Identification of functional $\alpha_2$- and $\beta$-adrenergic receptors in mammalian spermatozoa

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BACKGROUND: A recent study of several compounds, structurally related to amphetamine, provided evidence that mammalian spermatozoa might have adrenergic receptors able to regulate cAMP production. The present study investigated this possibility using physiological and immunochemical analyses of mouse and human spermatozoa.

METHODS: Antibodies specific for different receptor subtypes were used for Western blotting of mouse and human sperm lysates and for immunocytochemical evaluation of whole mouse and human spermatozoa. Uncapacitated and capacitated mouse spermatozoa were incubated with specific agonists and antagonists for $\alpha_2$, $\beta_1$, $\beta_2$ and $\beta_3$-adrenergic receptors for $\sim 35$ min and then assessed using chlortetracycline (CTC) fluorescence. RESULTS: Western blotting revealed proteins of the correct size for all these receptors; immunolocalization indicated their presence on the head, especially acrosomal and neck regions, and flagellum of both mouse and human spermatozoa. CTC results indicated significant responses to agonists for all of the $\beta$-receptors in uncapacitated cells, with agonist effectiveness being $\beta_1 > \beta_2 > \beta_3$; relevant antagonists blocked responses. In contrast, an agonist and antagonist for $\alpha_2$-receptors acted only on capacitated spermatozoa. CONCLUSION: These experiments provide the first good evidence that mammalian spermatozoa have both $\beta$-adrenergic receptors, known to stimulate cAMP production by membrane-associated adenylyl cyclases (mACs), and $\alpha_2$-adrenergic receptors, known to inhibit cAMP production by mACs. Responses are capacitation state dependent and provide a mechanism for inhibiting spontaneous acrosome reactions and helping to maintain fertilizing ability. These results suggest that the use of amphetamine-related compounds, either for medical or for social reasons, might have an unexpected positive impact on fertility.

Key words: amphetamine/cAMP/capacitation/fertility/membrane-associated adenylyl cyclase

Introduction

When first released from the male reproductive tract, mammalian spermatozoa are non-fertilizing. They require a further, species-dependent, period during which they complete maturation and acquire the capacity to fertilize, this is termed ‘capacitation’ (Austin, 1952). When fully capacitated, spermatozoa express hyperactivated motility and can interact with unfertilized oocytes, first undergoing the acrosome reaction that is triggered by specific molecular interactions between the spermatozoon and the zona pellucida and then fertilizing the oocyte. Spermatozoa that have undergone a spontaneous acrosome reaction before reaching an oocyte are unable to fertilize because they have lost the molecules present on the plasma membrane of the sperm head, which allow them to bind to the zona pellucida.

A recent study reported that cathine and norephedrine, phenylpropanolamines (PPAs) structurally related to amphetamine, significantly accelerated capacitation but inhibited spontaneous acrosome reactions in both mouse and human spermatozoa (Adeoya-Osiguwa and Fraser, 2005). These two compounds are metabolites of cathinone, a naturally occurring alkaloid found in leaves of the shrub Catha edulis (khat) that are often chewed recreationally; cathinone has euphoric, stimulatory properties, reflecting its structural similarities with amphetamine (Brenneisen et al., 1990; Kalix, 1992). Because some studies on somatic cell systems had suggested that these compounds might act by binding to adrenergic receptors (Rothman et al., 2003), both adrenaline and noradrenaline were also tested for effects on sperm function and found to elicit the same responses as PPAs. Initial experiments using chlortetracycline (CTC) analysis showed that responses to both adrenaline and noradrenaline were similar in magnitude, so only adrenaline was used in subsequent protocols. In particular, uncapacitated suspensions that were pre-incubated for only 15 min in adrenaline were significantly more fertile than untreated controls when analysed after 75 min co-incubation with oocytes, indicating stimulation of both hyperactivation and changes in the sperm head needed for successful fertilization. Furthermore, adrenaline was shown to stimulate cyclic adenosine monophosphate (cAMP) production in uncapacitated spermatozoa but to inhibit it in capacitated cells. These results suggested that mammalian...
spermatozoa might have adrenergic receptors able to regulate the activity of membrane-associated adenyl cyclases (mACs) and their production of cAMP. Adrenergic receptors are known to be G-protein coupled receptors (GPCRs), many of which have been shown to modulate the activity of mACs in somatic cells (Watling et al., 2001).

