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SALMON CALCITONIN INHIBITS HUMAN SPERM MOTILITY IN VITRO

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We have evaluated by a stroboscopic technique the in vitro effect of salmon calcitonin and human calcitonin on the motility of human migrated spermatozoa. We report here that human calcitonin is uneffective while salmon calcitonin is a potent inhibitor of the sperm motility. This salmon calcitonin action is abolished by the preincubation of the peptide with an anti-salmon calcitonin antiserum, demonstrating the specificity of the effect. In addition, we provide evidence that the release of intracellular calcium represents a necessary step for the action of the peptide. In fact, the salmon calcitonin effect is prevented in a dose-dependent way by dantrolene sodium which inhibits the release of calcium from intracellular stores while the calcium channel blocker verapamil is unefficacious. These results suggest a potential role for calcitonin in regulating human sperm motility.

It has been reported that CaM is a prominent costituent of mammalian spermatozoa (1) and that CaM antagonists inhibit sperm motility (2). From recent in vitro experiments it was concluded that sCT but not hCT is a potent CaM antagonist (3). Moreover, very high levels of immunoreactive CT have been detected in human semen (4) and an immunoreactive sCT like peptide has been found to coexist with hCT both in brain tissue (5) and in peripheral plasma (6) in man. The above observations have prompted us to investigate the effect of sCT and hCT on the motility of human spermatozoa. In the current study, we demonstrate that sCT is a potent and specific inhibitor of the human sperm motility. In addition, we show that this sCT effect is exerted through mobilization of calcium from intracellular stores.

MATERIALS AND METHODS

Pure synthetic sCT and hCT were gifts of Dr. Ron Orlowsky (Revlon Health Care Group). Rabbit anti-sCT antiserum was from Dr. James R. Harness (Meloy

Abbreviations: CT=calcitonin; sCT=salmon calcitonin; hCT=human calcitonin; CaM=calmodulin; DaNa=dantrolene sodium; PBS=phosphate buffered saline.

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Laboratories, Inc.). DaNa was a kind gift of Dr. Jacopo Meldolesi (University of Milan, Italy). Verapamil was purchased from Sigma Chem. Co. (St. Louis, MO 63178, USA). All other chemicals used were of analytical grade.

Human ejaculates were collected by masturbation from normal healthy donors (age 20-35 years) after a 3 day period of sexual abstinence. Each ejaculate was collected in a sterile reservoir and allowed to liquefy at room termperature (22-25°C). Only normal semen samples with volumes between 2.0 and 4.0 ml, counts of at least 40 million cells/ml, 60% normal forms and 60% forward motility were used for these studies. 0.5 ml of the specimens were transferred in plastic test tubes and carefully layered with 0.5 ml of a PBS containing NaCl (140 mM), KCl (3.5 mM), $CaCl_2$ (0.18 mM), $MgCl_2$ (0.18 mM), Na_2HPO_4 (12 mM), NaH_2PO_4 (2.2 mM); the pH was 7.4. The preparations were incubated at room temperature in a vertical position for 25-30 minutes, during which only well moving cells swam up into the buffer phase. At the end of the incubation period the buffer phases containing the migrated spermatozoa were gently aspirated and pooled separately for each subject. The final sperm concentration was 10 ± 3 million/ml.

Aliquots of the sperm suspensions were placed in plastic test tubes and mixed with sCT or hCT diluted in PBS in the ratio 1:1. The final concentrations of the peptides ranged from 4×10^{-10}M to 4×10^{-5}M. Equal volume of PBS was added to the control tubes. After 2 minutes of incubation, one drop of each sample was placed on a siliconized microscope slide and observed by phase contrast microscopy with stroboscoic illumination at a magnification of x400.

To evaluate the specificity of sCT effect 4×10^{-5}M sCT was preincubated for 12 hours at 4°C with a rabbit anti-sCT antiserum in 1:6000 dilution, warmed to room temperature and added in the 1:1 ratio to the migrated spermatozoa. The same dilution of the antiserum alone added in the same ratio to the migrated spermatozoa served as control.

