Testicular Expression of Adora3i2 in Adora3 Knockout Mice Reveals a Role of Mouse A3Ri2 and Human A3Ri3 Adenosine Receptors in Sperm

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Adenosine is a candidate modulator of sperm motility in the female reproductive tract that increases sperm flagellar beat frequency in vitro. Past work suggested that this acceleration may involve equilibrative (ENT) and concentrative (CNT) nucleoside transporters. Here we show that Slc29a1 (ENT-1) is the predominant nucleoside transporter expressed in the mouse testis. Unexpectedly, the beat of Slc29a1-null sperm still accelerates in response to 2-chloro-2′-deoxyadenosine (Cl-dAdo). Moreover, in wild-type sperm neither blockade of CNTs by removal of external Na⁺ nor inhibition of ENTs with nitrobenzylthioinosine, prevents acceleration of the sperm beat by Cl-dAdo. In contrast, pertussis toxin produces strong blockade, indicating involvement of a Gαo-coupled adenosine receptor. Although agonists selective for adenosine receptors A1R, A2aR, and A2bR are ineffective, A3R-selective agonists Cl-IB-MECA and IB-MECA do accelerate the beat. Consistent with this pharmacological profile, the predominant Adora transcripts in the testis are products of the nested Adora3i1 and Adora3i2 genes. Surprisingly, Cl-IB-MECA and Cl-dAdo still accelerate the beat of Adora3i1-null sperm indicating that the remaining Adora3i2 transcript produces an A3R that functions in sperm. When cloned Adora3i2 is heterologously expressed in tsA-201 cells, Cl-dAdo decreases forskolin-evoked accumulation of cAMP, indicating that Adora3i2 specifies a functional A3Ri2 adenosine receptor that couples through Gαo. Database mining reveals that mouse Adora3i2 is expressed primarily in testis, almost exclusively in spermatids. Expression of the orthologous Adora3i3 transcript also is most prominent in human testis; presumably producing an A3Ri3 receptor that is functional in sperm and that may be a target for development of male-directed contraceptives.

The only known roles of sperm are to reach the egg and deliver its genetic payload and to initiate early signaling events in the zygote. Although the list is short, the tasks are complex, and the sperm requires numerous specialized components to accomplish these goals. Our recent work has focused on how the sperm applies its specialized components to control swimming behavior. We have combined biophysical methods with a genetic approach, using sperm from null-mutant (knock-out) mice that carry targeted disruptions of the genes for several of the components of the signaling pathways that operate in the sperm flagellum. This approach has provided definitive evidence for required roles of the unique CatSper ion channel proteins (1–7), for the atypical sperm adenylyl cyclase SACY2 (3, 8), and for the sperm-specific Ca2+ catalytic subunit of PKA (9, 10). It also produced the unexpected finding that adenosine analogs engage CAMP-mediated signaling to increase flagellar beat frequency (10, 11).

In some somatic cells, adenosine activates cell surface G-protein-coupled adenosine receptors that couple to various conventional transmembrane adenylyl cyclases (ADCY1–9) to control synthesis of cAMP (12, 13). However, in sperm the predominant (perhaps the only) adenylyl cyclase is the atypical SACY (ADCY10) (8), which lacks transmembrane domains and is unaffected by G-proteins (14, 15). The apparent absence of conventional adenylyl cyclases led us to propose that adenosine analogs may enter sperm via cell surface nucleoside transporters to engage the SACY/PKA signaling pathway (10, 11). We now reconsider this proposal, evaluating the expression of Slc28a and Slc29a nucleoside transporters in adult mouse testis, and examining the phenotype of sperm from the Slc29a1 knockout mouse (16).

Some other past work reports finding adenosine receptor binding activity and immunoreactivity in sperm (17–20). We now also reexamine the expression of adenosine receptor genes in the testis and spermatogenic cells and explore the sperm phenotype of the Adora3 knockout mouse (21). The results provide new insights about the nature of the Adora3i2 tran-

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The abbreviations used are: SACY, sperm adenylyl cyclase; Cl-dAdo, 2-chloro-2′-deoxyadenosine; CGS21680, 2-[(p-carboxyethyl)phenylamino]-5-N6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamidoadenosine; CCPA, 2-chloro-N6-cyclopentyladenosine; IB-MECA, N6-(3-iodobenzyl)-9-(5-(methylcarbamoyl)-8-ribofuranosyl)adenosine; Cl-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamide; IBMX, isobutylmethylxanthine; NMDG, N-methyl-o-glucamine; Ptx, pertussis toxin; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter.
script in testis and of expression of A3R adenosine receptors in mouse sperm and of the orthologous ADORA3i3 transcript in human testis that presumably generates A3Rs for human sperm.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cAMP radioimmunoassay kit NEK 033 was from Perkin-Elmer (Shelton, CT). CGS21680, CCPA, IBB-MECA, and Cl-IB-MECA were from Calbiochem. Pertussis toxin B-oligomer was from List (Campbell, CA) and tagRFP vector was a generous gift from Dr. Roger Tsien (UCSD, San Diego, CA). Protease Inhibitor Mixture, pertussis toxin, and 2-chloro-2'-deoxyadenosine (Cl-dAdo) and all other chemicals were from Sigma-Aldrich, unless indicated otherwise.

