Adenosine and its analogues, possibly acting at A₂ receptors, stimulate mouse sperm fertilizing ability during early stages of capacitation

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Summary. Earlier studies have provided indirect evidence that the availability of endogenous adenosine can modulate the fertilizing ability of mouse spermatozoa during capacitation. More direct evidence has been sought by evaluating the effect of exogenous adenosine present during the early stages of capacitation. A concentration-dependent stimulation of in-vitro fertilizing ability was observed, with 10 µM- and 100 µM-adenosine significantly increasing the proportion of eggs fertilized compared with drug-free controls. The adenosine-induced stimulation was observed in the presence of 0.01 µM- and 0.1 µM-dipyridamole, an inhibitor of adenosine uptake, suggesting that adenosine is acting at an external site. Comparison of adenosine with its analogues 2'-deoxyadenosine and 2-chloroadenosine indicated that the analogues at 10 µM were able to stimulate fertilization in a manner similar to adenosine. While neither adenosine nor 2'-deoxyadenosine was consistently effective at 1 µM, 2-chloroadenosine significantly stimulated fertilization at both 1 µM and 0.1 µM. In addition, 5'-N-ethylcarboxamidoadenosine (NECA) and (R)-N⁶-phenylisopropyladenosine (R-PIA), potent analogues in somatic cell systems, proved to be so with mouse sperm suspensions, NECA being stimulatory at ≥ 0.01 µM and R-PIA at ≥ 0.1 µM. Subjective evaluation of motility patterns indicated that more cells exhibited hyperactivated motility in the presence of stimulatory concentrations of adenosine or analogues. Assessment of capacitation state using chlortetracycline fluorescence patterns indicated that incubation in 2'-deoxyadenosine resulted in significantly fewer cells expressing the uncapacitated F pattern and significantly more cells with the capacitated AR (acrosome-reacted) pattern, compared with drug-free counterparts. It is concluded that adenosine promotes capacitation by interacting with externally-directed receptors, possibly on adenylate cyclase to increase the intracellular availability of cyclic adenosine monophosphate (cAMP); cAMP is known to stimulate mouse sperm fertilizing ability. The greater sensitivity to NECA, 2-chloroadenosine and R-PIA, relative to adenosine and 2'-deoxyadenosine, is consistent with interaction at stimulatory A₂ adenosine receptors.

Keywords: adenosine; adenosine analogues; capacitation; A₂ adenosine receptors; NECA; R-PIA; mouse

Introduction

While evidence from a number of mammalian species suggests that cyclic adenosine monophosphate (cAMP) plays an important role in capacitation (Tash & Means, 1983; Fraser & Ahuja, 1988), mechanisms regulating sperm adenylate cyclase activity and hence cAMP biosynthesis are poorly understood. Stein & Fraser (1984) demonstrated that adenylate cyclase activity increases and phosphodiesterase activity decreases during capacitation *in vitro* of mouse spermatozoa. Such changes

could provide increased availability of cAMP, as demonstrated in hamster spermatozoa by White & Aitken (1989). Stein *et al.* (1986) subsequently reported that mouse sperm adenylate cyclase activity changed when adenosine deaminase was added to remove endogenous adenosine; these responses, modulated by the guanosine triphosphate (GTP) analogue 5'-guanylylimidodiphosphate (Gpp[NH]p), suggested receptor mediation of these effects. In sperm suspensions incubated for a short time and thus only partially capacitated, adenosine deaminase caused a decrease in adenylate cyclase activity which could be reversed by Gpp(NH)p; this suggested that endogenous adenosine stimulates adenylate cyclase during the early stages of capacitation. In contrast, the addition of adenosine deaminase to fully capacitated suspensions caused an increase in enzyme activity, again reversed by Gpp(NH)p; this suggested that endogenous adenosine inhibits adenylate cyclase when capacitation has been achieved.

