Acetylcholine causes an increase of intracellular calcium in human sperm

C. Bray¹, J.-H. Son and S. Meizel

Department of Cell Biology and Human Anatomy, School of Medicine, University of California at Davis, One Shields Avenue, Davis, CA, USA

¹To whom correspondence should be addressed at: Department of Cell Biology and Human Anatomy, School of Medicine, University of California at Davis, One Shields Avenue, Davis, CA, USA. E-mail: cmbray@ucdavis.edu

Sperm nicotinic acetylcholine receptors (nAChRs) can influence motility and the initiation of acrosome reaction (AR). We report that AR initiation by acetylcholine (ACh) in capacitated human sperm requires both Na⁺ and Ca²⁺ in the external medium. Pre-incubation with 50 μM 3-quinuclidinyl benzilate (QNB) or 50 nM strychnine failed to inhibit the ACh-initiated AR, demonstrating that muscarinic AChRs and nAChRs containing α9 subunits do not mediate this event. Choline (2.5, 5 and 10 mM), a highly specific but low potency agonist of the α7 nAChR initiated AR, with its effect blocked by the nAChR antagonist methyllycaconitine (MLA). ACh (50–400 μM) stimulated a small transient rise in the intracellular Ca²⁺ in sperm populations loaded with FURA-2, with 200 μM ACh being maximal (146 nM ± 23 SEM). The nAChR antagonists, α-bungarotoxin (α-BTX) and MLA, reduced the ACh-initiated Ca²⁺ rise by 75 and 78%, respectively, demonstrating the majority of the rise is mediated through nAChRs containing α7 or α9 subunits. Single cell imaging studies using FLYO-3 resolved two patterns of ACh-stimulated Ca²⁺ increase in the sperm head: 94% of responding sperm displayed a rise (59.6% ± 5.7 SEM increase from resting fluorescence intensity), returning to resting levels over a period of 2–3 min. The remaining sperm (6%) displayed a sharp spike of Ca²⁺ (~1 min; 86% ± 4.3 SEM change in fluorescence intensity), followed by abrupt loss of fluorescence, a pattern suggestive of AR. A Ca²⁺ influx in the sperm midpiece appeared to accompany the Ca²⁺ influx seen in the head. These observations confirm an ionotropic role for nAChRs in sperm function.

Key words: calcium/nicotinic acetylcholine receptors/sperm

Introduction

The acrosome is a large secretory vesicle located towards the anterior of the head of mammalian sperm. The acrosome reaction (AR) is an exocytotic event essential for fertilization. Upon binding to zona pelucida (ZP; a glycoprotein matrix surrounding the oocyte), the sperm undergoes the AR (Yanagimachi, 1994). During AR initiation a sperm’s plasma membrane fuses with the underlying outer acrosomal membrane leading to their fenestration and vesiculation, to release the acrosomal contents and induce modifications of the remaining sperm head plasma membrane (Yudin et al., 1988; Yanagimachi, 1994). Release of the acrosomal contents, coupled with thrust provided by the hyperactivated beating of its flagellum enable a sperm to penetrate the ZP (Yanagimachi, 1994).

The primary in vivo initiator of AR is believed to be a glycoprotein constituent of ZP, designated ZP3 (or ZPC) (Wassarman, 1999; Kopf, 2002), although a number of endogenous agents including progesterone (Osman et al., 1989), prostaglandin (E₂) (Schaefer et al., 1998) and acetylcholine (ACh) (Bray et al., 2002b) are capable of triggering acrosomal exocytosis in vitro. The nature of the receptors and channels on the sperm plasma membrane activated during ZP-initiated AR and complex-signalling events associated with the AR are still under investigation. The cations Na⁺ and Ca²⁺ are important for ZP-initiation of AR in capacitated mouse, hamster and bovine sperm (Yoshimatsu and Yanagimachi, 1988; Arnoult et al., 1999). For AR initiation in human sperm by recombinant human ZP3, the presence of Ca²⁺ and Na⁺ ions in the extracellular medium is essential (Bray et al., 2002a) (Jung-Ho Son, unpublished data). During AR initiation, influx of Na⁺ and Ca²⁺ through as yet unidentified poorly selective cation channels contributes to membrane depolarization (Forest et al., 1993; Florman et al., 1998). Following membrane depolarization a transient rise in [Ca²⁺]i is triggered through the opening of voltage-operated Ca²⁺ channels (VOCCs). This initial spike in intracellular Ca²⁺ concentration [Ca²⁺]i activates phospholipase Cγ (PLCγ) to generate inositol triphosphate (IP₃) and diacylglycerol. IP₃ acts upon IP₃ receptor channels located on the outer acrosomal membrane (Walesky and Snyder, 1995), to release calcium ions from the intra-acrosomal store (Herrick et al., 2005), leading to the opening of store-operated Ca²⁺ channels on the plasma membrane (Florman et al., 1998; Jungnickel et al., 2001). The calcium rise activates multiple downstream-signalling pathways ultimately leading the AR (Breitbart, 2002).

