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Inhibition of spermine on calcium influx during capacitation of guinea pig spermatozoa *in vitro*[1]

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ABSTRACT To investigate the mechanism of action of spermine, we measured the intracellular calcium ($[Ca]_i$) of guinea pig spermatozoa using a probe of fluorescence, Quin 2. Results showed that spermine (0.25-2.0 mmol· L^{-1}) suppressed the membrane permeability to Ca^{2+} during capacitation, which was similar to that of verapamil (a Ca^{2+} channel blocker). Furthermore, the rapid increase of $[Ca]_i$ induced by calcimycin (A-23187) was inhibited by spermine and verapamil, whereas trifluoperazine (an inhibitor of calmodulin) had no effect on it. The inhibition of the acrosome reaction caused by verapamil (5-100 μmol· L^{-1}) or trifluoperazine (1-60 μmol· L^{-1}) was reversed by calcimycin and cAMP, respectively. In addition, there was no effect on the initiation of the acrosome reaction when verapamil was added to capacitated spermatozoa. This result was in agreement with that of spermine. When addition of spermine (0.5 mmol· L^{-1}) was combined with trifluoperazine (5 μmol· L^{-1}), the acrosome reaction decline almost to zero, indicating that spermine might inhibit Ca^{2+} - sensitive channel during sperm capacitation.

KEY WORDS spermine; calcium; sperm capacitation; acrosome; calcium channel blockers; calmodulin; guinea pigs; spermatozoa; calcimycin (A-23187)

In previous studies, we suggested that spermine might be a potential inhibitor of *in vitro* sperm capacitation and fertilization in hamster[1], guinea pig, and human[2]. But how such inhibiting ability develops during capacitation remains unclear. This study was to investigate whether spermine was involved in blocking Ca^{2+} influx via calcium channel, calmodulin-dependent manner or both.

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MATERIALS

DL-Sodium lactate, sodium pyruvate, bovine serum albumin (BSA), tetrahydrochloride spermine, Quin 2 / AM (the acetoxymethyl derivative of Quin 2), (±)-hydrochloride verapamil, hydrochloride trifluoperazine, calcimycin (calcium salt), trypan blue, and EGTA were purchased from Sigma Chemical Co. Triton X-100 was obtained from Rohm-Mass Co. Dimethyl sulfoxide (Me_2SO) was a gift from the Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. All other chemicals were of AR.

METHODS

Sperm preparation Spermatozoa were freshly obtained from guinea pig (BW 710± *s* 40 g) vasa deferens and caudae epididymides. To provide an actively motile cleaned-up samples of spermatozoa, the sperms were subjected to a sperm-rise procedure which allowed a concentrated sample of spermatozoa to swim-up into an overlay of medium for 15 min at 39℃ in 5% CO_2 with 95% air. The supernatant was transferred to vials and was adjusted to (1-5)× 10^7 cells· ml^{-1}. According to the test of trypan blue exclusion, the viability of spermatozoa was not affected when spermatozoa were loaded with Quin 2 / AM.

Medium The standard medium was Ca^{2+}-deficient minimal capacitation medium (CaD-MCM, pH 8.0) which could induce guinea pig sperm capacitation *in vitro*, but did not support the acrosome reaction as previously described[3]. After the addition of $CaCl_2$ 2.0 mmol· ml^{-1}, the majority of spermatozoa underwent the acrosome reaction within 20 min. The medium was sterilized through a millipore filter (0.2 μm) before use.

