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Capacitation and Induction of the Acrosome Reaction in Bull Spermatozoa With Norepinephrine

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ABSTRACT: Identification of norepinephrine (NE) within the microenvironment of the bovine oviduct suggests a potential role for catecholamines in the events surrounding fertilization. Previous studies have shown that the catecholamines capacitate and induce the acrosome reaction in spermatozoa from several species. The current project was undertaken to investigate the role of catecholamines in bovine sperm capacitation and the acrosome reaction. Freshly ejaculated bovine spermatozoa were incubated in NE (0–1000 ng/mL) and induced to acrosome-react with lysophosphatidylcholine (LPC). Additionally, spermatozoa capacitated with heparin were incubated with NE (0–1000 ng/mL) to assess its ability to induce the acrosome-reaction in capacitated spermatozoa. Concentrations of NE were chosen on the basis of physiological concentrations previously determined for bovine oviductal fluid. NE at concentrations of 10 and 20 ng/mL capacitated bovine spermatozoa after 2 hours of incubation. Additionally, spermatozoa incubated for 2 hours with heparin were induced to acrosome-react with 10 and 20 ng/mL NE. Interestingly, higher concentrations of NE inhibited both capacitation and the acrosome reaction. Incubating spermatozoa with dopamine or epinephrine did not result in capacitation or the acrosome reaction, suggesting that the action of NE was specific to that catecholamine. The ability of NE to capacitate or induce the acrosome reaction appears to be dependent on the presence of another membrane-destabilizing factor. Although adrenergic receptors have not been identified on spermatozoa from any species, the action of NE on spermatozoa may be a receptor-mediated event. This study suggests a possible function for oviductal catecholamines in sperm preparation prior to fertilization.

Key words: Catecholamines, bovine.

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Previous studies have determined that epinephrine and norepinephrine (NE) induce the acrosome reaction in vitro in spermatozoa from many species (Bavister et al, 1976; Bavister and Yanagimachi, 1977; Cornett and Meizel, 1977, 1978; Meizel and Working, 1980; Bize and Santander, 1985). Hamster spermatozoa incubated in bovine adrenal medulla extracts, epinephrine, or NE exhibited a significant increase in motility and acrosome reactions (Cornett and Meizel, 1978), and hamster spermatozoa incubated in bovine adrenal gland extracts became capacitated, as shown by their ability to fertilize hamster oocytes (Cornett et al, 1974). Although these studies suggest that catecholamines may be important in preparing the sperm membrane for fertilization by either capacitation or the acrosome reaction, physiological concentrations of catecholamines found in oviductal fluid were not used. In order to evaluate the physiological relevance of these observations, it is necessary to determine whether oviductal concentrations of catecholamines are able to capacitate and induce the acrosome reaction in bovine spermatozoa. We have identified NE in bovine oviductal fluid by high-performance liquid chromatography in concentrations ranging from 0.828 to 1117 ng/mL (Way et al, 2001). The purpose of this study was to determine whether NE capacitates or induces the acrosome reaction in freshly ejaculated bovine spermatozoa, using a range of NE concentrations found in bovine oviductal fluid, and to determine if the effect observed was specific to NE.

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Methods

Catecholamine Preparation

Stock solutions of NE bitartrate (RBI N-113), epinephrine bitartrate (RBI E-104), and dopamine HCl (RBI D-019) were created by mixing each catecholamine in degassed, sterile distilled water (15230-147; Life Technologies, Gaithersburg, Md) and making dilutions of the stock solutions in modified Tyrode Medium (MTM; Parrish et al, 1988) just prior to each time point. Stock solutions were stored at −4°C and were protected from light throughout the experiment. The amount of NE bitartrate, epinephrine bitartrate, or dopamine HCl needed to equal the appropriate concentration of free base was calculated prior to preparation of the incubation tubes.

