Cathine and norephedrine, both phenylpropanolamines, accelerate capacitation and then inhibit spontaneous acrosome loss

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BACKGROUND: Cathinone, released when Catha edulis leaves (khat) are chewed, has euphoric, stimulatory properties. It is metabolized to the phenylpropanolamines (PPAs) cathine and norephedrine. This study investigated whether PPAs affect mammalian sperm function, using primarily mouse, but also human, spermatozoa.

METHODS: Uncapacitated sperm suspensions were treated with cathine, norephedrine, adrenaline and noradrenaline, then assessed using chlortetracycline (CTC) fluorescence. Cathine and adrenaline were also evaluated using in vitro fertilization. Capacitated suspensions were treated with PPAs ± progesterone and ± pertussis toxin. Finally, cAMP production was evaluated in uncapacitated and capacitated suspensions.

RESULTS: In uncapacitated mouse spermatozoa, cathine, norephedrine, adrenaline and noradrenaline all significantly accelerated capacitation; uncapacitated human spermatozoa responded similarly to cathine. Consistent with these results, cathine- and adrenaline-treated suspensions were significantly more fertile than controls. In capacitated spermatozoa, both PPAs inhibited spontaneous acrosome reactions (ARs) but progesterone could over-ride this inhibition. Pertussis toxin abolished cathine’s inhibition of ARs, suggesting G protein involvement. Finally, adrenaline/noradrenaline significantly stimulated cAMP production in uncapacitated suspensions, but significantly inhibited it in capacitated suspensions.

CONCLUSIONS: This is the first demonstration that PPAs can directly affect mammalian sperm function, accelerating capacitation and inhibiting spontaneous ARs. These responses correlated with initial stimulation and subsequent inhibition of cAMP production. Adrenaline/noradrenaline elicted similar responses, suggesting the presence of adrenergic receptors. Therefore, regulation of adenylyl cyclase/cAMP in a G protein-mediated fashion by PPAs may possibly involve adrenergic receptors. These results suggest that PPAs, at appropriate doses, might provide a novel approach to enhance natural fertility.

Key words: adrenaline/adrenergic receptors/cAMP/capacitation/G proteins

Introduction

Cathinone is a naturally occurring alkaloid found in leaves of the shrub Catha edulis (khat), which grows in East Africa and Southern Arabia. Inhabitants of these areas use khat as a recreational drug and the leaves are now becoming reasonably accessible in the UK and the rest of Europe (Al-Motarreb et al., 2002). Chewing of the leaves releases cathinone, which has a pharmacological profile resembling that of amphetamine and causes similar euphoric effects (Brenneisen et al., 1990; Kalix, 1992). Cathinone [S-(−)-α-aminopropiophenone] is fairly unstable, being metabolized relatively quickly to the more stable and less potent molecules of cathine [S,−(−)-pseudonoradrenaline] and norephedrine [R,−(−) norephedrine]. In four human subjects who chewed khat leaves for 1 h, then spat out the remaining material and subsequently had urine samples tested for the presence of cathinone and its metabolites, cathinone could be detected for up to ~26 h, but cathine and norephedrine could be detected for at least 80 h (Toennes and Kauert, 2002).

Cathinone, cathine and norephedrine are related to ephedrine, a stimulant present in prescription and over the counter medications and products, e.g. herbal dietary supplements for weight loss and treatment of asthma (Greenway, 2001), and together they are known as phenylpropylamines, with cathine, norephedrine and ephedrine being phenylpropanolamines (PPAs). Previous studies on PPAs have focused on developing methods to accurately detect these compounds in blood and urine samples (notably cathinone and its metabolites: Brenneisen et al., 1986; Toennes and Kauert, 2002; Toennes et al., 2003) or in the CNS, trying to understand how PPAs exert their psychoactive effects. Data from studies in the CNS suggested that these compounds can act at noradrenaline (norepinephrine) transporters; for example, ephedrine has been shown to act as an adrenergic agonist, activating
adrenergic receptors both directly and indirectly, via carrier-mediated exchange with norepinephrine (Rothman et al., 2003). An early study investigating the role of cathinone in brown tissue thermogenesis obtained evidence suggesting that beta adrenergic receptors might be involved in the responses obtained (Tariq et al., 1989).

Relatively few studies have addressed possible effects on reproductive health and these have focused on the male since the recreational use of khat is more prevalent in males than females (Al-Motarreb et al., 2002). Looking for possible mutagenicity caused by cathinone, Quereshi et al. (1988) orally administered aqueous solutions of the compound to adult male mice for 6 weeks, then tested their fertility by mating with females for 2 weeks; at the highest dose tested (40 mg/kg body weight) males appeared to be sterile in the first week of testing, but effects were short-lived, with none detected in the second week, and the underlying biology was very unclear. In another study, administration by injection of pure cathinone was said to cause degenerative changes in testicular morphology and reductions in plasma levels of testosterone in male rats (Islam et al., 1990) but this route of administration is quite different from that occurring when khat leaves are chewed. In a study involving humans (El-Shoura et al., 1995), semen parameters in two groups of Yemeni males, khat ‘addicts’ and ‘non-addicts’, were compared; sperm concentration, morphology and motility were reported to be significantly poorer in the ‘addicts’. However, the age ranges in both groups were wide, there were no details on amounts of khat ingested by the addicts and there was no information on the men’s intrinsic fertility, making it difficult to draw sound conclusions. More recently, the effects on the male reproductive tract were assessed after feeding rabbits for 3 months with a food to which different amounts of dried, ground Catha edulis leaves had been added (Al-Mamary et al., 2002). Unlike previous reports suggesting deleterious effects on the male reproductive tract, histopathological examination of sections of the male reproductive tract suggested that Catha edulis had stimulated spermatogenesis and the cauda epididymides and Leydig cells were normal, when compared with equivalent sections from untreated rabbits. In short, there is no consensus at present regarding the possible effects of PPAs on male reproductive function, perhaps reflecting the use of many different experimental designs.