Many neuronal receptors, including nicotinic acetylcholine receptors (nAChRs) have been implicated in sperm function (Meizel, 2004). In vertebrates, 10 α, 4 β and single δ, ε and γ nAChR subunits have been identified to date. Muscle type nAChRs conform to a strict stoichiometry of either (α1)₂ β1 γ δ (expressed in fetal tissue) or (α1)₂ β1 γ ε (adult tissue). Neuronal-type nAChRs are composed solely of α and β subunits, but subunit association is promiscuous, generating a high level of functional diversity in terms of pharmacological specificities, channel permeability and kinetics. The mechanisms governing the co-association of different nAChR subunits is poorly understood

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Human sperm express α3, α5, α7, α9 and β4 nAChR subunits, with expression localized to the midpiece, neck and post-acrosomal regions (Kumar and Meizel, 2005). Micromolar concentrations of ACh can initiate the AR in both capacitated human and mouse sperm (Bray et al., 2002b; Son and Meizel, 2003). The ACh-initiated AR is inhibited by separate pre-incubation with α-bungarotoxin (α-BTX), α-conotoxin IMI (α-CTX IMI) and methyllycaconitine (MLA) antagonists that all bind to α7 nAChRs. These antagonists also inhibited AR initiation by reconstituent human ZP3 or solubilized mouse ZP in human and mouse sperm, respectively (Bray et al., 2002b; Son and Meizel, 2003). Sperm nAChRs may play a role in the initiation of the ZP-initiated AR in vivo.

This work further studies the mechanism by which ACh initiates AR in capacitated human sperm. We investigated the requirement for the presence of Ca2+ and Na+ ions in the external medium for the ACh initiated AR. Muscarinic AChRs have been reported to be present on human sperm (Baccetti et al., 1995). We investigated the influence of quinuclidinyl benzilate (QNB) (an antagonist of muscarinic AChRs) upon ACh-initiated AR. To determine the potential contribution of α9 nAChRs to the ACh AR we incubated sperm with strychnine, an antagonist of α9 subunit containing nAChRs before AR initiation. We demonstrate that millimolar concentrations of choline, a highly specific but low potency α7 nAChR agonist are as potent as 200 μM in initiating the AR. Spectrofluorimetry was used to investigate whether ACh addition to capacitated human sperm populations could cause an increase in intracellular calcium concentrations [Ca2+]i and whether such changes were inhibitable by the absence of Na+ ions in the external medium or pre-incubation with α-BTX or MLA. Single-cell imaging studies were used to further resolve the calcium influx into human sperm upon ACh addition.

Materials and methods

Materials

Salts and metabolites used for incubation and wash media were of reagent grade and purchased from Fisher Scientific (Irvine, CA, USA), Mallinckrodt (Paris, KY, USA) or Sigma (St. Louis, MO, USA). Percoll was obtained from Amersham Biosciences (Uppsala, Sweden). ACh, choline chloride, N-methyl-d-glucamine, α-BTX, MLA, 3-QNB, strychnine chloride, ionomycin and 0.1% poly-L-lysine solution were purchased from Sigma. ConA-fluorescein isothiocynate (FITC) was purchased from Gallard-Schlesinguer Industries (New York, NY, USA). FURA-2-AM, 1,2 bis(aminophenoxy)ethane–N,N,N′,N′-tetraacetic acid (tetrasodium salt) (cell permeant) (BAPTA), FLUO-3-AM and a Live/Dead sperm viability kit were purchased from Molecular Probes Inc (Eugene, OR, USA). The deionized water used in experiments was purified to 18 MΩ-cm with a NANO-pure ion exchange system (Barnstead/Thermolyne, Dubuque, IA, USA).

Preparation and capacitation of human sperm

Protocols for human sperm studies were approved by the Human Subjects Committee at the University of California, Davis. Semen samples were obtained by masturbation from a pool of healthy donors. A population of >95% motile sperm was obtained by centrifugation of semen samples through a discontinuous Percoll gradient and subsequent washing in human sperm medium (HSM), as previously described (Suarez et al., 1986). For sperm to respond to AR initiators, they must first undergo a series of molecular changes collectively termed capacitation (Yanagimachi, 1994; Jaiswal and Eisenbach, 2002). Sperm suspensions were prepared by diluting the sperm to 6 × 10^6/ml in HSM containing 26 mg/ml bovine serum albumin (BSA) (HSM-26B) and capacitated by incubation of 500 μl aliquots for 18 h at 37°C and 5% CO2/95% air. In previous studies of Ca2+ changes in human sperm, we utilized a 24 h capacitation period (Bray et al., 2002b) but the 18 h capacitation period resulted in more sperm surviving the subsequent procedures used in this study. For experiments in this study examining AR initiation, a capacitation period of between 18 and 24 h was used.

AR initiation and assay

Capacitated human sperm were transferred into HSM-3B (Thomas and Meizel, 1988) and placed in microcentrifuge tubes for AR determination; 200 μl aliquots were used for experiments involving ACh. Initial experiments demonstrated that 200 μM ACh was optimal for the AR initiation following an 18 h capacitation period and was used for all subsequent studies. Following AR initiation, sperm were incubated at 37°C in 5% CO2/95% air for 25 min. Sperm were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), their acrosomes stained using ConA-FITC and mounted in fluoromount. Acrosomal status was assessed, with a minimum of two hundred sperm from each treatment scored in a blind fashion (Meizel and Turner, 1993).

ACh is a labile molecule and is hydrolysed by acetylcholinesterase (AChE; EC 3.1.1.7) and other cholinesterases. AChE is expressed on the neck, mid-piece and tail of human sperm (Mor et al., 2001). To confirm the increase in AR levels of capacitated sperm observed following incubation with ACh was due to the action of ACh and not to its breakdown products, we examined the influence of choline and acetate (200 μM) separately and together upon the levels of AR observed.

