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Involvement of Protein Kinase A and A Kinase Anchoring Protein in the Progesterone-Initiated Human Sperm Acrosome Reaction[1]

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ABSTRACT

The signal transduction pathways involved in the progesterone (P_4)-initiated mammalian sperm acrosome reaction (AR) are not fully understood. To investigate the role of the protein kinase A (PKA) pathway in the P_4-initiated AR, we probed this pathway by pretreating capacitated human sperm with reagents designed to either inhibit PKA activation or disrupt PKA/A kinase anchoring protein (AKAP) interactions. Preincubation with the stearated (membrane permeable) PKA inhibitor, PKI α 5-24 (S-PKI α 5-24), significantly inhibited the P_4-initiated AR at 10 μM as compared to stearated control peptide. In contrast, preincubation with 100 μM nonstearated PKI α 5-24 did not significantly inhibit versus solvent control. Preincubation with the PKA inhibitor Rp-8-Br-cAMP at 500 μM and 150 μM significantly inhibited the P_4-initiated AR versus 8-Br-cAMP and versus solvent. Preincubation with the anchoring inhibitory peptide S-Ht-31 significantly stimulated the P_4-initiated AR at 10, 3, and 1 μM versus inactive control peptide. The stimulation of the P_4-initiated AR by 3 μM S-Ht31 was significantly inhibited by the addition of 30 μM S-PKI α 5-24 prior to the addition of S-Ht31. Preincubation with S-PKI α 5-24 (30 μM) partially inhibited the ionomycin (50 μM)-initiated AR. A role for PKA in the P_4-initiated AR may exist both upstream and downstream of Ca^{2+} entry. Our studies present the first evidence for the participation of PKA in the P_4-initiated AR and also suggest that AKAPs are involved in the PKA-mediated events.

INTRODUCTION

The sperm acrosome reaction (AR) is a modified exocytotic event involving the acrosome, a large secretory granule-like organelle in the sperm head, and the overlying sperm plasma membrane [1]. The AR is required for penetration of the zona pellucida (ZP), an extracellular, glycoprotein egg envelope, and for sperm-egg plasma membrane fusion in eutherian mammals [2, 3]. The ZP via its glycoprotein ZP3 can initiate the AR in vitro and is generally considered the major or sole in vivo AR initiator [3, 4]. Progesterone (P_4) can also initiate the AR in vitro and is another possible physiological AR initiator given that it is probably secreted at high concentrations by the cumulus oophorus that surrounds the ovulated egg (and is still present at fertilization in the human and in most eutherian mammals) [5–7]. Despite a great deal of research, the signal transduction pathways involved in the P_4-initiated mammalian sperm AR are not yet fully understood.

The classic intracellular P_4 receptor does not exist in sperm, and P_4 appears to initiate the human AR via interaction with one or more types of plasma membrane receptors [8–11]. Interestingly, Cl^--deficient medium and/or antagonists of the gamma aminobutyric $acid_A$ ($GABA_A$) receptor/Cl^- channel prevent the P_4-initiated AR in several mammalian species including the human, suggesting the participation of a neuronal-like $GABA_A$ receptor/Cl^- channel (reviewed in [12]).

Ca^{2+} is a key second messenger in the P_4-initiated AR that has shown to be dependent on extracellular Ca^{2+} (reviewed in [13]). Unlike the ZP-initiated AR in which T-type Ca^{2+} channels appear to be involved [14], it is not clear whether or not T-type Ca^{2+} channels play a role in the P_4-initiated AR [13].

Protein phosphorylation is also important to the P_4-initiated AR. On the basis of studies utilizing specific PKC inhibitors, there is consensus that PKC is involved in the P_4-initiated AR [15, 16]. The involvement of a protein tyrosine kinase (PTK) in the P_4-initiated human AR has been strongly suggested by the results of several studies [17–19].

Inhibitor studies have suggested a role for the cAMP-dependent protein kinase (PKA) in the ZP-initiated AR [20], but the evidence suggesting the involvement of PKA in the P_4-initiated AR is even less direct. Preincubation with the PKA inhibitor KT5720 was shown to completely inhibit the follicular fluid-initiated AR of human sperm [21]. Since P_4 is the major AR-initiating activity in follicular fluid [6], the follicular fluid-initiated AR activity completely inhibited by KT5720 must have included that due to P_4.