Thus far there appear to have been no studies investigating whether cathinone and its metabolites have more subtle, direct effects on spermatozoa that might impact on fertility; such effects would probably require lower concentrations than those needed to affect organ function. Successful fertilization can only be achieved after spermatozoa have undergone post-release maturation, termed ‘capacitation’, which takes place in the female tract in vivo but can also be achieved in an appropriate capacitating medium in vitro. Although it is known that capacitation confers on spermatozoa the ability to fertilize an oocyte, the molecular mechanisms involved are still not fully understood (de Lamirande et al., 1997). Upon completion of numerous biochemical and physiological changes, capacitated spermatozoa have the capacity first to undergo the acrosome reaction, an obligatory pre-fertilization event that is usually triggered by the zona pellucida surrounding the oocyte, and then to fertilize the oocyte (Yanagimachi, 1994; de Lamirande et al., 1997). In vitro studies have shown that spermatozoa incubated in capacitating medium for an adequate period of time can complete capacitation and at least some may ‘over-capacitate’ and undergo spontaneous acrosome loss. This is very undesirable since already acrosome-reacted cells are unable to fertilize an oocyte because they have lost important ‘docking’ molecules needed for binding to the zona pellucida prior to undergoing the acrosome reaction.

The present study was designed to investigate, for the first time, whether cathine and norephedrine, the immediate metabolites of cathinone, have any detectable effects on mammalian sperm function. Both compounds were evaluated using uncapacitated and capacitated mouse spermatozoa and, in a small pilot study, uncapacitated human spermatozoa. Initial evaluations used chlortetracycline (CTC) fluorescence analysis to determine possible effects on capacitation per se and the acrosome reaction. Because results indicated that both compounds did elicit responses that should affect fertilizing ability, in vitro fertilization experiments were conducted. Finally, additional experiments were carried out to obtain information on the mechanism of action of PPAs. A short abstract containing some of the results obtained in this study was published in the abstract book of the 20th Annual Meeting of the European Society of Human Reproduction and Embryology (Adeoye-Osiguwa and Fraser, 2004).

Materials and Methods

Media and reagents

For mouse sperm suspensions, a standard modified Tyrode’s medium (Fraser, 1993) containing 1.8 mmol/l CaCl₂ and 4 mg/ml BSA was used. For human sperm suspensions, Earle’s medium with added penicillin (100 IU/ml) and human serum albumin at 4 mg/ml was used. All reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Fertilization promoting peptide (FPP), used as a positive control, was prepared and stored as described by Green et al. (1994); all working stock solutions were prepared fresh daily. Stock solutions of cathine hydrochloride in absolute ethanol and CGS-21680 and cyclopentyladenosine (CPA) in dimethylsulphoxide (DMSO) were prepared daily at 5 mM and diluted using standard medium containing the relevant serum albumin. Stock solutions of adrenaline and noradrenaline were also prepared daily using standard medium without albumin; these were diluted using albumin-containing medium. All working stock solutions were used at a 1/50 dilution to give desired final concentrations. Progesterone was prepared in DMSO and used at 1/100. Pertussis toxin was made up in distilled water to give a concentration of 100 μg/ml and stored refrigerated; for use, it was diluted 1/20 in Tyrode’s modified medium and then used at 1/50.

Sperm suspension preparation

Cauda epididymal mouse spermatozoa from mature TO males (Harlan, Bicester, UK) were released into a sterile dish (Nunc, Roskilde, Denmark) containing modified Tyrode’s medium; all sperm suspensions contained spermatozoa from at least 2–3 males or even more, depending on the amount of sample required for
the particular assay. To evaluate uncapacitated spermatozoa, suspensions were allowed to disperse for 5 min on a warming tray, then filtered through short columns containing Sephadex G-25 (medium grade; Amersham Biosciences, Little Chalfont, UK), pre-equilibrated with medium, to remove non-motile cells. Mouse spermatozoa cannot be centrifuged to obtain motile cells because this removes a decapacitation factor and so accelerates capacitation even before experimental treatment begins (Fraser, 1984). Filtrates were pooled, assessed briefly to check that motility was satisfactory (~90% motile), and then immediately treated as detailed in the Results.

In investigations requiring capacitated cells, spermatozoa were released into modified Tyrode’s medium. After dispersal and brief assessment of motility, the dishes were covered with equilibrated autoclaved liquid paraffin (Boots, Nottingham, UK) and incubated in an atmosphere of 5% CO₂:5% O₂:90% N₂ at 37 °C for a minimum of 90 min. Sperm suspensions were then filtered as described for the uncapacitated suspensions. Using this protocol, we have shown many times that mouse spermatozoa are capacitated as evidenced by chlortetracycline analysis (e.g. Fraser et al., 2001), in vitro fertilization (e.g. Fraser et al., 1997) and tyrosine phosphorylation analysis (Adeoya-Osiguwa and Fraser, 2000).

