Adenosine analogues with specificity for A₂ receptors bind to mouse spermatozoa and stimulate adenylate cyclase activity in uncapacitated suspensions

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Adenosine and its analogues, known to stimulate adenylate cyclase activity in somatic cells via A₂ receptors, can accelerate capacitation in mouse spermatozoa and thereby enhance fertilizing ability *in vitro*. Indirect evidence has suggested that adenosine can modulate mouse sperm adenylate cyclase, implicating this enzyme and cAMP in the observed functional responses. In the present study we provide evidence that [³H]5'-N-ethylcarboxamidoadenosine (NECA), an adenosine analogue with specificity for stimulatory A₂ adenosine receptors, can bind to mouse spermatozoa. This binding can be displaced by both unlabelled NECA and 2-chloroadenosine, another A₂ receptor agonist, but not by cyclopentyladenosine, an inhibitory A₁ receptor agonist, suggesting that the NECA binding is specific for A₂ receptors. The presence of S-(*p*-nitrobenzyl)-6-thioinosine, an adenosine transport inhibitor, did not affect binding, indicating an external site for interaction with sperm cells. Saturable specific binding of [³H]NECA to mouse spermatozoa incubated at 37°C was observed, with a $B_{\max}$ of 5.17 pmol mg⁻¹ protein and a K_d value of 930 nmol l⁻¹. Binding data were consistent with the presence of a single major class of receptor. In addition to demonstrable binding of [³H]NECA, both NECA and 2-chloroadenosine significantly stimulated adenylate cyclase activity in a concentration-dependent manner, with NECA being effective at a lower concentration. Furthermore, the hydrolysis-resistant GTP analogue Gpp(NH)p, alone and in the presence of either NECA or 2-chloroadenosine, also significantly stimulated enzyme activity. In somatic cells, expression of responses to adenosine usually requires GTP and G proteins. These results are consistent, therefore, with the hypothesis that adenosine-induced enhancement of sperm functional ability is due to stimulation of adenylate cyclase, via A₂ adenosine receptors, and hence increasing availability of intracellular cAMP. They also suggest that G proteins play a role in the receptor-mediated response.

Introduction

Current evidence suggests that cyclic nucleotides, particularly cAMP, play a role in mammalian sperm capacitation, acrosome reaction and motility (reviewed by Garbers and Kopf, 1980; Tash and Means, 1983; Fraser, 1984; Fraser and Monks, 1990). Introduction of exogenous cyclic nucleotides and their analogues has been shown to promote sperm capacitation and fertilization (e.g. dibutyryl cAMP: Fraser, 1981) but mechanisms regulating sperm adenylate cyclase activity and hence endogenous cAMP production are poorly understood. Capacitation-related increases in adenylate cyclase activity and decreases in cyclic nucleotide phosphodiesterase activity have been demonstrated in mouse spermatozoa (Stein and Fraser, 1984; Monks *et al.*, 1986; Monks and Fraser, 1987). Consistent with the implications of such changes for intracellular cAMP, White and Aitken (1989) have detected capacitation-dependent increases in cAMP in hamster

spermatozoa. When adenosine deaminase was added at the time of assay to remove endogenous adenosine, Stein *et al.* (1986) observed that adenylate cyclase activity changed. The fact that these responses were modulated by the GTP analogue 5'-guanylylimidodiphosphate (Gpp[NH]p) suggested receptor mediation of these effects. The responses were consistent with adenosine stimulation of adenylate cyclase early in capacitation and adenosine inhibition late in capacitation. In a variety of somatic cells, adenosine has been shown to modulate adenylate cyclase activity (Fain and Malbon, 1979) through two functionally distinct external adenosine receptors (Londos *et al.*, 1983) which can either inhibit (A₁) or stimulate (A₂) the enzyme (Van Calcar *et al.*, 1979). Given the mouse sperm enzyme responses to adenosine deaminase, and hence to endogenous adenosine, Stein *et al.* (1986) hypothesized the presence of stimulatory A₂ receptors on uncapacitated cells and inhibitory A₁ receptors on capacitated cells.

Indirect support for this hypothesis was obtained by altering the availability of endogenous adenosine through the inclusion

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of specific inhibitors of enzymes either producing adenosine or metabolizing it during sperm incubation. In mouse sperm suspensions used for *in vitro* capacitation and fertilization, 5'-nucleotidase, which produces adenosine from 5'-AMP, is present as an ecto-enzyme and adenosine deaminase, which metabolizes adenosine to inosine, is almost entirely present in the extracellular fluid (Monks and Fraser, 1988a). Thus an extracellular site of action for adenosine is feasible. Inhibition of 5'-nucleotidase with α,β -methylene adenosine diphosphate, and hence reduction in endogenous adenosine production, produced a concentration-dependent inhibition of fertilization in partially capacitated suspensions. In contrast, inhibition of adenosine deaminase with coformycin, which would increase available adenosine, stimulated fertilization in partially capacitated suspensions (Monks and Fraser, 1988b). Such responses are consistent with the presence of stimulatory A_2 adenosine receptors on mouse spermatozoa during the initial stages of capacitation.

