Stimulation of in vitro activation and the acrosome reaction of hamster spermatozoa by catecholamines

(capacitation/adrenergic receptors/epinephrine/membranes)

LAWRENCE E. CORNETT AND STANLEY MEIZEL

Department of Human Anatomy, School of Medicine, University of California, Davis, California 95616

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ABSTRACT Capacitation and the acrosome reaction of mammalian spermatozoa are essential for fertilization. In vitro results are presented that demonstrate that catecholamines stimulate activation (a whiplash flagellar movement characteristic of capacitated hamster spermatozoa) and the acrosome reaction. Protein-free ultrafiltrates of bovine adrenal cortex and medulla preparations stimulated motility, activation, and acrosome reactions of hamster spermatozoa in the presence of bovine serum albumin. The medulla preparation was more effective than the cortex preparation in the stimulation of activation and acrosome reactions. Epinephrine (0.5-50 μM) and norepinephrine (50.0 μM) in the presence of bovine serum albumin and a partially purified protein-free cortex preparation also stimulated activation and the acrosome reactions. Both activation and acrosome reactions in the presence of epinephrine were inhibited by the adrenergic antagonists phentolamine and propranolol, suggesting the involvement of α- and β-adrenergic receptors in the stimulation of capacitation and the acrosome reaction. In addition, phenylephrine, an α-adrenergic agonist, was as potent as epinephrine in the stimulation of acrosome reactions, but activation was reduced. Isoproterenol, a β-adrenergic agonist, was as potent as epinephrine in the stimulation of activation, but acrosome reactions were reduced. High percentages of both activation and acrosome reactions were observed only in the presence of epinephrine, norepinephrine, or phenylephrine and isoproterenol together.

Sperm capacitation (subtle cellular changes occurring within the female reproductive tract or in vitro) and the resulting acrosome reaction (a fusion and vesiculation of the outer acrosomal membrane and its overlying sperm head plasma membrane) are essential for mammalian fertilization (1, 2). The molecular events of capacitation and of the acrosome reaction, including its initiation, are only partially understood (3). Capacitation and the acrosome reaction can be induced in hamster spermatozoa in vitro by detoxified follicular fluid (4, 5) or by blood sera (6). A nondialyzable serum or follicular fluid factor (labile at 90°) was required for the acrosome reaction to occur and a dialyzable heat-stable factor derived from serum or follicular fluid was necessary for sperm motility, activation (the whiplash flagellar movement characteristic of capacitated hamster spermatozoa), and the occurrence of the optimum number of acrosome reactions (4–6). It has now been shown that certain serum albumins can replace the nondialyzable factor (6–9) and that the bovine follicular fluid factor is serum albumin (9).

Recently, it has been reported that the dialyzable factor can be replaced by a "motility factor" of less than 1000 molecular weight obtained from extracts of hamster adrenal gland (10) or hamster, guinea pig, or human spermatozoa (11, 12). The motility factor derived from spermatozoa also seems to maintain hamster sperm viability upon dilution in vitro (12). In the present paper the bovine adrenal gland was chosen as a starting point for identification of the motility factor because the large size of the bovine adrenal gland enabled us to separate the adrenal cortex and medulla (two very different functional tissues) for testing as sources of the factor(s). The results presented in this paper demonstrate that catecholamines are one of the components of the adrenal gland motility factor and that catecholamines can stimulate activation and the acrosome reaction of hamster spermatozoa in vitro. This work has been reported in preliminary form (10).

MATERIALS AND METHODS

Preparation of Motility Factor from Bovine Adrenal Glands. The medulla and cortex of several steer adrenal glands, trimmed of fat (Pel-Freez Biologicals), were separated by dissection and homogenized in 0.9% NaCl (0.3 ml of saline per 30 mg wet weight) in a VirTis homogenizer (15,000 rpm for 3 min at 4°). The homogenate was centrifuged at 27,000 × g for 30 min, and the supernatant was heated to 56° for 60 min to destroy complement and then centrifuged once again at 27,000 × g for 30 min. The supernatant was then passed through a Millipore PSAC ultrafiltration membrane (cutoff 1000 molecular weight at 30 pounds/inch² (200 kPa) with N2 gas in an Amicon cell). The protein-free ultrafiltrate was treated with Florisil, a steroid absorbent, that had previously been washed with twice-distilled H2O to remove fine particles (14). Florisil (60 mg/ml) was added to the ultrafiltrate, and the suspension was vortex mixed for 60 sec. The supernatant was removed and passed through a Millipore filter (0.45 μm pore diameter) into sterile tubes and stored at −20°.