**Sperm Preparation and Incubation**—Sperm were prepared as in prior work (1, 2, 22, 23). Briefly, after euthanasia by CO₂ asphyxiation, caudae epididymides, and vasa deferentia were asphyxiated, excised tissue was washed twice, then dispersed, and stored at 1–2°C. Sperm were allowed to swimout/capacitation medium (HS with 5 mg BSA/ml and 15 mM NaHCO₃). Semen was allowed to exude (15 min at 37°C, 5% CO₂) from several small incisions.

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**Adenosine Analogs, Pharmacological Agonists, and Transport Inhibitors**—Stock solutions of Cl-dAdo (1 mM in medium HS) and other adenosine analogs, agonists, and inhibitors (1–30 mM in DMSO) were stored frozen. After final dilution the DMSO was <1% (v/v). A parallel solvent-only control was performed when addition of stocks produced DMSO >0.1%.

**RNA Extraction and Gene Expression**—Mouse tissue was rapidly frozen in liquid nitrogen, transferred to 6 m guanidinium chloride and sonicated. Mouse testis RNA was isolated using the RNAeasy™ kit (Qiagen, Germantown, MD) per the manufacturer’s instructions. Human testis RNA was purchased from Origene (Rockville, MD). RNA concentrations were determined from absorbance at 260 nm. Quantitative RT-PCR reactions were performed using 25 ng of RNA in Brilliant II Syber Green Master Mix™ with reverse transcriptase (Stratagene, La Jolla, CA) using gene-specific primers with matched annealing temperatures and similar amplicon lengths. Mouse primers: Slc29a1 (F: 5'-CACGCTTGGCAGTTGTCATTT-3', R: 5'-CCGTTGAGAAGACGAGACA-3'), Slc29a2 (F: 5'-TGGCTCCTACACCAAGGAGTGG-3', R: 5'-GGACGTAGGACAGGATGAC-3'), Slc29a2 (F: 5'-ATGGTTAGCTCCTGGAAAGA-3', R: 5'-ATCTGATATCCAGCGACCTG-3'), Slc29a3 (F: 5'-TGGATCTGCCTTTCTACATCT-3', R: 5'-GGC- 

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**Protein Extraction**—Tissues and sperm were extracted with the TNE buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, 1× protease inhibitor mixture) for 45 min on ice with vortexing every 5 min. Samples were clarified by centrifugation (10,000 × g, 5 min), and protein concentration was determined using the BCA protein assay reagent (Thermo Scientific).

**VideoMicroscopy and Waveform Analysis**—As in past work (22), time series of stop-motion, phase-contrast images of loosely-tethered sperm were recorded while a gravity-fed, selenoid-controlled local-perfusion device applied and removed test solutions. After adjustments of image size and orientation using Metamorph (Molecular Devices, Toronto, Canada) and Igor Pro (WaveMetrics, Lake Oswego, OR) the flagellar location in each image was captured by automated determination of intensity maxima and minima in a line-scan perpendicular to the axis of the flagellar beat. The most prominent peak in the
power spectrum of the Fourier transform of the flagellar location over time then directly reported flagellar beat frequency.

Cloning and Recombinant Protein Expression—Cloning of Adora3i2 was performed by RT-PCR amplification of the full-length transcript from testis RNA using gene-specific primers (Forward: 5’-TATCGGCTCTGCCCACATGGATTCCTCCTCCTCTTCT-3’, Reverse: 5’-GATACGGAATCCCTCAAATCCCTGGAAAAAGTTATG-3’) as described (6). EcoRI and HindIII restriction sites were incorporated into the 5’-ends of the forward and reverse PCR primers (underlined), to facilitate cloning into the EcoRI and HindIII sites in the tagRFP vector. A consensus Kozak sequence, CGCCAAC, was inserted upstream of the Adora3i2 start codon to ensure efficient translation. The full-length, PCR-amplified template was then digested with EcoRI and HindIII and ligated into the tagRFP vector. The ligated plasmids were then transformed into chemically competent DH5α Escherichia coli. Plasmid DNA isolated from bacterial clones was fully sequenced to verify the identity of the inserted gene. Recombinant adenosine receptor Adora3i2 was expressed in tsA-201 cells in culture by transient transfection with Fugene 6 (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Cells were grown in DMEM containing 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin at 37 °C. Cells were transfected at 50% confluency and assayed at 100% confluency ~24–36 h after transfection. Control cells were transfected with cytosolic GFP (Invitrogen).

Cell Treatments and cAMP Radioimmunoassay—Transfected cells were washed with and equilibrated for 15 min in assay buffer (in mM): 110 NaCl, 5 NaHCO3, 1.2 Na2HPO4, 5 KCl, 2.7 CaCl2, 1 MgSO4, 20 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH. Incubation continued for 15 min in the presence of 1 mM IBMX. Triplicate wells were further treated for 15 min with the same buffer containing 1 mM IBMX alone or with 3 μM forskolin. An additional 6 wells were treated with IBMX and 30 μM CI-dAdo. In the final 15 min of incubation, forskolin was added to 3 of these 6 wells. All remaining wells received no further additions. Buffer was then carefully aspirated, and cells were lysed by addition of 0.1% Triton X-100 in 0.1 M NaOH (150 μl) followed by an equal volume of 0.1% Triton X-100 in 0.1 M HCl. The neutralized cell lysates were assayed for cAMP content using a CAMP radioimmunoassay kit per manufacturer’s instructions for non-acetylated samples.