More direct evidence for A_2 receptors was obtained by adding exogenous adenosine and adenosine analogues to sperm suspensions and evaluating the effect on sperm function. A concentration-dependent stimulation of fertilizing ability was obtained with adenosine and several analogues, including 5'-N-ethylcarboxamidoadenosine (NECA), (R)- N^6 -phenylisopropyladenosine (R-PIA) and 2-chloroadenosine. This stimulation was not reduced when dipyrindamole, an inhibitor of adenosine uptake, was included, suggesting action at an external site. The use of chlortetracycline fluorescence patterns to assess the capacitation state of spermatozoa treated with an adenosine analogue indicated a significant shift towards the capacitated pattern (Fraser, 1990). Thus adenosine and its analogues accelerated sperm capacitation and the greater sensitivity of the responses to NECA, R-PIA and 2-chloroadenosine than to adenosine is consistent with action at A_2 receptors (Daly *et al.*, 1987). However, a direct involvement of adenylate cyclase in the response has yet to be demonstrated.

The present study has been designed to determine whether adenosine can modulate adenylate cyclase activity in mouse spermatozoa during the early stages of capacitation. We have examined the binding of [3 H]NECA to intact sperm cells, to match as closely as possible the earlier functional assessments, and then the ability of two adenosine deaminase-resistant analogues, NECA and 2-chloroadenosine, to alter sperm adenylate cyclase activity in the absence and presence of the GTP analogue Gpp(NH)p.

Materials and Methods

Media

A modified Tyrode's medium containing 5.56 mmol glucose l^{-1} (Fraser, 1987) was prepared using water purified by reverse osmosis and deionization (Milli-Q: Millipore, Molsheim), analytical grade chemicals (BDH, Poole) and crystalline BSA (Sigma, Poole) at 4 mg ml^{-1} . For the binding studies, calcium-deficient medium was prepared by omitting the $CaCl_2$. For the enzyme assay studies, standard calcium-containing medium (1.80 mmol $CaCl_2$ l^{-1}) was used. Stock solutions for NECA (Boehringer-Mannheim, Lewes), 2-chloroadenosine (Sigma) and

cyclopentyl-adenosine (Sigma) were prepared in dimethyl sulfoxide (DMSO) and then diluted in culture medium or assay buffer, as appropriate; the final concentration of DMSO was <0.15% in enzyme assays and <0.6% in binding assays. Because of the higher DMSO concentrations in the latter, DMSO was also included in all control assays.

[3 H]NECA binding assay

The binding assay used was a modification of those described by Bruns *et al.* (1986) and Monaco and Conti (1986). [3 H]NECA, specific activity 25–26 Ci $mmol^{-1}$, was obtained from Amersham International (Little Chalfont, Bucks). A Tris–NaCl buffer was prepared which contained 50 mmol Tris–HCl l^{-1} (Trizma pH 7.9 preset crystals, Sigma) and 103 mmol NaCl l^{-1} . The NaCl was included to avoid osmotic shock to the whole, live sperm cells used in the binding assay. Adenosine deaminase (Type VII, Sigma) stock solution at 150 U ml^{-1} was prepared in the Tris–NaCl buffer and stored frozen; aliquots were thawed and added to sperm suspensions to give 1 U ml^{-1} .

Sperm suspensions were prepared by extruding the contents of the epididymides of three to five mature male TO mice (Harlan-Olac, Bicester) into 3–5 ml Ca^{2+} -deficient, BSA-containing medium in 30 mm sterile plastic tissue culture dishes (Sterilin, Teddington). This medium was chosen because, while maintaining viability and motility, it permits only partial capacitation (Fraser, 1987) and hence should block any loss of A_2 adenosine receptors that might accompany completion of capacitation (Stein *et al.*, 1986). After incubation at 37°C for 5 min to allow sperm dispersal, a sample was removed, fixed in buffered formalin and counted on a haemocytometer. The sperm suspension was then diluted to 20×10^6 cells ml^{-1} , adenosine deaminase was added, and the suspension was incubated for a further 5 min before use in assays. Typically, 25 min elapsed between preparation of sperm suspension and beginning of binding incubation.

