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CHANGES IN INTRACELLULAR CALCIUM OF PORCINE SPERM DURING IN VITRO INCUBATION WITH SEMINAL PLASMA AND A CAPACITATING MEDIUM

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The intracellular free Ca^{2+} concentration in ejaculated, porcine sperm was determined with a fluorescent, Ca^{2+}-specific probe, Fura 2. Following suspension of sperm in a medium capable of sustaining capacitation and the acrosome reaction, the intracellular [Ca^{2+}] increased from an initial value of about 75 nM to a peak value of 130 nM, after about 4 to 5 h of incubation. Within this period of time, a peak value of 246 nM was attained when sperm was incubated in seminal plasma. Ca^{2+} uptake is presumably not associated with membrane potential-dependent channels. The results indicate that a pronounced increase in intracellular free Ca^{2+} occurs towards the end of the incubation period when rather synchronous acrosome reactions take place in the sperm population, either in capacitating medium or in seminal plasma.
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Immediately following release from the male reproductive tract, mammalian sperm are incapable of fertilizing eggs. A series of sequential processes, namely capacitation and the acrosome reaction, must take place to confer upon sperm the full fertilizing potential (1). In the course of sperm activation the cellular surface is profoundly altered, in part by the interaction of macromolecules and ions present in the female reproductive tract (1,2,3). For some mammalian species, in vitro systems have been established (1), thus facilitating research on mechanisms involved in the capacitation and acrosome reaction phases of sperm activation. In the coordination of sequential phases Ca^{2+} appears to play a key role. Extracellular Ca^{2+} is essential for triggering the acrosome reaction (1) while Ca^{2+} has only recently been implicated to be involved at the end of the capacitation phase as opposed to early stages (4). Our knowledge about biochemical mechanisms of Ca^{2+} entry and regulation in

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sperm is rather fragmentary. Concerning Ca^{2+} uptake, Na^{+}/Ca^{2+} antiports (5), calcium channels (6), and calmodulin-dependent, energy-requiring Ca^{2+} transporters (7) may be responsible. These types of uptake mechanisms may operate alone or in unison during various phases of sperm activation. Evidently, information is needed on the role of Ca^{2+} in integrating sequential processes of sperm activation.

The current study was therefore designed to examine temporal changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, of ejaculated boar spermatozoa during incubation in a capacitating medium and in seminal plasma. Employing a highly selective fluorescent Ca^{2+} indicator, Fura 2, $[Ca^{2+}]_i$ was found to vary dramatically after 4 to 5 h of incubation, especially in the presence of seminal fluid.

MATERIAL AND METHODS

Media and reagents: (a) Physiological medium (PM) comprises 145 mM NaCl, 0.5 mM $MgSO_4$, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES buffer, pH 7.4. (b) Capacitation medium (CM) comprises 88 mL medium 199 (GIBCO, Gaithersburg, MD), 12 mL foetal calf serum, 2.3 g bovine serum albumin, 2.9 mM calcium lactate, 7.5 mg penicillin G (1670 U/mg), 5 mg streptomycin (750 U/mg), 100 mM HEPES, pH 7.8. Boar sperm reportedly acquired fertilizing ability after 4 to 5 h of incubation in this medium (8).

The fluorescent Ca^{2+} chelator, Fura 2/AM, was purchased as the cell- permeant ester, {1-[2-(carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2- (2'-amino-5'-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acid, pentaacetoxy- methyl ester}, from Molecular Probes (Eugene, OR). Fura 2/AM was maintained as a 1 mM stock solution in dimethyl sulfoxide, -20°C. All chemicals were of the highest grade available. Doubly glass-distilled water was used to prepare the buffer systems.

Boar sperm collection: Freshly ejaculated sperm were collected at room temperature from 2 years old Yorkshire boars (weight about 270 kg) at the Swine Research Center of Michigan State University. Semen samples were immediately filtered through Miracloth (Calbiochem, La Jolla, CA) to remove semen gel, and maintained at 38°C. Using a Makler counting chamber (Sefi-Medical Instruments, Haifa), sperm concentration and motility were measured with a Zeiss bright field microscope.

Filtered sperm were washed twice with physiological medium (PM), followed by further incubation in the same medium, or in a capacitating medium (CM). In experiments dealing with the effect of seminal fluid on Ca^{2+} uptake, filtered sperm were first centrifuged followed by incubation with seminal fluid. Incubated sperm (3.4×10^7 cells/ ml) were maintained at 38°C in an atmosphere of 5 percent carbon dioxide in air.

Intracellular Ca^{2+}: The relative fluorescence intensity was measured with a Perkin-Elmer luminescence spectrometer, model LS-5B. The excitation wavelength was set at 339 nm with 5 nm slits; emission was recorded at 500 nm with 10 nm slits.