In many somatic cells, adenosine has been shown to modulate adenylate cyclase activity through two functionally and pharmacologically different external receptors (Londos *et al.*, 1983), one being inhibitory (A_1) and the other stimulatory (A_2) (Van Calker *et al.*, 1979). Stein *et al.* (1986) therefore proposed that the results with adenosine deaminase suggested the presence of stimulatory A_2 adenosine receptors in uncapacitated cells and inhibitory A_1 receptors in capacitated cells.

If this hypothesis is correct, then alteration in availability of adenosine should modify the demonstrable fertilizing ability of sperm suspensions. The epididymal sperm suspensions used for fertilization *in vitro* have been shown to contain enzymes involved in adenosine metabolism. Both 5'-nucleotidase, which synthesizes adenosine from 5'-AMP, and adenosine deaminase, which metabolizes adenosine to inosine, are present primarily in the extracellular compartment (adenosine deaminase) or as an ecto-enzyme (5'-nucleotidase) rather than within the cells themselves (Monks & Fraser, 1988a). An extracellular site for adenosine action is therefore possible. Inhibition of 5'-nucleotidase in partially capacitated suspensions by introduction of α,β -methylene adenosine diphosphate, to reduce production of endogenous adenosine, resulted in a concentration-dependent inhibition of fertilization (Monks & Fraser, 1988b). Inhibition of adenosine deaminase in similar suspensions by introduction of coformycin, to increase available endogenous adenosine by preventing its breakdown, stimulated the fertilizing ability (Monks & Fraser, 1988b). These responses are consistent with the presence of stimulatory A_2 adenosine receptors on mouse spermatozoa during the early stages of capacitation.

To obtain more direct evidence of adenosine involvement, the present study has examined the effects of exogenous adenosine on fertilizing ability. Because analogues are frequently more potent agonists than adenosine itself, several of these, including 2'-deoxyadenosine, 2-chloroadenosine, 5'-N-ethylcarboxamidoadenosine (NECA) and (R)-N⁶-phenylisopropyladenosine (R-PIA), have also been evaluated. While current evidence suggests that adenosine uptake in mammalian spermatozoa is minimal (Brown & Casillas, 1984; Vijayaraghavan & Hoskins, 1986), it was felt important to exclude the possibility of an intracellular site of action. Therefore the effect of dipyrindamole, an inhibitor of adenosine transport systems in somatic cells (Cass *et al.*, 1987), was evaluated. Finally, chlortetracycline (CTC) fluorescence was used to assess the capacitation state of spermatozoa (Ward & Storey, 1984) incubated in the absence and presence of an adenosine analogue.

Materials and Methods

Media. A modified Tyrode's medium containing 5.56 mM-glucose (Fraser, 1984) and BSA (crystalline; Sigma, Poole, Dorset, UK) at 4 mg/ml was used. Stock solutions of adenosine, 2'-deoxyadenosine and 2-chloroadenosine (Sigma) were prepared in the standard medium so that 5 μ l stock were added to 245 μ l sperm suspension to achieve desired final concentrations. NECA and R-PIA (Boehringer-Mannheim, Lewes, East Sussex, UK) were dissolved in DMSO and diluted in standard medium to prepare stocks for use as detailed above; the final concentration of DMSO was $\leq 0.02\%$.

In-vitro fertilization. Mature TO female mice (> 8 weeks of age) were induced to superovulate by i.p. injection of 7.5 i.u. PMSG (Intervet, Cambridge, UK) and, 48–54 h later, 5 i.u. hCG (Intervet). At 14 h after hCG, unfertilized

cumulus-intact eggs were released into 4 ml standard medium in a 30-mm sterile plastic culture dish (Sterilin, Teddington, Middx, UK), overlaid with autoclaved liquid paraffin (Boots, Nottingham, UK).