[3 H]NECA, diluted in Tris–NaCl as appropriate, was added to 1.5 ml microcentrifuge tubes. When unlabelled adenosine analogues were used, these were added next to the [3 H]NECA and mixed by vortexing. All analogue stock solutions were prepared on the day of assay; DMSO was added to control tubes to give the same final concentration in all samples. The binding assays were initiated by adding well-mixed sperm suspension to the tubes (5 parts sperm suspension to 1 part analogue–Tris–NaCl) and gently mixing by low speed vortexing. All suspensions were incubated in a shaking water bath at 37°C for 60 min (shown by a preliminary study to give optimal binding).

Assays were terminated by filtering duplicate 200 μl aliquots through 2.4 cm Whatman GF/B filters. The filters were washed with 3×2 ml ice cold Tris–NaCl. Filters were dried and put into scintillation vials; 3 ml of scintillant (Ultima Gold, Canberra Packard, Pangbourne) was added and samples were then counted in a Packard Tri-Carb scintillation counter.

Binding was calculated as fmol [3 H]NECA bound mg^{-1} protein. Before carrying out binding assay, sperm suspensions were prepared in BSA-free medium (4 mg polyvinyl alcohol ml^{-1} was included to maintain membrane integrity) and the protein content was measured using the method of Bradford (1976); whole cells were used to estimate surface protein content. A mean value of about 19 μg protein per 10^6 sperm cells was obtained

from measurements on three separate suspensions. This figure, in conjunction with the sperm counts for suspensions used in the binding assays, was used to calculate protein content in assays. One microlitre aliquots of [^3H]NECA solutions were spotted directly onto filters and then radioactivity was counted to determine mean c.p.m. fmol^{-1} [^3H]NECA.

Adenylate cyclase assay

Sperm suspensions were prepared by releasing the contents of the epididymides of two mature male TO mice into 2 ml complete Tyrode's medium. After 5 min incubation at 37°C for dispersal, a sample was removed as described above, fixed and counted in a haemocytometer. The sperm concentration was adjusted to about 20×10^6 cells ml^{-1} and the suspension was frozen in liquid N_2 within 20–25 min of sperm release into medium. After thawing, the sample was divided into 37.5 μl aliquots which were frozen in liquid N_2 , lyophilized and stored at -20°C until needed for assay. One aliquot was reconstituted, diluted in formalin and counted on a haemocytometer to obtain an accurate final sperm concentration. This method of sample preparation has been shown to give enzyme activity comparable to that obtained with fresh, sonicated cells (Stein and Fraser, 1984). It has the added advantages that exact timing of samples is possible and the assays can be carried out whenever convenient.

Adenylate cyclase activity was assayed by measuring cAMP formed from unlabelled ATP (Albano *et al.*, 1973). Aliquots of spermatozoa were reconstituted in 50 mmol Tris-HCl l^{-1} , pH 7.5. Adenosine deaminase (Type VII, Sigma) stock solutions were prepared in Tris-HCl and used at a final concentration of 1 U ml^{-1} . Adenosine analogue stock solutions were prepared daily in DMSO, then diluted in Tris-HCl so that the final concentration in the assay was 1, 10, 100 and 1000 nmol l^{-1} . A stock solution of the GTP analogue Gpp(NH)p (Sigma) was prepared in Tris-HCl and frozen; after thawing, it was added where required to give a final concentration of 50 $\mu\text{mol l}^{-1}$. The total volume of reconstituted sperm suspension, adenosine deaminase, analogues and Gpp(NH)p was 100 μl . After a 3 min preincubation in a shaking water bath at 37°C , 200 μl of reaction mixture made up in Tris-HCl was added to give final concentrations of 1 mmol ATP l^{-1} , 2 mmol MnCl_2 l^{-1} , 5 mmol phosphocreatine l^{-1} and 7.5 units creatine phosphokinase ml^{-1} . Mouse sperm adenylate cyclase is much more active when assayed in the presence of Mn^{2+} than Mg^{2+} (Stein and Fraser, 1984). After 8 min with shaking at 37°C , the reaction was stopped by adding 140 μl of enzyme assay solution to 1.4 μl of 0.4 mol EDTA l^{-1} , in duplicate, and heating to 60°C for 3 min. Samples were stored at -20°C until cAMP assay. A cyclic nucleotide phosphodiesterase inhibitor, such as caffeine or isobutylmethylxanthine, was not included. Under the adenylate cyclase assay conditions used, phosphodiesterase activity was very low: degradation of cAMP in the presence or absence of isobutylmethylxanthine was less than 2% (Monks, 1987). Since these inhibitors can interact with adenosine receptors (Daly *et al.*, 1987), they were not included in the assay.

cAMP assay

A protein binding technique (Brown *et al.*, 1972), using a cAMP binding protein (BDH) and [$8\text{-}^3\text{H}$]cAMP (29 Ci mmol l^{-1} ;