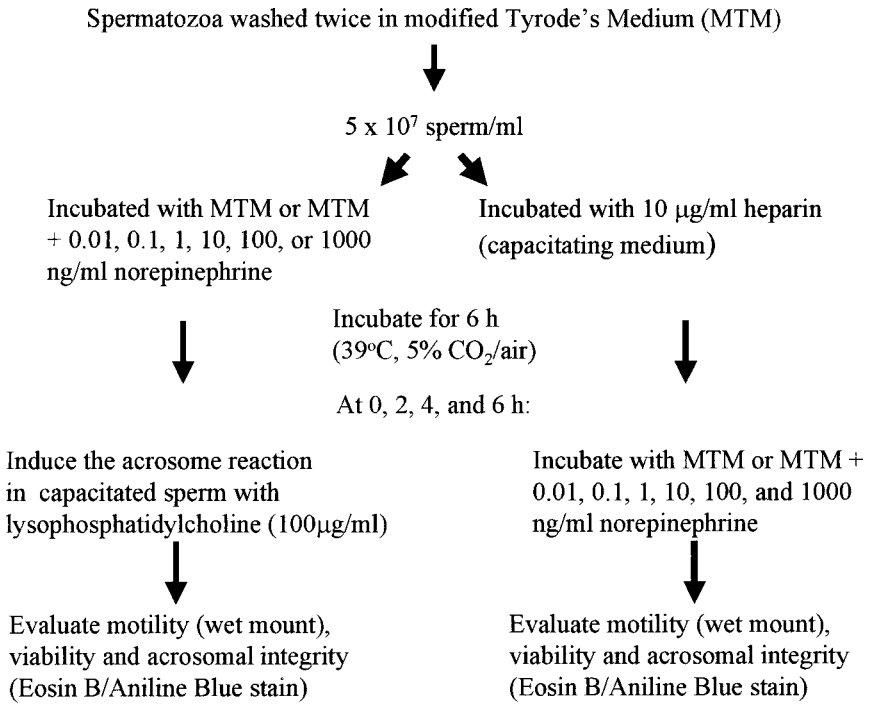

Figure 1. Schematic of experiment incubating spermatozoa in norepinephrine (NE) to determine if it acts as a capacitating or acrosome reaction-inducing agent.

Collection and Preparation of Spermatozoa

Ejaculated semen was collected from 3 mature Holstein bulls by artificial vagina. The same 3 bulls were used for all experiments. Semen (1 mL) from each bull was suspended in 10 mL MTM and centrifuged to separate the spermatozoa from seminal plasma (500 × *g*, 10 minutes). After centrifugation, the supernatant was removed, and spermatozoa were suspended in an additional 10 mL MTM and centrifuged. After the supernatant was removed, spermatozoa were gently suspended in any MTM remaining in the tube and counted by hemocytometer. Equal numbers of spermatozoa from each bull were pooled to yield a concentration of 5×10^7 spermatozoa/mL.

Incubation of Spermatozoa in NE, Epinephrine, and Dopamine

Spermatozoa were incubated in MTM containing 0, 0.01, 0.1, 1.0, 10, 100, and 1000 ng/mL NE for 6 hours (39°C, 5% CO_2/air) to determine if NE acted as a capacitating agent (Figure 1). Every 2 hours, an aliquot of sperm suspension was incubated with either MTM (negative control) or 100 μg/mL lysophosphatidylcholine (LPC) (L-5004; Sigma Chemical Co, St Louis, Mo) for 10 minutes at 39°C to induce the acrosome reaction in capacitated spermatozoa (McNutt and Killian, 1991). To determine if NE was capable of inducing the acrosome reaction in capacitated spermatozoa, 5×10^7 sperm/mL were incubated in 10 μg/mL heparin (H-3393; Sigma) in MTM to capacitate (Parrish et al, 1988) or in MTM alone (negative control). Every 2 hours, aliquots of sperm suspension were challenged with either MTM or NE at concentrations of 0.01, 0.1, 1.0, 10, 100, or 1000 ng/mL (Figure 1). Spermatozoa capacitated with 10 μg/mL heparin for 6 hours and then induced to acrosome react with LPC served as the positive control. Samples were subjectively assessed for percentage progressive motility, as well as for viability and acrosomal integrity, using eosin B/aniline blue staining and differential interference contrast microscopy (Way et al, 1995). The population of acrosome-reacted live spermatozoa in each treatment was considered to represent functionally capacitated spermatozoa (Way et al, 2000). Experiments to evaluate capacitation and the acrosome reaction were conducted on the same day.

A second set of experiments was conducted with NE in which spermatozoa were incubated in either MTM or NE at concentrations of 5, 10, 20, 40, 60, or 80 ng/mL NE for 2 hours and then exposed to LPC to induce the acrosome reaction in any spermatozoa capacitated by NE. This experiment was designed to further define the optimum range of NE concentrations capable of eliciting a response in spermatozoa. Spermatozoa and NE were prepared as described above and evaluated for motility, viability, and acrosomal integrity. To determine if NE exerted a specific effect on spermatozoa, additional experiments were conducted as described for NE to evaluate whether epinephrine or dopamine capacitated or induced the acrosome reaction in spermatozoa. In those experiments, both catecholamines were incubated individually with spermatozoa as described for NE, and spermatozoa were assessed for capacitation and the acrosome reaction.