Motile human sperm suspensions were obtained using discontinuous Percoll gradient centrifugation, followed by washing and resuspension in Earle’s medium as described in Fraser and Osiguwa (2004) and adjusted to 5 × 10⁴ cells/ml. The use of human semen samples for this research has received ethical approval from the King’s College London Research Ethics Committee. Samples were incubated with/without catinge for 1 h in an atmosphere of 5% CO₂:5% O₂:90% N₂ at 37 °C; they were then stained with the vital dye Hoechst bis-benzimide 33258 followed by chlortetracycline (using methodology for staining and slide preparation as described by Green et al., 1996a) and assessed. Because human spermatozoa generally capacitate more slowly than mouse spermatozoa, we have found that incubating suspensions in test compounds for 1 h allows us to detect stimulatory responses reliably (e.g. Fraser and Osiguwa, 2004).

Chlortetracycline fluorescence analysis

The functional state of both mouse and human spermatozoa was assessed using the chlortetracycline (CTC) fluorescence assay as described in Green et al. (1994, 1996a). Assessment was carried out using an Olympus BX41 microscope (Olympus, UK) equipped with phase contrast and epifluorescent optics. CTC analysis used the U-MWVB2 fluorescence cube (wide blue-violet). In each mouse sperm sample, 100 spermatozoa were classified into three staining patterns: F, with fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact spermatozoa; B, with a fluorescence-free band in the postacrosomal region, characteristic of capacitated, acrosome-intact spermatozoa; and AR, with dull or absent fluorescence over the entire head, characteristic of acrosome-reacted spermatozoa.

For human spermatozoa, the live/dead status was assessed first, using Hoechst bis-benzimid 33258 and the U-MWU2 fluorescence cube (wide ultraviolet), and then the CTC pattern was assessed. CTC results were obtained for 100 live cells in each sample. There were very few dead cells (<5%).

cAMP assay

The amount of cAMP produced in live, intact cells was determined using a non-radioactive enzyme immunoassay kit from Amersham Biosciences. Assays (in triplicate or quadruplicate for each treatment in each replicate experiment) were performed as described in the instruction booklet provided with the kit.

In vitro fertilization

Mature female TO mice were induced to superovulate by intraperitoneal injections of 7.5 IU PMSG (Folligon, Intervet, Cambridge, UK) followed by 5 IU hCG (Chorulon, Intervet) administered 48–54 h later. Approximately 14–15 h post-hCG, cumulus masses were released from oviducts into standard medium covered with liquid paraffin. Sperm suspensions were prepared as described previously, but not filtered. After 5 min for dispersal, they were divided into aliquots and treated as detailed in the Results. All suspensions were incubated for 15 min, then diluted 1/10 into the medium used for pre-incubulation; 400 µl droplets from each sample were prepared under liquid paraffin and cumulus masses were added. Dishes were gassed with the mixture used for sperm suspensions and incubated at 37 °C for 60 min. Oocytes were transferred to fresh droplets of standard medium and then fixed at 75 min with buffered formalin (4% formaldehyde in PBS). After 30 min fixation, oocytes were stained with 0.75% aceto-orcein, mounted on clean slides and assessed. Oocytes were considered to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head (Fraser, 1993). This approach, with only a short period being allowed for gamete interaction, allows us to test the sperm functional state at the time of gamete mixing; longer incubations would allow spermatozoa that had not completed capacitation within the 75 min to fertilize oocytes.

Statistical analysis

CTC data were analysed using Cochran’s modification of the χ² test (Snedecor and Cochran, 1980). This test compares responses within each replicate and then sums the values; in order to have a significant difference, there must be a sufficiently large difference between the control and experimental values in each replicate and responses must be consistent among the replicates. cAMP determinations were analysed using a paired t-test (Sigma Stats; Jandell Scientific International, Chicago, IL).

Results

Series I: does cathine have a detectable effect on uncapacitated mouse and human spermatozoa?

(A) For investigations using mouse spermatozoa, the pooled filtered suspension was divided immediately into six aliquots and treated: (1) untreated (control), (2) 100 nmol/l FPP (positive control), (3–6) increasing concentrations of cathine from 0.01–10 µmol/l. All suspensions were incubated for 30 min at 37 °C and then analysed using CTC (n = 4).

All four concentrations of cathine tested significantly accelerated capacitation, as evidenced by significantly fewer cells exhibiting the uncapacitated F pattern and significantly more cells exhibiting the capacitated, acrosome-intact B pattern than observed in the untreated controls (Figure 1). There was a concentration-dependent increase in the stimulatory responses, with 1 and 10 µmol/l cathine eliciting responses of a similar magnitude to that obtained with FPP, the positive control. Although capacitation was stimulated by both cathine and FPP, the acrosome reaction was not; no significant changes were observed in the proportion of AR pattern cells.
A pilot study \((n = 3)\) was carried out using human spermatozoa from donors with a good semen profile; one was a retired DI donor and so of proven fertility. As can be seen in Figure 2, 1 \(\mu\)mol/l cathine significantly accelerated capacitation but had no effect on the acrosome reaction. The degree of stimulation was similar to that seen in 1 \(\mu\)mol/l cathine-treated mouse spermatozoa (Figure 1).