Amersham International), was used to determine cAMP content in the above samples. The assay buffer was 50 mmol Tris-HCl l^{-1} , pH 7.5, containing 4 mmol EDTA l^{-1} . The assay mixture included 50 μl cAMP standard (2–16 pmol) or unknown sample, 50 μl [^3H]cAMP (about 10 000 c.p.m.) and 100 μl diluted binding protein. After mixing well, assay samples were incubated at 4°C for between 90 and 120 min. Bound and unbound labelled cAMP were separated by charcoal adsorption; 100 μl ice-cold charcoal suspension (3% w/v 'Norit GSX charcoal' (BDH), 2% fraction V BSA (BDH)) were added and mixed well. Within 4 min, tubes were centrifuged at 11 600 g_{max} for 5 min in a microcentrifuge at 4°C ; 200 μl of supernatant was mixed with 2 ml Ultima Gold scintillant (Packard) and radioactivity was counted.

Statistical analysis

Results of adenylate cyclase activities assayed in the absence and presence of adenosine analogues and Gpp(NH)p were analysed using a paired *t* test.

Results

[^3H]NECA binding

In the first binding studies, the ability of unlabelled NECA and cyclopentyladenosine (CPA) to inhibit binding of [^3H]NECA was investigated. CPA has a high specificity for inhibitory A_1 adenosine receptors, with very high affinity (very low nanomolar range; Daly *et al.*, 1987). In contrast, analogues with specificity for stimulatory A_2 receptors have a lower affinity (nanomolar – low micromolar range). Bruns *et al.* (1986) demonstrated that 50 nmol CPA l^{-1} can be used to eliminate binding of NECA to A_1 receptors. When spermatozoa were incubated with 50 nmol [^3H]NECA l^{-1} , the inclusion of 50 nmol CPA l^{-1} had little effect on binding: 447.2 ± 22.3 fmol [^3H]NECA bound per mg protein in the absence of CPA and 407.5 ± 37.3 in its presence (mean $\pm$ SEM for three replicate experiments, $n = 3$). In contrast, the inclusion of 20 μmol unlabelled NECA l^{-1} , whether or not CPA was present, resulted in markedly reduced binding: 160.4 ± 17.9 fmol [^3H]NECA bound mg^{-1} protein in the absence of CPA and 145.0 ± 10.8 in its presence (Fig. 1). Since CPA would block binding of [^3H]NECA to inhibitory A_1 receptors, the lack of a major effect on binding in the presence of CPA suggests that the [^3H]NECA is binding to stimulatory A_2 receptors. Therefore CPA was not included in the subsequent experiments.

In the second series of experiments ($n = 3$), the effect of S-(*p*-nitrobenzyl)-6-thioinosine (NBTI), an adenosine transport inhibitor (Torres *et al.*, 1990), on binding of [^3H]NECA to sperm cells was investigated. Two concentrations of [^3H]NECA (125 and 250 nmol l^{-1}) and three concentrations of NBTI (1, 10 and 100 nmol l^{-1}) were used. Torres *et al.* (1990) reported that NBTI markedly inhibited the uptake of adenosine (150 nmol l^{-1}) by chromaffin cells, with 80% inhibition observed in the presence of 10 nmol NBTI l^{-1} and total inhibition with 100 nmol l^{-1} . The inclusion of NBTI had no detectable effect on the binding of [^3H]NECA to mouse sperm cells (Fig. 2). Values for 125 nmol

Fig. 1. [^3H]5'-N-ethylcarboxamidoadenosine (NECA) binding to live mouse sperm cells (20×10^6 cells ml^{-1}) incubated for 60 min in Ca^{2+} -deficient medium at 37°C in the presence of: (a) 50 nmol [^3H]NECA l^{-1} ; (b) 50 nmol [^3H]NECA l^{-1} plus 50 nmol unlabelled cyclopentyladenosine (CPA) l^{-1} ; (c) 50 nmol [^3H]NECA l^{-1} plus 20 μmol unlabelled NECA l^{-1} ; (d) 50 nmol [^3H]NECA l^{-1} plus 50 nmol unlabelled CPA l^{-1} plus 20 μmol unlabelled NECA l^{-1} . Data are shown as fmol [^3H]NECA bound mg^{-1} protein; the mean $\pm$ SEM of three replicate experiments is given for each condition.

Fig. 2. [^3H]5'-N-ethylcarboxamidoadenosine (NECA) binding to live mouse sperm cells (20×10^6 cells ml^{-1}) incubated for 60 min in Ca^{2+} -deficient medium at 37°C in the presence of 125 (■) or 250 (▨) nmol [^3H]NECA l^{-1} and S-(p-nitrobenzyl)-6-thioinosine (NBTI) at 0–100 nmol l^{-1} . Data are shown as pmol [^3H]NECA bound mg^{-1} protein, with the mean $\pm$ SEM of three replicate experiments.