Millimolar concentrations of choline act as a highly specific but low potency agonist of α7 nAChRs (Mandzely et al., 1995; Papke et al., 1996). To investigate whether activation of α7 nAChRs alone was sufficient to initiate the AR, we incubated aliquots capacitated human sperm with millimolar concentrations of choline (2.5, 5 and 10 mM final concentrations; dissolved in PBS) using the conditions described. To confirm that choline’s action was through α7 nAChRs, we pre-incubated sperm with the nAChR antagonist MLA (100 nM final concentration; in PBS; 10 min) before the addition of choline and AR assessment. As MLA is prepared as a citrate salt, we also determined whether pre-incubation with this salt alone (100 nM final concentration; in PBS) influenced AR levels.

In all AR studies, aliquots of sperm (5 μl) were removed before and following treatment and the motility of the sample determined. The percentage of motile sperm was determined by examination using phase contrast microscopy (100–200 sperm per treatment) and a subjective score was assigned to the quality of sperm motility using a scale from 1 (twitching, non-progressive motion) to 4 (vigorous forward motility) (Thomas and Meizel, 1988).

Investigation of the requirement for extracellular Na+ and Ca2+ for the ACh-initiated AR

For Na+-free studies, aliquots of capacitated sperm were washed twice and re-suspended in a media osmotically identical to HSM-3B, designated NaB (Na+-containing medium: 126 mM NaCl, 25 mM KHCO3, 0.36 mM KH2PO4, 2.5 mM CaCl2, 0.5 mM MgCl2, 2 mM glucose, 0.25 mM pyruvate, 19 mM lactate 0.034 streptomycin, 0.21 mM K+ penicillin and 3 mg/ml of BSA) and NMDG-B (Na+-deficient: 126 mM N-methyl-d-glucamine, 25 mM KHCO3, 0.36 mM KH2PO4, 2.5 mM CaCl2, 0.5 mM MgCl2, 2 mM glucose, 0.25 mM pyruvate, 19 mM lactate 0.034 streptomycin, 0.21 mM K+ penicillin and 3 mg/ml) (Garcia and Meizel, 1996). The pH and [Cl–] of the NMDG-B were made equal to that of NaB through the addition of HCl.

We also wished to determine whether the transfer of sperm into Na+-free medium interfered with their ability to undergo AR even when intracellular Ca2+ was increased by means of a Ca2+ ionophore. For that experiment, we attempted to initiate the AR by incubation of sperm with ionomycin [3 μM; 0.05% dimethylsulphoxide (DMSO); 10 min] in Na+-free medium. The total number of live sperm was determined for control and ionomycin-treated sperm with the Molecular Probes Live/Dead sperm viability kit.
To remove extracellular Ca\(^{2+}\), sperm were washed and re-suspended in a Ca\(^{2+}\)-free medium of HSM-3B supplemented with a final concentration of 7.5 mM BAPTA (Bray et al., 2002b) immediately before the start of the experiment. AR initiation by 200 μM ACh and assessment of AR were performed.

**Effect of the AChR antagonists QNB and Strycnione on ACh-initiated AR**

QNB (50 μM) can antagonize muscarinic-type AChRs. QNB (50 μM final concentration) dissolved in DMSO (0.05% DMSO final concentration) or solvent vehicle was added to aliquots of capacitated sperm 1 h before AR initiation by ACh (200 μM) and AR assessment. Strychnine (50 μM) can antagonize nAChRs containing θ9 subunits (Verbitsky et al., 2000). Strychnine (50 mM in PBS) or the solvent vehicle (PBS) was added to aliquots of capacitated sperm 10 min before AR initiation by 200 μM and AR assessment.

**Investigation of the ACh-stimulated [Ca\(^{2+}\)]\(_i\), rise by spectrofluorimetry**

Capacitated sperm suspensions in HSM-26B were prepared for cuvette based [Ca\(^{2+}\)]\(_i\) studies by pooling aliquots and loading the acetoxymethyl ester of FURA-2 (2 μM final concentration; in DMSO 0.05% final concentration; 40 min; 37°C) and transfer into HSM-3B (Thomas and Meizel, 1988).

Measurement of [Ca\(^{2+}\)]\(_i\) was performed every 0.5 s using an Hitachi F-2000 spectrofluorometer (excitation wavelength of 364/385 nm; emission wavelength of 510 nm and a 5 nm excitation/emission band pass) (Garcia and Meizel, 1999). Measurements were performed on 600 μl aliquots of FURA-2 loaded sperm. Samples were stirred magnetically and temperature maintained at 37°C. A constant flow of 5% CO\(_2\) was passed over the top of the sample to maintain pH.

Following equilibration and the establishment of a stable baseline, additions were made using a pre-positioned Hamilton syringe. Additions were made using a syringe specific for a given reagent, at 1/200 v/v. Following ACh addition changes in sperm, [Ca\(^{2+}\)]\(_i\) was measured for an additional 120 s. Each experiment was ended with a calibration, performed by the sequential addition of 20 μM digitonin and 25 mM Tris–EGTA (final concentrations). Calculation of [Ca\(^{2+}\)]\(_i\) was performed as described previously (Gryniewicz et al., 1985), using a $K_D$ of 224 nM for FURA-2 loaded human sperm at 37°C (Harper et al., 2003). The calibration step was omitted occasionally, for sperm to be removed for visual assessment of motility (Thomas and Meizel, 1988).