**Series II: does norephedrine have any effect on uncapacitated spermatozoa?**

Norephedrine is the metabolite of cathine and both compounds appear to be quite stable, being detected in urine as long as 80 h after a khat chewing session (Toennes and Kauert, 2002). Therefore, the effect of norephedrine on mouse spermatozoa was assessed. The experimental protocol was essentially that used for cathine: filtered suspensions were divided into five aliquots and then treated: (1) untreated (control), (2–5) increasing concentrations of norephedrine from 0.01–10 \(\mu\)mol/l. All suspensions were incubated for 30 min at 37 °C and then analysed using CTC \((n = 3)\). As with cathine, norephedrine significantly accelerated capacitation at all concentrations tested but had no detectable effect on the acrosome reaction (Figure 3). The magnitude of the response obtained with 0.01–1 \(\mu\)mol/l norephedrine was slightly less than that obtained with cathine, consistent with the former being the metabolite of cathine.

**Series III: does cathine have any effect on capacitated spermatozoa?**

Sperm suspensions were prepared and incubated for 90 min as described above, then filtered to remove non-motile cells. The pooled filtered suspension was divided into six aliquots and treated as described in Series I. Suspensions were incubated for a further 30 min (120 min total incubation time), then prepared for CTC evaluation \((n = 5)\). As in Series I, 100 \(\mu\)mol/l FPP was included as a positive control. In the untreated control samples, \(\sim 33\%\) of cells had undergone the acrosome reaction by the time of assessment while only \(\sim 17\%\) of FPP-treated cells had done so, consistent with FPP’s demonstrated ability to inhibit the spontaneous acrosome reaction (Green et al., 1996b). Interestingly, the data indicate that cathine used at all four concentrations from 0.1 to 10 \(\mu\)mol/l also significantly inhibited spontaneous acrosome reactions, compared with untreated control suspensions. There appears to be a concentration-dependent response since 0.01 \(\mu\)mol/l was slightly less effective than the higher

![Figure 1](https://academic.oup.com/humrep/article-abstract/20/1/198/671572/3?download=true)

**Figure 1.** Cathine significantly accelerates capacitation in uncapacitated mouse sperm suspensions incubated for 30 min in the absence/presence of cathine (Cat) at 0.01–10 \(\mu\)mol/l and 0.1 \(\mu\)mol/l FPP (positive control), then analysed using chlortetracycline (CTC) fluorescence. Data are presented as % cells (mean ± SE; \(n = 4\)) expressing the F pattern (⊗), B pattern (□) and AR pattern (□□□) of CTC fluorescence. **\(P < 0.025\), ***\(P < 0.01\) compared with untreated control suspensions (Con).

![Figure 2](https://academic.oup.com/humrep/article-abstract/20/1/198/671572/4?download=true)

**Figure 2.** Cathine significantly accelerates capacitation in uncapacitated human sperm suspensions incubated for 1 h in the absence/presence of 1 \(\mu\)mol/l cathine (Cat), then analysed using CTC fluorescence. Data are presented as % cells (mean ± SE; \(n = 3\)) expressing the F pattern (⊗), B pattern (□□□) and AR pattern (□□□□□) of CTC fluorescence. **\(P < 0.025\) compared with untreated control suspensions (Con).

![Figure 3](https://academic.oup.com/humrep/article-abstract/20/1/198/671572/5?download=true)

**Figure 3.** Norephedrine significantly accelerates capacitation in uncapacitated mouse sperm suspensions incubated for 30 min in the absence/presence of norephedrine (Nor) at 0.01–10 \(\mu\)mol/l, then analysed using CTC fluorescence. Data are presented as % cells (mean ± SE; \(n = 5\)) expressing the F pattern (⊗), B pattern (□□□) and AR pattern (□□□□□□) of CTC fluorescence. **\(P < 0.025\), ***\(P < 0.01\) compared with untreated control suspensions (Con).
Figure 4. Cathine significantly inhibits spontaneous acrosome reactions in capacitated mouse sperm suspensions incubated in the absence/presence of cathine (Cat) at 0.01–10 μmol/l and 0.1 μmol/l FPP (positive control) for 30 min, then analysed using CTC. Data are presented as % cells (mean ± SE; n = 5) expressing the F pattern (□), B pattern (■) and AR pattern (□) of CTC fluorescence. *P < 0.05, **P < 0.025, ***P < 0.01 compared with untreated control suspensions (Con).

Figure 5. Progesterone overcomes cathine’s inhibition of the spontaneous acrosome reaction. Capacitated suspensions were divided into two, 1 μmol/l cathine (Cat) was added to one aliquot and both were incubated for another 30 min (120 min total incubation). The cathine-treated sample was then divided into two and progesterone (20 μmol/l; Prog) was added to one. After a further 15 min (135 min total incubation), all samples were analysed using CTC. Data are presented as % cells (mean ± SE; n = 3) expressing the F pattern (□), B pattern (■) and AR pattern (□) of CTC fluorescence. *P < 0.05, **P < 0.025 compared with untreated control suspensions at 120 min (Con 120); 1P < 0.05 compared with cathine-treated samples (Cat 135).

concentrations in inhibiting the acrosome reaction and the higher concentrations stimulated capacitation more effectively than the lower ones (Figure 4). In experiments detailed in Series IV, norephedrine was also shown to inhibit the spontaneous acrosome reaction.

Series IV: can progesterone overcome the inhibition of spontaneous acrosome reactions elicited by PPAs?

The fact that PPAs inhibit the spontaneous acrosome reaction, as shown in Series III, raises the question whether PPA-treated spermatozoa are still able to undergo an acrosome reaction in response to a physiological agent. This question was addressed in two ways: (1) determining whether progesterone would trigger an acrosome reaction when added to suspensions containing a PPA (this series); and (2) determining whether PPA-treated sperm suspensions were demonstrably fertile (Series VII). In this series, sperm suspensions were incubated for 90 min to allow capacitation, filtered and a sample was taken immediately for CTC assessment. The remaining suspension was divided into two, one half being treated with 1 μmol/l cathine and the other half remaining untreated. After a further incubation for 30 min at 37 °C (total of 120 min), samples were taken for CTC assessment; both the control and the cathine-treated suspensions were then divided into two, with one sample receiving 20 μmol/l progesterone and the other, nothing. Incubation was continued for an additional 15 min (total incubation time of 135 min) and then cells were assessed with CTC (n = 3).