For those experiments testing the effect of catecholamines and adrenergic agonists and antagonists, the protein-free ultrafiltrate derived from the cortex was not extracted with Florisil, but was treated by exposure to alumina as described for adsorption of plasma catecholamines (15) and then was extracted twice with 4 vol of chloroform for 60 min with constant shaking. The aqueous fraction resulting from this extraction was then lyophilized overnight, redissolved to the original volume with twice-distilled H2O, passed through a Millipore filter (0.45 μm) into sterile tubes, and stored at −20°.

Sperm Washing Procedure. Spermatozoa recovered from the cauda epididymides of 3- to 4-month-old golden hamsters weighing 130-150 g were washed by the method of Bavister and Yanagimachi (11) except for the following modifications: the distal tubule of one epididymis was punctured, the contents were carefully layered on 10 ml of washing medium (equal volumes of Dulbecco's phosphate-buffered saline and isotonic sucrose) containing penicillin at 100 units/ml in a conical centrifuge tube, and the spermatozoa were allowed to disperse


* To whom reprint requests should be addressed.
until homogeneous. After the second centrifugation step, the sperm pellet was gently resuspended in 0.5 ml of washing medium, but the suspension was not centrifuged again.

An aliquot of the washed sperm suspension was diluted 1:14 with modified Tyrode's buffer (containing 25.0 mM bicarbonate, glucose at 0.9 mg/ml, 0.2 mM pyruvate, 10.0 mM lactate, and penicillin at 200 units/ml and adjusted to pH 7.4 with CO2 prior to sperm addition) to a concentration of 5.3-6.8 X 10^6 sperm/ml. Aliquots of this sperm suspension were used in all incubations.

**Sperm Incubation Procedure.** Incubations were carried out at 37° in a humidified 95% air/5% CO2 atmosphere in sterilized 1.5-ml polypropylene Eppendorf tubes (Brinkmann) in the absence of oil (16). In all cases, the pH of the incubation mixtures was maintained at 7.5-7.6 throughout the incubation. In experiments testing the medulla and cortex preparations, incubation tubes contained 50 μl of sperm suspension, 40 μl of bovine serum albumin dissolved in Tyrode's buffer (pH 7.4, 12 mg/ml), 5 μl of either cortex preparation or medulla preparation (Florisil treated), and 5 μl of phosphate-buffered saline. When the two preparations were tested together, 5 μl of each was used and the phosphate-buffered saline was omitted.

In experiments testing epinephrine, norepinephrine, and adrenergic agonists, the incubation tubes contained 50 μl of sperm suspension, 40 μl of bovine serum albumin, 5 μl of the alumina-treated and chloroform-extracted cortex preparation (AC cortex preparation), and 5 μl of a stock solution of the compound being tested. The compounds were dissolved in phosphate-buffered saline and passed through Nucleopore filters (0.45 μm), immediately before addition to the incubation tubes.

In experiments testing the adrenergic antagonists, the incubation tubes contained 50 μl of sperm suspension, 40 μl of bovine serum albumin, 5 μl of the AC cortex preparation, 5 μl of a stock solution of epinephrine, and 5 μl of a stock solution of the antagonist being tested. The antagonists were dissolved in twice-distilled H2O and passed through Nucleopore filters (0.45 μm) immediately before addition to the incubation tubes.

**Determination of Sperm Motility, Activation, and Acrosome Reactions.** At various times during the incubation, individual tubes were removed from the incubator and the percentage of motile spermatozoa and the percentage of motile spermatozoa showing activation at 37° by phase-contrast microscopy were estimated (16). Activated spermatozoa were identified by a vigorous whiplash flagellar movement characteristic of capacitation (17). The percentage of motile spermatozoa that had undergone an acrosome reaction was determined by phase-contrast microscopy, using the morphological criteria established by Yanagimachi (4) and observing at least 100 motile spermatozoa.