**Effect of ACh on sperm [Ca\(^{2+}\)]\(_i\)**

Sperm suspensions were prepared for Ca\(^{2+}\) measurement and placed in the spectrofluorimeter, and ACh (50 μM to 20 nM final concentrations; dissolved in PBS) was added to the cuvette. To limit potential hydrolysis of ACh, each solution was made immediately before addition. Initial experiments demonstrated 200 μM ACh was the optimal concentration to obtain the maximal change in [Ca\(^{2+}\)]\(_i\) (Figure 4B), and, therefore, this concentration was used for all subsequent Ca\(^{2+}\) studies.

**Effect of nAChR antagonists on ACh-stimulated [Ca\(^{2+}\)]\(_i\), rise**

Sperm were pre-incubated with a 100 nM final concentration of α-BTX (an antagonist binding to α1, α7, ε8 and ε9 nAChR subunits; 45 min pre-incubation; in PBS) or MLA (an antagonist binding to α7 and ε9 nAChR subunits; 20 min; dissolved in PBS). Concentrations of the antagonists used inhibited the onset of the ACh-initiated AR in human sperm (Bray et al., 2002b). As MLA is prepared as a citrate salt, control experiments were undertaken to determine whether pre-incubation with this salt alone (100 nM final concentration; in PBS) influenced either the basal level of Ca\(^{2+}\) in FURA-2 loaded cells or the change in [Ca\(^{2+}\)]\(_i\) (Δ[Ca\(^{2+}\)]\(_i\)) by ACh.

**Analysis of spectrofluorimetric data**

Experiments included in the data set were selected upon the criteria that each maintained a steady baseline over the control period, and basal levels of [Ca\(^{2+}\)]\(_i\) were comparable (within 50 nM of each other). Basal calcium levels were calculated from mean [Ca\(^{2+}\)]\(_i\) over the control period before ACh addition. The peak value was taken as the highest value within 20 s following the addition of ACh (with single peak points excluded). Overall change in [Ca\(^{2+}\)]\(_i\) (Δ[Ca\(^{2+}\)]\(_i\)) was calculated using the formula Δ[Ca\(^{2+}\)]\(_i\) = [Ca\(^{2+}\)]\(_i\) (peak) – [Ca\(^{2+}\)]\(_i\) (basal).

In experiments using nAChR antagonists, % inhibition of the Δ[Ca\(^{2+}\)]\(_i\) was calculated using the equation % inhibition = ([Ca\(^{2+}\)]\(_i\) Ag – [Ca\(^{2+}\)]\(_i\) C / [Ca\(^{2+}\)]\(_i\) (ACh) – Δ[Ca\(^{2+}\)]\(_i\) C) × 100, where [Ca\(^{2+}\)]\(_i\) (ACh) denotes the change in intracellular Ca\(^{2+}\) level following the addition of ACh. Δ[Ca\(^{2+}\)]\(_i\), Ag denotes the change in intracellular calcium levels initiated by ACh in the presence of an nAChR antagonist and Δ[Ca\(^{2+}\)]\(_i\), C change in intracellular Ca\(^{2+}\) levels by the solvent vehicle (PBS).

**Investigation of the ACh-initiated [Ca\(^{2+}\)]\(_i\), rise by single-cell imaging**

Following capacitation, 100 μl aliquots of sperm were loaded with FLUO-3-AM (5 μM final concentration; in DMSO 0.01% F-127 pluronic; final concentration of DMSO 0.05%) for 30 min at 37°C 5% CO\(_2\). For the final 15 min of dye loading, sperm were transferred to a temperature-controlled (37°C) imaging chamber (volume 200 μl; Warner Instruments, Hamden, CT, USA) and cells adhered to the upper surface of the vential coverslip (coated with poly-l-lysine; 0.01% in ddH\(_2\)O and air dried). Excess probe and unattached sperm were removed by washing fresh HSM-3B through the chamber. Cells were imaged on a Zeiss/Axiowert S100 microscope fitted with an XF104-ALPHA filter set (Omega Optical Inc, Brattleb, VT, USA), a fast DX-1000 optical switching system (Solamere technology group, UT, USA) and illuminated with a 75-W xenon source. Image capture was controlled by Openlab 3.5.1 software (Improvision, Coventry, UK) running on a G4 Macintosh computer. Image capture was performed every 10 s for a total of 600 s, using either x400 and x630 magnification through a XR GEN III+ ICCD camera (Stanford Photonics, CA, USA) and digitalized (CG-7 color frame grabber; Scion Corporation, MD, USA). ACh was dissolved in HSM-3B immediately before the start of each experiment and injected into the chamber following a control period of 20 capture cycles (2.0 μl 1/100 v/v). Addition of ACh was performed using a Hamilton syringe connected to a remote air piston at the edge of the chamber, over a period of 10 s, allowing gentle diffusion of ACh over the adherent sperm. An outflow port on the imaging chamber was kept open to ensure the added volume caused no change in internal pressure. Images were captured for a further 400 s (40 cycles). Control experiments demonstrated that injection of HSM-3B caused no change in calcium levels of the imaged sperm (data not shown).