After a total incubation time of 120 min, ~30% of cells in the untreated controls had acrosome-reacted (Figure 5), significantly more than the ~15% AR pattern cells seen at 90 min. In contrast, after 30 min incubation in the presence of 1 μmol/l cathine there were only ~13% AR cells, essentially the same as observed in controls at 90 min; these results are consistent with the inhibition obtained in Series III (Figure 4). However, when progesterone was added to cathine-treated cells, there was a significant stimulation of the acrosome reaction (~34% AR), compared with cathine-treated suspensions not receiving progesterone (~18% AR). Thus, even though cathine inhibits spontaneous acrosome loss, progesterone, a physiological initiator of the acrosome reaction, is able to overcome this inhibition and trigger the response. Control suspensions treated with progesterone had ~38% AR cells, similar to that obtained in the cathine + progesterone group. Both those values were higher than the ~27% AR cells seen in the controls without progesterone at 135 min. The control data ± progesterone have not been included in Figure 5 because the answer to the question asked in these experiments is obtained from comparing responses in cathine-treated suspensions with and without progesterone, not from comparing cathine + progesterone with control + progesterone.

Norephedrine was also evaluated using the same protocol (n = 4) and the results mirrored those obtained with cathine: norephedrine significantly inhibited the acrosome reaction in capacitated cells, but the addition of progesterone was able to trigger the acrosome reaction in a significant proportion of cells (data not shown).

Series V: does pertussis toxin inhibit responses to cathine in capacitated spermatozoa?

Pertussis toxin, which blocks responses involving many inhibitory Go subunits, has been shown to abolish the inhibitory effects of FPP in capacitated cells when assessed using CTC and cAMP analyses (Fraser and Adeoya-Osiguwa, 1999), as well as protein tyrosine phosphorylation (Adeoya-Osiguwa and Fraser, 2000). Given the similarity in responses obtained with cathine and FPP in both uncapacitated and capacitated spermatozoa, it seemed possible that inhibitory Go subunits might be involved in responses to PPAs in capacitated spermatozoa. Earlier
studies demonstrated that pertussis toxin had no detectable effect on either uncapacitated or capacitated suspensions when used alone and it only inhibited responses to FPP in capacitated cells (Fraser and Adeoya-Osiguwa, 1999). Since responses to PPAs mimic responses to FPP, we felt there was no need to test pertussis toxin on its own again.

Capacitated suspensions were preincubated for 90 min and prepared as detailed in Materials and methods, then divided into two. In order to allow time for pertussis toxin to enter the cells, one half of the suspension was incubated in the presence of 100 ng/ml pertussis toxin for 10 min while the other half remained untreated. After 1 μmol/l cathine had been added to the pertussis toxin-treated sample and to half of the untreated sample, all suspensions were incubated for a further 30 min (total incubation time of 130 min), then assessed using CTC (n = 4).

Consistent with results obtained in Series III, cathine significantly inhibited spontaneous acrosome reactions, compared with the untreated control. However, the presence of pertussis toxin abolished this inhibition: the proportion of acrosome-reacted cells in this treatment group was essentially the same as that observed in the untreated controls at the same time point and significantly higher than that seen in cathine-only treated suspensions (Figure 6). These results suggest that Gαi subunits are involved in responses to cathine.

**Series VI: might responses to cathine and norephedrine involve adrenergic receptors?**

Several studies have suggested that cathine and other PPAs might act as agonists of adrenergic receptors and/or mimic noradrenaline and displace it from its receptor in nerve cells (Rothman et al., 2003). Therefore, in this series the effects of adrenaline and noradrenaline on uncapacitated spermatozoa were evaluated using CTC. Immediately after filtration, uncapacitated suspensions were treated with/without 1 and 10 μmol/l norephedrine (positive controls), adrenaline or noradrenaline for 30 min at 37 °C (n = 4). CTC assessment of spermatozoa revealed that both adrenaline and noradrenaline, like norephedrine, were able to significantly accelerate capacitation, compared with untreated controls (Figure 7). It is not clear why 1 μmol/l noradrenaline was more effective than 10 μmol/l; perhaps 1 μmol/l is the optimal concentration for obtaining a positive response, with higher concentrations being slightly less effective. As shown earlier with cathine and norephedrine, neither adrenaline nor noradrenaline stimulated the spontaneous acrosome reaction.

**Series VII: do cathine and adrenaline stimulate in vitro fertilization?**

The CTC results obtained in Series I, II and VI, indicating that both PPAs and adrenaline/noradrenaline significantly accelerate capacitation in uncapacitated spermatozoa, suggested that these compounds would stimulate fertilizing ability in vitro. This hypothesis was tested by undertaking in vitro fertilization experiments using sperm suspensions that had been preincubated for ~15 min with no additions (control), 1 μmol/l cathine or 1 μmol/l adrenaline. After a 1/10 dilution of sperm suspensions in the same medium used for preincubation, unfertilized oocytes were added and gametes were co-incubated for a total of 75 min. Oocytes were fixed, stained and assessed for the presence of a decondensing sperm head (n = 4).