**Chemicals Used.** Pyruvic acid sodium salt, L-(-)-lactic acid (30%), D-(+)-glucose, penicillin G sodium salt, (-)-epinephrine, (-)-norepinephrine, (-)-phenylephrine-HCl, Florisil, and alumina (grade 1, neutral) were purchased from Sigma Chemical Co. Sucrose was purchased from J. T. Baker. Phen tolamine-HCl, (-)-propranolol-HCl, and (-)-isoproterenol (+)-bitartrate were gifts from Ciba Pharmaceutical Co., Ayerst Laboratories, and Sterling-Winthrop Research Institute, respectively. Pentex fraction V bovine serum albumin was obtained from Miles Laboratories.

**RESULTS**

**Florisil-Treated Bovine Adrenal Cortex and Medulla Preparations.** While the percentage of spermatozoa alive during an incubation (% motility) was constant irrespective of which source of "motility factor" was used, sperm motility was more vigorous in the presence of the medulla preparation (data not shown). The protein-free ultrafiltrates of the bovine adrenal gland vary in their ability to induce activation (Fig. 1A). The cortex preparation was ineffective in inducing activation; only a low percentage of activation (never more than 20%) was observed during 4.5 hr of incubation. However, the medulla preparation induced a high degree of activation, reaching a plateau at 50% after only 3.5 hr. When the cortex and medulla preparations were present together, a higher percentage of activation (40%) was seen early in the incubation and the plateau (60%), also reached at 3.5 hr, was higher.

The protein-free ultrafiltrates of the bovine adrenal gland also differ in their ability to stimulate the acrosome reaction (Fig. 1B). The Florisil-treated cortex preparation never stimulated more than 20% acrosome reactions up to 4 hr (although as many as 40% were detected at 4.5 hr). However, in the presence of Florisil-treated medulla preparation, acrosome reactions were observed as early as 2.5 hr and the percentage increased to greater than 70% by 4.5 hr. High numbers of acrosome reactions (80% at 4 hr) also were seen in spermatozoa incubated in the presence of both the medulla and cortex preparations.

**Epinephrine and Norepinephrine.** Because epinephrine and norepinephrine are present in the adrenal medulla in high amounts, each was tested in place of the medulla preparation. However, spermatozoa did not survive in the presence of bovine serum albumin and catecholamine alone but required the presence of the cortex preparation (data not shown). The AC cortex preparation was ineffective in inducing activation (Figs. 2A and 3A). Only 10-20% of motile incubated spermatozoa were activated during a 4.5-hr incubation. Those spermatozoa that were motile were less vigorous than spermatozoa incubated
in the presence of added epinephrine or norepinephrine (data not shown). The results in Fig. 2A also show that epinephrine, in the presence of AC cortex preparation, stimulated activation. The highest concentration tested, 50.0 µM, gave a high percentage of activation (50%) early in the incubation and reached a plateau at 60%. In the presence of lower concentrations, 5.0 µM and 0.5 µM, the same plateau was reached by 4.5 hr, but at earlier times the percentage of activation was lower than that seen with 50.0 µM epinephrine.

Epinephrine in the presence of the AC cortex preparation also stimulated acrosome reactions (Fig. 2B). With 50.0 µM epinephrine, the percentage of acrosome reactions increased from 10% at 3 hr to 80% at 4.5 hr. In the absence of added epinephrine, 20% acrosome reactions were seen at 4.0 hr and the percentage increased to over 40% at 4.5 hr. In the presence of 5.0 µM and 0.5 µM epinephrine, there were 70% acrosome reactions at 4.5 hr.

Norepinephrine also stimulated activation in the presence of the AC cortex preparation (Fig. 3A). With 50.0 µM norepinephrine, activation was high (30%) early in the incubation and reached a plateau (60%) at 4 hours. The lower concentrations of norepinephrine, while better than the AC cortex preparation alone, were less effective at inducing activation than equal concentrations of epinephrine (Figs. 2A and 3A).