[^3H]NECA l^{-1} ranged from 0.84 to 0.87 pmol bound mg^{-1} protein and for 250 nmol [^3H]NECA l^{-1} , 1.65–1.72 pmol bound mg^{-1} protein.

In the third series of experiments ($n = 3$), binding of [^3H]NECA over the range 62.5–2000 nmol l^{-1} , in the absence and presence of 100 μmol unlabelled 2-chloroadenosine l^{-1} , was determined. Nonspecific binding was defined as binding observed in the presence of 100 μmol 2-chloroadenosine. Specific binding was total binding minus nonspecific binding. Data for a representative experiment (Fig. 3) show that specific binding increased linearly up to 500 nmol [^3H]NECA l^{-1} , then reached a plateau, indicating that specific binding was saturable.

Fig. 3. [^3H]5'-N-ethylcarboxamidoadenosine (NECA) binding to live mouse sperm cells (20×10^6 cells ml^{-1}) incubated for 60 min in Ca^{2+} -deficient medium at 37°C . Binding in the absence (total binding; Δ) and presence (nonspecific binding; $\circ$) of excess (100 μmol l^{-1}) unlabelled 2-chloroadenosine was measured over the range of 62.5–2000 nmol [^3H]NECA l^{-1} . Specific binding ($\square$) was total-binding minus nonspecific binding. These data from duplicated determinations in one experiment are representative of the three replicates carried out. The theoretical curve (—) for the saturation binding function $B = B_{\text{max}}(x/(x + K_d))$, where x is the [^3H]NECA concentration, B is the bound [^3H]NECA, $K_d = 930$ nmol l^{-1} and $B_{\text{max}} = 5.17$ pmol mg^{-1} protein (the last two values obtained from Scatchard analysis of the data; shown in Fig. 4), is very close to the experimentally derived curve, indicating a single class of binding site.

Fig. 4. Scatchard analysis of data from the experiment presented in Fig. 3. Bound [^3H]5'-N-ethylcarboxamidoadenosine (NECA) is plotted against bound/free [^3H]NECA. B_{max} value is 5.17 pmol mg^{-1} protein and K_d value is 930 nmol l^{-1} .

The nonspecific binding was linear over the concentration range evaluated, with absolute values exceeding those for specific binding at 1000 and 2000 nmol l^{-1} . This in part probably reflects the temperature (37°C) at which binding was assayed. Binding assays using membrane preparations are usually carried out at 0° or 25°C (e.g. Bruns *et al.*, 1986). However, we wished to investigate binding under conditions as close as possible to those used for sperm function evaluations (Fraser, 1990) and hence chose 37°C .

Scatchard analysis (Fig. 4) of the specific binding data in Fig. 3 yielded a B_{max} value of 5.17 pmol mg^{-1} protein and a K_d value

Table 1. Stimulatory effect of NECA on adenylylase activity of mouse spermatozoa

NECA (nmol l ⁻¹)	Adenylylase, specific activity (pmol cAMP min ⁻¹ 10 ⁻⁶ cells)	% Stimulation
0	5.78 ± 0.87 ^a	—
1	6.65 ± 0.78***	15.0
10	7.06 ± 0.78***	22.1
100	6.40 ± 0.78***	10.7
1000	6.58 ± 1.30**	13.9

^aData expressed as means ± SEM for six replicate assays on five frozen-thawed, lyophilized sperm samples. Adenylylase assays measured cAMP production, in the absence and presence of NECA, from unlabelled ATP in the presence of MnCl₂, phosphocreatine and creatine phosphokinase. cAMP content was determined with a protein binding technique which used a cAMP binding protein [³H]cAMP. ***P* < 0.01 and ****P* < 0.001 compared with control.

NECA: 5'-N-ethylcarboxamidoadenosine.

Table 2. Stimulatory effect of 2-chloroadenosine on adenylylase activity of mouse spermatozoa

2-Chloroadenosine (nmol l ⁻¹)	Adenylylase, specific activity (pmol cAMP min ⁻¹ 10 ⁻⁶ cells)	% Stimulation
0	5.97 ± 0.78 ^a	—
1	6.21 ± 0.70***	4.0
10	7.78 ± 0.86**	30.3
100	7.75 ± 0.77***	29.8
1000	7.65 ± 0.94***	28.1

^aData expressed as means ± SEM for six replicate assays on the same five frozen-thawed, lyophilized sperm samples used to obtain data in Table 1. Conditions for assay are as given in Table 1, except that 2-chloroadenosine was present. ***P* < 0.01 and ****P* < 0.001 compared with control.

of 930 nmol l⁻¹. Linear regression analysis using the method of least squares revealed a regression coefficient of -0.969, indicating a good fit to a straight line; this suggests a single class of binding site for [³H]NECA. A similar conclusion was reached when the values obtained for *B*_{max} and *K*_d were used to calculate the theoretical curve of the saturation binding function $B = B_{\max} (x / (x + K_d))$ where *x* is the [³H]NECA concentration and *B* is the theoretical concentration of bound ligand. There is close correlation between the observed specific binding and the theoretical binding (Fig. 3), which is consistent with the presence of a single major species of receptor.