Both cathine and adrenaline significantly stimulated fertilizing ability, compared with untreated controls (Table 1). In the untreated group, only ~45% of oocytes had a fertilizing spermatozoon, while ~79% of the cathine-treated and ~83% of the adrenaline-treated oocytes had been fertilized. These results therefore confirm the conclusion drawn from CTC analyses, namely that capacitation is accelerated in spermatozoa treated with PPAs and adrenaline.
Table I. In vitro fertilizing ability of mouse sperm suspensions preincubated for 30 min in medium with or without 1 mmol/l cathine or 1 mmol/l adrenaline and then incubated with unfertilized oocytes for 75 min. Oocytes were fixed and assessed for the presence of a fertilizing spermatozoon.

<table>
<thead>
<tr>
<th>Preincubation treatment</th>
<th>Oocytes fertilized/total oocytes</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>92/216, 44.9%</td>
<td>27–60</td>
</tr>
<tr>
<td>1 mmol/l cathine</td>
<td>148/187, 79.1%***</td>
<td>72–90</td>
</tr>
<tr>
<td>1 mmol/l adrenaline</td>
<td>136/163, 83.4%***</td>
<td>76–89</td>
</tr>
</tbody>
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***P < 0.01 compared with control suspensions (n = 4)

Series VIII: do responses to cathine and adrenaline involve capacitation state-dependent changes in cAMP production?

Many investigations have shown that cAMP plays a major role in modulating mammalian sperm function. Recently, several small molecules found in seminal plasma, including FPP, adenosine and calcitonin, have been shown to accelerate capacitation and then inhibit spontaneous acrosome loss by regulating the activity of membrane-associated adenyl cyclases (mAC) and consequent production of cAMP (Fraser et al., 2003). Since PPAs and adrenaline/noreadrenaline appear to mimic responses to FPP/adenosine/calcitonin, the possibility that modulation of cAMP is involved was investigated. To do this, both uncapacitated and capacitated suspensions were assessed.

At least 3–4 male mice were used per replicate in order to have sufficient volume of suspension for these assays. Uncapacitated filtered sperm suspensions, with ~1 × 10⁷ cells/ml, were prepared and Ro-20-1724, a general phosphodiesterase inhibitor, was added to give a final concentration of 5 μmol/l. The suspension was divided into four aliquots and treated for 2 min with: (1) nothing (control), (2) 500 nmol/l CGS-21680, a specific stimulatory A₁ adenosine receptor agonist that served as a positive control, (3) 1 μmol/l cathine and (4) 1 μmol/l adrenaline. After treatment, reactions were stopped by transferring 200 μl aliquots into tubes containing EGTA and protease inhibitors to give final concentrations of 4 mmol/l EDTA, 400 μg/ml trypsin inhibitor and 0.2 μg/ml leupeptin and samples were then frozen in liquid N₂. After thawing at room temperature, cAMP was extracted with ice-cold ethanol and assayed (n = 3–4). As stated in Materials and methods, each treatment sample in each replicate was assayed in triplicate or quadruplicate.

Capacitated sperm suspensions were preincubated for 90 min, filtered and Ro-20-1724 was added. Suspensions were divided into four aliquots and treated for 5 min with: (1) nothing (control), (2) 5 nmol/l CPA, a specific inhibitory A₁ adenosine receptor agonist that served as a positive control, (3) 1 μmol/l cathine and (4) 1 μmol/l adrenaline. Samples were extracted and assayed as above (n = 3–4).

When uncapacitated suspensions were evaluated, CGS-21680, as expected, significantly stimulated cAMP production, compared with untreated controls (Figure 8A). Both cathine and adrenaline also significantly stimulated cAMP; indeed, the mean amount of cAMP produced in spermatozoa treated with these two compounds was greater than that in suspensions treated with CGS. With capacitated suspensions, CPA, as expected, significantly inhibited cAMP production, compared with cAMP in untreated controls (Figure 8B). Interestingly, cathine and adrenaline also significantly inhibited cAMP production. Thus responses to both cathine and adrenaline involve modulation of cAMP production, initially stimulating it in uncapacitated cells and then inhibiting it in capacitated cells.

Discussion

The aim of the present study was to investigate whether cathine and norephedrine have detectable direct effects on sperm function, rather than on semen parameters that are not directly related to sperm fertilizing ability. Because the initial results obtained indicated that these PPAs have biologically
significant effects on both mouse and human spermatozoa, further experiments to investigate possible mechanisms of action were carried out. The results obtained have provided new and novel insights into mechanisms regulating sperm function that might have practical applications relating to fertility enhancement.

The first experimental question posed was whether cathine and norephedrine have effects on uncapacitated sperm suspensions, determined by CTC analysis. When a wide range of concentrations (0.01 – 10 μmol/l) of each PPA was tested, both were seen to have a concentration-dependent stimulatory effect on mouse spermatozoa, with cathine being somewhat more potent (Figures 1 and 3). In a pilot study, 1 μmol/l cathine also significantly accelerated capacitation in uncapacitated human spermatozoa, confirming other evidence that mouse and human spermatozoa often respond similarly to specific molecules (e.g. Fraser and Osiguwa, 2004). In experiments evaluating cathine, 100 nmol/l FPP was included as a positive control since it has been shown to stimulate capacitation (Fraser et al., 2003); the magnitude of responses to 1 and 10 μmol/l cathine and FPP were very similar (Figure 1). Thus this study has provided the first evidence that PPAs can have a direct and biologically significant effect on uncapacitated mammalian spermatozoa.