Epididymal sperm suspensions were prepared by releasing the contents of each cauda epididymidis from 2 mature male TO mice (>8 weeks) into 2 ml standard medium. After 5 min to allow sperm dispersal, 245 μ l aliquants were transferred to small sterile plastic tubes (Sterilin) and 5 μ l medium or drug solution were added. Suspensions were then quickly gassed with 5% CO₂–5% O₂–90% N₂, tubes were capped and incubated at 37°C. At 25–30 min after release, suspensions were diluted 10-fold to $\sim 2 \times 10^6$ cells/ml in the same medium used for initial incubation. Droplets of 400 μ l were prepared in 30-mm culture dishes and overlaid with liquid paraffin. Cumulus-intact eggs were then added, with approximately equal numbers of eggs in the various suspensions.

Fertilization assessment. At 65 min after gamete mixing, eggs were transferred to small droplets of fresh medium; at 75 min, phosphate-buffered formalin (pH 7.4, 4% formaldehyde) was added in excess to each droplet. After a minimum of 30 min, eggs were stained with 0.75% aceto-orcein and assessed. Eggs were considered to be fertilized if they had resumed meiosis II and contained a decondensing sperm head. The rapidity of sperm penetration was assessed by calculating the proportion of fertilized eggs which had reached telophase II/second polar body and possessed a fully decondensed sperm head. This is the most advanced stage observed after a 75 min incubation (Fraser, 1983). Diluted sperm suspensions were also assessed subjectively for the proportion of motile cells and the expression of hyperactivated motility (Fraser, 1977).

CTC fluorescence patterns. A modification of the method of Ward & Storey (1984) was used to evaluate the capacitation state of spermatozoa. The sperm suspension (5 μ l) was mixed with 5 μ l staining solution consisting of 750 μ M-CTC (Sigma) in a buffer of 20 mM-Tris, 130 mM-NaCl and 5 mM-cysteine (final pH 7.8) on a clean warm slide. After 30 sec, 0.2 μ l of 12.5% paraformaldehyde in 0.5 M-Tris buffer (final pH 7.4) was added with mixing. Finally, a drop of 0.22 M-1,4-diaza-bicyclo (2,2,2)-octane (DABCO; Sigma) in glycerol was mixed in to retard fading of fluorescence. After addition of a coverslip, the slide was gently compressed between paper tissues. This step was needed to remove excess fluid so that sufficient cells were in an appropriate orientation for assessment. Spermatozoa were evaluated on an Olympus BHS microscope equipped with both phase-contrast and epifluorescence optics. The excitation beam was passed through a 405 nm band pass filter and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

In each sample, 100 cells (50 on each of 2 slides) were classified as having 1 of 3 staining patterns: (1) 'F', with uniform fluorescence over the entire head, which is characteristic of acrosome-intact uncapacitated spermatozoa; (2) 'B', with a fluorescence-free dark band in the postacrosomal region, which is characteristic of acrosome-intact capacitated spermatozoa; (3) 'AR', with fluorescence absent from the head, which is characteristic of acrosome-reacted spermatozoa. The presence/absence of the acrosomal cap was verified with phase-contrast illumination (Fraser, 1987).

Statistical analysis. All data were analysed using Cochran's test for the combination of 2×2 contingency tables (Snedecor & Cochran, 1989). Because this utilizes comparisons within individual replicates, a consistent pattern of response is required to obtain significant differences.

Results

Series I: adenosine stimulates fertilizing ability of partially capacitated mouse sperm suspensions

The effects of adenosine at 1, 10 and 100 μ M were evaluated in 4 replicate experiments ($n = 4$). Fertilizing ability of the 30-min preincubated control samples was moderate, with 43.7% of egg fertilized (Table 1). In the presence of 1 μ M-adenosine, 60.9% of eggs were fertilized; although this is considerably higher than values obtained with control suspensions, it was not significant because an elevated response was only obtained in 2 of the 4 replicates. However, significantly more eggs were fertilized in the presence of both 10 μ M-adenosine (81.7%; $P < 0.01$) and 100 μ M-adenosine (74.5%; $P < 0.025$). In this series, the control suspensions were also assessed after 120 min preincubation to ensure that complete capacitation was eventually achieved; 99.2% of eggs were fertilized, demonstrating the time-dependent nature of capacitation under standard conditions.