Adenylylase activity

In the next series of experiments, the effects of NECA and 2-chloroadenosine on adenylylase activity were investigated; both compounds had significantly stimulated sperm fertilizing ability *in vitro* (Fraser, 1990). Each was assessed at 1, 10, 100 and 1000 nmol l⁻¹.

The introduction of NECA significantly stimulated (*P* < 0.01–<0.001) adenylylase activity. All concentrations stimulated enzyme activity, the effect ranging from

+10.7 to +22.1% (Table 1); stimulation was greatest with 1 and 10 nmol l⁻¹, but all concentrations increased activity. Data are presented as mean specific activity (pmol cAMP min⁻¹ (10⁶ sperm cells)⁻¹) for five different sperm samples and a total of six assays. A similar, significant (*P* < 0.01–<0.001) response to 2-chloroadenosine was also observed (six assays on five samples). Here an increase was again observed with all concentrations, although the response with 1 nmol 2-chloroadenosine l⁻¹ was only 4% above the control value; 10–1000 nmol l⁻¹ stimulated activity by 28–30% (Table 2).

In the final series of experiments, the effect of including the hydrolysis-resistant GTP analogue, Gpp(NH)p, on adenylylase activity was investigated. As in the previous series, NECA and 2-chloroadenosine at 1–1000 nmol l⁻¹ were also added. The inclusion of Gpp(NH)p and NECA significantly (*P* < 0.001) stimulated enzyme activity. Interestingly, Gpp(NH)p itself consistently increased adenylylase by about 11%. With NECA this increased further, up to a maximum of about 16% (Table 3). In the 2-chloroadenosine series, a similar pattern was seen: the inclusion of Gpp(NH)p, with or without 2-chloroadenosine, significantly (*P* < 0.01–*P* < 0.001) stimulated activity. Gpp(NH)p itself stimulated activity by about

Table 3. Stimulatory effect of Gpp(NH)p and NECA on adenylate cyclase activity of mouse spermatozoa

NECA (nmol l ⁻¹)	Gpp(NH)p	Adenylate cyclase, specific activity (pmol cAMP min ⁻¹ 10 ⁻⁶ cells)	% Stimulation
0	—	9.19 ± 0.46 ^a	—
0	+	10.14 ± 0.31***	10.9
1	+	10.38 ± 0.61***	12.9
10	+	10.61 ± 0.58***	15.5
100	+	10.20 ± 0.46***	11.0
1000	+	10.48 ± 0.52***	14.0

^aData expressed as means ± SEM for seven replicate assays on five frozen-thawed, lyophilized sperm samples. Adenylate cyclase assays measured cAMP production, in the absence and presence of 50 µmol Gpp(NH)p l⁻¹ and NECA, from unlabelled ATP in the presence of MnCl₂, phosphocreatine and creatine phosphokinase. cAMP content was determined using a cAMP binding protein and [³H]cAMP. ***P < 0.001 compared with control.

NECA: 5'-N-ethylcarboxamidoadenosine. Gpp(NH)p: 5'-guanylylimidodiphosphate.

Table 4. Stimulatory effect of Gpp(NH)p and 2-chloroadenosine on adenylate cyclase activity of mouse spermatozoa

2-Chloroadenosine (nmol l ⁻¹)	Gpp(NH)p	Adenylate cyclase, specific activity (pmol cAMP min ⁻¹ 10 ⁻⁶ cells)	% Stimulation
0	—	9.63 ± 0.75 ^a	—
0	+	11.08 ± 1.1***	15.1
1	+	11.12 ± 1.1***	15.5
10	+	11.53 ± 1.1***	19.7
100	+	11.26 ± 1.4**	16.9
1000	+	11.78 ± 1.23***	22.3

^aData expressed as means ± SEM for seven replicate assays on the same five frozen-thawed, lyophilized sperm samples used to obtain data in Table 3. Conditions for assay are as given in Table 3, except that 2-chloroadenosine and 50 µmol Gpp(NH)p l⁻¹ were used. **P < 0.01 and ***P < 0.001 compared with control.

Gpp(NH)p: 5'-guanylylimidodiphosphate.

