. 4. Immunofluorescence micrographs of mouse (*a–c*), rat (*d*), and monkey (*e*) testis after incubation with CCK antiserum (*a, b, d,* and *e*) or control serum (*c*). In all three species dot-like structures containing CCK immunoreactivity can be observed in the tubules. (*a* and *b*) Some tubule profiles (asterisk) seem to lack CCK immunoreactivity, and others show only weak reaction (arrowheads). (*c*) No positive structures are seen after incubation with control antiserum. (*d*) In the rat, the CCK immunoreactivity (arrowhead) is irregularly distributed. (*e*) Very strong CCK immunoreactivity is seen in the monkey, sometimes as fine dots (small arrowhead), but mostly as larger, more irregular puncta (big arrowhead). (Bars = 50 μm; magnifications are the same in *a, c,* and *e* and in *b* and *d*.)

immunoreactivity respectively, predominantly during certain phases of spermatogenesis, whereby CCK mRNA was found in earlier stages than CCK immunoreactivity. In addition, the number of cells containing CCK mRNA and peptide, and their level of mRNA and peptide, varied between different tubules. Thus, the combined results of *in situ* hybridization and immunohistochemistry suggest expression of CCK during spermatogenesis with CCK mRNA in spermatogonia/spermatocytes and accumulation of peptide at a somewhat later stage, perhaps corresponding to the appearance of spermatids. The presence of CCK peptide in spermatids was confirmed by immuno electron microscopy, which showed the presence of CCK immunoreactivity in the acrosomal granule.

The majority of the CCK peptides in rat testis and epididymis corresponded to nonsulfated, larger pro-CCK forms. Only sulfated and carboxyamidated CCK peptides have so far been shown to cause gallbladder contraction and to stimulate secretion of pancreatic enzymes (32). In testis and epididymis, the previously identified biologically active form represented 2.2% and 10.4%, respectively, of the CCK peptides in these tissues. In epididymis, the processing of the CCK precursor peptides appeared further progressed than in the testis, suggesting a higher demand for biologically active CCK peptides in the distal part of the genital tract.

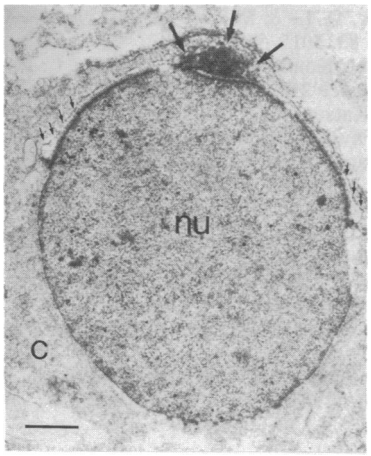

FIG. 5. Electron micrograph of CCK immunoreactivity in monkey spermatid. CCK immunoreactivity is localized to the acrosomal granule (large arrows). The acrosomal vesicle (small arrows) and the cytoplasm (c) are not stained. nu, Nucleus. (Bar = 1 μm.)

Several studies have suggested that opioid peptides and peptides derived from proopiomelanocortin play a role in spermatogenesis. The mRNAs for each of the protein precursors of these peptides have been identified in the testis of several species (33–40). Proopiomelanocortin mRNA and peptides are predominantly found in interstitial Leydig cells, where they have been shown to suppress Sertoli-cell growth and to modulate testosterone secretion by Leydig cells (41). The opioid peptide precursor proenkephalin A, on the other hand, is predominantly expressed in developing germ cells, and proenkephalin-derived peptides have been suggested to function as hormones or autocrine/paracrine factors for spermatogenesis (42). Systemic administration of CCK has shown that the peptide can act as an opiate antagonist (43, 44). Whether or not testicular CCK peptides may influence the putative testicular function of opioid peptides remains to be elucidated.

Our data show that CCK, a neuroendocrine peptide, is localized in the acrosomal granule of spermatogenic cells. The acrosomal granule contains enzymes of lysosomal nature as well as the trypsin-like proteolytic enzyme acrosin (45). The enzyme-rich contents of the acrosome are released during the acrosome reaction, which is believed to facilitate sperm entry into the egg (45). The spermatozoon remains actively motile during its penetration, and a role of CCK could therefore be related to its known action in stimulating intestinal motility and contraction of the gallbladder (46). Hence, if CCK is released during the acrosome reaction, this strong contractive peptide may facilitate sperm motility during penetration.

However, of more interest in relation to our findings are the results recently reported by Moriarty *et al.* (47), who showed that application of 2 μM CCK to *Xenopus laevis* oocytes

induced a depolarizing inward current that was similar in shape, time course, and reversal potential to the Cl^- current produced by intracellular injection of inositol 1,4,5-trisphosphate ($InsP_3$). Thus, CCK receptors linked to the $InsP_3$ second-messenger system appear to be present on oocytes. In fact, fertilization appears to activate a guanine nucleotide-binding (G) protein, leading to $InsP_3$ production (48, 49). Furthermore, Kline *et al.* (50) recently showed that application of serotonin or acetylcholine to oocytes, into which the corresponding receptors had been introduced, caused several responses characteristic of fertilization. They postulated the presence of an endogenous receptor in the egg membrane activated by sperm to initiate fertilization. Our finding of CCK in the acrosomal granule of sperm, raises the possibility that CCK may be one of the components that activate the oocyte for fertilization through interaction with CCK receptors on the oocyte. Validation of this hypothesis will require demonstration of CCK peptides in sperm after ejaculation as well as its release from sperm.

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