Data for rapidity of sperm penetration indicated no obvious differences among the various 30 min preincubated suspensions, whether or not adenosine was present. About 35–40% of fertilized eggs had reached advanced stages of development. In contrast, 94% of eggs fertilized by the 120 min preincubated suspension had fully decondensed sperm heads.

Subjective assessment of motility patterns in diluted suspensions revealed that a higher proportion of cells expressed hyperactivated motility in the presence of 10 μ M- and 100 μ M-adenosine (~ 15 –20% of cells) than in 0.1 μ M-adenosine-treated samples or in control suspensions ($< 10\%$). This is consistent with the fertilization data.

Table 1. Adenosine stimulates in-vitro fertilizing ability of mouse spermatozoa

Preincubation (min)	[Adenosine]	Eggs fertilized/ total eggs, %	Range, %
30	0	69/158, 43.7	27–63
	1 μM	112/184, 60.9	20–78
	10 μM	188/230, 81.7***	56–98
	100 μM	117/157, 74.5**	37–91
120	0	131/132, 99.2***	97–100

Values are for 4 replicates.

** $P < 0.025$, *** $P < 0.01$ compared with adenosine-free 30 min suspensions.

Table 2. Dipyridamole, an adenosine uptake inhibitor, does not affect in-vitro fertilizing ability of mouse spermatozoa evaluated after a 30-min preincubation

[Dipyridamole]	Eggs fertilized/ total eggs, %	Range, %
0	55/129, 42.6	27–66
0.01 μM	53/106, 50.0	22–70
0.1 μM	37/100, 37.0	21–63

Values are for 3 replicates.

Table 3. Dipyridamole does not reduce the ability of exogenous adenosine to stimulate in-vitro fertilizing ability of mouse spermatozoa evaluated after a 30-min preincubation

[Adenosine]	[Dipyridamole]	Eggs fertilized/ total eggs, %	Range, %
0	0	72/236, 30.5	0–70
10 μM	0	137/269, 50.9*	14–85
10 μM	0.01 μM	66/109, 60.5*	11–82
10 μM	0.1 μM	143/266, 53.8*	20–87

Values are for 6 replicates.

* $P < 0.05$ compared with drug-free control suspensions.

Series II: dipyridamole, an inhibitor of adenosine uptake, does not affect fertilizing ability

The continuous presence of dipyridamole at 0.01 μM and 0.1 μM was assessed for any detectable effect on in-vitro fertilizing ability of sperm suspensions after 30 min preincubation ($n = 3$). No significant differences were detected among the control and treated suspensions (37.0–52.7% of eggs fertilized; Table 2). No differences in rapidity of sperm penetration or motility patterns were noted.

Series III: dipyridamole does not reduce the ability of adenosine to stimulate fertilizing ability

In Series I, 10 μM -adenosine had the greatest stimulatory effect on fertilization. Therefore, this concentration of adenosine in the absence or presence of dipyridamole at 0.01 and 0.1 μM was

Table 4. Adenosine and its analogues 2'-deoxyadenosine and 2-chloroadenosine show a concentration-dependent stimulation of mouse sperm in-vitro fertilizing ability after a 30-min preincubation

Drug		No. of replicates	Eggs fertilized/ total eggs, %	Range, %
None (control)	0	4	74/191, 38.7	0–78
Adenosine	1 μM	4	76/122, 62.3	31–81
	10 μM	4	100/189, 52.9**	22–94
2'-deoxyadenosine	1 μM	4	83/170, 48.8	4–85
	10 μM	4	109/164, 66.5**	18–95
2-chloroadenosine	1 μM	4	130/247, 52.6*	16–90
	10 μM	4	130/210, 61.9*	26–77
2-chloroadenosine	0.1 μM	2	34/94, 36.2††	27–44
None (control)	0	2 ^a	2/90, 2.2	0–4

^aThese 2 replicates are among the 4 shown earlier in this Table.