RESULTS

Adenosine Transporters in Acceleration of the Sperm Flagellar Beat—Our past work suggested that adenosine and related analogs may enter sperm via equilibrative and concentrative nucleoside transporters (ENTs and CNTs) to engage a cAMP-mediated signaling pathway that increases sperm flagellar beat frequency (10, 11). For the mouse, the Slc28a1–3 genes encode ENT1–4 (26). Since Slc29a1-4 genes encode ENT3, which is predominantly mitochondrial (27), Although the adenosine analog increases beat frequency of the prominent Slc29a1 mRNA is 160- and 32-fold greater than mRNA of Slc29a4 and Slc29a2, the products of the genes for the other surface membrane ENT transporters (the Slc29a3 encodes ENT3, which is predominantly mitochondrial (27)). Consequently, our focus turns to Slc29a1, the nucleoside transporter gene that is most highly expressed in the testis.

To test directly for a required role of ENT1 in sperm responses to adenosine analogs, we used the Slc29a1-null mouse (16). In the experiments of Fig. 1B, the averaged beat frequencies of resting sperm from wild-type and Slc29a1-null mice were ~3 Hz. Upon challenge with 25 μM CI-dAdo, the beat of wild-type sperm accelerated by 2.3 Hz min⁻¹ reaching 5.6 Hz at 90 s of stimulus. In comparison, the Slc29a1-null sperm accelerated by 1.4 Hz min⁻¹, reaching 4.3 Hz at 90 s. Although the adenosine analog increases beat frequency of the Slc29a1-null sperm, the flagellar waveform is abnormal (see supplemental videos and Fig. S1). Specifically, we consistently observe an apparent stiffness of the proximal flagellum so that the flagellar beat appears to initiate in the principal piece rather than in the proximal flagellum. We interpret this flagellar wave-
form abnormality to be a probable result of a developmental
defect introduced into the flagellum by the absence of the
ENT-1 transporter in spermatogenic cells or in the Sertoli cells
that support them during spermiogenesis. The same develop-
mental defect may explain the diminished ability of the flagel-
um of mature Slc29a1-null sperm to respond to accelerating
stimuli. We conclude that, although decreased in extent, the
ability of Cl-dAdo to accelerate the beat of Slc29a1-null sperm
is inconsistent with a required role for ENT1.

We also applied a pharmacological approach to examine
the role of Slc29a family transporters. Nitrobenzylthioinosine
(NBTI) rapidly inhibits both ENT1 and ENT2 at 10 μM and
likely also inhibits ENT3 and ENT4 (26). In the experiments of
Fig. 2A, 12.5 μM Cl-dAdo accelerated the beat of wild-type
sperm by 2.1 Hz min⁻¹, reaching 5.1 Hz after 90 s of stimulus. In
the presence of 10 μM NBTI, Cl-dAdo accelerated the beat by
2.3 Hz min⁻¹, reaching 5.4 Hz at 90 s. The inability of this broad
spectrum ENT inhibitor to diminish sperm response to
Cl-dAdo suggests that ENT-1 and other Slc29a-family equili-
brate nucleoside transporters are not required for flagellar
response to Cl-dAdo.

Fig. 2B examines possible involvement of the Slc28a family
of concentrative nucleoside transporters in sperm. Because CNTs
are driven by the transmembrane [Na⁺] gradient, we replaced
external Na⁺ with N-methyl-D-glucamine (NMDG⁺). Sperm
were perfused with Na⁺-containing HS medium or with
NMDG-substituted (Na⁺-deficient) medium for 1 min, then
challenged with the same medium containing 25 μM Cl-dAdo.
The acceleration of the beat (4.4 Hz and 4.2 Hz min⁻¹) was
indistinguishable, reaching 8.7 and 8.5 Hz after 90 s of stimulus.
A final challenge with 15 mM HCO₃⁻ produced little or no addi-
tional increase. Because removal of sodium has little impact on
sperm response to Cl-dAdo, we conclude that Slc28a family
(CNT) transporters also are not required.

Table 1

| Treatment          | Beat frequency (Hz) | n  |
|--------------------|--------------------|----|
| None               | 3.45 ± 0.19        | 97 |
| DMSO (1%)          | 3.27 ± 0.17        | 59 |
| Adenosine analog   |                    |    |
| Cl-dAdo (25 μM)    | 6.32 ± 0.51        | 71 |
| A1R agonist        |                    |    |
| CCPA (100 μM)      | 3.65 ± 0.18        | 62 |
| A2R agonist        |                    |    |
| CGS21680 (40 μM)   | 2.50 ± 0.14        | 20 |
| A3R agonist        |                    |    |
| IB-MECA (100 μM)   | 5.06 ± 0.37        | 53 |
| Cl-IB-MECA (30 μM) | 5.87 ± 0.53        | 42 |

a These groups did not differ (p > 0.05).
b–d These groups differed from a and from one another (p < 0.05).