Norepinephrine also stimulated acrosome reactions (Fig. 3B). With 50.0 µM norepinephrine, the percentage of acrosome reactions increased from 5% at 3 hr to nearly 80% at 4.5 hr. The lower concentrations tested, 5.0 µM and 0.5 µM, did not stimulate acrosome reactions above the control values.

Adrenergic Agonists. The α-adrenergic agonist phenylephrine and the β-adrenergic agonist isoproterenol were tested for their ability to induce the acrosome reaction and activation in the presence of the AC cortex preparation (Table 1). With epinephrine, which is known to interact with both α- and β-adrenergic receptors (18), the percentage of acrosome reactions and activation is greatly increased over the control values (AC cortex preparation only) at the three concentrations tested. The α-adrenergic agonist at 50.0 µM and 5.0 µM was as effective as epinephrine in the induction of the acrosome reaction, but the percentage of activated spermatozoa was reduced. Spermatozoa incubated in the presence of isoproterenol showed a high degree of activation equal to that seen with spermatozoa incubated in the presence of epinephrine at the three concentrations tested. However, isoproterenol only increased the percentage of acrosome reactions over control values at the highest concentration tested, 50.0 µM. When phenylephrine and isoproterenol were present together, the percentage of activated spermatozoa was equal to that with epinephrine at all three concentrations tested and the percentage of acrosome reactions was equal to that seen in tubes with epinephrine presented except at the highest concentration, 50.0 µM.

Adrenergic Antagonists. The α-adrenergic antagonist phentolamine and the β-adrenergic antagonist propranolol were tested for their ability to inhibit activation and acrosome reactions in the presence of 10.0 µM epinephrine (Table 2). Phentolamine, 25.0 µM and 10.0 µM, reduced acrosome reactions and activation during a 4.5 hr incubation. However, with 1.0 µM phentolamine, there was no effect on either acrosome reactions or activation. Propranolol, 25.0 µM, reduced acrosome reactions and completely inhibited activation. With 10.0 µM and 1.0 µM propranolol, activation was reduced, but there was no effect on acrosome reactions. The percentages of acrosome reactions obtained in the presence of either 25.0 µM phentolamine or 25.0 µM propranolol (Table 2) were also lower than the percentage of acrosome reactions in the presence of bovine serum albumin and the AC cortex preparation (Fig. 2B).
Table 1. Effect of adrenergic agonists on the hamster sperm acrosome reaction

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>% motile, % activated, % AR, SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (13)</td>
<td>(50 (40–60) 0 (0–20) 27 ± 4</td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td></td>
</tr>
<tr>
<td>50.0 μM (12)</td>
<td>(60 (40–70) 70 (50–90) 67 ± 4</td>
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<tr>
<td>5.0 μM (5)</td>
<td>(50 (50) 50 (40–60) 48 ± 7</td>
</tr>
<tr>
<td>0.5 μM (4)</td>
<td>(50 (50) 50 (40–60) 41 ± 4</td>
</tr>
<tr>
<td>(-)-Phenylephrine</td>
<td></td>
</tr>
<tr>
<td>50.0 μM (4)</td>
<td>(50 (50) 40 (20–40) 68 ± 4</td>
</tr>
<tr>
<td>5.0 μM (4)</td>
<td>(50 (50) 20 (10–40) 46 ± 6</td>
</tr>
<tr>
<td>0.5 μM (4)</td>
<td>(50 (50) 20 (10–30) 26 ± 7</td>
</tr>
<tr>
<td>(-)-Isoproterenol</td>
<td></td>
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<tr>
<td>50.0 μM (4)</td>
<td>(60 (60) 80 (80–90) 53 ± 4</td>
</tr>
<tr>
<td>5.0 μM (4)</td>
<td>(50 (50–60) 50 (30–70) 25 ± 2</td>
</tr>
<tr>
<td>0.5 μM (4)</td>
<td>(50 (50–60) 30 (30–40) 23 ± 3</td>
</tr>
<tr>
<td>(-)-Phenylephrine + (-)-Isoproterenol</td>
<td></td>
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<tr>
<td>25.0 + 2.5 μM (6)</td>
<td>(60 (60–70) 80 (70–80) 53 ± 8</td>
</tr>
<tr>
<td>2.5 + 2.5 μM (5)</td>
<td>(60 (50–70) 50 (40–70) 47 ± 7</td>
</tr>
<tr>
<td>0.25 + 0.25 μM (5)</td>
<td>(50 (50–70) 40 (40–50) 44 ± 3</td>
</tr>
</tbody>
</table>