During the 1970s and 1980s, there was considerable interest in the possible presence of adrenergic receptors on mammalian spermatozoa because various catecholamines, especially adrenaline (epinephrine) and noradrenaline (norepinephrine), appeared to have biological effects on hamster sperm function. Both adrenal gland extracts, containing catecholamines, and individual catecholamines were reported to stimulate hamster sperm motility (either general or hyperactivated), capacitation, acrosome reactions and fertilizing ability in vitro (Bavister et al., 1976; Cornett and Meizel, 1978; Bavister et al., 1979; Cornett et al., 1979; Meizel and Working, 1980; Bize and Santander, 1985). Although incubation of hamster spermatozoa in the continuous presence of adrenaline and noradrenaline resulted in high levels of acrosome loss (Cornett and Meizel, 1978), pre-incubation for 3.5 h in the absence of catecholamines, followed by their addition, failed to trigger acrosome loss; this observation led the authors to suggest that these compounds acted mainly on capacitation (Meizel and Turner, 1983). Relatively, high concentrations of catecholamines, about 20–50 μmol/l, were used in those various studies. Recently, Way et al. (2001) found evidence for the presence of noradrenaline, at moderate nanomolar to low micromolar concentrations, in bovine oviductal fluid and subsequently investigated whether this compound had any effect on bovine spermatozoa. By using rather complicated experimental protocols, Way and Killian (2002) reported that low concentrations of noradrenaline, but not adrenaline, stimulated capacitation and induced the acrosome reaction, whereas high concentrations appeared to inhibit these responses. They suggested that there might be two different populations of receptors but had no direct experimental evidence to support this possibility. Earlier studies, using a fluorescent β-adrenergic receptor antagonist probe (Cornett and Meizel, 1980) and ligand-binding techniques (Falkay et al., 1989), were unable to obtain conclusive data demonstrating the presence of adrenergic receptors on mammalian spermatozoa. In reviewing existing evidence for the presence of membrane receptors on mammalian spermatozoa, originally thought to be neuron-specific, Meizel (2004) recently concluded that the indications for adrenergic receptors were not strong.

However, it is likely that those earlier failures to identify adrenergic receptors reflected the scarcity of adequate tools to address the question. We reasoned that the better reagents now available, including specific antibodies for the different receptor subtypes and a greater array of specific agonists and antagonists, should make it easier to look for and to find these receptors if they are present. Because both PPAs and adrenalin/noradrenaline appeared able to regulate the production of cAMP, we hypothesized that mammalian spermatozoa have two populations of adrenergic receptors involved in these responses: β-receptors being involved in the stimulatory response and α2 in the inhibitory response. These are the adrenergic receptors known to stimulate (β) and inhibit (α2) cAMP production in somatic cells; it seemed unlikely that α2-receptors would be involved. Inclusion of pertussis toxin abolished cathine’s ability to inhibit spontaneous acrosome reactions in capacitated mouse spermatozoa (Adeoya-Osiguwa and Fraser, 2005), consistent with evidence that α2-receptors interact with pertussis toxin-sensitive inhibitory Gα subunits to inhibit mAC activity (Watling et al., 2001). In contrast, α1-adrenergic receptors usually stimulate phospholipase C via pertussis toxin-insensitive Gαq/11 to produce InsP3 and diacylglycerol (Watling et al., 2001).

The present study was designed to test the hypothesis that mammalian spermatozoa have both α1- and β-adrenergic receptors. Specific antibodies were used to identify the presence of receptor proteins with Western blotting and to determine where the receptors might be located on the cell, whereas specific agonists and antagonists were used to look for capacitation-dependent responses. Although the study focused primarily on mouse spermatozoa, in several instances, it was possible to use human spermatozoa as well.