15%, and the adenosine analogue augmented the response, up to 22% at 1000 nmol l⁻¹ (Table 4). A total of seven assays were performed on five different sperm samples. These were different samples from those used in the preceding series (Table 1 and 2) and the adenylate cyclase activity was somewhat higher. Direct comparisons of specific enzyme activity can therefore only be made within a series, not between series.

Discussion

The present study has demonstrated that a labelled adenosine analogue, [³H]NECA, can bind to mouse spermatozoa and that the binding can be displaced by either unlabelled NECA or 2-chloroadenosine. Both analogues have greater affinity for stimulatory A₂ receptors than for inhibitory A₁ receptors, compared with other analogues (Daly *et al.*, 1987), suggesting that the compounds are binding to A₂ receptors of mouse spermatozoa. Furthermore, the inclusion of cyclopentyladenosine, with high affinity for A₁ receptors (Bruns *et al.*, 1986; Daly *et al.*, 1987), did not inhibit [³H]NECA binding, indicating that the NECA and

cyclopentyladenosine were not competing for the same sites. Saturable specific binding of [³H]NECA in the presence of excess 2-chloroadenosine was demonstrated; Scatchard analysis of these data yielded a B_{max} value of 5.17 pmol mg⁻¹ protein and a K_d value of 930 nmol l⁻¹. Further analysis of the specific binding data suggested that a single major class of receptors was involved in the binding. Daly *et al.* (1983) proposed that there are two subclasses of A₂ receptor, one with high affinity for adenosine (0.1–1 µmol l⁻¹) which stimulates adenylate cyclase activity in broken cell preparations from brain and one with low affinity (about 10 µmol l⁻¹) which does not stimulate enzyme activity. Our data would suggest that the mouse sperm A₂ receptor belongs to the high-affinity subclass of adenosine receptors (Bruns *et al.*, 1988). Because intact cells incubated at 37°C were used in the original studies which indirectly indicated the presence of adenosine receptor-mediated effects on sperm function (e.g. Monks and Fraser, 1988b; Fraser, 1990), it was deemed most appropriate to use intact cells and similar conditions during the binding studies. For more detailed investigations of binding parameters, membrane preparations might provide more homogeneous samples for analysis. Individual

sperm suspensions will show some intrinsic biological variation and their binding characteristics may alter during the course of the assay. The latter is less likely with membrane preparations made at a single point in time. In addition, a lower temperature would reduce the level of non-specific binding, particularly with the highest concentrations of [3 H]NECA.

The detection of [3 H]NECA binding is consistent with the presence of externally directed binding sites. In an earlier study, the inclusion of dipyrindamole, an inhibitor of adenosine uptake, did not interfere with exogenous adenosine stimulation of sperm function in uncapacitated cells (Fraser, 1990). In the present study, the inclusion of S-(*p*-nitrobenzyl)-6-thioinosine, another inhibitor of adenosine uptake (Torres *et al.*, 1990), did not alter binding of [3 H]NECA to sperm cells. Both results are consistent with an external site of action. 5'-Nucleotidase which generates adenosine from 5'-AMP appears to be an ecto-enzyme in mouse spermatozoa (Monks and Fraser, 1988a) and hence endogenously generated adenosine would be produced at the cell surface. The fact that inhibition of this enzyme inhibited fertilizing ability (Monks and Fraser, 1988b) suggests that under normal conditions, sperm-generated adenosine helps to promote sperm function.

Although adenosine has been shown to modulate various cell functions, e.g. calcium fluxes (Daly *et al.*, 1987), its best documented effects have been on adenylyate cyclase activity and hence cAMP production. A link between external adenosine receptors and adenylyate cyclase activity was proposed when adenosine was found to stimulate cAMP formation in brain slices (Sattin and Rall, 1970). In addition to these stimulatory (A_2) receptors, external A_1 receptors which inhibit cAMP formation and an intracellular inhibitory site, the P site, have been demonstrated (Londos and Wolff, 1977). Because indirect evidence had linked adenosine and adenylyate cyclase activity in mouse spermatozoa, direct evidence was sought. Adenosine deaminase was used in all assays to try to minimize endogenously produced adenosine which could mask the effects of exogenously supplied compounds (Bruns *et al.*, 1986; Monaco and Conti, 1986). The adenosine deaminase-resistant adenosine analogues chosen, NECA and 2-chloroadenosine, had already been shown to accelerate significantly sperm fertilizing ability *in vitro*. The concentration range of 1–1000 nmol l $^{-1}$, used for both compounds in the present study, included concentrations previously demonstrated to stimulate fertility. Recent studies have reported that sperm adenylyate cyclase is susceptible to degradation by a membrane-bound trypsin-like protease (Okamura *et al.*, 1990). Since the assay used here did not include proteinase inhibitors, it is possible that the adenylyate cyclase activity, in the presence and absence of these compounds, was higher than the values reported.