Determination of $[Ca]_i$ The method of Mahanes *et al*[4] was slightly modified in this study. Briefly, spermatozoa were loaded with Quin 2/AM prepared in Me_2SO to a sperm suspension containing (1–5) $\times 10^7$ cells·ml^{-1} of CaD–MCM. The final concentrations of Quin 2/AM and DMSO were 10 μmol·ml^{-1} and <0.2%, respectively. After a 10-min incubation with Quin 2/AM, the spermatozoa were diluted with fresh CaD–MCM (1 : 1, vol : vol) and incubated an additional 1 h prior to fluorescence measurement so as to allow the intracellular Quin 2/AM to be converted to Quin 2. By the end of 1 h, the spermatozoa were centrifugated at 250× *g* for 5 min and the resulting pellets were resuspended in fresh CaD–MCM lacking Quin 2/AM (5×10^7 cells·ml^{-1}). Fluorescence readings were made using Hitachi model MPF–4 spectrofluorometer equipped for warming (37℃). The excitation and emission wavelengths were 339 nm and 486–492 nm with slit of 5 nm and 20 nm, respectively. $[Ca]_i$ was calculated as Reference 4 while F_{max} was determined by adding 18 mg·L^{-1} triton X–100 to the cell suspension and F_{min} was subsequently determined by adding $MnCl_2$ 30 mmol·ml^{-1}. Fluorescence of extracellular Quin 2 was assessed by adding EGTA 0.5 mmol·L^{-1} and Tris–base 40 mmol·L^{-1} to the cell suspension. The immediate decrease in fluorescence caused by chelation of extracellular free calcium by EGTA was measured by the % of fluorescence signal due to extracellular Quin 2.

In vitro **capacitation** Spermatozoa were capacitated *in vitro* as described previously[3] using CaD–MCM containing BSA 3 mg·ml^{-1}. Capacitation was evidenced by a rapid synchronization of the ability of the acrosome reaction 15 min following the addition of Ca^{2+}.

RESULTS

Spermatozoa $[Ca]_i$ During *in vitro* capacitation, samples were prepared for fluorescence readings at intervals of incubation in CaD–MCM. At the end of 6 h, the acrosome-reacted spermatozoa ranged from 40%–50% and did not appear to be modified by Me_2SO. Results from 10 experiments showed that $[Ca]_i$ increased proportionally with the prolonged capacitation period ($r=0.986$). $[Ca]_i$ in fresh guinea pig spermatozoa was calculated to be 129±67.2 nmol·L^{-1}. After a 6 h incubation, it increased to over 1 μmol·L^{-1}.

Effect of spermine on $[Ca]_i$ Spermatozoa were added with spermine (0.25–2.0 mmol·L^{-1}) to CaD–MCM and incubated for capacitation. Spermine 0.25 mmol·L^{-1} inhibited the Ca^{2+} influx during capacitation in CaD–MCM ($P<0.05$). The $[Ca]_i$ of spermatozoa maintained at a level similar to normal level (164.4 nmol·L^{-1}) when incubated for 6 h with spermine. There was no concentration–response relationship of spermine. The ability that spermine suppressed the rise in cytoplasmic Ca^{2+} normally occurred with the increase of capacitation period (Fig 1). These results were consistent with the acrosome reaction data in other series of experiments.

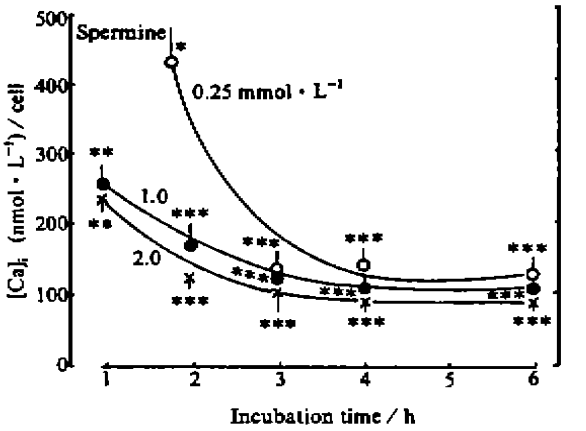

Fig 1. Effect of spermine on $[Ca]_i$ of guinea pig spermatozoa incubated in CaD–MCM. $n=3$, $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **spermine-free medium.**

Acrosome reaction, $[Ca]_i$ and their interaction Spermine had been shown to have a biphasic effect on the acrosome reaction *in vitro*, which was similar to that of verapamil. The response of verapamil was also similar to that of spermine (Fig 2).