Materials and methods

Media and reagents

For mouse sperm suspensions, a modified Tyrode’s medium (Fraser, 1993) containing 1.8 mmol/l CaCl2, 25 mM NaHCO3 and 4 mg/ml bovine serum albumin was used. For human sperm suspensions, Earle’s medium with added penicillin (100 IU/ml) and human serum albumin (4 mg/ml) was used. Unless otherwise stated, all reagents were purchased from Sigma (Poole, Dorset, UK). All stock solutions were prepared daily at 5 mmol/l and diluted using standard culture medium. The solvents used to prepare stock solutions were: DL-noradrenaline hydrochloride, procaterol hydrochloride, BRL 37344 and SKF 86466—culture medium; ICI 118551—absolute ethanol; CGP 20712A, SR 59230A and UK 14304—dimethylsulphoxide. All stock solutions were then diluted 1000-fold or more in medium and used at 1/50; we have found that these final concentrations of non-aqueous solvents have no detectable effect on sperm function. All antibodies and blocking peptides (denoted by P) were from Santa Cruz Biotechnology (CA, USA): α2A (sc-1478, no blocking peptide was available), α2B (sc-1479), α2C (sc-1480), β1 (sc-568 and sc-568 P), β2 (sc-570 and sc-570 P) and β3 (sc-1473 and sc-1473 P for mouse and sc-1472 and sc-1472 P for human).

Sperm suspension preparation

For each replicate, cauda epididymal mouse spermatozoa from at least 2–3 mature TO males (Harlan, Bicester, UK), depending on the amount of suspension required, were released into sterile plastic dishes (Nunc, Roskilde, Denmark) containing modified Tyrode’s medium (approximately 0.8 ml/pair of epididymides). For uncapsulated cells, suspensions were allowed to disperse for 5 min on a warming tray and then filtered through short columns of Sephadex G-25 (medium grade; Amersham Biosciences, Little Chalfont, UK), pre-equilibrated with medium, to remove non-motile cells. Centrifugation is not an option with mouse spermatozoa because this removes a decapacitation factor, leading to accelerated capacitation even before experimental treatment begins (Fraser, 1984). Filtrates were pooled, assessed to determine that motility was satisfactory (approximately 90% motile), and then immediately treated as detailed in the Results.

For capacitated cells, spermatozoa were released into modified Tyrode’s medium, allowed to disperse and assessed for motility; suspensions were then covered with equilibrated autoclaved liquid
paraffin (Boots, Nottingham, UK) and incubated in a gas phase of 5% CO₂:5% O₂:90% N₂ at 37°C for a minimum of 90 min. Suspensions were then filtered as described. This protocol has been shown many times to produce capacitated suspensions, as evidenced by CTC analysis (Fraser et al., 1997), IVF (Fraser et al., 1997) and protein tyrosine phosphorylation analysis (Adeoya-Osiguwa and Fraser, 2000).

Motile human spermatozoa were obtained using discontinuous Percoll gradient centrifugation, washing and resuspension in Earle’s medium (Fraser and Osiguwa, 2004). The use of human semen samples for this research has received ethical approval from the King’s College London Research Ethics Committee. Whole cells were used for immunocytochemical and sperm lysates for Western blotting.

**CTC fluorescence analysis**

The functional state of mouse spermatozoa was assessed using the CTC fluorescence assay (Green et al., 1994). An Olympus BX41 microscope (Olympus Optical, London, UK) equipped with phase-contrast and epifluorescent optics was used for assessment, with the U-MW BV2 fluorescence cube (wide blue–violet), the one suitable for CTC fluorescence. In each treatment sample in each replicate, 100 spermatozoa were classified into three staining patterns: F, with fluorescence over the entire head, characteristic of uncomplicated, acrosome-intact spermatozoa; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact spermatozoa; and AR, with dull or absent fluorescence over the entire head, characteristic of acrosome-reacted spermatozoa.