Statistical Analysis

Experiments incubating spermatozoa in NE, epinephrine, and dopamine were repeated 3 times, and data from each experiment were analyzed by repeated-measures analysis for each population

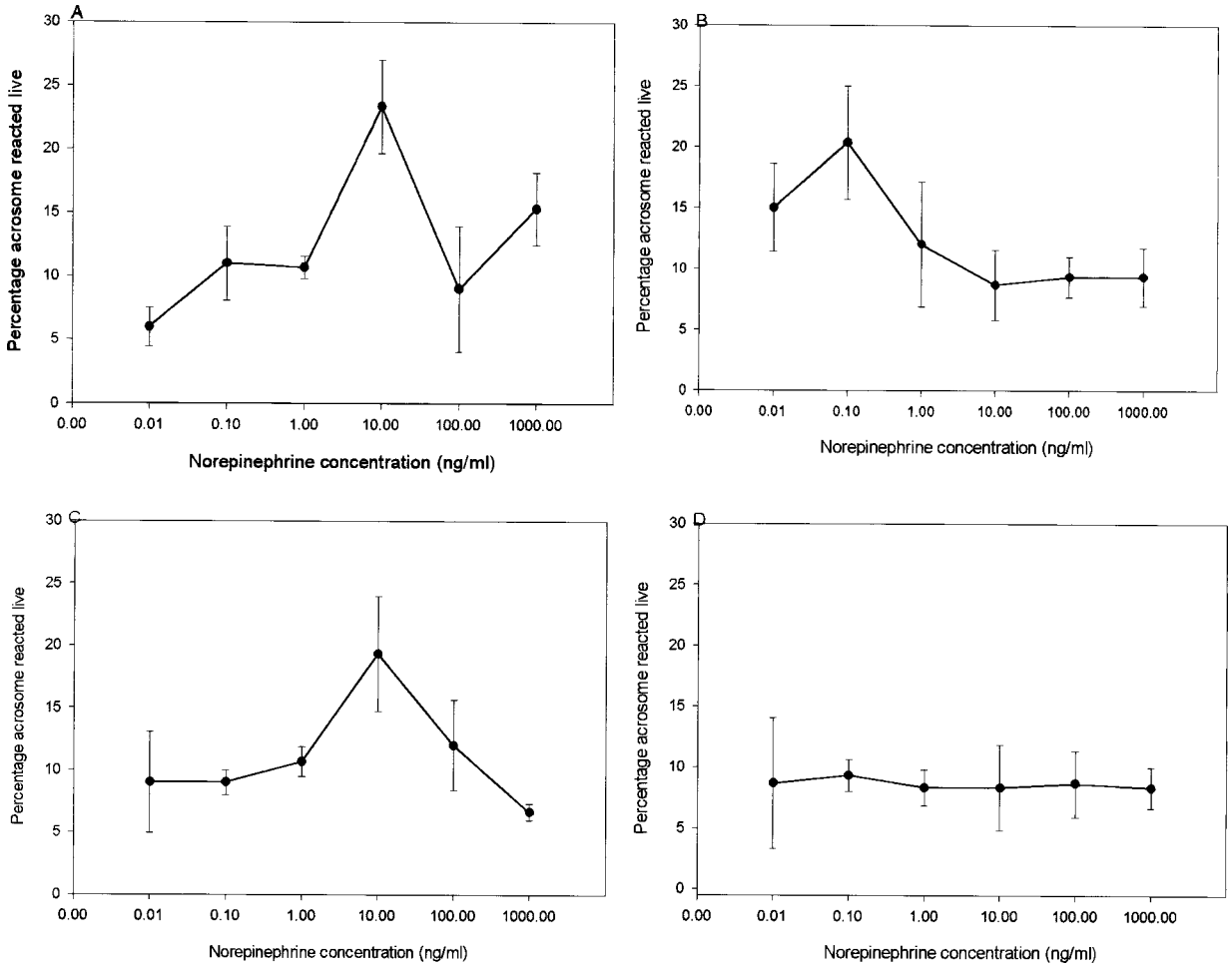

Figure 2. Mean percentage of acrosome-reacted live spermatozoa plus or minus standard error of the mean for spermatozoa incubated for 2 hours in **(A)** norepinephrine (NE) (0.01–1000 ng/mL) and induced to acrosome react with lysophosphatidylcholine (LPC); **(B)** NE (0.01–1000 ng/mL) with no LPC induction; **(C)** heparin and induced to acrosome react with NE (0.01–1000 ng/mL); and **(D)** modified Tyrode Medium (MTM) and induced to acrosome react with NE (0.01–1000 ng/mL).

of live and acrosome-reacted spermatozoa. Differences among treatments were determined by least square means comparisons with a significance level of $P < .05$ (SAS 6.12 for Windows, Statistical Analysis Systems, 1989).