Sperm suspensions were incubated with Fura 2/AM at a final concentration of 1 µM, 38°C, 1 h. 3 mL of Fura 2-loaded sperm were transferred to a quartz cuvette (final concentration 1.7×10^7 cells/ml), thermostatically maintained at 38°C. The concentration of dimethyl sulfoxide in the Fura 2/AM loading

solution was 0.25 percent (w/w). Control experiments showed no detectable influence of dimethyl sulfoxide on sperm motility and intracellular $[Ca^{2+}]$, at the solvent concentrations used. Regardless of the incubation media (PM, CM, seminal fluid) and time employed, subsequent dye loading (duration 1 h) and fluorescence studies were always performed in the presence of PM medium.

In dye-loaded cells, the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was determined following a procedure recently described (9). Briefly, the presence of extracellular Fura 2 (including leakage of Fura 2) was ascertained by addition of EGTA (3.3 mM) and then Tris (20 mM), at pH 8.2. The fluorescence minimum, F_{min}, was obtained by dye release from sperm in the presence of digitonin (0.06 mg/mL). Following addition of HCl (20 mM), the fluorescence maximum, F_{max}, was measured in the presence of 3.3 mM Ca^{2+} added, pH 7.5 (Fig. 1). Autofluorescence was spectrally subtracted. Generally the autofluorescence intensity was less than 20 percent that of F_{max}; autofluorescence intensity remained constant regardless of the presence of EGTA, Tris, digitonin, HCl, and Ca^{2+}. The intracellular concentration of Ca^{2+} was calculated from the relation $[Ca^{2+}]_i = k_d (F - F_{min})/(F_{max} - F)$, where k_d = 224 nM is the dissociation constant of calcium binding to Fura 2 (10).

RESULTS

Time course of $[Ca^{2+}]_i$ during incubation (Fig. 1). At the onset of incubation, $[Ca^{2+}]_i$ amounted to 70-80 nM. With an increase in incubation time, $[Ca^{2+}]_i$ increased concomitantly, its associated rate and peak value depen-

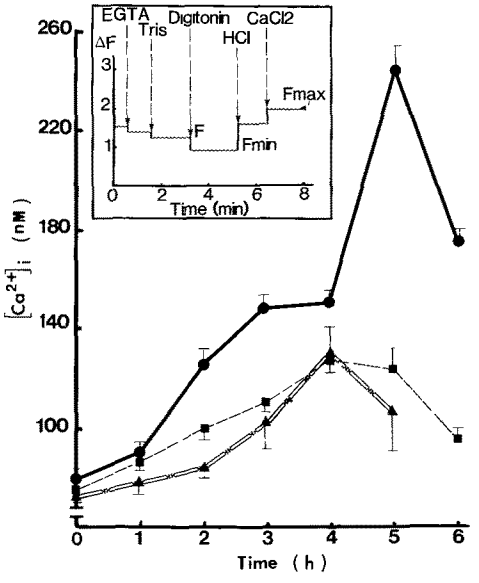

Fig. 1. Dependence of the intracellular free Ca^{2+} concentration in ejaculated porcine sperm on incubation time, in the presence of a physiological medium (■), a capacitating medium (▲), or seminal plasma (●), respectively. 1.7 x 10^7 cells/ml were loaded with a Ca^{2+}-specific, fluorescent chelator, Fura 2 (1 µM), 38°C. Following incubation in the respective medium, spectral measurements were always performed on cells suspended in physiological medium. Insert: Typical time dependence of changes in relative fluorescence intensity (ΔF) of Fura 2-loaded sperm following addition of EGTA (3.3 mM), Tris (2.2 mM), digitonin (0.05 mM), and HCl (20 mM). From these values the minimum and maximum values of fluorescence intensity were derived.