**Western blotting**

Western blotting was carried out using specific antibodies and blocking peptides for α₂A-, α₂B-, α₂C-, β₁-, β₂- and β₃-adrenergic receptors. Mouse and human sperm lysates were prepared by freeze–thawing suspensions in liquid N₂, pelleting the cells and resuspending in phosphate-buffered saline containing 40 mg/ml trypsin inhibitor and 0.2 μg/ml leupeptin. Proteins were resolved using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Amersham Biosciences). These were blocked in 5% milk in Tris-buffered saline plus 0.1% Tween (TBST) for 1 h at room temperature and then incubated overnight at 4°C in antibodies diluted in TBST-containing 0.5% milk; the dilutions ranged from 1:1000 for β₁ to 1:200 for β₃. Antibody specificity was checked by pre-incubating the different primary antibodies with the appropriate blocking peptide (1:5) for at least 1 h before use. The membranes were washed (3 × 5 min in TBST) and then incubated at room temperature for 1 h in peroxidase-linked secondary antibody (depending on the primary antibody, either 1:20 000 donkey anti-goat IgG or 1:20 000 goat anti-rabbit IgG in TBST plus 0.5% milk). After further washing (6 × 5 min in TBST), proteins were detected using an enhanced chemiluminescence (ECL)-Plus Western blotting detection system on ECL Hyperfilm (Amersham Biosciences).

**Immunolocalization**

Filtered mouse sperm suspensions and Percoll-prepared human sperm suspensions (resuspended to 4 × 10⁷ cells/ml) were prepared for immunocytochemistry using the techniques described by Gibbons et al. (2005), including permeabilization. Suspensions were mixed 1:1 with 4% (w/v) sucrose help to stabilize cellular structure, permeabilized by freezing in liquid N₂ and thawing, and then fixed with 2.5% (w/v) paraformaldehyde; cells needed to be permeabilized because all antibodies were directed against epitopes in the intracellularly located C-terminus region of the proteins. The cells were left to bind to the poly-L-lysine-coated slides for 10 min. The primary antibodies were used at 1:25, and the fluorescein isothiocyanate-labelled secondary antibodies (either donkey anti-goat or goat anti-rabbit IgG, depending on the primary antibody) were used at 1:200. Normal goat or donkey serum at 3% was used as a blocker.

**Statistical analysis**

CTC data were analysed using Cochran’s modification of the χ² test, which compares responses within each replicate and then sums the values (Snedecor and Cochran, 1980). To obtain a statistically significant difference, the responses must be consistent and the difference in responses between the experimental and control values must be sufficiently large in each replicate.

**Results**

**Series I: Western blotting identification of α₂A- and β-adrenergic receptor proteins in mouse and human sperm lysates**

When mouse sperm lysates were probed for the presence of adrenergic receptor proteins, proteins of the correct size were found for α₂A-, β₁-, β₂- and β₃-adrenergic receptors (Figure 1); no proteins were identified using the α₂B and α₂C antibodies. Pre-incubation of the specific antibodies with the relevant blocking peptide abolished the signal for these proteins (no peptide was available for α₂A); however, some lower molecular weight bands were still present, indicating some non-specific binding of the secondary antibody. Similarly, evaluation of human sperm lysates with the β₁ antibody also showed the presence of a protein of the appropriate size (data not shown) and the blocking peptide abolished the signal; subsequent immunolocalization results confirmed the presence of this and other adrenergic receptor proteins in human spermatozoa. These results supported the hypothesis being tested and so further investigations were undertaken to determine where the receptors are located within the sperm cells and whether they are functional.
Series II: Immunolocalization of adrenergic receptors on mouse and human spermatozoa

Using uncapacitated mouse spermatozoa for the main investigations, a strong signal for $\alpha_{2A}$-adrenergic receptors was seen in the head, especially in the acrosomal cap, post-acrosomal and neck regions, with fainter fluorescence along the flagellum (Figure 2). Although the $\alpha_{3B}$ and $\alpha_{5C}$ antibodies failed to detect any protein bands when used in Western blotting, they were still used for immunolocalization because some antibodies work well in one application but not in another. However, there was no evidence for positive staining with either antibody, leading us to conclude that neither $\alpha_{2B}$- nor $\alpha_{2C}$-adrenergic receptors are present.