Adenosine Receptors in Testis and Sperm—With strong indi-
cations that adenosine analog action does not require entry via
cell surface adenosine transporters, we applied pharmacology
to evaluate the alternate hypothesis that analog action involves
cell surface adenosine receptors. Table 1 compares averaged
beat frequencies of sperm bathed (1–5 min) in HS medium
alone, with Cl-dAdo, or with agonists selective for the 3 families
(A1R, A2R, and A3R) of adenosine receptors that have been
defined by pharmacological response and binding profiles (13).
The resting beat frequency (in HS alone) was ~3–3.5 Hz, and
increased ~2-fold in the presence of CI-dAdo, consistent with
Figs. 1 and 2. The A1R-selective agonist CCPA (100 μM) did not
change the beat frequency significantly, and the A2aR- and
A2bR-selective agonist CGS21680 (40 μM) slightly slowed the
beat (to 2.5 Hz). By contrast, with the A3R-selective agonist
CI-IB-MECA (30 μM) the mean beat was 5.9 Hz, indistinguish-
able from that with Cl-dAdo. The related A3R agonist
IB-MECA (100 μM) also was effective; however the mean beat
with IB-MECA (5.1 Hz) was marginally slower than with either
Cl-dAdo or CI-IB-MECA. Fig. 3 shows the concentration
dependence of the extent and time-course of response of the
flagellar beat to CI-IB-MECA. With 1 to 30 μM CI-IB-MECA,
the beat accelerated linearly for 60–90 s before reaching a sus-
tained, concentration-dependent, elevated rate. The potency of
CI-IBMECA shown here (EC₅₀ ~5 μM) is consistent with its
published potency in A3R-mediated responses in mast cells (28). Subsequent replacement of 30 μM CI-IB-MECA with 15 mM NaHCO₃ did not further increase the sustained rate. The agonist response profiles of Table 1 and Fig. 3 indicate that an A3R receptor may initiate acceleration of the flagellar beat evoked by adenosine and its analogs.

The mouse genome has 3 documented isoforms of Adora3. Adora3i1 and Adora3i2 share no coding regions. The newly-recognized Adora3i3 shares exons with both Adora3i1 and Adora3i2, but shares coding exons only with Adora3i1 (Fig. 4A). If an adenosine receptor is required for Cl-dAdo-evoked acceleration of sperm, then that receptor should be expressed in their spermatogenic cell precursors. Using primers to amplify full-length transcripts, PCR reported that only Adora3i1 and Adora3i2 transcripts are expressed in the mouse testis (Fig. 4B). The mobility of the Adora3i1 and Adora3i2 PCR products indicated they were the appropriate size, and sequencing confirmed their identification. Using an approach similar to that in Fig. 1, we applied qRT-PCR to examine abundance of Adora1, -2a, -2b, and -3 mRNA in the adult testis. Fig. 4C shows that mRNA for Adora3i2 is 13-fold more abundant than mRNA for Adora3i1, and 250-fold more abundant than mRNA for Adora1, -2a, and -2b.

**Sperm Phenotype of the Adora3 Knock-out Mouse**—Anticipating use of the Adora3 knock-out mouse to test for a required role of A3 receptors in sperm, we performed PCR on testes from Adora3⁻/⁻ mice and from age-matched Adora3+/⁺ mice of the same genetic background. Fig. 5A shows that RNA from wild-type testis produces Adora3i1 and Adora3i2 PCR products. As expected, the Adora3⁻/⁻ testis lacks the Adora3i1 transcript. Surprisingly, the Adora3i2 transcript was still present. Hence, we will refer to these animals as Adora3i1⁻/⁻ or Adora3i1⁻/₋. The second exon of the Adora3 gene is absent in the Adora3i1⁻/₋ mouse. The second exon is the only coding exon in the Adora3i3 transcript, therefore the Adora3i1⁻/₋ mouse also does not express Adora3i3⁻/₋.

**FIGURE 3. Acceleration of the flagellar beat by A3R agonist CI-IB-MECA.** Individual wild-type sperm were perfused for 120 s with Medium HS containing 0–30 μM CI-IB-MECA as indicated, then for 45 s with HS fortified with 15 mM NaHCO₃. Shown are mean beat frequencies for cells sampled at 0, 30, 60, 90, 120, and 165 s (n = 24–36 cells in three independent experiments). Mean acceleration determined by regression analysis of the initial 60 s of response was 0.08 ± 0.05, 2.3 ± 0.9, 2.8 ± 1.1, and 4.8 ± 1.0 Hz min⁻¹. Perfusion with 30 μM CI-IB-MECA for 90 s increased beat frequency significantly (p = 0.021).

**FIGURE 4. Adora gene expression in the adult testis.** A, organization of the mouse Adora gene, present on chromosome 3: 105673825–105726959. Approximate scale indicated by scale bar. Numbers at the right indicate exon useages. B, PCR for full-length Adora3 transcripts in the testis; Adora3i1 (black arrowhead) and Adora3i2 (white arrowhead) were amplified. C, relative expression of Adora3 transcripts in the testis reported by quantitative RT-PCR of testicular cDNA with isoform-specific primers for Adora1, Adora2a, Adora2b, Adora3i2, and primer for the conserved coding region of Adora3i1 and Adora3i3. PCR products were confirmed by sequencing and predicted product length was verified by agarose gel electrophoresis (lower panel).