**Processing of single-cell data**

Data from single-cell experiments were processed using Openlab 3.1.5 software (Improvision). Following background subtraction, regions of interest (ROIs) were drawn around each sperm head within the field of view and eight-bit grey-scale measurements were extracted. For sperm adhered to the cover slip with the midpiece firmly attached and measurements of Ca\(^{2+}\) changes in the midpiece were made. Any cell that moved out of its ROI during the course of the experiment was excluded from the analysis. Raw intensity values were imported into an Excel spreadsheet (Microsoft, Seattle, WA, USA), converted from greyscale values and normalized using the equation $R = ([F - F_{min}] / F_{max}) \times 100$ (Kirkman-Brown et al., 2000), where $R$ is the normalized fluorescence intensity, $F$ is the fluorescence intensity at a given time point and $F_{max}$ is the mean fluorescence intensity derived from the 20 time points measured during the control period.

**Statistical analysis**

All AR percentage data were transformed to the arc sine of their square roots (Sokal and Rohlf, 1981). The Duncan’s new multiple range test (SAS, 1994) was used for the comparison of group mean differences. Statistical significance was set as $P \leq 0.05$.

**Results**

**Extracellular Na\(^+\) and Ca\(^{2+}\) ions are required for the ACh-initiated AR**

Removal of Na\(^+\) ions from the external medium significantly inhibited stimulation of the AR by 200 μM ACh (Figure 1, a). From this data it was calculated that the level of ACh-initiated AR observed in Na\(^+\) free medium represents 89% inhibition of AR when compared to that seen in Na\(^+\)-containing medium. No difference was observed between untreated cells and solvent controls, indicating the transfer of the cells into Na\(^+\)-deficient medium or the solvent vehicle did not influence
The incubation of capacitated sperm with 50 nM strychnine before the addition of ACh (200 μM) did not significantly inhibit AR initiation when compared to its solvent vehicle (PBS). The following data were obtained (n = 3, % AR ± SEM; P ≤ 0.05 for all results compared to PBS + PBS): PBS + PBS 13.2 ± 2.5%; strychnine (50 nM) + PBS 11.5 ± 2.1%; PBS + ACh (200 μM) 27.6 ± 3.2%; strychnine (50 nM) + ACh (200 μM) 25.4 ± 2.9%.

Choline can initiate the AR at mM concentrations

The addition of 2.5, 5 and 10 mM concentrations of choline to sperm capacitated for between 18 and 24 h resulted in a significant increase in AR, when compared to AR levels in the PBS solvent control (Figure 2). To confirm the action of choline was through α7 nAChRs, we pre-incubated sperm with the nAChR antagonist MLA (100 nM final concentration; in PBS) before the addition of choline and AR assessment. MLA is prepared as a citrate salt; we have previously determined that no difference was observed in AR between untreated cells (Ca2+ present) and solvent controls, indicating removal of extracellular Ca2+ and the solvent vehicle did not influence the basal AR (Figure 1, b).

ACh addition causes a rise in intracellular calcium in human sperm populations

Of the experiments that showed a measurable change in [Ca2+]i upon ACh addition, only those that maintained a stable initial baseline throughout the subsequent series of experiments and exhibited comparable baseline levels of [Ca2+]i were included in the data set described here (20/52 experiments). The mean calculated basal [Ca2+]i from all experiments was 210 ± 7 nM (n = 47). Basal levels of [Ca2+]i were comparable to that reported previously for human sperm following an equivalent capacitation period (Patrat et al., 2000). ACh addition caused a rise in [Ca2+]i; Figure 3A shows a typical experimental trace. Time taken to reach the peak value was 8–15 s following addition, after which the transient decayed over ~60 s. All concentrations of ACh (50, 100, 200 and 400 μM) tested caused a significant increase in [Ca2+]i when compared to the PBS control (Figure 3B) (Duncan’s new multiple range test; P ≤ 0.05) (Figure 3). Amplitude of Δ[Ca2+]i was

QNB and strychnine do not influence the ACh-initiated AR

The incubation of capacitated sperm with QNB (50 μM) followed by the addition of ACh (200 μM) did not significantly inhibit AR initiation when compared to its solvent vehicle (0.05% DMSO) and ACh. The following data were obtained (n = 3, % AR ± SEM; P ≥ 0.05 for all results compared to PBS + DMSO and P ≥ 0.05 for QNB + ACh

results compared to DMSO + ACh): 0.05% DMSO + PBS 7.9 ± 0.8%; 0.05% DMSO + ACh 200 μM 16.9 ± 0.9%; QNB 50 μM + ACh 200 μM 15.3 ± 0.2%.

Figure 1. The acetylcholine (ACh)-initiated acrosome reaction (AR) in capacitated human sperm is dependent on the presence of both extracellular Na+ and Ca2+ ions. Capacitated sperm (6 × 10⁶/ml) were incubated with ACh (200 μM) for 30 min in medium containing or deficient in either Na+ (a) or Ca2+ (b) ions. The level of AR was then assessed as described in Materials and methods. Each column represents the mean value from five different experiments; bars on each column represent the ±SEM of the mean; different letter superscripts denote a significant difference between treatments (P ≤ 0.05).