$\beta$-Adrenergic receptors appear to be located in the same general regions as the $\alpha_{2A}$ receptors, with a strong fluorescent signal for $\beta_1$-receptors being observed on the head, especially in the acrosomal cap, equatorial segment and neck regions, and weaker fluorescence on the flagellum (Figure 2). $\beta_2$-Receptors also appear on both the head (acrosomal cap and neck) and the flagellum, the fluorescent signal on the head being the stronger. For $\beta_3$-receptors, fluorescence was consistently seen in the acrosomal cap and post-acrosomal regions as well as on the flagellum, particularly, in the midpiece. Although immunolocalization data are not quantitative, the fluorescent signals were stronger for $\beta_3$-receptors than for the other two $\beta$-receptors. This is consistent with our finding that higher concentrations of $\beta_2$ and $\beta_3$ agonists were required to obtain significant responses compared with the $\beta_1$ agonist. The distributions shown in Figure 2 are typical of the large majority of cells in 3–4 replicates. When antibodies were pre-incubated with the appropriate blocking peptides, no fluorescence was observed, indicating the specificity of the antibodies. To illustrate this, both fluorescence and phase-contrast photographs of a spermatozoon incubated with peptide-blocked antibody to the $\beta_3$-receptor are shown in Figure 2; the other peptide-blocked antibodies gave similar results.

Because the responses to specific agonists indicated that these receptors have capacitation state-dependent functionality (Series III–V), with $\beta$-receptors only active in uncapacitated cells and $\alpha_{2A}$ receptors only active in capacitated cells, we investigated possible capacitation state-dependent differences in the staining patterns. We did this because such changes were observed in our recent study of adenosine receptors, which also have capacitation state-dependent functionality (Adeoya-Osiguwa and Fraser, 2002). An aliquot of sperm suspension was incubated for about 25 min and then centrifuged to remove endogenous decapacitation factor (DF) proteins and so accelerate capacitation (method described by Gibbons et al., 2005). Aliquots of DF-depleted spermatozoa were treated with $\beta_1$ and $\alpha_{2A}$ antibodies and then evaluated; when these cells were compared with antibody-treated uncapacitated cells from the same suspensions, there was no clear evidence for different patterns or intensity of staining with either antibody (data not shown). This probably means that any changes in protein conformation that might be involved in altering receptor function do not change the accessibility of the relevant antigenic epitopes.

Figure 2. Immunolocalization of $\alpha_{2A}$- and $\beta$-adrenergic receptors on mouse spermatozoa. Specific staining was seen with antibodies directed against $\alpha_{2A}$-, $\beta_1$-, $\beta_2$- and $\beta_3$-adrenergic receptors; fluorescence and phase-contrast photographs of individual cells are shown. With all antibodies, a fairly bright fluorescent signal was seen in the acrosomal cap and neck regions of the head, along with less intense fluorescence on the flagellum. Strong staining was also seen in the post-acrosomal region with the $\alpha_{2A}$ antibody and in the equatorial segment with the $\beta_1$ antibody, whereas a weaker signal was seen in the post-acrosomal region with the $\beta_3$ antibody. Inclusion of the relevant blocking peptide blocked the signal with all antibodies; results are shown for the $\beta_1$ antibody. Results are typical of the majority of cells in 3–4 replicates; the bar represents 10 $\mu$m.
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Figure 3. Immunolocalization of \( \alpha_{2A} \) and \( \beta \)-adrenergic receptors on human spermatozoa. These receptors appeared to be located in similar regions to those seen in mouse spermatozoa; fluorescence and phase-contrast photographs of individual cells are shown. Strong fluorescence for \( \alpha_{2A} \) receptors was seen throughout the sperm head, with less intense fluorescence along the flagellum. Using antibodies for \( \beta_1 \) and \( \beta_3 \) receptors, there was strong staining in the acrosomal and neck regions of the head, with less-bright staining along the flagellum; there was also bright fluorescence in the post-acrosomal region with the \( \beta_1 \) antibody. \( \beta_3 \) receptors are presumed to be located in both the head and the flagellum, as for mouse spermatozoa (the required antibody was unavailable when these experiments were undertaken). Inclusion of the relevant blocking peptide blocked the signal with all antibodies; results are shown for the \( \beta_1 \) antibody. Results are typical of the majority of cells in 2 replicates; the bar represents 5 \( \mu \)m.