* $P < 0.05$, ** $P < 0.025$ compared with drug-free controls where $n = 4$.

†† $P < 0.025$ compared with drug-free controls where $n = 2$.

Table 5. NECA and 2-chloroadenosine stimulate in-vitro fertilizing ability of mouse spermatozoa evaluated after a 30-min preincubation

Drug		Eggs fertilized/ total eggs, %	Range, %
None (control)	0	43/140, 30.7	11–59
NECA	0.01 μM	134/210, 63.8***	37–80
	0.1 μM	91/127, 71.6**	48–100
2-chloroadenosine	0.01 μM	98/195, 50.2	28–75
	0.1 μM	83/145, 57.2**	30–91

Values are for 5 replicates except for 0.1 μM -NECA where $n = 4$.

** $P < 0.025$, *** $P < 0.01$ compared with drug-free controls.

Table 6. NECA and R-PIA stimulate in-vitro fertilizing ability of mouse spermatozoa evaluated after a 30-min preincubation

Drug		Eggs fertilized/ total eggs, %	Range, %
None (control)	0	82/220, 37.3	17–63
NECA	0.1 μM	161/220, 73.2***	49–94
R-PIA	0.1 μM	122/203, 60.1*	23–81
	1 μM	120/198, 60.6*	28–97

Values are for 5 replicates.

* $P < 0.05$, *** $P < 0.01$ compared with drug-free controls.

evaluated ($n = 6$). The in-vitro fertilizing ability of 30-min preincubated suspensions was significantly higher in the presence of adenosine alone (43.9% of eggs fertilized; $P < 0.05$) or with dipyrindamole at 0.01 μM (60.5%; $P < 0.05$) or 0.1 μM (46.6%; $P < 0.05$) compared with drug-free control suspensions (25.5% of eggs fertilized; Table 3). These results suggest that adenosine-related stimulation of sperm function does not require adenosine uptake.

Hyperactivated motility was observed more frequently in the presence of adenosine (~ 15 – 20% of cells) than in its absence ($< 10\%$). No differences in the rapidity of penetration were noted.

Series IV: adenosine, 2'-deoxyadenosine and 2-chloroadenosine stimulate fertilizing ability of partially capacitated sperm suspensions

Since adenosine had a stimulatory effect on fertilization (Series I, III), two analogues which have been shown to stimulate bull sperm motility more effectively than adenosine itself (Vijayaraghavan & Hoskins, 1986) were assessed. In these experiments, both 1 and $10\ \mu\text{M}$ -adenosine or analogue were present continuously during sperm preincubation and sperm-egg incubation ($n = 4$). In the last 2 replicates, $0.1\ \mu\text{M}$ -2-chloroadenosine, reported to have a lower K_m for stimulating A_2 adenosine receptors than adenosine (Daly *et al.*, 1987), was also assessed.

As in Series I and III, adenosine significantly stimulated fertilization (52.9% of eggs fertilized; $P < 0.025$) compared with control samples (38.7% ; Table 4). Although 62.3% of eggs were fertilized in the $1\ \mu\text{M}$ -adenosine suspensions, this was not significantly different from control values because marked stimulation was only observed in 2 of 4 replicates. With 2'-deoxyadenosine, $10\ \mu\text{M}$ significantly stimulated fertilization (66.5% ; $P < 0.025$) while $1\ \mu\text{M}$ did not (48.8%). A difference in response was noted with 2-chloroadenosine: all concentrations tested had a stimulatory effect. The higher concentrations of $1\ \mu\text{M}$ and $10\ \mu\text{M}$ were similar (52.6% and 61.9% , respectively; $P < 0.05$). Although only 2 replicates included $0.1\ \mu\text{M}$ -2-chloroadenosine, the 36.2% of eggs fertilized was significantly higher than the 2.2% of eggs fertilized in the corresponding control suspensions ($P < 0.025$). Therefore, 2-chloroadenosine is a more potent stimulator of fertilizing ability in partially capacitated suspension than either adenosine or 2'-deoxyadenosine.