Results

Effects of NE on Spermatozoa

For all experiments conducted with 0, 0.01, 0.1, 1, 10, 100, and 1000 ng/mL NE, the only differences observed were at 2 hours. Spermatozoa incubated in 10 ng/mL NE for 2 hours followed by LPC (capacitating experiment) had significantly more acrosome-reacted live spermatozoa than spermatozoa incubated in other concentrations of NE or controls ($P < .05$) (Figure 2A), with the exception of the spermatozoa incubated in 0.1 ng/mL NE followed by incubation in MTM (Figure 2B). The 0.1-ng/mL treatment in the absence of LPC had significantly more acrosome-reacted live spermatozoa than spermatozoa incubated in 10, 100, and 1000 ng/mL NE followed by incubation in MTM ($P < .05$) (Figure 2B). In addition, this treatment had significantly more acrosome-reacted live spermatozoa than spermatozoa incubated in 0.01, 0.1, 1, and 100 ng/mL NE and subsequently challenged with LPC ($P < .05$) (Figure 2A).

Spermatozoa capacitated with heparin for 2 hours and challenged with 10 ng/mL NE (acrosome reaction experiment) had significantly more acrosome-reacted live spermatozoa than those incubated in 0.01, 0.1, and 1000 ng/mL NE or controls ($P < .05$) (Figure 2C and D). There were more acrosome-reacted live spermatozoa in the 10-ng/mL NE treatment compared to 1 and 100 ng/mL NE, but these differences were not significant. At 2 hours, the percentages of acrosome-reacted live spermatozoa incubated in 10 ng/mL NE and induced to acrosome react with LPC (23.33% ± 3.67%) and spermatozoa incubated in

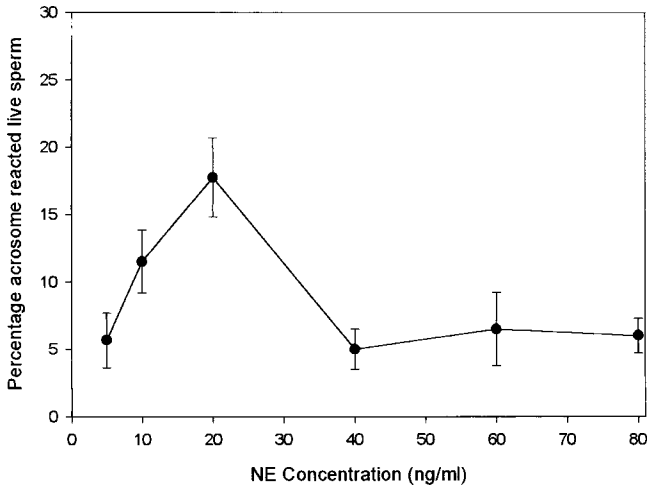

Figure 3. Mean percentage of acrosome-reacted live spermatozoa plus or minus standard error of the mean for spermatozoa incubated in 5, 10, 20, 40, 60, and 80 ng/mL norepinephrine (NE) and induced to acrosome react with lysophosphatidylcholine (LPC).

heparin and induced to acrosome react with NE (19.33% ± 4.63%) were comparable to spermatozoa capacitated with heparin for 6 hours and induced to acrosome react with LPC (18.33% ± 5.67%).

When spermatozoa capacitated with heparin were challenged with NE at 4 or 6 hours of incubation, no significant increase over the controls was observed. The same was true for spermatozoa incubated in NE and induced to acrosome react with LPC at 4 and 6 hours. Viability ranged from 78.67% plus or minus 2.03% to 92.67% plus or minus 3.93% for the capacitation experiment and from 79.00% plus or minus 7.81% to 92.00% plus or minus 4.36% for the acrosome reaction experiment. None of the NE treatments adversely affected viability.