In human spermatozoa, the adrenergic receptors appear to be located in similar regions to those seen in mouse spermatozoa. A strong signal for \( \alpha_{2A} \)-adrenergic receptors was seen throughout the head, with somewhat less intense fluorescence on the flagellum. A strong fluorescent signal for \( \beta_1 \)-receptors was seen in the acrosomal, post-acrosomal and neck regions of the head, whereas somewhat less intense fluorescence was seen along the flagellum (Figure 3). \( \beta_3 \)-receptors appear to be located in the acrosomal and neck regions of the head and along the flagellum. At the time that these experiments were undertaken, the appropriate \( \beta_2 \) antibody was unavailable; since results with the other three antibodies were similar to those obtained using mouse spermatozoa, we presume that \( \beta_3 \)-adrenergic receptors are present on the head and flagellum of human spermatozoa. The distributions shown in Figure 3 are typical of the large majority of cells in 2 replicates. Again, pre-incubation of antibodies with blocking peptides resulted in no fluorescence; results for peptide-blocked antibody to the \( \beta_1 \)-receptor are shown in Figure 3, and the other blocked antibodies gave similar results.

Series III: \( \beta \)-Adrenergic receptor agonists accelerate capacitation in uncapacitated mouse sperm suspensions but an \( \alpha_2 \) agonist does not

Having obtained evidence for the presence and location of several adrenergic receptor subtypes, we then looked for evidence that the receptors are functional. The specific agonists used were noradrenaline, procaterol, BRL 37344 and UK 14304 for \( \beta_1 \)-, \( \beta_2 \)-, \( \beta_3 \) and \( \alpha_2 \)-adrenergic receptors, respectively (Watling et al., 2001). In our earlier study (Adeoya-Osiguwa and Fraser, 2005), noradrenaline was tested at both 1 and 10 \( \mu \)mol/l; the higher concentration was slightly less effective than the lower one, leading us to suspect that the optimal concentration did not exceed 1 \( \mu \)mol/l. Therefore, in this study, all specific \( \beta \)-adrenergic receptor agonists were tested at 1, 10 and 100 nmol/l, whereas the \( \alpha_2 \)-adrenergic receptor agonist was tested at 1 nmol/l; all reagents were evaluated in each of the 3 replicates (n = 3). Suspensions were prepared as described and divided into aliquots, and agonists were added. After ~35 min, samples were stained with CTC and fixed, and then slides were prepared and evaluated. At the end of the treatment period, samples were quickly evaluated for general motility to ensure that cells were still moving and that the agonists had not adversely affected motility. In all samples, motility was still high (≥80%) and those treated with the \( \beta \) agonists had more vigorous movement than the untreated controls, consistent with our earlier demonstration that adrenaline stimulates cAMP in uncapacitated suspensions (Adeoya-Osiguwa and Fraser, 2005). At all three concentrations tested, noradrenaline significantly (\( P < 0.025–0.001 \)) accelerated capacitation but had no effect on acrosome reactions; there was a clear concentration-dependent response, the greatest response being elicited by 100 nmol/l (Figure 4A). Procaterol appeared to be less effective because a concentration of 10 nmol/l was needed to get a significant response, but the response to 100 nmol/l was similar to that obtained with 100 nmol/l noradrenaline (Figure 4B). BRL 37344 proved to be the least effective of the agonists tested: only 100 nmol/l elicited a significant response (Figure 4C) and this was similar in magnitude to that obtained with 1 nmol/l noradrenaline. Thus, the responsiveness to the three \( \beta \)-receptor agonists appears to be \( \beta_1 > \beta_2 > \beta_3 \).

UK 14304, the \( \alpha_2 \)-adrenergic receptor agonist, had no detectable effect on uncapacitated mouse spermatozoa when used at 1 nmol/l (Figure 4D), a concentration that had a significant effect on capacitated suspensions (Series V). This result suggests that only \( \beta \)-adrenergic receptors are active in uncapacitated cells.

Series IV: \( \beta \)-Adrenergic receptor antagonists block responses to agonists in uncapacitated mouse sperm suspensions

Because 100 nmol/l of all three \( \beta \)-receptor agonists elicited a significant response, this concentration was evaluated on uncapacitated suspensions with or without specific antagonists for each of the three \( \beta \)-receptors. The antagonists were used at concentrations reported to be effective in somatic cells: 100 nmol/l CGP 20712A (\( \beta_1 \)), 10 nmol/l ICI 118551 (\( \beta_2 \)) and 100 nmol/l SR 59230A (\( \beta_3 \)). Each of the antagonists was able to completely block responses to the specific agonists (Figure 5A–C), providing further support for the presence of all three \( \beta \)-adrenergic receptors and their functional activity in uncapacitated spermatozoa.