The second question posed was whether PPAs have any detectable effect on capacitated spermatozoa. The same range of cathine concentrations (0.01 – 10 μmol/l) was tested and all were found to significantly inhibit the spontaneous acrosome reaction, the magnitude of the response again being concentration-dependent (Figure 4). FPP was included as a positive control since it has been shown to inhibit the acrosome reaction in capacitated spermatozoa from several species (Fraser et al., 2003). All but the lowest concentration of cathine elicited responses similar to FPP. In other experiments, norephedrine was shown to have a similar effect, also significantly inhibiting spontaneous acrosome loss. These results provide the first evidence that PPAs can also have a direct and biologically significant effect on capacitated mammalian spermatozoa.

An important question arising from these results was whether the inhibitory effects of PPAs would interfere with the ability of cells to undergo an acrosome reaction in response to a physiological agent known to trigger an acrosome reaction in capacitated cells. Subsequent experiments demonstrated that the presence of cathine and norephedrine did not inhibit the ability of capacitated spermatozoa to undergo a progesterone-induced acrosome reaction (Figure 5). These results are similar to those of Green et al. (1996b) who showed that FPP-treated capacitated suspensions could respond to progesterone in the presence of FPP. Consistent with the progesterone data, cathine-treated capacitated suspensions were highly fertile in vitro, despite cathine’s inhibition of spontaneous acrosome reactions (Table 1).

These capacitation-dependent responses to PPAs are reminiscent of those obtained with FPP, adenosine and calcitonin, small molecules known to be present in seminal plasma, when evaluated using both mouse and human spermatozoa (Fraser et al., 2003; Fraser and Osiguwa, 2004). Thus it would appear that all these molecules, despite their varied molecular structures, can regulate capacitation, first accelerating it, then preventing ‘over-capacitation’ and the spontaneous acrosome reaction and so maintaining spermatozoa in the potentially fertilizing state.

The similarity of responses to PPAs and FPP/adenosine/calcitonin suggested that these might reflect similarities in their mechanisms of action. The latter molecules have been shown to act via G protein-coupled receptors (GPCRs) and mouse and human spermatozoa have been shown to have both Gαs and several Gαi3 subunits (Baxendale and Fraser, 2003). Stimulatory responses to FPP/adenosine/calcitonin involve stimulatory G proteins and inhibitory responses involve inhibitory G proteins (Fraser et al., 2003). One way to address the possibility that responses to PPAs involve G proteins was to determine whether pertussis toxin had any effect on specific responses. Since the inhibitory responses to cathine (those most likely to involve inhibitory Gα subunits) occurred in capacitated suspensions, the important question was whether the inclusion of pertussis toxin would affect capacitated sperm responses to cathine. When these experiments were carried out, it was quite clear that pertussis toxin abolished cathine’s ability to inhibit the acrosome reaction: the proportion of acrosome reacted cells in the cathine + pertussis toxin treatment group was essentially the same as in the untreated controls (Figure 6). Again, these results are very similar to earlier ones showing that pertussis toxin could abolish responses in capacitated suspensions to FPP (Fraser and Adeoya-Osiguwa, 1999; Adeoya-Osiguwa and Fraser, 2000) and calcitonin (Fraser et al., 2001). Therefore, these results supported the evolving hypothesis that responses to PPAs may involve GPCRs, but the next question needing to be addressed was identification of plausible receptors for cathine and norephedrine.

Earlier studies attempting to identify a mechanism of action for PPAs in somatic cells focused mainly on either responses in the central nervous system or effects on ventricular contraction. Amphetamine derivatives and cathinone were shown to potentiate the actions of noradrenaline on rat right ventricle contraction; it was suggested that this occurred as a result of cathinone preventing the uptake of noradrenaline from the nerve terminal by an action that involved competitive blockade of the noradrenaline transporter (Cleary et al., 2002; Cleary and Docherty, 2003). Another study surveyed the interaction of ephedrine-related stereoisomers, including cathine and norephedrine, at both biogenic amine transporters and a large battery of cloned human adrenergic receptors; results indicated that these related compounds could act as substrates of the noradrenaline transporter and had only a weak affinity for adrenergic receptors (Rothman et al., 2003). However, ephedrine is known to exert both direct and indirect agonist responses via adrenergic receptors; Rothman et al. (2003) and Tariq et al. (1989) obtained evidence suggesting that β-adrenergic receptors were involved in cathinone- and amphetamine-induced thermogenesis in brown adipose tissue. These findings all provide some support for the possibility that cellular responses induced by cathinone and its metabolites might involve adrenergic receptors.
Several investigations have looked for evidence that β-adrenergic receptor agonists/antagonists and catecholamines (adrenaline and noradrenaline) might have an effect on mammalian sperm function in vitro, but the data have been inconclusive. Earlier studies reported that these catecholamines could induce the acrosome reaction (e.g. Cornett and Meizel, 1978) and stimulate capacitation capacity in hamster spermatozoa in vitro (e.g. Cornett et al., 1979; Leibfried and Bavister, 1982) and that the β-adrenergic receptor agonist isoprenaline could stimulate human sperm motility (Semczuk, 1987, 1988). More recently, noradrenaline, but not adrenaline, at low concentrations was reported to stimulate capacitation and induce the acrosome reaction in bovine spermatozoa, but higher concentrations appeared to inhibit these responses; the authors suggested that there might be two populations of adrenergic receptors but provided no experimental evidence for this (Way and Killian, 2002). Attempts to identify adrenergic receptors per se using a fluorescent β-adrenergic receptor antagonist probe (Cornett and Meizel, 1980) and ligand-binding techniques (Falkay et al., 1989) failed to provide conclusive evidence for the presence of these receptors in mammalian spermatozoa.