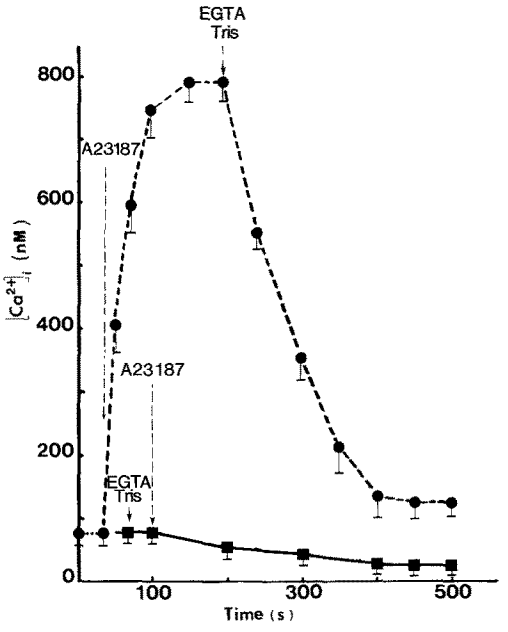

Fig. 2. Response of the intracellular free Ca^{2+} concentration in ejaculated porcine sperm, incubated in a physiological medium, to the application of a Ca^{2+}-specific ionophore, A23187 (25 µM), then followed by the application of EGTA (3.3 mM) and Tris (2.2 mM), respectively. These sperm were washed twice after collection. Without any preceding incubation, sperm were loaded with Fura 2/AM (duration 1 h), followed by A23187 treatment. Bottom: Response of intracellular $[Ca^{2+}]$ when the order of application of these agents was reversed.

dent on the type of incubation medium employed. Upon incubation in seminal plasma for 5 h, a peak value of $[Ca^{2+}]_i$ =246±10 nM was obtained which was almost twice as high as the corresponding peak values in the presence of the artificial media tested.

As determined in a physiological medium (PM), the peak value of $[Ca^{2+}]_i$ was independent of the presence or absence of monovalent Na^+ or K^+ in the incubation medium. This was illustrated, for example, by findings that in a PM medium devoid of K^+ (150 mM Na^+ added to maintain osmolarity), the maximal value of $[Ca^{2+}]_i$ was found to be 126±17 compared with the corresponding value of 129±4 measured after 4 h of incubation in the full PM medium.

Experiments were also performed regarding $[Ca^{2+}]_i$ in response to incubation in a medium deficient in Ca^{2+}; to this end 3.3 mM EGTA were added. Following incubation in EGTA-containing seminal plasma for 5 h, $[Ca^{2+}]_i$ was 108±7 nM, as opposed to 246±10 nM in the absence of the Ca^{2+} chelator. A

similar reduction in $[Ca^{2+}]_i$, viz., from 129 nM to 82 nM, was measured in sperm incubated in PM supplemented with EGTA (3.3 mM).

Intracellular $[Ca^{2+}]$ following induction of acrosome reaction. Since the acrosome reaction was reportedly (11) inducible by the application of a Ca^{2+} specific ionophore, A23187, the intracellular Ca^{2+} concentration was determined in response to the application of A23187 (25 µM) to the sperm suspension in PM (Fig. 2). These sperm were washed twice after collection. Without any preceding incubation, sperm were loaded with Fura 2/AM (duration 1 h, PM). Starting at a basal value of 75±5 nM, upon A23187 application $[Ca^{2+}]_i$ promptly increased (in about 2 min) to a maximal value of 791±37 nM. Subsequent administration of EGTA (3.3 mM) and Tris (2.2 mM) diminished $[Ca^{2+}]_i$ to a value of 125±23 nM, within less than 4 min. The latter Ca^{2+} concentration, 125 nM, is higher than that evaluated prior to the application of the ionophore, viz. 75 nM. Presumably, some Ca^{2+} became trapped in intracellular compartments following application of the Ca^{2+} chelator and Tris which enhances Ca^{2+} chelation at slightly alkaline pH. Reversing the sequence of treatment, viz., Tris/EGTA first, then A23187, yielded a value of 22±5 nM for $[Ca^{2+}]_i$ after about 6 min (Fig. 2). The presence of the ionophore apparently facilitated Ca^{2+} efflux, thus lowering the interior $[Ca^{2+}]$.

Sperm motility. Since sperm acquire unique motility patterns in the course of capacitation and the subsequent acrosome reaction (2, 12), sperm motility was evaluated. Irrespective of the incubation medium employed, 80-90 percent of sperm was found to be motile at the onset of incubation. Sperm motility declined to about 40-50 percent after 5 h of incubation in artificial media (PM and CM). In the presence of EGTA, motility further declined to a value of 30-40 percent (5 h). The observed motility decline with time is in accord with recent findings on boar sperm in a similary reconstituted medium 199 (13). Upon incubation of sperm in seminal plasma (5 h), however, motility remained at 70-80 percent, a value only slightly lower than that found at the onset of incubation. When cell viability was assessed with the Trypan blue assay (14), the initial viability was about 85 percent, and after 5 h of incubation (PM, SM) approximately 80 percent.

At the onset of incubation, the sperm motility pattern was characterized by linear swimming. After 5 h of incubation, this pattern was converted into one typified by nonprogressive, tumbling sperm movements, called "hyperactivation" (1,2,12).

As judged by motility and viability criteria, the micromolar Fura 2 concentration used exerted no detectable toxic influence upon sperm. This observation is consistent with motility studies employing a closely related dye, quin 2, for measurements of $[Ca^{2+}]$ in boar epididymal sperm (15).