Figure 2. Millimolar concentrations of choline can initiate the acrosome reaction (AR) in capacitated human sperm through nicotinic acetylcholine receptors (nAChRs). Capacitated sperm (6 × 10⁶/ml) were incubated with acetate (ACh) (200 μM) or choline (Cho) 2.5–10 mM in the absence and presence of methyllycaconitine (MLA; 100 nM). The level of AR was then assessed as described in Materials and methods. Each column represents the mean value of three different experiments; bars on each column represent the ±SEM of the mean; different letter superscripts denote a significant difference between treatments (P ≤ 0.05).
dose dependent, 200 μM causing change of 146 ± 23 nM (n = 8). 400 μM ACh caused a smaller rise, with higher concentrations of 2 and 20 mM ACh-inhibiting Ca²⁺ changes (data not shown). Addition of solvent vehicle (PBS) caused a small but statistically insignificant increase in [Ca²⁺]I: PBS Δ[Ca²⁺]I 13.3 ± 2.5 nM (n = 10). Addition of ACh (400 μM) to sperm alone caused no change in autofluorescence.
α-BTX and MLA can reduce the ACh-stimulated Ca²⁺ rise

Pre-incubation of FURA-2 loaded sperm with either α-BTX (100 nM) or MLA (100 nM), significantly reduced the size of the transient Ca²⁺ influx seen upon addition of 200 μM ACh (Figure 4A and C). By subtracting the mean value of Δ[Ca²⁺]ᵢ from parallel experiments in which PBS alone was added, we were able to remove the contribution of the solvent vehicle from the overall Ca²⁺ change and calculate the efficiency of each antagonist in blocking the Ca²⁺ influx initiated by ACh. The presence of α-BTX effected a 75% reduction of the mean peak value of the Ca²⁺ influx initiated by ACh (Figure 4B). Pre-incubation with MLA lead to a 78% reduction of the mean peak value of the Ca²⁺ influx initiated by ACh (Figure 4D).

Single-cell imaging of the ACh-stimulated Ca²⁺ rise

Addition of the solvent vehicle (HSM-3B) had no effect on the FLUO-3 fluorescence. From 19 experiments, using cells from six different experimental donors we measured increases in [Ca²⁺]ᵢ in 125 sperm (18% of a total of 685 cells examined with 20.2% ±6.4 SEM cells responding per experiment) upon the addition of ACh (200 μM). The changes in Ca²⁺ in responding sperm occurred immediately or shortly after (within 1 min) addition of ACh into the imaging chamber. The variation in onset of the response may reflect heterogeneous activation states of the sperm within the experimental population and inherent variation in the sensitivity of a given cell ability to respond to ACh.

ACh addition initiated two distinct patterns of increase in [Ca²⁺]ᵢ in the sperm head (Figure 4). The most frequently observed pattern of Ca²⁺ influx into the head following ACh addition was a rise to peak and decline back to basal levels (94% of responding cells; n = 117 cells). From initiation this pattern of Ca²⁺ typically took 10–20 s to reach a peak and was followed by a decline phase lasting between 100 and 200 s (Figure 4A). The mean amplitude of the change of [Ca²⁺]ᵢ was 59.6% ±5.7 SEM increase in fluorescence intensity relative to resting levels. This mean value was derived from 20 randomly picked cells within the sample.

A second, less frequently observed pattern of Ca²⁺ influx in the sperm head following ACh addition was a rapid rise to a spike, followed by abrupt loss of fluorescence to below the initial resting level (Figure 4B) (6% of total cells responding; n = 8 cells). The mean amplitude of the spike was 86% ±4.3 SEM; n = 8 cells) increase in fluorescence intensity relative to resting levels. Previous imaging studies during AR initiation in human sperm have reported similar events, with the sudden loss of fluorescence attributed to the dispersal of fluorophore from the sperm head as the plasma membrane and outer acrosomal membranes fuse, indicating the onset of the AR (Tesarik et al., 1996; Meizel et al., 1997).

Most sperm demonstrating ACh-initiated Ca²⁺ influx retained flagellar motility when adhered to the poly-l-lysine cover slip, which was apparent by movements of the midpiece region throughout each experiment. However, of the few sperm with both adherent heads and midpiece that responded to ACh (n = 4 cells), we observed with a rise in [Ca²⁺]ᵢ in the midpiece, concurrent to that seen in sperm head. Figure 5 shows an ACh-initiated Ca²⁺ rise in the midpiece, concurrent to the spike and loss of fluorescence pattern in the sperm head indicative of AR.

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<th>Sperm motility</th>
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<td>The percentage of motile sperm in all experimental samples except ionomycin additions was the same as the controls (range was 70–80% in different experiments). No differences were found between the quality of motility in experimental and control tubes in any single experiment. Motility decreased after ionomycin additions but viability staining (with SYBR14 and propidium iodide dyes; Molecular Probes Inc.) indicated comparable levels of live and dead sperm in experimental and control treatments [n = 3, % live sperm ± SEM; P ≥ 0.05 (DMSO solvent control 74.1 ± 0.3%; ionomycin 69.2 ± 1.1%)]</td>
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Discussion

Micromolar concentrations of ACh can initiate AR in capacitated human sperm, and nAChR antagonists inhibit the ACh-initiated AR (Bray et al., 2002b; Son and Meizel, 2003). Expression of nAChRs has been demonstrated on both mouse and human sperm (Bray et al., 2005; Kumar and Meizel, 2005).