In another set of experiments the migrated spermatozoa were pretreated with 1.5 mM verapamil or with DaNa in concentrations ranging between 0.12 and 1 mM then sCT was added to the suspension and sperm motility was assessed after 2 minutes of incubation.

RESULTS AND DISCUSSION

Concentrations of sCT ranging from 4×10^{-9}M to 4×10^{-8}M induced a decrease in the percentage of motile cells and the appearance of cells showing abnormal swimming pattern such as curled tails and/or asymmetric beating of the flagella. At sCT concentrations of 4×10^{-7}M or above motility was inhibited completely, tails remained curled and the beating stopped; hCT had no effect (Fig. 1). Moreover, the preincubation of the sCT with the anti-sCT antiserum completely prevented the effect of the peptide on sperm motility and the antiserum alone did not affect the motile behavior of the cells (data not shown).

Recent observations have shown that calcium uptake by washed boar sperm suspensions is markedly stimulated by the CaM antagonists and that this

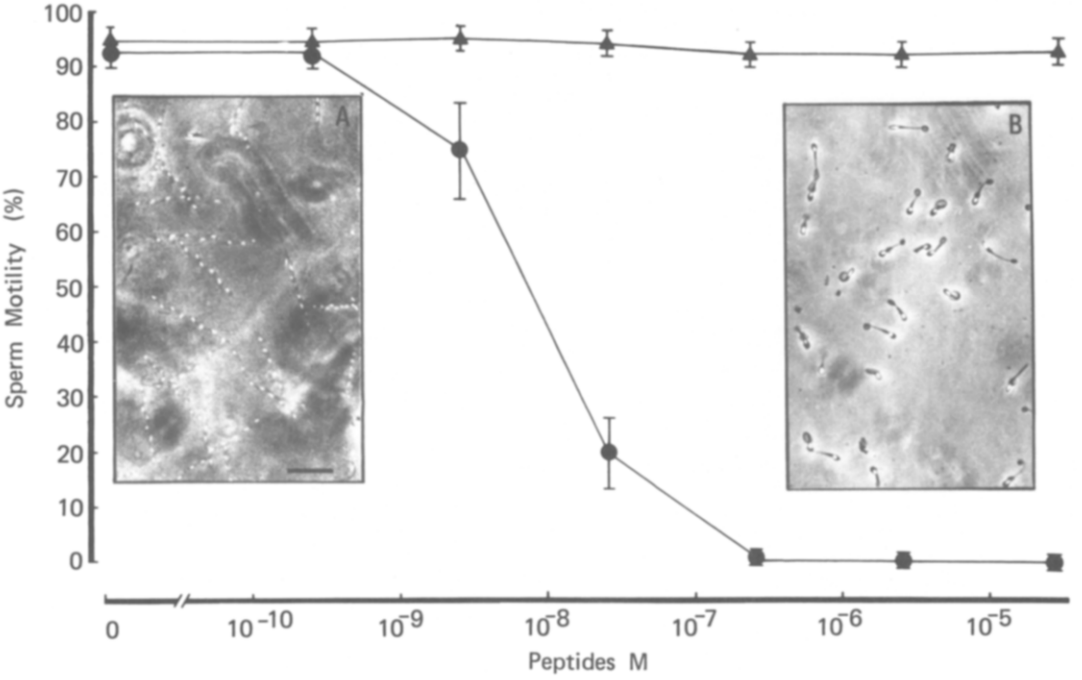

FIGURE 1: Percentage of motile sperm as a function of sCT (●) and hCT (▲) concentration. The spermatozoa were prepared as described in the text. Sperm motility was assessed two minutes after the addition of the peptides. Results are expressed as means ± S.E. of four different determinations each in triplicate. Insert A shows a phase-contrast stroboscopic micrograph of the typical sperm motility pattern of the controls; movement is evident. Insert B shows the effect of the higher sCT concentration on the motility of human migrated spermatozoa; no evidence of movement. Bar in A indicates 50 um.

effect is blocked by 1 mM verapamil (7). It is proposed that CaM is involved in the control of calcium entry in boar spermatozoa and in contrast to the uptake mechanism, ATP-dependent calcium extrusion does not appear to be regulated by CaM (7). We then investigated whether the ability of sCT to inhibit the sperm motility could be prevented by the calcium channel blocking agent verapamil (8). The verapamil alone was without any noticeable effect on sperm motility, as reported by others (9), and did not alter the immobilizing effect of sCT (Table 1a). Thus, we conclude that the effect of sCT on the motility of human migrated spermatozoa is not correlated to an increase of calcium uptake.