Evidence now exists for the presence not only of PKA but also of A kinase anchoring protein (AKAP) in the mammalian sperm head [22–24]. AKAPs appear to serve multiple roles in cells. At a minimum, AKAPs act to tether the regulatory subunit of PKA to subcellular sites in such a way as to functionally compartmentalize the kinase [25]. In addition, some AKAPs may coordinate activities of other signal transduction molecules such as calmodulin [26, 27], calcineurin [28], and protein kinase C (PKC) [29] via the binding of these molecules to the AKAP. It was the aim of this study, therefore, to examine the involvement of PKA and AKAPs in the P_4-initiated AR using newly available inhibitors for both PKA and AKAPs.

MATERIALS AND METHODS

Materials

Salts and metabolites used for incubation and wash media were of reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA), Irvine Scientific (Irvine, CA), Mallinckrodt (Paris, KY), or Sigma (St. Louis, MO). Percoll was purchased from Sigma. The water uti-

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lized throughout these studies was deionized water further purified through a NANA-pure system (Barnstead/Thermolyne, Dubuque, IA) to greater than 18 MΩ-cm. Fraction V bovine albumin (Pentax #81-066, lot 59) was purchased from Miles (Kankakee, IL). The PKA inhibitor Rp-8-Br-cAMP and the cAMP analogue 8-Br-cAMP were purchased from Alexis (La Jolla, CA). Dr. S. Goueli from Promega Corporation (Madison, WI) generously provided the PKA inhibitors stearated PKI α 5-24 (S-PKI) and nonstearated PKI α 5-24; the stearated PKI control peptide, stearated calcineurin-binding peptide (S-CaNBP); and the stearated AKAP peptide Ht31 (S-Ht31) and its inactive stearated control Ht31-P (S-Ht-31-P). The peptides had the following sequences: S-Ht31, N-stearate-DLIEEAASRIVDAVIEQVKAAGAY; S-Ht31-P, N-stearate-DLIEEAASRPVDAVPEQVKAAGAY; S-CaNBP, N-stearate-SLKRLVTRRKRSESSKQQKPLE; PKI α 5-24, TYADFIASGRTGRRNAI, and S-PKI α 5-24, N-stearate-TYADFIASGRTGRRNAI. P_4 (4-pregnen-3, 20-dione) and ionomycin were purchased from Calbiochem (San Diego, CA). The reagents used for fixation and staining included formaldehyde from Sigma, fluorescein isothiocyanate-concanavalin A (FITC-ConA) lectin from EY Labs (San Mateo, CA), and Vectashield from Vector Labs (South San Francisco, CA). Viability dyes SYBR-14 and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). Both 15-ml polypropylene centrifuge tubes from Applied Scientific (Hayward, CA) and 1.5-ml siliconized, polypropylene microcentrifuge tubes from United Scientific Products (San Leandro, CA) were utilized in the studies.

Sperm Preparation and Capacitation

The human semen used in this study was produced by masturbation from a pool of five healthy donors. Donors were included in the study if the sperm in their semen had a motility greater than 70% and a concentration greater than 100×10^6/ml. After incubation at 37°C for 30 min, the semen was centrifuged over a discontinuous (40%/80%) Percoll gradient for 25 min at $500 \times g$. The supernatant was discarded and the sperm pellet was subsequently washed twice at $300 \times g$ for 12 min in wash medium. The wash medium consisted of a modified Tyrode's buffer with 25 mM bicarbonate containing lactate, pyruvate, glucose, streptomycin, penicillin G, and 3 mg/ml albumin [30].

In order for sperm to respond to AR initiators in vitro or in vivo they must first undergo molecular changes collectively called capacitation [3, 31, 32]. After the last centrifugation, the washed sperm were resuspended in capacitation medium (identical to wash medium except that it contained 26 mg/ml BSA [30]), counted, examined for motility (see below), brought to a concentration of 6×10^6/ml, and aliquoted at 200 μl per tube in either 15-ml centrifuge tubes or 1.5-ml microcentrifuge tubes. If the sperm had both motility greater than 95% and vigorous forward motility, they were then capacitated by incubation for 22–24 h in a humidified 5% CO_2 and 95% air atmosphere incubator [33].