In experiments conducted with 0, 5, 10, 20, 40, 60, and 80 ng/mL NE, spermatozoa incubated in 20 ng/mL NE and then induced to acrosome react with LPC had significantly more acrosome-reacted live spermatozoa than any other concentration of NE or the control ($P < .05$) (Figure 3). Viability was not affected by any of the treatments.

Effects of Epinephrine and Dopamine on Spermatozoa

The acrosome reaction profiles for spermatozoa incubated in epinephrine or dopamine did not resemble those of spermatozoa incubated in NE (data not shown). Viability was not affected by epinephrine or dopamine, and it does not appear that these catecholamines exert the same effect on bovine spermatozoa as NE. At 2 hours, spermatozoa incubated in heparin could be induced to acrosome react with 10 ng/mL epinephrine, resulting in 11.67% plus or minus 1.45% acrosome-reacted live spermatozoa. After 6 hours of incubation in 10 ng/mL epinephrine, 15% plus or minus 5.69% spermatozoa could be induced to acrosome react with LPC (data not shown). Both of these values were lower than those observed for NE at 2 hours. Incubation of spermatozoa with dopamine did not induce capacitation or the acrosome reaction at any time point.

Discussion

Several studies have suggested a role for catecholamines in capacitation of spermatozoa (Bavister et al, 1976; Leibfried and Bavister, 1982; Stanger, 1983; Bize and Santander, 1985). Hamster epididymal spermatozoa incubated with epinephrine and taurine for 3.5 hours were capable of acrosome reacting following exposure to LPC (Llanos and Ronco, 1994). It is also possible that capacitation is a prerequisite for binding of catecholamines to spermatozoa on the basis of studies in which hamster spermatozoa were stimulated to acrosome react after exposure to catecholamines following preincubation in the capacitating agents taurine and bovine serum albumin (Cornett and Meizel, 1978; Mrsny et al, 1979).

The majority of experiments conducted with catecholamines and spermatozoa suggest that catecholamines act as acrosome reaction-inducing agents (Bavister and Yanagimachi, 1977; Cornett and Meizel, 1977, 1978; Meizel and Working, 1980). Hamster spermatozoa incubated in adrenal medulla extracts, epinephrine, or NE exhibited a significant increase in motility and acrosome reactions, and hamster spermatozoa incubated in hamster adrenal gland extracts became capacitated, as shown by their ability to fertilize hamster oocytes (Bavister et al, 1976). Later studies determined that catecholamines were one of the sperm motility factors in adrenal gland extracts (Cornett and Meizel, 1978). In bovine spermatozoa, NE induces a head-to-head association in vitro (Lindahl, 1978). Because the majority of these studies were conducted using nonphysiological concentrations of catecholamines, it was unknown how physiological concentrations of catecholamines affected spermatozoa.

With the identification of NE in bovine oviductal fluid, it is not surprising to find that NE facilitates capacitation and the acrosome reaction in bovine spermatozoa. At 2 hours of incubation, spermatozoa that were either capacitated with heparin and then induced to acrosome react with 10 ng/mL NE or incubated with NE and induced to acrosome react with the fusogenic lipid LPC had a higher percentage acrosome-reacted live population than spermatozoa incubated in MTM or heparin for 6 hours and then induced to acrosome react with LPC. This increase in acrosome-reacted live spermatozoa was observed for both 10 and 20 ng/mL NE but not for other concentrations of NE or at other time points. If spermatozoa capacitated with heparin were challenged with NE at 4 or 6 hours of incubation, no significant increase over the controls was

observed. The same was true for spermatozoa incubated in NE and induced to acrosome react at 4 and 6 hours with LPC. Concentrations of NE above and below the 10- to 20-ng/mL range were either less effective or completely ineffective in increasing the percentage of acrosome-reacted live spermatozoa. The ability of NE to shorten the length of time required for bull sperm capacitation to take place is similar to what was observed for hamster spermatozoa in which incubation with epinephrine and hypotaurine shortened the time required to capacitate from 3–4 hours to 2 hours (Leibfried and Bavister, 1982).

With the exception of the 0.01-ng/mL NE treatment, NE appears to facilitate capacitation and the acrosome reaction if another membrane-destabilizing substance such as LPC or heparin is present. This complementary effect of NE with LPC and heparin is similar to what was described for epinephrine with hamster spermatozoa (Leibfried and Bavister, 1982). Epinephrine alone was unable to capacitate or acrosome react hamster spermatozoa. It required a cofactor to facilitate either capacitation or the acrosome reaction. Incubation with 20 μM penicillamine or other chelators and 0.5 nM epinephrine stimulated more spermatozoa to acrosome react than if spermatozoa were incubated in epinephrine or the chelator alone (Meizel and Working, 1980). The β-amino acid hypotaurine capacitates hamster spermatozoa in vitro (Meizel et al, 1980), and prior exposure of hamster spermatozoa to hypotaurine is a prerequisite for the action of epinephrine (Leibfried and Bavister, 1982).