Results indicate that both NECA and 2-chloroadenosine significantly stimulated adenylyate cyclase activity over the concentration range assessed. 2-Chloroadenosine had relatively little effect at 1 nmol l $^{-1}$, but stimulated enzyme activity by about 30% at the other concentrations. NECA was stimulatory at all concentrations, although the maximal stimulation observed, about 22% with 10 nmol NECA l $^{-1}$, was slightly lower than that obtained with 2-chloroadenosine. The ability of NECA to stimulate at lower concentrations than 2-chloroadenosine is consistent with the potency of these compounds in somatic cells, where NECA > 2-chloroadenosine (Daly *et al.*, 1987). The degree of stimulation observed in these experiments is in a range similar to that obtained

in other studies using somatic cells (e.g. platelets: Londos and Wolff, 1977; luteal cells: Billig *et al.*, 1989).

In somatic cells, adenylyate cyclase has three components: (1) receptors for molecules capable of modulating enzyme activity (e.g. hormones, fluoride, cholera toxin); (2) coupling GTP-binding (G) proteins, either stimulatory (G_s) or inhibitory (G_i), which are heterotrimeric (α , β , γ subunits) and act as substrates for ADP-ribosylation by cholera toxin (G_{so}) or pertussis toxin (G_{io}); (3) a catalytic subunit responsible for conversion of ATP to cAMP (Gilman, 1984; Londos, 1988; Pfeuffer and Helmreich, 1988). Experimental evidence has suggested that sperm adenylyate cyclase differs from its somatic cell counterpart, being insensitive to most hormones, fluoride and cholera toxin (Hoskins and Casillas, 1975; Cheng and Boettcher, 1982; Stengel *et al.*, 1982) and reportedly lacking both G_s and G_i (Hildebrandt *et al.*, 1985). More recently, a G protein with the characteristics of G_i has been identified in mammalian spermatozoa (Bentley *et al.*, 1986; Kopf *et al.*, 1986) but a role for it in regulating adenylyate cyclase has yet to be demonstrated (Kopf, 1990). In the present study, the introduction of Gpp(NH)p consistently stimulated enzyme activity. When NECA or 2-chloroadenosine and Gpp(NH)p were used in combination, further modest stimulation was obtained. This positive effect of a GTP analogue on adenylyate cyclase activity is consistent with a receptor-induced response mediated via GTP and G proteins. The fact that stimulation was obtained in the absence of added Gpp(NH)p indicates that the sperm cells had sufficient endogenous GTP to permit expression of adenosine effects. Because Gpp(NH)p is resistant to hydrolysis, it would ensure a continuous stimulation at available sites (Pfeuffer and Helmreich, 1988), even in the absence of adenosine. There have been several earlier reports that GTP or Gpp(NH)p can stimulate sperm adenylyate cyclase (e.g. bull: Casillas *et al.*, 1980; human: Cheng and Boettcher, 1979; Hyne and Lopata, 1982; mouse: Monks *et al.*, 1986; Stein *et al.*, 1986), although no direct evidence for the mechanism involved was obtained.

The present results strongly suggest the presence of stimulatory A_2 adenosine receptors on uncapacitated mouse sperm cells: adenosine analogues can bind to mouse spermatozoa and stimulate adenylyate cyclase activity in cells. The stimulation by Gpp(NH)p, alone and in conjunction with the analogues, is consistent with adenosine-elicited responses in somatic cells and would therefore suggest that, in mouse spermatozoa as in somatic cells, the adenosine receptors are coupled to the catalytic subunit of adenylyate cyclase by a G protein, either G_s or one with analogous function. Vijayaraghavan and Hoskins (1986) observed increases in intracellular cAMP in bull spermatozoa incubated in the presence of adenosine analogues, a response consistent with a mechanism of action via adenosine receptors and adenylyate cyclase. The fact that earlier studies have failed to detect responses to the known modulators of adenylyate cyclase in somatic cells suggests that the range of effectors for the sperm enzyme may be much more restricted.

In conclusion, adenosine and its analogues can stimulate fertilizing ability of mouse spermatozoa by accelerating capacitation (Fraser, 1990). On the basis of evidence obtained in the present study, we propose that this response is elicited via adenylyate cyclase, leading to an increase in intracellular cAMP. Given that the sperm cells themselves can generate adenosine at an extracellular site and that adenosine is present in micromolar concentrations in female reproductive tract fluid (Samuelson *et al.*,

1985) and follicular fluid released at ovulation (Eppig *et al.*, 1985), we suggest that adenosine acts *in vivo* as an endogenous regulator of sperm function.

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