* Incubations carried out in the presence of the AC cortex preparation for 4 hr.
† Approximate percentage of motile sperm at 37°C.
‡ Approximate percentage of motile sperm at 37°C with whiplash flagellar movement characteristic of capacitated sperm.
§ Percentage of motile sperm (at least 100 observed) that had undergone an acrosome reaction, observed by phase contrast microscopy.

It should be noted that, in the presence of 25.0 μM propranolol, abnormalities of the sperm head occurred in approximately 30% of the motile spermatozoa. In these spermatozoa the tip of the acrosomal cap assumed a swollen blunt shape, making it impossible to determine if an acrosome reaction had occurred. These spermatozoa were not included in the acrosome reaction counts. As yet we do not know whether the abnormalities of the sperm head represent a stage of inhibited membrane fusion or nonspecific damage of the membranes of some spermatozoa.

Table 2. Effect of adrenergic antagonists on the hamster sperm acrosome reaction in the presence of (-)-epinephrine

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>% motile, SEM</th>
<th>% activated, SEM</th>
<th>% AR, SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 10.0 μM</td>
<td>(50 (50–70) 70 (50–80) 67 ± 3</td>
<td></td>
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<tr>
<td>(-)-epinephrine (11)</td>
<td></td>
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<tr>
<td>Phentolamine + 10.0 μM epinephrine</td>
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<tr>
<td>25.0 μM (4)</td>
<td>(50 (30–50) 10 (0–20) 11 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 μM (4)</td>
<td>(40 (30–50) 40 (10–60) 34 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 μM (4)</td>
<td>(50 (50) 60 (50–80) 63 ± 8</td>
<td></td>
<td></td>
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<tr>
<td>(-)-Propranolol + 10.0 μM epinephrine</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>25.0 μM (4)</td>
<td>(40 (30–50) 0 (0) 20 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 μM (4)</td>
<td>(50 (50–60) 40 (30–50) 64 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 μM (4)</td>
<td>(50 (50–60) 50 (30–80) 69 ± 8</td>
<td></td>
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</tr>
</tbody>
</table>

* Incubations carried out in the presence of the AC cortex preparation for 4.5 hr.
† Approximate percentage of motile sperm at 37°C.
‡ Approximate percentage of motile sperm at 37°C with whiplash flagellar movement characteristic of capacitated sperm.
§ Percentage of motile sperm (at least 100 observed) that had undergone an acrosome reaction, observed by phase contrast microscopy.

DISCUSSION

We have shown in this paper that the "motility factor" previously found in hamster adrenal glands (10) is also present in bovine adrenal glands. The results of the experiments indicated that the adrenal medulla contained factors that sustained normal motility and also stimulated both activation and acrosome reactions. The adrenal cortex preparation did not stimulate acrosome reactions or activation as well as the medulla preparation, but did support motility and seemed to facilitate the activity of the medulla preparation. The cortex preparation was treated with Florisil or extracted with chloroform to remove several steroids (cortisol, progesterone, testosterone, and estrogen) that in high concentrations (10–100 μM) inhibit in vitro acrosome reactions of hamster spermatozoa (unpublished data).

The adrenal gland motility factor is probably more than one molecule, because spermatozoa did not survive in the presence of bovine serum albumin and catecholamine alone but required the presence of the cortex preparation.