Series V: An \( \alpha_2 \)-adrenergic receptor agonist inhibits spontaneous acrosome reactions in capacitated mouse sperm suspensions and a specific antagonist blocks this response

To test the working hypothesis further, capacitated suspensions were evaluated for responses to an \( \alpha_2 \)-adrenergic receptor agonist and antagonist. Suspensions were prepared, incubated and filtered as described. Prior to starting incubation with test reagents, a sample of the just-filtered capacitated suspension was immediately stained with CTC and fixed; this provided...
information on the proportions of F, B and AR pattern cells before treatment began. The remaining suspension was divided into aliquots and incubated for ~35 min with or without 1 and 10 nmol/l UK 14304, a specific \( \alpha_2 \)-adrenergic receptor agonist, and with 10 nmol/l UK 14304 + 10 nmol/l SKF 86466 (a specific antagonist) and then assessed using CTC. In each replicate (\( n = 3 \)), procaterol at 100 nmol/l and noradrenaline at 10 nmol/l were also evaluated. We hypothesized that procaterol, a \( \beta_2 \)-receptor agonist, would have no effect on capacitated cells, but that noradrenaline would have because it is also able to act at \( \alpha_2 \)-receptors. As with the uncapacitated suspensions, motility was still high in all samples at the end of treatment.

Results showed that spontaneous acrosome loss in the control suspensions had increased considerably during the 35 min treatment period (Figure 6). In contrast, UK 14304 significantly inhibited the acrosome reaction, with both concentrations being equally effective, but the inclusion of SKF 86466 completely blocked this response. Procaterol had no detectable effect, the proportion of acrosome-reacted cells being essentially the same as seen in the untreated control suspensions and those treated with UK 1304 + SKF 86466. In contrast, noradrenaline significantly inhibited the acrosome reaction, similar to the inhibition seen with UK 14304. Thus, the \( \alpha_2 \)-adrenergic receptors identified by Western blotting appear to be active in capacitated spermatozoa, whereas the \( \beta \)-receptors appear to be inactive in the same cells.

**Series VI: 2',5'-Dideoxyadenosine blocks response to noradrenaline**

As mentioned in the *Introduction*, adrenergic receptors are GPCRs that, in somatic cells at least, are known to interact...
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The responses in uncapacitated suspensions to noradrenaline were blocked by the inclusion of 2′,5′-ddAdo, a specific inhibitor of mACs. This supports our hypothesis that the sperm adrenergic receptors, like those in somatic cells, are regulating the activity of mACs. Although mammalian spermatozoa have been shown to have another, soluble, isoform of AC (sAC) (Buck et al., 1999), sAC is reported to be unaffected by reagents such as ddAdo and G protein inhibitors that can inhibit the functioning of mACs (Wuttke et al., 2001).

The presence of β- and α2A-adrenergic receptors in both the head and the flagellum is consistent with the ability of adrenergic receptors to affect the function of both compartments of spermatozoa. In particular, the presence of α2A-receptors in the acrosomal region is consistent with our evidence that an α2 receptor-specific agonist inhibits spontaneous acrosome reactions. In addition, receptors located on the flagellum would allow for local production of cAMP in response to agonists; this would support and promote the development of hyperactivated motility required for successful fertilization. The fact that uncapacitated mouse spermatozoa pre-incubated with catheine and adrenaline for a short time were significantly more fertile in vitro than untreated controls indicates that more treated cells were expressing hyperactivated motility (Adeoya-Osiguwa and Fraser, 2005). In other studies, we have demonstrated the presence in spermatozoa of stimulatory and inhibitory Gα subunits (Fraser and Adeoya-Osiguwa, 1999; Baxendale and Fraser, 2003b), as well as several different mAC isoforms (Baxendale and Fraser, 2003a), particularly in the regions where the adrenergic receptors are also found. Thus, all components required for the signal transduction pathways are present in the same places.