Preincubation of Capacitated Sperm with Inhibitors, Addition of AR Initiators, and AR Determination

Three different protocols were used with PKA inhibitors and AKAP peptides. The most generally used protocol involved preincubation of sperm with the stearated peptides and their controls for 5 min prior to the addition of P_4 (3.18 μM), ionomycin (50 μM, a concentration of ionomycin that was determined in preliminary experiments to maximize the AR and minimize cell death in the presence of the high BSA concentration, 26 mg/ml, in the capacitation medium), or solvent control (0.1% dimethyl sulfoxide, DMSO). A second protocol was used with Rp-8-Br-cAMP because of the high affinity of this compound for albumin. In this protocol the capacitated sperm were pooled, spun at $300 \times g$ for 12 min, recounted, resuspended in wash medium (3 mg/ml BSA), realiquoted at 200 μl per tube, and incubated for 30 min in the incubator. After the 30-min incubation, sperm were preincubated with Rp-8-Br-cAMP or its controls for 5 min prior to addition of P_4 (3.18 μM) or DMSO. The third protocol consisted of sequential preincubations of sperm with two different sets of stearated peptides or their controls. In this protocol, the first set of stearated peptides was added 5 min prior to the addition of the second set, which was added 5 min prior to the final addition of P_4 or DMSO.

In all protocols, P_4, ionomycin, or DMSO was added to the sperm for 5 min, at which point the reaction was stopped with the addition of 600 μl of 4% formaldehyde in PBS (pH 7.4). After a minimum of 1 h, the sperm were spun at $500 \times g$ for 15 min, the supernatant was removed, and the pellet was resuspended with 200 μl of fresh 4% formaldehyde. The sperm were stained with FITC-ConA, an inner acrosomal membrane-binding lectin, and a minimum of 200 sperm per treatment were counted [34]. Sperm were considered acrosome reacted when fluorescence in the anterior head had either a speckled (early or incomplete reaction) or fully fluorescent caplike shape (completed reactions). All PKA inhibitors, AKAP peptides, and controls were dissolved in H_2O.

Motility Determination

Since the PKA inhibitors, AKAP peptides, and ionomycin used are known to affect sperm motility, quality, and in some cases viability [30, 35], it was important to monitor the motility, quality, and viability of key samples in all experiments. For logistical reasons it was not possible to monitor all these parameters in the tubes used for AR assessment. As a result, a separate set of tubes was used for determining motility, quality, and viability. The protocols for these determinations were identical to those discussed above except that 5 min after P_4, ionomycin, or solvent addition, the viability dyes SYBR-14 and PI were added in accordance with the directions provided by Molecular Probes in the LIVE/DEAD sperm viability kit. Subsequently, the percentages of motile and viable sperm and the sperm quality were assessed using 600× magnification under visible and UV illumination. Sperm quality was assessed using the following subjective scale: 0 (not moving at all) and 1 (''twitching,'' nonprogressive motion) to 4 (vigorous forward motility, as prior to incubation). At least 100 and 200 sperm, respectively, were examined in motility and viability determinations.

Statistics

Two different statistical methods were used. For examination of the statistical relationship between treatment and controls using raw AR data (e.g., the number of acrosome-reacted sperm counted vs. the total number of sperm counted), the GENMOD linear regression protocol was utilized (Statistical Analysis System Ver. 6.12, Cary, NC; 1996). For examination of the statistical relationship between treat-

ment and controls using percentage inhibition or stimulation data (which were derived from the raw data), Duncan's new multiple range test was utilized (Statistical Analysis System Ver. 6.12). *P* values less than 0.05 were considered significant.

RESULTS

S-PKI, PKI, and the P_4-Initiated AR

PKI α is a potent and specific inhibitor of PKA, and the majority of its active site is contained in the N-terminal residues 6–22 [36, 37]. Whereas the K_i of the full-length molecule is approximately 0.2 nM, the PKI α peptide used in this study, PKI α 5-24, is approximately 10-fold less potent [38]. On the basis of the sequence of PKI α and PKI α 5-24, and the reported isoelectric point for PKI of 4.2, it is probable that these molecules would be highly charged at physiological pH [38, 39]. Therefore, the likelihood that these molecules would be able to penetrate into an intact sperm is theoretically small. The addition of a stearate moiety to the N-terminal of the peptide was designed to facilitate the entry of PKI α 5-24 into the sperm [35].