Again, more cells expressed hyperactivated motility in the presence of stimulatory concentrations of adenosine and analogues (~ 15 – 20%) compared with the other conditions. No obvious differences in the rapidity of sperm penetration were noted.

Series V: NECA and 2-chloroadenosine stimulate fertilizing ability of partially capacitated sperm suspensions

Since NECA has been shown to have an even lower K_m for stimulating A_2 adenosine receptor sites in somatic cells than 2-chloroadenosine (Daly *et al.*, 1987), the effects of these two adenosine analogues were investigated ($n = 5$ except for NECA at $0.1\ \mu\text{M}$ for which $n = 4$). At $0.1\ \mu\text{M}$, both compounds significantly stimulated fertilization (NECA, 58.1% of eggs fertilized and 2-chloroadenosine, 57.2% ; $P < 0.025$) when compared with control suspensions (30.7% ; Table 5). This is consistent with the positive effects of $0.1\ \mu\text{M}$ -2-chloroadenosine observed in Series IV (Table 4). At $0.01\ \mu\text{M}$, NECA again significantly stimulated fertilizing ability (63.8% ; $P < 0.01$); although 2-chloroadenosine-treated suspensions consistently fertilized a higher proportion of eggs (50.2%) than did control suspensions, the difference was not quite sufficient to be statistically significant ($\chi^2 = 3.73$).

Evaluation of hyperactivated motility again indicated a greater expression in the presence of adenosine analogues than in their absence. A slightly higher proportion ($\sim 10\%$) of motile cells was usually observed in the analogue-treated suspensions compared with the untreated control suspensions. No differences in the rapidity of penetration were noted.

Series VI: a comparison of the abilities of NECA and R-PIA to stimulate fertilizing ability

In somatic cells possessing stimulatory A_2 adenosine receptors, NECA is a more potent analogue than R-PIA (Daly *et al.*, 1987). In a preliminary investigation comparing the effects of these two on mouse sperm suspensions, NECA again proved to be stimulatory at $0.01\ \mu\text{M}$ (as in Series V) but R-PIA was not (data not presented). Therefore the analogues were compared at higher concentrations, NECA at $0.01\ \mu\text{M}$ and R-PIA at 0.1 and $1\ \mu\text{M}$ ($n = 5$). Consistent with Series V, NECA

Fig. 1. CTC fluorescence patterns in mouse sperm suspensions incubated in the absence or presence of 10 μ M-2'-deoxyadenosine. Data are the mean of 5 replicates $\pm$ s.e.m. * P < 0.05, ** P < 0.025 compared with drug-free control suspensions.

significantly stimulated fertilizing ability (73.2% of eggs fertilized; P < 0.01) when compared with control suspensions (37.3%; Table 6). R-PIA also had a stimulatory effect at both concentrations (0.1 μ M, 60.1%; 1 μ M, 60.6%), although the level of significance (P < 0.05) was lower than with NECA. This reflected a less consistently high stimulation in the replicates by R-PIA than by NECA.

There was a slightly higher proportion ($\sim$ 5–10%) of motile cells and increased expression of hyperactivated motility in the presence of the analogues. No differences in the rapidity of penetration were observed.

Series VII: 2'-deoxyadenosine advances the capacitation state as determined by CTC fluorescence

Sperm suspensions were allowed to disperse and 2 aliquants were removed, one receiving 10 μ M-2'-deoxyadenosine (n = 5). After incubation for 60–75 min, slides were prepared and assessed. This timing was chosen since it is approximately the time when sperm–egg interaction would be occurring (Fraser, 1983). Significant differences were noted between the two treatments, with fewer cells exhibiting the uncapacitated acrosome-intact F pattern (P < 0.025) and more cells with the AR acrosome-reacted pattern (P < 0.05) in the presence of 2'-deoxyadenosine than in its absence (Fig. 1). No differences in the staining patterns on the tail were noted: the midpiece fluoresced brightly in all samples.