Despite those failures, the fact that PPAs are structurally related to adrenaline and noradrenaline made it reasonable to investigate whether responses to the latter two compounds could be detected when evaluated using the same assay systems used to test PPAs. When this was done, both adrenaline and noradrenaline significantly accelerated capacitation but did not stimulate the acrosome reaction, responses similar to those obtained with norephedrine which was included to inhibit these responses; the authors suggested that there might be two populations of adrenergic receptors but provided no experimental evidence for this (Way and Killian, 2002). Attempts to identify adrenergic receptors per se using a fluorescent β-adrenergic receptor antagonist probe (Cornett and Meizel, 1980) and ligand-binding techniques (Falkay et al., 1989) failed to provide conclusive evidence for the presence of these receptors in mammalian spermatozoa.

In conclusion, this study has provided the first convincing evidence that PPAs and adrenaline/noradrenaline, like FPP/adenosine/calcitonin, can act as ‘first messengers’ to regulate the production of the ‘second messenger’ cAMP in mammalian spermatozoa in a capacitation state-dependent manner. It has also provided the first biochemical evidence suggesting the possible presence of two populations of adrenergic receptors on mammalian spermatozoa that function in a capacitation-dependent manner. Given the structural and functional similarities between cathine/norephedrine and adrenaline/noradrenaline, it is plausible that both are working via adrenergic receptors. Furthermore, the concentrations of cathine and norephedrine used in this study were within the range of concentrations of noradrenaline reported to be in oviductal fluids by Way et al. (2001).

Initial experiments in the present study used CTC to assess treated sperm suspensions; since CTC results indicated that PPAs and adrenaline/noradrenaline significantly accelerated capacitation, it was hypothesized that treated cells would become fertile more quickly. This was tested by carrying out in vitro fertilization experiments. Even though the initial pre-incubation was short, sperm suspensions treated with 1 μmol/l cathine or 1 μmol/l adrenaline were significantly more fertile than the untreated controls. These results provide firm physiological data to support the conclusions drawn from the CTC analyses and demonstrate that inhibition of the spontaneous acrosome reaction by these molecules does not interfere with oocyte-induced acrosome reactions in fertilizing spermatozoa. Furthermore, the positive responses elicited by adrenaline are consistent with the presence of adrenergic receptors on these spermatozoa.

If the similarities in responses to PPAs, adrenaline/noradrenaline and FPP/adenosine/calcitonin are considered further, the members of the last group have been shown to act via GPCRs that regulate membrane-associated adenyl cyclases (mACs), first stimulating mAC activity and then inhibiting it (Fraser et al., 2003). Many adrenergic receptor subtypes act via mAC/cAMP, with β receptors often acting in conjunction with Gαs, to activate mACs and α2 receptors often acting in conjunction with Gαi, to inhibit mAC/cAMP (Watling et al., 1995). Therefore, it seemed reasonable to investigate the effects of cathine and adrenaline on cAMP production in both uncapacitated and capacitated suspensions. Results clearly demonstrated that both compounds have a capacitation state-dependent effect on cAMP production, significantly stimulating it in uncapacitated cells and then significantly inhibiting it in capacitated cells. These results would be consistent with the presence of both β and α2 adrenergic receptors. α1 receptors are less likely to be involved since they usually act on phospholipase C via the non-G protein linked Gq/11 to increase availability of the second messengers InsP3 and diacylglycerol (DAG; Watling et al., 1995). Our present results, demonstrating that pertussis toxin abolished responses to PPAs, suggest that G proteins are involved, and previous results have shown that rises in DAG are associated with stimulation, rather than inhibition, of the acrosome reaction (O’Toole et al., 1996). Furthermore, it is unlikely that PPAs are acting via soluble AC since that isoform is not regulated by G proteins (Buck et al., 1999).

In conclusion, this study has provided the first convincing evidence that PPAs and adrenaline/noradrenaline, like FPP/adenosine/calcitonin, can act as ‘first messengers’ to regulate the production of the ‘second messenger’ cAMP in mammalian spermatozoa in a capacitation state-dependent manner. It has also provided the first biochemical evidence suggesting the possible presence of two populations of adrenergic receptors on mammalian spermatozoa that function in a capacitation-dependent manner. Given the structural and functional similarities between cathine/norephedrine and adrenaline/noradrenaline, it is plausible that both are working via adrenergic receptors. Furthermore, the concentrations of cathine and norephedrine used in this study were within the range of those detected in blood samples from individuals who chewed a fixed amount of khat leaves for only 1 h (Toennes et al., 2003). Although it is possible that continued exposure to high concentrations of PPAs might be deleterious to general and reproductive health (e.g. Quereshi et al., 1988; El-Shoura et al., 1995), our results suggest that moderate levels of PPAs, especially in the female reproductive tract, could have a positive effect on natural fertility. We have shown that these compounds accelerate capacitation and then inhibit the spontaneous acrosome reaction in vitro, actions that would preserve the intrinsic fertilizing ability of capacitated spermatozoa in vivo in the uterine tubes. Given that PPAs related to cathine and norephedrine are already used in a range of over-the-counter and prescription products (Greenway, 2001), it is plausible that products containing cathine, norephedrine or related molecules could be developed that might enhance natural fertility.

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References

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