The signalling molecule ACh can interact with two different types of receptors: nicotinic and muscarinic. Immunocytochemical studies have suggested the presence of muscarinic AChRs on human sperm (Baccetti et al., 1995). An antagonist of muscarinic AChRs, QNB, shown to inhibit the ZP-initiated AR in mouse sperm at 50 μM (Florman and Storey, 1981), had no influence on the AR initiated by ACh in human sperm, suggesting muscarinic type AChRs do not play an role...
in the ACh-initiated AR. Both α-BTX and MLA block AR (Bray et al., 2002b) and a substantial portion of the Ca2+ influx initiated by ACh. α-BTX binds the α1, α7, α8 and α9 nAChR subunits; however, the α1 subunit is only found in muscle and the α8 is not a mammalian subunit (Hogg et al., 2003; Funke, 2004). MLA is an antagonist of both α7 and α9 nAChRs. Human sperm express both α7 and α9 nAChR subunits, localized to the posterior sperm head and the midpiece, respectively (Kumar and Meizel, 2005). Our previous work has shown that the human sperm α7 nAChR is involved in the AR initiated by ACh or by recombinant human ZP3 (Bray et al., 2002b). Here, we demonstrate that strychnine, an antagonist of α9 subunit containing nAChRs at the concentration used here (Verbitsky et al., 2000), has no influence on the level of AR initiated by ACh, suggesting activation of the α7 nAChR is solely responsible. As choline, a specific agonist of α7 nAChRs initiates the AR in capacitated human sperm to levels equivalent to those achieved with ACh, and the action of choline is blocked by MLA, we have confirmed the ACh initiated AR is mediated through α7 nAChRs.

The requirement for Ca2+ influx during AR initiation has been demonstrated in sperm from many species (Yanagimachi, 1994). Previous histochemical studies have reported micromolar concentrations of ACh, and nicotine induce Ca2+ uptake in hypotonically washed ram sperm (Stewart and Forrester, 1979) and bovine sperm cells (Nelson et al., 1982). In this study, AR initiation by ionomycin was not blocked by α-BTX, demonstrating nAChR functioning is upstream of Ca2+ influx. The ACh-initiated AR is dependent on the presence of extracellular Na+ and Ca2+ ions. We have demonstrated that ACh initiates a rapid calcium influx into capacitated human sperm. Both α-BTX and MLA block AR (Bray et al., 2002b) and a substantial portion of the Ca2+ influx initiated by ACh. MLA is an antagonist of both α7 and α9 nAChRs (Verbitsky et al., 2000). Human sperm express both α7 and α9 nAChR subunits (Kumar and Meizel, 2005); it is likely each antagonist could exert their influence on sperm function reported in this study through α7 nAChRs, α9 nAChR subunits or both. As human sperm also express α3, α5 and β4 nAChR subunits (Kumar and Meizel, 2005), which form heteromeric nAChRs in vivo (in either α3α5β4 or α3α5β4 stoichiometry) and are insensitive to both α-BTX and MLA (Hogg et al., 2003), the α-BTX and MLA resistant component of the ACh-initiated rise in [Ca2+]i may be due to activation of these nAChRs. Additionally, as the activation of muscarinic AChRs can also activate cellular Ca2+ influx pathways (Akerman et al., 2004), we cannot discount that such receptors potentially present on sperm also contribute in part to the observed Ca2+ rise, although we can discount them from a significant role in the ACh-initiated AR.

Human sperm α7 nAChRs are localized to the neck and post-acrosomal regions (Kumar and Meizel, 2005); activation of these receptors might lead to Ca2+ increases in the sperm head and midpiece. Two distinct patterns of Ca2+ increase were observed in the head following ACh addition: The most frequent was a rise to peak and decline back to basal levels. The less frequent pattern is characterized by rapid rise and abrupt loss of fluorescence to below the initial resting level. This ‘spike’ pattern is probably due to dispersion of fluorophore from the sperm head as the plasma membrane and outer acrosomal membranes fuse and fuseanstrate, at the onset of the AR. An increase in the [Ca2+]i of the sperm midpiece was detected following ACh addition (Figure 6). Due to the interval of sampling we could not resolve whether the Ca2+ changes seen in both the head and midpiece were concurrent or one event preceded the other. Adherence of the sperm midpiece and loss of flagellar beating may indicate the approach of senescence and this data may have been obtained from moribund cells. We have not as yet further characterized this event or its relationship to sperm function; however, as nAChRs are present upon the midpiece of human sperm, we felt this observation was worthy of inclusion in this study.

Homomeric nAChRs heterologously expressed in vitro display a high permeability to Ca2+ ions when compared to other nAChRs, with flux through these channels per se being enough to elevate intracellular calcium levels in vivo (Funke, 2004). However, a number of recent studies have demonstrated that the Ca2+ permeability of α7 nAChRs is much lower in vivo and their activity is regulated in a cell-specific manner (Fayuk and Yakul, 2005). In addition, the rapid deactivation time of nAChR channels (~100 ms for α7 nAChRs; Fayuk and Yakul, 2005) is irreconcilable with the temporal kinetics of the Ca2+ changes we observed in both our fluorimetric and single-cell studies. One of the best characterized functions of neuronal nAChRs has been the post-synaptic propagation of action potentials by NaA/μC2+ flux, leading to membrane depolarization (Ashcroft, 2000). If the current activated through sperm nAChRs is sufficient to depolarize the plasma membrane, recruitment of VOCCs may lead to further Ca2+ influx. Such coupling mechanisms have been described in other cell types (Furubach et al., 2005). Recruitment of VOCCs following membrane depolarization during AR initiation by ZP is a widely accepted model (Florman et al., 1998; Kirkman-Brown et al., 2002). A role for α7 nAChR signalling in the pathways activated during ZP-initiated AR has been suggested (Bray et al., 2002a; Son and Meizel, 2003).