Using this new insight about the Adora3 knock-out mouse, we could test specifically whether the Adora3i1 isoform is required for evoked acceleration. In the experiments of Fig. 5B we challenged Adora3i1⁻/₋ and Adora3i1+//+ sperm with A3R agonists. With 25 μM CI-dAdo, the flagellar beat of Adora3i1⁻/₋ sperm accelerated by 1.4 Hz min⁻¹, reaching 4.9 Hz at 90 s of stimulus. Adora3i1+/+ sperm accelerated by 1.9 Hz min⁻¹, reaching 5.5 Hz at 90 s. With CI-IB-MECA (30 μM), the beat of Adora3i1⁻/₋ sperm accelerated by 1.0 Hz min⁻¹ reaching 4.1 Hz after 90 s, and the beat of Adora3i1+/+ sperm accelerated by 1.9 Hz min⁻¹ reaching 5.0 Hz at 90 s. The ability of CI-dAdo and CI-IB-MECA to accelerate the beat of Adora3i1-null sperm is inconsistent with a required role of the Adora3i1. It suggests instead that the A3R2 protein product of Adora3i2 initiates responses to adenosine analogs.

**Coupling of Adenosine Receptors to Gαi Proteins in Sperm**—For somatic cells, much past work reports that adenosine A3 receptors activate Gαi to decrease production of cAMP (13). If Gαi proteins are required for the accelerating action of adenosine analogs on sperm, then inactivation of Gαi₆, proteins with pertussis toxin should block acceleration. Fig. 6 examines sperm responses after incubation in medium HS alone, or medium containing pertussis toxin (Ptx), heat-inactivated Ptx,
or with the inactive B-oligomer of the toxin. Sperm from each treatment group were challenged with the agonists Cl-dAdo (25 \( \mu \)M), Cl-IB-MECA (30 \( \mu \)M), or NaHCO\(_3\) (15 mM). Shown are mean beat frequencies for sperm of wild-type (n = 18–24 cells in three experiments) and Adora3i1/−/− mice (n = 12–30 cells in three experiments), sampled at t = −60, 0, 30, 60, 90, 120, and 180 s. Mean acceleration determined by regression analysis of the initial 60 s of response to Cl-dAdo and Cl-IB-MECA. Wild-type and Adora3i1/−/− sperm showed no significant difference in beat frequency after 90 s of perfusion with Cl-dAdo (p = 0.23), but the beat was slightly slower (p < 0.01) for Adora3i1/−/− than for wild-type sperm after 90 s of perfusion with Cl-IB-MECA.

**Functional Expression of Adenosine Receptors in Cells Transfected with Adora3i2**—We have established that the sperm of Adora3i1/−/− mice respond to A3R-selective agonists via a G\(_\alpha_i\)-coupled mechanism, and that their testes express Adora3i- and Adora3b-selective mRNA. We sought direct demonstration that Adora3i2 produces a functional A3R that has not been characterized previously. For this purpose, we expressed the cloned Adora3i2 heterologously in tsA-201 cells, which lack detectable mRNA for endogenous adenosine receptors (as determined by PCR; data not shown). Fig. 7 compares the cAMP content of cells co-transfected either with a RFP-tagged construct containing the full-length Adora3i2 transcript, or mock-transfected with a vector containing only an expression marker. Both groups of cells were challenged with medium alone, with Cl-

**FIGURE 7. Adenosine reduces forskolin-evoked cAMP rise in cultured cells expressing Adora3i2.** tsA-201 cells expressing cloned mouse Adora3i2 tagged with RFP (Adora3i2) or expressing cytosolic GFP alone (Mock) were treated with medium that was supplemented at (t = 0) with either: no additions (None), 30 \( \mu \)M Cl-dAdo, 3 \( \mu \)M forskolin (FSK), or with 3 \( \mu \)M forskolin after addition of 30 \( \mu \)M Cl-dAdo at t = −5 min (Cl-dAdo + FSK). At t = 15 min, assays were terminated and cAMP content of the neutralized extracts was determined by radioimmunoassay. Protein content was determined on assay of parallel cultures. The right axis shows responses normalized to the mean basal content and the mean content of cells treated with forskolin alone. The cAMP content of Adora3i2-expressing cells was reduced significantly (−60%) for cells receiving Cl-dAdo prior to addition of forskolin compared with cells challenged with forskolin alone. **Double asterisk** indicates p < 0.002.
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dAdo, with forskolin, or with Cl-dAdo then forskolin in the continued presence of Cl-dAdo. The basal cAMP contents of mock-transfected controls and Adora3i2-expressing cells were indistinguishable. This basal content (~40 pmol/mg protein) was unchanged by treatment with Cl-dAdo, confirming an absence of endogenous Goi2-coupled adenosine receptors. Forskolin-evoked stimulation of endogenous adenylyl cyclase(s) increased the cAMP content ~4-fold for both mock- and Adora3i2-transfected cells. For mock-transfected cells, the forskolin-evoked increase in cAMP content was not significantly changed by Cl-dAdo. By contrast, for Adora3i2-transfected cells, co-challenge with Cl-dAdo reduced the forskolin-evoked rise in cAMP content by ~60% (p = 0.002). Thus, Adora3i2 reduces evoked accumulation of cAMP, presumably by coupling to Goi2. Functional expression of Adora3i2 is demonstrated.

Database Mining for Tissue-selective Expression of Mouse Adora3i2 and Human ADORA3 Splice Variants—Four publicly available, searchable gene-array databases (29–32) provide complementary information about the tissue expression profiles for the adenosine receptor genes of rat, mouse, and human. Our searches of these databases (see supplemental Figs. S2–S5) found indications of highly restricted expression of Adora3i2 in spermatogenic cells of mouse testis, where it likely produces an A3R2 protein that functions in sperm. A similar prominent expression of rAdora3 (a paralog of mAdora3i2) was indicated in rat round spermatids. Interpretation of the data for human tissue was more complicated, also indicating restricted expression of ADORA3(s) in testis, but not establishing isoform identity.