Although activation has only been observed in hamster and guinea pig spermatozoa, the occurrence of acrosome reactions in motile hamster and guinea pig spermatozoa together with a high percentage of activation is generally considered to be an indication that capacitation and subsequent physiological acrosome reactions have occurred (17, 19, 20). In the present results, the acrosome reactions stimulated by catecholamines in the presence of bovine serum albumin and the cortex preparation do appear to be physiological. Unpublished results (D. Friend and S. Meizel) indicate that the ultrastructural morphology of the hamster sperm acrosome reaction induced in the presence of bovine serum albumin and a protein-free preparation of the bovine adrenal gland is typical of the physiological acrosome reaction, by previously established criteria (1). In addition, spermatozoa incubated in the presence of bovine serum albumin, the AC cortex preparation, and 20.0 μM epinephrine fertilize a high percentage of hamster eggs, while spermatozoa incubated in the presence of albumin and the AC cortex preparation only did not fertilize eggs (L. E. Cornett, B. Bavister, and S. Meizel, unpublished data).

Because phentolamine and propranolol in the presence of added epinephrine reduced the percentage of acrosome reactions and activation below that obtained in the presence of only bovine serum albumin and the cortex preparations, it is likely that low levels of catecholamines remaining in the AC cortex preparation or bound to albumin were responsible for the induction of acrosome reactions and low activation in the absence of added catecholamines. The ultrastructural morphology of these acrosome reactions observed in the absence of activation will have to be studied before we can determine whether or not such acrosome reactions are physiological.

Ahlquist (21) divided adrenergic responses into two types on the basis of relative potencies of six catecholamines on different physiological preparations. Moran and Perkins (22) were able to define two types of adrenergic antagonists that supported Ahlquist's findings. The nature of the behavior of the agonists and antagonists led to a definition of an α-adrenergic receptor and a β-adrenergic receptor to explain the many different responses to catecholamines.

Our results with adrenergic agonists and antagonists suggest the involvement of both α- and β-adrenergic receptors in the stimulation of activation and the acrosome reaction. Both activation and acrosome reactions were inhibited by either antagonist. The α-adrenergic agonist was as potent as epinephrine in the stimulation of acrosome reactions, but activation was reduced. The β-adrenergic agonist was as potent as epinephrine in the stimulation of activation but had a reduced ability to stimulate acrosome reactions. A high percentage of activation
and acrosome reactions was seen only when epinephrine or norepinephrine was used or when the α- and β-adrenergic agonists were used together. Epinephrine is known to have both α- and β-adrenergic effects, and norepinephrine acts predominantly on α-adrenergic receptors but has some effect on β-adrenergic receptors (18).

Obviously an increased number of capacitated spermatozoa could lead to an increased number of acrosome-reacted spermatozoa (provided an acrosome reaction initiator was also present). Further experiments will be necessary in order to determine in a definitive manner whether catecholamines stimulate the acrosome reaction primarily through some direct effect, indirectly through stimulation of capacitation, or through both mechanisms.

The expression of β-adrenergic receptors is usually mediated through increased cyclic AMP levels (23). There is evidence that cyclic AMP may be involved in the capacitation process (24). The expression of α-adrenergic receptors is not as well understood, but there is evidence that α-adrenergic activation leads to a calcium influx in hepatocytes (25) and in parotid gland (26). Calcium has been shown to be essential for activation and the acrosome reaction of mammalian spermatozoa (20, 27). The manner in which putative increased levels of sperm cyclic AMP or calcium might then stimulate activation or acrosome reactions is not yet known. Several such mechanisms for the acrosome reaction have been suggested, involving cyclic AMP and calcium stimulation of sperm hydrolytic enzymes (3).

It having been established that epinephrine and norepinephrine interact with hamster spermatozoa in vitro to stimulate activation and the acrosome reaction, it will be important to determine whether or not catecholamines stimulate capacitation and/or the acrosome reaction of spermatozoa of other species and in vivo. Although the catecholamine content of whole oviduct and uterus (the sites of in vivo capacitation) have been determined in several mammalian species (28, 29), no such assay has been made of a female reproductive tract fluid. In addition, catecholamines have been demonstrated to be present in unfertilized rat ova (30). It will be of interest to determine if the motility factor derived from mammalian spermatozoa (11) contains catecholamines.

In conclusion, these results demonstrate a positive effect of a hormone on activation (an indication of hamster sperm capacitation) and the acrosome reaction of mammalian spermatozoa. Further experiments may add to our understanding of the molecular events essential for mammalian fertilization and future extension of these results to other species may lead to new approaches to contraception.

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