Human sperm α7 nAChR subunits, most likely assembled as homomeric α7 nAChRs are localized to the neck and post-acrosomal regions of the sperm head (Kumar and Meizel, 2005); the VOCCs responsible for the Ca2+ wave associated with the onset of AR are also localized to post-acrosomal region (Goodwin et al., 1997). Sperm α7 nAChRs may share identity with the non-specific cation channels responsible in part for membrane depolarization during AR-initiation by ZP and progesterone (Foresta et al., 1993; Florman et al., 1998). The portion of the change in sperm [Ca2+]i initiated by ACh and inhibited by MLA and α-BTX may be due to the additive influx of Ca2+ through both nAChRs and VOCCs. Although the requirement for extracellular Na+ in the ACh initiated AR is suggestive of a membrane depolarization component in this pathway, further experiments using membrane potential dyes and calcium channel blockers need to be undertaken to further resolve the sequence of events associated with the observed Ca2+ rise.

Activation of sperm α7 nAChRs alone by ACh or choline is enough to trigger AR in our in vitro culture system. Our previous work has shown that the α7 nAChR is essential to the mouse AR initiated by mouse ZP (Meizel and Son, 2005) and the human AR initiated by human recombinant ZP3 (Bray et al., 2002b). However, it is important to stress that we do not believe nAChRs are the sole arbiters of the AR in vivo. This in vitro study serves to demonstrate nAChR-signalling pathways exists in sperm which potentially contributes to the ionotropic regulation of sperm function in vivo. Two other sperm neurotransmitter receptor/ion channels are also involved in the AR: the glycine receptor/chloride channel in the ZP-initiated but not the progesterone-initiated AR (Melendrez and Meizel, 1995; Sato et al., 2000; Bray et al., 2002a) and the GABA<sub>A</sub> receptor/chloride channel (Wistrum and Meizel, 1993;
Shi and Roldan, 1995) in the progesterone-initiated but not the ZP-initiated AR. It has been speculated that these neurotransmitter receptor/ion channels play roles in membrane potential changes associated with the onset of the AR in vivo (Meizel et al., 1997; Bray et al., 2002a). The ZP-initiated AR, requires activation of both α7 nAChR and glycine receptor (Meizel, 2004). Their compound and simultaneous opening may generate sufficient ionic fluxes to bring about the membrane potential changes that herald the onset of the AR.

The concentration of ACh used in this study and the mode in which it was presented to sperm is unlikely to be representative of how sperm encounter endogenous cholinergic signals within the female reproductive tract. ACh can be synthesized by the enzymes choline acetyltransferase (ChAT) and carnitine acetyltransferase. ACh synthesis, ChAT, and carnitine acetyltransferase have been reported in human sperm (Goodman et al., 1984; Ibáñez et al., 1991). Within the female reproductive tract ACh, secretion has been detected from cells of the vaginal mucosa, granulosa cells of the cumulus oophorus and the ovary (Mayerhofer et al., 2003; Wessler et al., 2003). ACh generated by sperm or the tissues of the female reproductive tract may influence sperm behaviour in a paracrine or autocrine manner, potentially acting to provide low level tonic stimulation of cholinergic receptors on sperm. Sperm do, however, encounter levels of choline equivalent to those used in this study within the female reproductive tract; the mean concentration of choline in human cervical mucus has been reported to be 5.25 mM (Sahrburcher et al., 2002). The ability of cervical mucus to increase hyperactivated motility (Zhu et al., 1992) might be due in part to choline acting through α7 nAChRs.

The distinct regionalization of different nAChR subtypes on human sperm suggests cholinergic signalling may influence additional aspects of sperm function other than that described in this study. Ca2+ signalling plays a role in many aspects of sperm function, including motility, capacitation, hyperactivation and AR (Breitbart et al., 2002; Jaiswal and Eisenbach, 2002; Suarez and Ho, 2003). Segregation of the complex Ca2+ signals associated with these phenomena is achieved in part by compartmentalization and the close spatial anchoring of downstream-signalling elements to sources of Ca2+ flux. It was previously reported that nanomolar and low micromolar concentrations of ACh stimulate sperm motility through nAChRs in a number of species, including human (Dwivedi and Long, 1989). Recently, we demonstrated that mice sperm express α7 nAChRs on their midpiece and flagella and that mouse deficient in the α7 nAChR produce sperm with impaired motility (Bray et al., 2005). The influence of nAChRs upon these diverse aspects of sperm function would depend on their functional characteristics, distribution on the plasma membrane and the subcellular compartments into which they exert their ionotropic effect.

Acknowledgements

The authors thank Dr J. Kirkman-Brown (University of Birmingham, UK) for advice on single-cell imaging and Alex Bray for help with the Openlab automation. Supported by NIH grant HD33688 to SM and a Lalar Foundation Fellowship to CB.

References


Meizel S and Son J-H (2005) Studies of sperm from mutant mice suggesting that two neurotransmitter receptors are important to the zona pellucida-initiated acrosome reaction. Mol Reprod Dev 72,250–258.


Submitted on June 7, 2005; accepted on November 11, 2005