Selective Expression of ADORA3i3 in Human Testis—To identify the adenosine receptors expressed in human testis we performed qRT-PCR on human testis RNA using primers specific for the 3 splice variants of ADORA3 shown in Fig. 8A. With primers designed to span exons 1–2 of human ADORA3, ADORA3i3 was the predominant ADORA3 transcript, ~8-fold more abundant than ADORAi2 (Fig. 8B). No expression of ADORA3i1 was detected. The lower panel of Fig. 8B shows that with primers designed to amplify full-length transcripts, the PCR products of human testis cDNA report expression only of transcripts of ADORA3i2 and ADORA3i3. It is noteworthy that ADORA3i3 is orthologous to Adora3i2 in mouse. Thus, the pattern of expression in the human testis parallels that in the mouse testis.

At the amino acid level, the A1R, A2aR, and A2bR of rat and mouse are more closely related to each other than to the human A1R, A2aR, and A2bR (supplemental Fig. S6). Similarly, the mouse A3i1R is closer in sequence to rat A3R than to human A3R2. Curiously, the rat apparently lacks a testis-specific Adora3 isoform. Rat spermatogenic cells express the same Adora3 transcript (29) that presumably is expressed in somatic cells.

DISCUSSION

Over the last decade, the targeted-disruption (“knock-out”) approach has contributed substantially to our understanding of the signaling processes that operate in sperm. Examples include the complete loss-of-function phenotypes in the SACY2−/− (8) and Ca2+−/− (9) mice that provided definitive evidence for required roles of SACY and the Ca2+ subunit of PKA in the stimulatory actions of the HCO3− anion on the flagellar beat. In the work that we now report, absence of the expected loss-of-function phenotype for sperm of the Sclc29a1−/− mouse also has been informative, providing evidence that ENT-1 is not required for adenosine analogs to speed the flagellar beat.

The nonessential role of ENT-1 does not support our past proposal (11) that adenosine may act intracellularly following its entry into sperm via ENT and CNT transporters. This inconsistency prompted additional study of the pharmacological profile of responses to adenosine analogs. The results strongly suggested that we reconsider involvement of a cell surface A3R adenosine receptor. The blockade of A3R agonist action on sperm by Ptx provided additional incentive to examine this possibility. Sensitivity to Ptx is consistent with the known coupling of A3R and A1R to Goi2 in some somatic cells (13) and with the prominent presence of Goi2 in sperm (33).

Unexpectedly, we found that sperm of the A3R-knock-out mouse still respond to A3R agonists. This lack of the expected loss-of-function phenotype also has been informative. Clarification arose from demonstration that transcripts of Adora3i1 are absent, but that Adoras3i2 transcripts are still produced in the knock-out testis. The apparent explanation is that although the targeting construct for the A3R knock-out replaced the segment between the initiation codon through the third transmembrane domain of Adora3i1 and part of the intron, the construct terminated in 78.5 kb of genomic DNA that contained the second exon (21). Thus the upstream promoter region and first exon of Adora3i2 are unperturbed, and transcription of
exons 2–7 is not prevented. Therefore, the “A3R-knock-out” is more accurately the “A3Ri1-knock-out” mouse.

Two lines of indirect evidence suggest that Adora3i2 transcripts in spermatogenic cells produce an A3R that initiates sperm responses to Cl-dAdo and A3R-selective agonists. First, we find that Adora3i2 is the predominant Adora family transcript in adult wild-type testis. Moreover, Adora3i2 is by far the most prominent transcript in postmeiotic germ-line cells. Second, we show that recombinant Adora3i2 forms functional Ga_i-coupled receptors in cultured cells. For now, we lack direct evidence for the presence and localization of A3Ri2 protein on the sperm surface.

Some recent work reports immunological evidence of A1R in sperm and finds that A1R-selective agonist CCPA evokes Ca^{2+} entry into sperm of wild-type, but not A1R-knock-out mice (34). Although we find that CCPA does not accelerate the flagellar beat, we cannot exclude the possibility that it evokes other A1R-mediated responses. We do find however that Adora1 transcripts are much less abundant than those of Adora3i2 in the adult testis, and available gene-array data finds only minimal signals for Adora1 in post-meiotic germ-line cells of the mouse (31) or rat (29).

If we accept that the accelerating actions of Cl-dAdo and A3R-selective agonists are initiated by the putative A3Ri2 of mouse sperm, then we must ask what events couple A3Ri2 to the cAMP-mediated, SACY- and PKA-requiring pathway that speeds the flagellar beat. Several reports indicate that in somatic cells A3Rs engage a signaling cascade involving ERK1/2. Examples include the human A3R expressed in CHO cells, where βy subunits released from Gi apparently engage PI3K, Ras, and MEK to phosphorylate ERK1/2 (35, 36). Similarly in mast cells, A3R also couples through G_i3 to engage ERK1/2 signaling (28). Recently a pertussis toxin-sensitive phosphorylation of ERK1/2 also was observed in sperm (34) but was attributed to activation of A1Rs.