The adrenergic receptor locations are also similar to those found in both mouse and human spermatozoa for a DF receptor (DF-R) that we have recently identified as phosphatidylserine-binding protein-1 (PEBP-1; Gibbons et al., 2005).
The relative fluorescent intensity was seen with a specific antiserum to DF-R/PDBP-1 altered, depending on the presence or absence of the DF binding to this receptor, and may reflect conformational changes in the DF-R. We have hypothesized that these changes may cause significant alterations in membrane lipid architecture and lead to altered functionality of many membrane-associated proteins, including GPCRs such as adenosine receptors. For example, addition of purified DF to capacitated mouse sperm suspensions, which will decapacitate the spermatozoa and render them non-fertilizing, resulted in inactivation of inhibitory adenosine receptors that function only in capacitated cells and re-activation of stimulatory adenosine receptors that function only in uncapacitated cells (Adeoya-Osiguwa and Fraser, 2002). Because the present study provides evidence that mammalian spermatozoa also have both stimulatory and inhibitory adrenergic receptors whose function is capacitation state dependent, it is plausible that the change from active stimulatory β-adrenergic receptors in uncapacitated cells to active inhibitory α2-adrenergic receptors in capacitated cells involves the following: loss of DF → changes in DF-R conformation → alterations in plasma membrane architecture → changes in the accessibility of adrenergic receptor binding sites.

Although some of our results are consistent with those reported in earlier studies of catecholamines as mentioned in the Introduction, it is difficult to reconcile others. Results in our earlier study confirm that adrenaline and noradrenaline can stimulate capacitation and fertilization in vitro. However, why did so many earlier studies report that adrenaline, noradrenaline and/or adrenal gland extracts stimulated acrosome reactions, when the present results indicate the opposite, namely, that activation of β-adrenergic receptors accelerates capacitation but not acrosome loss and activation of α2-receptors inhibits acrosome reactions? The simplest explanation would appear to be that those studies used high concentrations of specific catecholamines, usually ~20–50 μmol/l, that probably masked or overrode the responses we have obtained. As we previously noted with noradrenaline (Adeoya-Osiguwa and Fraser, 2005), concentrations above 1 μmol/l gave suboptimal stimulatory responses, so perhaps even higher concentrations have non-specific effects. The report by Way et al. (2001) suggested that noradrenaline can be present at low–moderate concentrations in the oviducal secretions around the time of ovulation, at least in cows. The mean values obtained from 6 cows were generally in the range of 20–45 ng/ml (~100–220 nmol/l); the two highest single values were ~494 and 1117 ng/ml (~2.4 and 5.4 μmol/l, respectively), but all other individual values were <75 ng/ml. Therefore, even moderate micromolar concentrations are probably unphysiological, being much higher than those that spermatozoa might be likely to encounter in vivo.

The present results are consistent with the recent demonstration that adrenaline stimulates cAMP production in uncapacitated cells but then inhibits it in capacitated cells (Adeoya-Osiguwa and Fraser, 2005). Because the responses to adrenaline and noradrenaline, as well as PPAs, involve regulation of the production of the ‘second messenger’ cAMP, they all fit the definition of ‘first messengers’. More important, if the biphasic responses they elicit in vitro also occur in vivo, especially in females where capacitation and fertilization occur, all these compounds could play an important role in helping to maintain sperm fertilizing potential by inhibiting the spontaneous acrosome reaction. This inhibition does not interfere with the triggering of the acrosome reaction in fertilizing spermatozoa by the zona pellucida, as demonstrated by enhanced fertilizing ability in vitro of mouse sperm suspensions pre-incubated for a short time in the presence of either adrenaline or cathine (Adeoya-Osiguwa and Fraser, 2005).

Currently, many individuals use amphetamine-related compounds for medical and/or social reasons. Given the presence of functional adrenergic receptors on mammalian spermatozoa, the frequent use of (i) khat leaves for recreational purposes, (ii) amphetamine-related drugs for social (e.g. Ecstasy) or addictive (e.g. Dextedrine and Benzedrine) purposes and (iii) amphetamine-related drugs for medical conditions (e.g. salbutamol to relieve the symptoms of asthma) might have a positive effect on fertility in vivo, even though this may well not have been the intended result. The results obtained in this study suggest that further investigations should be undertaken to determine whether these amphetamine-related compounds, especially when used by females, increase the possibility of conception, planned or otherwise.

References


Submitted on October 14, 2005; resubmitted on December 21, 2005; accepted on January 5, 2006