S-PKI α 5-24 did inhibit the P_4-initiated AR in a concentration-dependent manner (Fig. 1A). In these experiments, S-CaNBP, an AKAP79-based peptide that binds calcineurin (protein phosphatase 2B) and is known to have no inhibitory effect on PKA, was used as a stearated peptide control in addition to H_2O. Expressed as the percentage inhibition of the P_4-initiated AR versus that with DMSO, pretreatment with S-PKI α 5-24 at 10 μM caused a 66.1 ± 8.0% ($P < 0.0001$) inhibition of the P_4-initiated AR as compared to the 10 μM S-CaNBP control and a 71.9 ± 7.3% ($P < 0.0001$) inhibition as compared to the H_2O control. Significant inhibition was not observed at 3 or 1 μM S-PKI α 5-24. Despite the clear effect on the P_4-initiated AR at 10 μM, neither S-PKI α 5-24 nor its controls had an effect on percentage motility or viability at that concentration or at the lower concentrations tested (Fig. 1B).

In order to compare the effectiveness of stearated versus nonstearated PKI α 5-24, and to examine the effects of a higher concentration of S-PKI α 5-24 on quality and percentage motility and viability, we compared the effect of 100 μM S-PKI α 5-24 versus 100 μM PKI α 5-24 on the P_4-initiated AR. As seen in Figure 2A, 100 μM S-PKI α 5-24 caused a dramatic inhibition of the P_4-initiated AR versus 100 μM S-CaNBP. In contrast, 100 μM PKI α 5-24 did not significantly inhibit versus H_2O. In terms of percentage inhibition of the P_4-initiated AR versus DMSO, S-PKI α 5-24 and PKI α 5-24 sperm pretreatment at 100 μM resulted in a 76.9 ± 6.8% ($P < 0.0004$) inhibition by S-PKI α 5-24 versus 100 μM S-CaNBP, in contrast to a 7.3 ± 5.4% ($P > 0.25$) inhibition with PKI α 5-24 versus H_2O. Clearly, the presence of the stearate moiety had a profound impact on the inhibition of the P_4-initiated AR. Interestingly, although the higher concentrations of S-PKI α 5-24 and PKI α 5-24 had no effect on percentage viability, percentage motility and quality were significantly reduced with S-PKI α 5-24 (Fig. 2B).

Rp-8-Br-cAMP and the P_4-Initiated AR

Rp-8-Br-cAMP is a cell-permeable competitive antagonist of cAMP that inhibits PKA activation [40, 41]. In contrast to 8-Br-cAMP, a cell permeable cAMP agonist that had no effect on the P_4-initiated AR, Rp-8-Br-cAMP significantly inhibited the AR (Fig. 3A). In addition, 8-Br-cAMP had no stimulatory effect on the AR when added 5 min prior to DMSO. Expressed as a percentage inhibition of the P_4-initiated AR versus DMSO, Rp-8-Br-cAMP at 500 μM and 150 μM caused a 78.9 ± 2.9% ($P < 0.0001$) and 42.2 ± 12.4% ($P < 0.0023$) inhibition of the P_4-initiated AR, respectively, versus the 8-Br-cAMP control. When compared to the H_2O control, 500 μM Rp-8-Br-cAMP inhibited the P_4-initiated AR by 77.4 ± 2.2% ($P < 0.0002$). There was no significant AR inhibition at 150 μM Rp-8-Br-cAMP compared to the H_2O control or at 50 μM Rp-8-Br-cAMP compared to the 8-Br-cAMP or H_2O control. Neither Rp-8-Br-cAMP nor 8-Br-cAMP had significant effects on percentage viability and quality, but Rp-8-Br-cAMP did significantly decrease the percentage motility as compared to the H_2O control (Fig. 3B).