The presence and possible roles of ERK1/2 in sperm have been studied by several groups, but remain controversial. Almog et al. (37) found prominent ERK1/2 immunoreactivity in the human sperm flagellum and showed that stimulation with OAG promoted phosphorylation of ERK1/2 and produced modest increases in motility. De Lamirande and Gagnon (38) also found that a transient rise in ERK1/2 activity during capacitating incubations of human sperm. By contrast, Nixon et al. (39) did not find ERK1/2 immunoreactivity, nor observe any changes in motility for mouse sperm treated with inhibitors of MAPK signaling. Nixon also noted that no fertility defect was reported for ERK1/−− mice (40). ERK2/−− mice are embryonic lethal (41). In addition, Jha et al. (42) found that inhibitors of ERK1/2 failed to block the proline-directed Ser/Thr phosphorylation that occurs during capacitating incubation of mouse sperm.

Past work on both mouse Adora3 and human ADORA3 genes has overlooked an unusual shared feature, both contain a nested pair of isoforms. Adora3i2 is the host for Adora3i1, and ADORA3i3 is host for ADORA3i2. The nested Adora3i1 and ADORA3i2 genes each have 2 exons, contained entirely within an intron of their host, and are on same strand as their host. They share no exons with their host, thus could be considered as separate genes rather than splice variants.

The significance of such nested gene pairs is unclear. Yu et al. (43) found ~340 pairs of nested host-guest gene pairs in the mouse genome. Of the 158 guests that were potentially expressed, 2/3 had hosts on opposite strands, only 5 had hosts with similar functions. For the 74 nested genes for which expression data were then available, 16 had tissue-specific expression. Thus, the Adora3i1/i2 (and the ADORA3i1/i3) pair is somewhat unusual in its co-localization on the same strand and in having a shared, related function. At least one previous precedent exists for a nested gene pair with these traits. Berse and Blusztajn (44) found that the S18a3 gene for the vesicular acetylcholine transporter VACHT (which stores acetylcholine) is nested within the first intron of the Chat gene for the choline acetyltransferase, which synthesizes acetylcholine. For the S18a3/Chat pair, nesting may ensure advantageous co-expression of functionally-linked proteins. For the Adora3i1/i2 pair, nesting may be related to the tissue-selective expression of the Adora3i1 to mast cells, and of Adora3i2 to post-meiotic germ-line cells of the mouse. The similar architecture of the human ADORA3i1/ADORA3i3 pair may have a similar role relating to restricted expression of ADORA3i3 in the human testis.

The expression of Adora3i1 in testis found here by PCR (Fig. 4) and reported by Affymetrix gene arrays (supplemental Fig. S2) is consistent with a previous report of testis expression by tissue profiling with qRT-PCR using Taqman probes and primers closely-spaced within exon 2 of Adora3i1 (45). The nesting of Adora3i1 within Adora3i2 makes it likely that the Taqman probe also reported production of the more abundant Adora3i2 primary (unspliced) transcript. This interpretation also may explain the highly testis-restricted expression profile observed by the Taqman method.

In conclusion, our study of the S1c29a1 and Adora3i1 knock-out mice did not find definitive loss-of-function sperm phenotypes. However, our work does strongly indicate that expression of testicular Adora3i2 and its ortholog ADORA3i3 produces murine A3Ri2 and human A3Ri3 receptors that allow adenosine and its analogs to speed the flagellar beat of sperm. Our work also suggests that generation of an Adora3i2 knock-out mouse will provide a valuable tool to define the uncharacterized Ga_i-coupled signaling pathway for the accelerating action of adenosine analogs, and to determine whether sperm A3Ri2 has a required role in fertilization. A requirement of mouse A3Ri2 for male fertility would provide strong incentive to consider the highly similar human ADORA3i3 as a potential candidate target for male-directed contraception.