Discussion

The present study has demonstrated that exogenous adenosine and some of its analogues can stimulate the in-vitro fertilizing ability of mouse sperm suspensions when present during the early stages of capacitation. Suspensions preincubated for 30 min are generally poorly fertile: a minority of eggs are penetrated and relatively few of these have fully decondensed sperm heads (present study; Fraser, 1981, 1983, 1984). A comparison of suspensions preincubated for 30 min in the absence or presence of micromolar concentrations of adenosine indicated that the nucleoside at 10 and 100 μ M significantly increased the proportion of fertilized eggs; the response to 1 μ M-adenosine was less consistent. The analogue 2'-deoxyadenosine was stimulatory at 10 μ M but not at 1 μ M, while 2-chloradenosine was effective at 0.1, 1 and 10 μ M, NECA at 0.01 and 0.1 μ M (the only concentrations evaluated) and R-PIA at 0.1 and 1 μ M. The fact that only a minority of fertilized eggs in these various treatment groups had fully decondensed sperm heads suggests that adenosine and its analogues increased the proportion of spermatozoa undergoing the initial stages of capacitation rather than accelerating capacitation in the relatively few already at later stages. If the latter

had been the case, most of the fertilized eggs would have had fully decondensed sperm heads, as seen following incubation with fully capacitated spermatozoa preincubated for 120 min (Series I). Consistent with this enhanced fertilizing ability were the results of CTC fluorescence and motility assessments. Incubation in 2'-deoxyadenosine resulted in a higher proportion of cells exhibiting the B and AR patterns of CTC fluorescence associated with the capacitated state than in drug-free counterparts. Similarly, more cells expressed hyperactivated motility following incubation in the concentrations of adenosine or analogues which stimulated fertilization.

These results are consistent with our recent study (Monks & Fraser, 1988b) utilizing inhibitors of the enzymes involved in adenosine synthesis (5'-nucleotidase) and breakdown (adenosine deaminase). When endogenous adenosine synthesis was inhibited, fertilization was significantly inhibited, yet when adenosine breakdown was inhibited, fertilization was stimulated. Thus increased endogenous adenosine availability had a positive effect on sperm function. The presence of these enzymes, 5'-nucleotidase and adenosine deaminase, in the extracellular compartment suggested that adenosine could be exerting its effect at an external site on the sperm cell. This would be consistent with the reported inability of bull spermatozoa to take up adenosine from the surrounding medium (Brown & Casillas, 1984; Vijayaraghavan & Hoskins, 1986). Dipyridamole is an inhibitor of adenosine transport systems involved in uptake in somatic cells (Cass *et al.*, 1987). For example, the ability of pig aortic endothelial cells to take up 10 μM -adenosine was inhibited by >90% when 0.1 μM -dipyridamole was present (Pearson *et al.*, 1983). When dipyridamole was added to mouse sperm suspensions, it had no detectable effect on fertilizing ability. Furthermore, its inclusion at 0.01 and 0.1 μM in the presence of 10 μM -adenosine did not reduce the adenosine-induced increase in fertilization. From these results it is concluded that the sperm responses to adenosine involve action at an external, rather than an intracellular, site. This is further supported by the evidence that NECA and 2-chloroadenosine are very potent stimulators of fertilization. These compounds are quite stable, generally being poor substrates for adenosine transport systems and for adenosine deaminase (Daly *et al.*, 1987).