Several hormones and neurotransmitters raise they cytosolic free calcium concentration by mobilizing stored calcium (10). Recent advances have clari-

TABLE 1

Effect of sCT on the motility of human migrated spermatozoa treated with verapamil or DaNa.

	Pretreatment	Addition	Motility%
a			
	none	buffer	94 ± 2.4
	none	sCT 4 x 10^{-6}M	0
	verapamil 1.5 mM	buffer	93 ± 1.9
	verapamil 1.5 mM	sCT 4 x 10^{-6}M	0
b			
	none	sCT 4 x 10^{-6}M	0
	DaNa 1 mM	buffer	95.8 ± 2.1
	DaNa 1 mM	sCT 4 x 10^{-6}M	85.1 ± 8.2
	DaNa 0.5 mM	sCT 4 x 10^{-6}M	40.3 ± 4.6
	DaNa 0.25 mM	sCT 4 x 10^{-6}M	20.7 ± 3.8
	DaNa 0.12 mM	sCT 4 x 10^{-6}M	0

Human spermatozoa were prepared as stated in the text. Tubes containing sperm were incubated for ten minutes at room termperature with verapamil (a) or DaNa (b) dissolved in PBS to raise the final concentrations of the substance indicated. Two minutes after the addition of sCT or of the buffer, the percentage of sperm motility was assessed as previously described. Results are expressed as mean ± S.E. of four different determinations each in triplicate.

fied that the biochemical mechanisms triggered by the hormones that increase cytosolic calcium is their induction of phosphatidylinositol breakdown (11,12) and polyphosphoinositide hydrolysis as a consequence of receptor activation (13-16); in some tissues this provokes, within seconds, a marked accumulation of the water-soluble products, inositol-1,4-diphosphate and inositol-1,4,5-triphosphate (17-20), which might be necessary for calcium mobilization from internal pools to occur (19,20). To investigate the possibility that the sCT inhibitory effect observed was due to a release of calcium from intracellular stores we treated spermatozoa with DaNa which inhibits the calcium movements at intracellular storage sites (21,22). Our data show that DaNa alone does not modify the sperm motility while it is able to prevent in a dose-dependent way the sCT immobilizing effect (Table 1b). As a consequence it seems likely that sCT acts, at sperm level, by increasing calcium ion concentrations through a release of the ions from internal stores. Since it is well established that calcium, when elevated intracellularly, inhibits flagellar motility (23) an increase of intracellular calcium could justify the immobilizing effect of sCT. However, the data now available do

not allow us to explain the intimate mechanism through which calcium modulates the sperm movements.

The results described here demonstrate that sCT is a potent inhibitor of human sperm motility with an IC_{50} in the nanomolar range while hCT is uneffective. The intracellular calcium release seems to be a necessary step in sCT action. The mechanism by which sCT determines the rise of cytosolic calcium remains to be determined and until now we cannot ascribe a precise biological significance to the sCT immobilizing effect. However, the recent discovery of the coexistence of an immunoreactive sCT like peptide with hCT in human peripheral plasma (6) and brain tissue (5), where CT has been suggested as a possible transmitter or modulator (24-29), could add more physiological meaning to our findings. In fact, it has been demonstrated that human semen contains very high levels of neuroactive peptides like beta-endorphin (30), met-enkephalin, leu-enkephalin, substance P (31) and CT (4) suggesting for these substances important local regulatory functions. Thus, we are now evaluating the presence of a sCT like peptide in human seminal plasma and the involvement of phosphatidylinositol metabolites in the action of sCT at sperm level.

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