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REFERENCES
1. Carlson, A. E., Quill, T. A., Westenbroek, R. E., Schuh, S. M., Hille, B., and Babcock, D. F. (2005) J. Biol. Chem. 280, 32238–32244
2. Carlson, A. E., Westenbroek, R. E., Quill, T., Ren, D., Clapham, D. E., Hille, B., Garbers, D. L., and Babcock, D. F. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 14864–14868
3. Hess, K. C., Jones, B. H., Marquez, B., Chen, Y., Ord, T. S., Kamensky, M., Miyamoto, C., Zippin, J. H., Kopf, G. S., Suarez, S. S., Levin, L. R., Williams, C. I., Buck, J., and Moss, S. B. (2005) Dev Cell 9, 249–259
4. Jin, J., Jin, N., Zheng, H., Ro, S., Tafolla, D., Sanders, K. M., and Yan, W. (2007) Biol. Reprod. 77, 37–44
5. Quill, T. A., Ren, D., Clapham, D. E., and Garbers, D. L. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 12527–12531
6. Carlson, A. E., Burnett, L. A., del Camino, D., Quill, T. A., Hille, B., Chong, J. A., Moran, M. M., and Babcock, D. F. (2009) PLoS One 4, e6844
7. Qi, H., Moran, M. M., Navarro, B., Chong, J. A., Krapivinsky, G., Krapivinsky, L., Kirichok, Y., Ramsey, I. S., Quill, T. A., and Clapham, D. E. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 1219–1223
8. Xie, F., Garcia, M. A., Carlson, A. E., Schuh, S. M., Babcock, D. F., McMa-
9. Nolan, M. A., Babcock, D. F., Wennemuth, G., Brown, W., Burton, K. A., and McKnight, G. S. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 13483–13488
10. Schuh, S. M., Carlson, A. E., Quill, T. A., Babcock, D. F. (2006) Biol. Reprod. 74, 492–500
11. Schuh, S. M., Hille, B., and Babcock, D. F. (2007) Biol. Reprod. 77, 960–969
12. Alexander, S. P., Mathie, A., and Peters, J. A. (2006) Br. J. Pharmacol. 147, Suppl. 3, S1–S16
13. Fredholm, B. B., Arslan, G., Haldner, L., Kull, B., Schulte, G., and Waterman, W. (2000) Nauyn Schmiedebergs Arch. Pharmacol. 362, 364–374
14. Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Kopf, J. (2000) Science 289, 625–628
15. Jaiswal, B. S., and Conti, M. (2001) J. Biol. Chem. 276, 31698–31708
16. Choi, D. S., Cascini, M. G., Mailliard, W., Young, H., Paredes, P., McMa-
17. Adonya-Osiguwa, S. A., and Fraser, L. R. (2002) Mol. Reprod. Dev. 63, 245–255
18. Minelli, A., Allegretti, C., Piomboni, P., Mannucci, R., Llus, C., and Franco, R. (2000) J. Histochem. Cytochem. 48, 1163–1171
19. Shen, M. R., Linden, J., Chen, S. S., and Wu, S. N. (1993) Clin. Exp. Phar-
20. Shen, M. R., Linden, J., Chiang, P. H., Chen, S. S., and Wu, S. N. (1993) J. Pharm. Pharmacol. 45, 650–653
21. Salvatore, C. A., Tilley, S. L., Latour, A. M., Fletcher, D. S., Koller, B. H., and Jacobson, M. A. (2000) J. Biol. Chem. 275, 4429–4434
22. Wennearth, G., Carlson, A. E., Harper, A. J., and Babcock, D. F. (2003) Development 130, 1317–1326
23. Westenbroek, R. E., and Babcock, D. F. (1999) Dev. Biol. 207, 457–469
24. Sanz, E., Yang, L., Su, T., Morris, D. R., McKnight, G. S., and Amieux, P. S. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 13939–13944
25. Gray, I. H., Owen, R. P., and Giacomini, K. M. (2004) Pflugers Arch. 447, 728–734
26. Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E., and Young, J. D. (2004) Pflugers Arch. 447, 735–743
27. Govindarajan, R., Leung, G. P., Zhou, M., Tse, C. M., Wang, J., and Unad-
28. Baram, D., Dekel, O., Mekori, Y. A., and Sagi-Eisenberg, R. (2010) J. Im-
29. Johnston, D. S., Jelinsky, S. A., Zhi, Y., Finger, J. N., Kopf, G. S., and Wright, W. W. (2007) Ann. N.Y. Acad. Sci. 1120, 36–46
30. Johnston, D. S., Wright, W. W., Dicandeloro, P., Wilson, E., Kopf, G. S., and Jelinsky, S. A. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 8315–8320
31. Shima, J. E., McLean, D. J., McCarrey, J. R., and Griswold, M. D. (2004) Biol. Reprod. 71, 319–330
32. Su, A. I., Wiltshire, T., Batalov, S., Lapp, H., Ching, K. A., Block, D., Zhang, J., Soden, R., Hayakawa, M., Kreiman, G., Cooke, M. P., Walker, J. R., and Hogenesch, J. B. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 6062–6067
33. Kopf, G. S., Woolkalis, M. J., and Gerton, G. L. (1986) J. Biol. Chem. 261, 7327–7331
34. Minelli, A., Bellezza, I., Collodel, G., and Fredholm, B. B. (2008) Biochem. Pharmacol. 75, 931–941
35. Schulte, G., and Fredholm, B. B. (2000) Mol. Pharmacol. 58, 477–482
36. Schulte, G., and Fredholm, B. B. (2002) Mol. Pharmacol. 62, 1137–1146
37. Almog, T., Lazar, S., Weiss, N., Etkovitz, N., Rahamim, N., Dobkin-Bekman, M., Rotem, R., Kalina, M., Ramon, J., and Jelinsky, S. A. (2008) J. Biol. Chem. 283, 14479–14489
38. de Lamirande, E., and Gagnon, C. (2002) Mol. Hum. Reprod. 8, 124–135
39. Nixon, B., Bielawicz, A., Anderson, A. L., Walsh, A., Hall, T., Mc-
40. Pagès, G., Guérin, S., grill, D., Bonino, F., Smith, A., Anjuere, F., Aubergier, and Pouyssegur, J. (1999) Science 286, 1374–1377
41. Aouadi, M., Binekrey, B., Caron, L., Le Marchand-Brustel, Y., and Bost, F. (2006) Biochimie 88, 1091–1098
42. Almeida, P., Arceley, F., Chertihin, O., Kumari, S., Herr, J. C., and Visconti, P. E. (2006) Mol. Hum. Reprod. 12, 781–789
43. Yu, P., Ma, D., and Xu, M. (2005) Genomics 86, 414–422
44. Bester, B., and Blusztajn, J. K. (1995) J. Biol. Chem. 270, 22101–22104
45. Regard, J. B., Sato, I. T., and Coughlin, S. R. (2008) Cell 135, 561–571