Verapamil 100 μmol·L^{-1} suppressed the increase of $[Ca]_i$ during capacitation in CaD–MCM

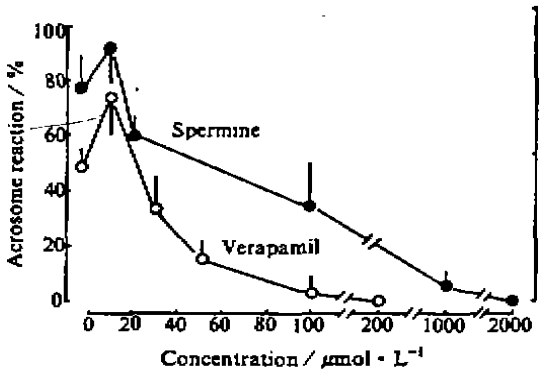

Fig 2. Inhibition of acrosome reaction in guinea pig spermatozoa caused by spermine and verapamil. $n=5$, $\bar{x}\pm s$.

($P<0.01$), the concentration of $[Ca]_i$ being 122.0 nmol· L^{-1} by the end of 6 h. Trifluoperazine has no effect on $[Ca]_i$ even at high concentration (300 μmol· L^{-1}) by which the acrosome reaction was abolished. Moreover, the inhibitory effects of both spermine and verapamil were reversed by calcimycin 1.0 μmol· L^{-1}, while the action of trifluoperazine was only antagonized by cAMP 8 mmol· L^{-1}. Spermine 0.5 mmol· L^{-1} combined with trifluoperazine 5 μmol· L^{-1} reduced the acrosome reaction to zero, suggesting that they acted synergistically.

Effect of spermine, verapamil, and trifluoperazine on calcimycin-induced rapid increase of $[Ca]_i$ With fresh guinea pig spermatozoa, spermine inhibited the calcimycin-induced increase of $[Ca]_i$ (Fig 3).

The same result was obtained with verapamil 100 μmol· L^{-1}, $[Ca]_i$ remained at 182.6 nmol· L^{-1}. However, trifluoperazine 60 μmol· L^{-1} showed no effect on calcimycin-induced increase of $[Ca]_i$. After spermatozoa were mixed with spermine or verapamil for 5 min, the $[Ca]_i$ of it declined significantly before the addition of calcimycin. If spermatozoa were treated with spermine for 6 h, the $[Ca]_i$ was also increased after the addition of calcimycin, indicating that the inhibitory effect of spermine on capacitation was reversible.

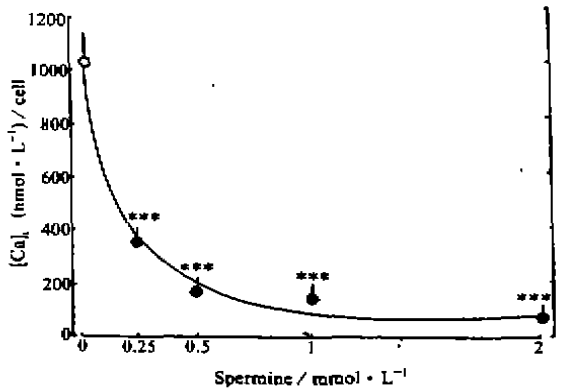

Fig 3. Effects of spermine on elevation of $[Ca]_i$ caused by calcimycin 0.8 μmol· L^{-1}. $n=3$, $\bar{x}\pm s$. $^{***}P<0.01$ *vs* **calcimycin alone.**