In earlier studies we proposed that during initial stages of capacitation mouse spermatozoa possess stimulatory A_2 adenosine receptors (Stein *et al.*, 1986; Monks & Fraser, 1988b). In somatic cells in which such receptors have been evaluated in some detail, various adenosine and analogues with modifications in either the purine ring or the ribose moiety have been shown to be significantly more potent than adenosine at A_2 receptors, e.g. NECA > 2-chloroadenosine > R-PIA > adenosine (Daly *et al.*, 1987). Compounds with modifications at the 2' position in the ribose moiety, e.g. 2'-deoxyadenosine, have shown weak activity at A_2 sites (Londos & Wolff, 1977). Vijayaraghavan & Hoskins (1986) have reported that 2'-deoxyadenosine stimulated bull sperm motility at lower micromolar concentrations than did adenosine. Aitken *et al.* (1986) observed a stimulation of motility in cryostored human sperm suspensions by 2'-deoxyadenosine, but at millimolar concentrations. In those studies, it is unclear whether the responses were elicited via external adenosine receptors. In the present study, NECA, 2-chloroadenosine and R-PIA were the most potent stimulators of fertilizing ability, being effective at concentrations as low as 0.01 μM (NECA) and 0.1 μM (2-chloroadenosine and R-PIA). In comparison, adenosine and 2'-deoxyadenosine were only consistently effective at 10 μM . At 1 μM , adenosine was stimulatory in half the replicates, whereas 2'-deoxyadenosine had little effect. Therefore, a ranking order of potency would be NECA > 2-chloroadenosine $\gg$ adenosine > 2'-deoxyadenosine and NECA > R-PIA. Such an order is consistent with results obtained for A_2 receptor-mediated effects in other cell types (Daly *et al.*, 1987). The requirement for micromolar concentrations of adenosine is consistent with the K_m displayed by stimulatory A_2 adenosine receptors, in contrast with inhibitory A_1 receptors with affinities in the nanomolar range (Van Calker *et al.*, 1979). It is therefore proposed that the present results provide further evidence for the presence of A_2 receptors on mouse spermatozoa.

The initial proposal that adenosine receptors on the cell surface were linked to adenylate cyclase activity came from the observation of Sattin & Rall (1970) that adenosine stimulated cAMP formation in brain slices. Since then it has been shown that inhibitory adenosine receptors (A_1),

which inhibit cAMP formation, also exist as well as a further inhibitory site, the P site, which is located intracellularly (Londos & Wolff, 1977). In addition, there is some evidence that adenosine receptors may affect other cell functions (e.g. calcium fluxes) as well as cAMP levels (Daly *et al.*, 1987). However, given our earlier observation that introduction of adenosine deaminase caused a decrease in adenylate cyclase activity (Stein *et al.*, 1986) and the recent evidence that adenosine analogues capable of stimulating bull sperm motility also significantly increased intracellular cAMP levels (Vijayaraghavan & Hoskins, 1986), it is reasonable to conclude that the adenosine receptors are able to modulate mouse sperm adenylate cyclase activity and hence cAMP availability. This would be consistent with earlier demonstrations that both caffeine (Fraser, 1979) and dibutyryl cAMP (Fraser, 1981) were able to stimulate fertilizing ability of suspensions preincubated for a short time.

Although the sperm adenylate cyclase appears to differ from that in somatic cells, e.g. being insensitive to a range of hormones and apparently lacking guanine nucleotide regulatory subunits (discussed by Fraser & Ahuja, 1988), there is some evidence that guanine nucleotides can modulate enzyme activity (Cheng & Boettcher, 1979; Casillas *et al.*, 1980; Hyne & Lopata, 1982; Stein *et al.*, 1986). The latter appears to be a requisite for responsiveness to adenosine at adenosine receptors (Fain & Malbon, 1979). Furthermore, other studies have demonstrated that sperm adenylate cyclase activity can be modified by adenosine (Hyne & Lopata, 1982; Brown & Casillas, 1984; Stein *et al.*, 1986). Therefore, the adenosine-induced effects observed in this study may have involved stimulation of mouse sperm adenylate cyclase.

As a final point of consideration, it is likely that adenosine could modulate sperm function *in vivo*. 5'-Nucleotidase is present as an ecto-enzyme (Monks & Fraser, 1988a) and hence extracellular adenosine production by the cells themselves is possible. Additionally, evidence suggests that adenosine is present in micromolar concentrations both in the female reproductive tract (Samuelson *et al.*, 1985) and in follicular fluid released at the time of ovulation (Eppig *et al.*, 1985).

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