Dye compartmentation: Since a spermatozoon is compartmentalized, the question arises as to the intracellular localization of free Ca^{2+} which is monitored in boar sperm by the fluorescent dye, Fura 2. Using a fluorescence microscope, examination of freshly Fura 2-loaded spermatozoa suggested that the sperm head was uniformly stained with the dye. This qualitative result is consistent with data obtained in response to digitonin application (Fig. 1). At the low concentration used, this membrane-disruptive agent reportedly released constituents of cytoplasmic rather than mitochondrial origin (16). It seems therefore that Fura 2 mainly monitored the Ca^{2+} concentration in the cytosol of the sperm head. In this context it appears noteworthy that recent ultrastructural findings demonstrated that bound Ca^{2+} can be detected in the anterior regions of the spermatozoon head during various stages of the acrosome reaction (17).

DISCUSSION

In this study we demonstrated temporal changes of intracellular $[Ca^{2+}]$ in ejaculated porcine sperm exposed to a capacitating medium, or to seminal plasma. Compared with $[Ca^{2+}]_i$ at the onset of incubation, enhancement of $[Ca^{2+}]_i$ is pronounced after 4 to 5 h in the media tested, especially upon incubation in seminal plasma. This period of time is in accord with recent data on boar sperm capacitation (8, 13). The dramatic increase of $[Ca^{2+}]_i$ of sperm in seminal plasma is perhaps related to several claims asserting that sperm capacitated in vivo are more effective in fertilizing ova than sperm capacitated in artificial media (1).

The pronounced increase (4-5 h) in $[Ca^{2+}]_i$ appears to coincide with the time required to achieve maximal fertilizing ability of boar sperm incubated in vitro (8). A relationship between enhanced $[Ca^{2+}]_i$ and the acrosome reaction is indicated by the manifold increase of $[Ca^{2+}]_i$ in the presence of A23187, a Ca^{2+} ionophore known to rapidly trigger the acrosome reaction in sperm in the presence of extracellular Ca^{2+} (2,11). Besides morphological parameters (hyperactivation, acrosome exocytosis), the enhanced $[Ca^{2+}]_i$ may thus be a useful biochemical marker suggestive of sperm fertilizing ability.

The virtual independence of $[Ca^{2}]_i$ on the presence of extracellular $[K^+]$ argues against a major involvement of voltage-gated channels in Ca^{2+} uptake by boar sperm because at the millimolar $[K^+]$ employed depolarization of the K^+-dependent membrane potential is to be expected (18).

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REFERENCES

1. Yanagimachi (1988) In The Physiology of Reproduction (Knobil, E. and Neill, J.D., eds), vol. 1, pp. 135-185. Raven Press, New York.
2. Blottner, S. (1989) Biol. Zentr. bl. 108, 41-62.
3. Blaquier, J.A., Cameo, M.S., Cuasnicu', P.S., Echeverria, M.F.G., Pineiro, L., Tezon, J.G., and Vazquez, M.H. (1988) Reprod. Nutr. Dévelop. 28, 1209-1216.
4. Fraser, L.R., and McIntyre, K. (1989) J. Reprod. Fertil. 86, 223-233.
5. Bradley, M. P. and Forrester, I.T. (1980) FEBS Lettr. 121, 15-18.
6. Breitbart, T., Rubinstein, S., and Nass-Arden, L. (1985) J. Biol. Chem. 260, 11548-11553.
7. Silvestroni, L. and Menditto, A. (1989) Arch. Androl. 23, 87-96.
8. Saxena, N., Peterson, R.N., Saxena, N.K., and Russell, L.D. (1986) J. Reprod. Fertil. 78, 601-614.
9. Capponi, A.M., Lew, P.D., Schlegel, W., and Pozzan, T. (1986) Meth. Enzymol. 124, 116-135.
10. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
11. Byrd, W. (1981) J. Exp. Zool. 215, 35-46.
12. Lindemann, C.B. and Kanous, K.S. (1989) Arch. Androl. 23, 1-22.
13. Berger, T. and Horton, M.B. (1988) Gamete Res. 19, 101-111.
14. Shi, B. and Haug, A. (1990) J. Neurochem. 55, 551-558.
15. Simpson, A.M. and White, I.G. (1988) Cell Calcium 9, 45-56.
16. Akerman, K.E.A. (1989) FEBS Lettr. 242, 337-340.
17. Ruknudin, A. (1988) Gamete Res. 22, 375-384.
18. Chou, K., Chen, J., Yuan, S. and Haug, A. (1989) Biochem. Biophys. Res. Comm. 165, 58-64.