DISCUSSION

In the present study, we established a reliable method to determine $[Ca]_i$ in guinea pig spermatozoa. To achieve complete disruption of guinea pig sperm membrane, we used triton here instead of inomycin which was reported previously[4], as triton is inexpensive and easy to be obtained. Besides, higher concentration of $MnCl_2$ was needed for the extinction of fluorescence, which might be due to more fluorescent impurities in reagents used in this study than that in Mahanes', and the conversion of Quin 2/AM to Quin 2 in guinea pig spermatozoa also seemed to be much slower. The viability of spermatozoa was essential to $[Ca]_i$ determination and it was greatly influenced by temperature, vibration, and incubation period. The rest level of $[Ca]_i$ was 129.0± 67.2 nmol· L^{-1}, which was in agreement with direct measurement of $[Ca]_i$ in many other cell types, for example, pancreatic acinar cells, lymphocytes, and parietal cells.

Singh *et al*[6] demonstrated that a net uptake of Ca^{2+} by the spermatozoa occurred in 2 distinguishable phases. The present work might provide a direct evidence for the change of Ca^{2+} in the secondary Ca^{2+} uptake during guinea pig sperm capacitation. The increase in $[Ca]_i$ could not be attributed to the fluorescence of extracellular Quin 2 as the spermatozoa were washed twice and resuspended

in fresh CaD–MCM immediately prior to each reading. It could not be due to the leakage of Ca^{2+} from mitochondrial membrane. Alternatively, the change of $[Ca]_i$ of spermatozoa incubated in CaD–MCM might probably be due to the little amount of Ca^{2+} existing in distilled water and other reagents (about 2 $\mu mol \cdot L^{-1}$). The results obtained from CaD–MCM confirmed that the change in cell membrane permeability to Ca^{2+} did occur during capacitation of guinea pig spermatozoa. Because of the limitation of the measurement, we could not detect the tremendous change of $[Ca]_i$ after the spermatozoa had undergone the acrosome reaction.

It was reported that many physiological processes in fertilization depend upon Ca^{2+} [7,8]. But it remains unknown how Ca^{2+} penetrates the spermatozoa membrane in guinea pig. Roldan *et al*[9] found that the entering of extracellular Ca^{2+} into spermatozoa was not mediated by Ca^{2+} channels. In this study, we found verapamil could inhibit the elevation of $[Ca]_i$ during capacitation of spermatozoa, resulting in decrease of the acrosome reaction. It could be concluded that Ca^{2+}–channel played an important role in regulating the Ca^{2+} level in spermatozoa. This discrepancy may represent difference in the dose of verapamil used. Whether the action was brought into playing with or without the participation in calmodulin both inside and outside the acrosomal membrane remained unknown. Although calmodulin antagonist had no effect on $[Ca]_i$, the role of calmodulin–mediated Ca^{2+} function could not be excluded. Feinstein *et al*[10] indicated that cAMP acted primarily through stimulation of Ca^{2+} transportation and / or calcium binding. The decrease of free Ca^{2+} in the spermatozoa might affect the internal system that generated cAMP, which in part, was controlled by calmodulin. Further study is needed to determine whether spermine has some direct effects on calmodulin and its related systems in guinea pig spermatozoa.

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141–144 (12)

精胺抑制豚鼠体外精子获能中的钙离子内流[1]

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摘要 精胺(SP)能降低豚鼠精子获能过程中$[Ca]_i$的升高, 并抑制 A–23187 引起的钙内流, 与维拉帕米(Ver)一致. 三氟啦嗪(Tri)无类似作用. SP 和 Ver 对已获能精子顶体反应(AR)无影响, 抑制作用均呈双向可逆并受 A–23187 拮抗. Tri 对 AR 的抑制受 cAMP 逆转, 与 SP 有明显的协同作用. 据此推断 SP 通过抑制钙通道降低精子膜对钙的通透性, 从而阻遏 AR 和受精.

关键词 精胺; 钙; 精子获能; 顶体; 钙通道阻滞剂; 钙调蛋白; 豚鼠; 精子; 卡西霉素