In the present study, neither dopamine nor epinephrine was able to capacitate or induce the acrosome reaction at 2 hours of incubation. Spermatozoa incubated with 10 ng/mL epinephrine did exhibit an increase in acrosome reactions, but that increase was not as great as that observed for NE. Dopamine was unable to capacitate or induce the acrosome reaction at any concentration or time point. What is striking about these results is that the influence of NE on spermatozoa does not represent a typical response of NE binding to an adrenergic receptor. As the concentration of available catecholamine increases, the measured activity also increases until a plateau is reached where no increase in activity can be observed. This was not observed for spermatozoa. It is possible that higher concentrations of NE are stimulating an inhibitory β-adrenergic receptor, and the acrosome reactions observed at the lower concentrations represent stimulation of an α-adrenergic receptor. The presence of 2 receptors on the sperm plasma membrane certainly could explain the contradictory effects observed here. The possibility of both α- and β-adrenergic receptors existing on spermatozoa is supported by several studies that found that catecholamine-induced capacitation and the acrosome reaction can be inhibited by both α- and β-antagonists (Cornett et al, 1974; Cornett and Meizel, 1978; Bavister et al, 1979; Leibfried and Bavister, 1982). This indirect evidence suggests that spermatozoa have adrenergic receptors, although a receptor has not been identified (Cornett and Meizel, 1980; Falkay et al, 1989). Attempts to identify adrenergic receptors on bovine spermatozoa using a variety of assays were unsuccessful (data not shown).

It is possible that the mechanism of action of catecholamines on spermatozoa is not mediated by an adrenergic receptor. Catecholamines act as chelators (Fagan and Racker, 1977; Hexum, 1977) and/or phosphodiesterase inhibitors (Goren and Rosen, 1972; Ain-Shoka and Buckner, 1978). Fagan and Racker (1977) and Hexum (1977) have shown that catecholamines can stimulate somatic cell Na^+-K^+ adenosine triphosphatase (ATPase) by chelating metal ions. Inhibitors of Na^+-K^+ ATPase inhibit activation and the acrosome reaction in hamster spermatozoa, and phosphodiesterase inhibitors stimulate activation and the acrosome reaction (Mrsny and Meizel, 1980). Alvarez and Storey (1983) found that epinephrine inhibits lipid peroxidation and loss of motility of rabbit spermatozoa, likely by acting as a scavenger for superoxide (Misra and Fridovich, 1972). Any of these nonhormonal mechanisms could provide an explanation for what was observed in the present study, although the concentration range of 10–20 ng/mL NE is considerably lower than what was used in previous studies. However, those concentrations are higher than the 152 pg/mL NE normally present in bovine plasma (Buhler et al, 1978) and may be sufficient to act via an alternative mechanism.

Without direct evidence to disprove the existence of adrenergic receptors in spermatozoa, it is difficult to conclusively rule out a classical method of action of NE, and with no evidence to prove an alternative mechanism, the manner in which NE affects spermatozoa can only be speculated upon. It is still possible that capacitation of spermatozoa by NE is an adrenergic receptor-mediated event. If receptors present on spermatozoa are unmasked for only a brief period of time, it is very likely that previous attempts were not optimized to identify a receptor. If those receptors are available to bind NE for a specific time during capacitation, this may explain why spermatozoa were able to respond to NE after only 2 hours of incubation. Prolonged incubation in NE may promote degradation or down-regulation of an adrenergic receptor on spermatozoa. Incubation in NE after stimulation of an excitatory adrenergic receptor may result in stimulation of an inhibitory receptor and inhibition of capacitation and the acrosome reaction. A nonhormonal mode of action is still possible for catecholamines and spermatozoa, and this is supported by studies on both bovine and rodent spermatozoa (Misra and Fridovich, 1972; Peterson and Freund, 1973; Stanger, 1983). Regardless of the mechanism, NE at physiological concentrations previously determined to exist in bovine oviductal fluid were capable

of both capacitating and inducing the acrosome reaction in bovine spermatozoa. More investigation into this area should help to elucidate the mechanism of action of NE on spermatozoa.

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