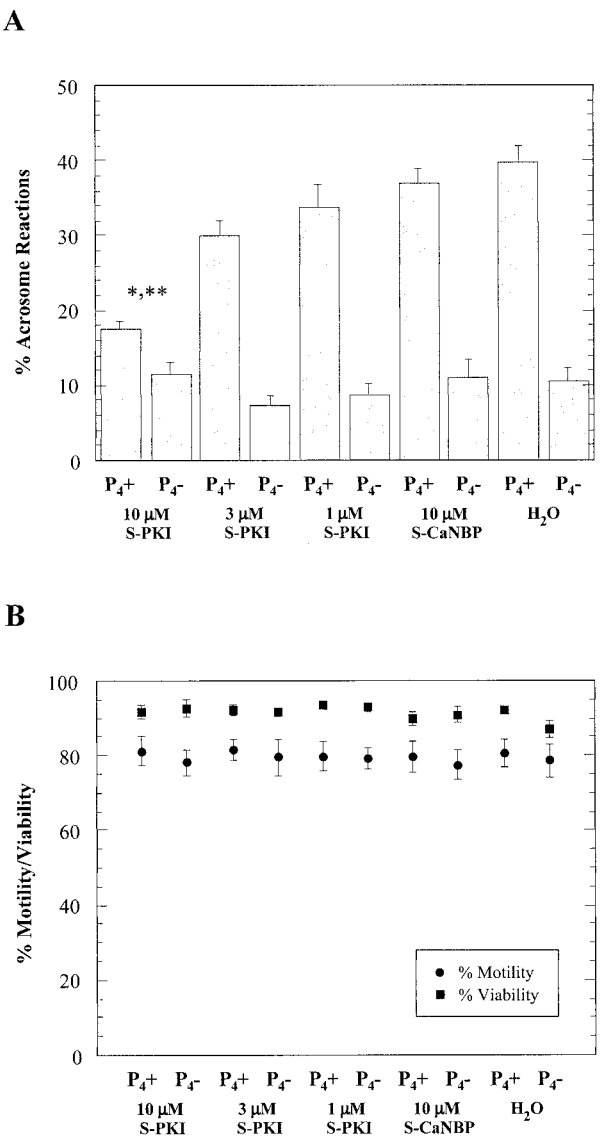

FIG. 1. Effect of S-PKI α 5-24 and P_4 on capacitated human sperm. Aliquots of sperm (200 μl, 6×10^6/ml) were preincubated with S-PKI α 5-24, control peptide S-CaNBP, or H_2O for 5 min prior to the addition of P_4 (3.18 μM) or solvent (0.1% DMSO). P_4 or solvent control H_2O samples were then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility and percentage viability. *Inhibitory effect of S-PKI α 5-24 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of S-CaNBP control (P_4+ and P_4−). **Inhibitory effect of S-PKI α 5-24 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of H_2O control (P_4+ and P_4−).

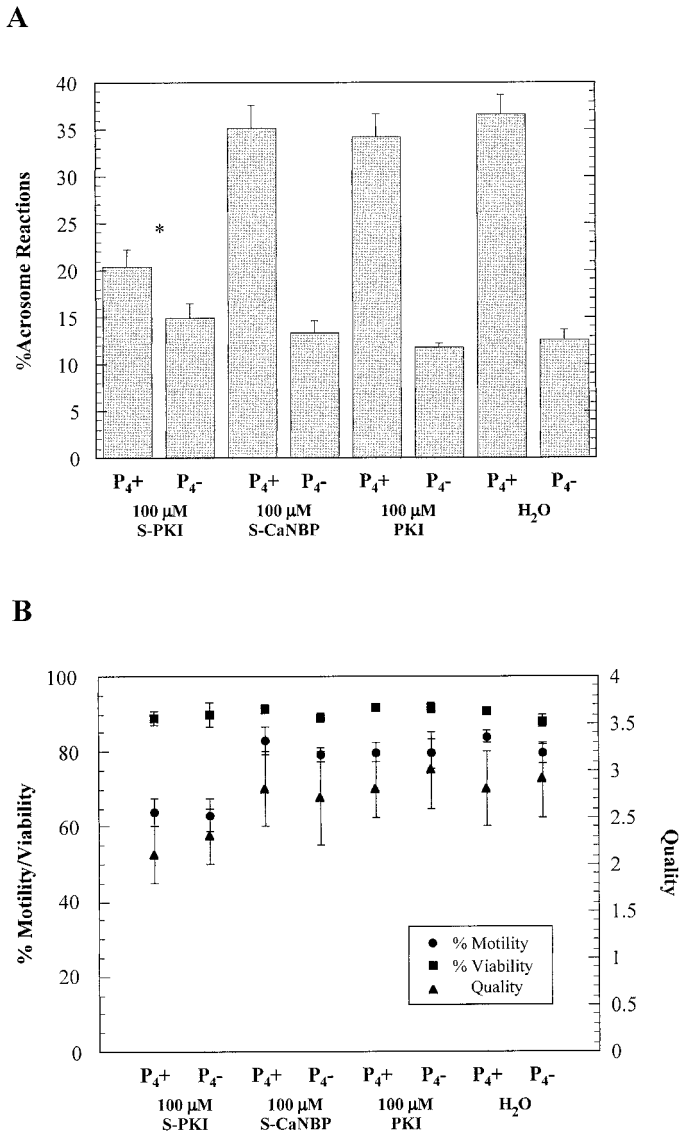

FIG. 2. Effect of S-PKI α 5-24 or PKI α 5-24 and P_4 on capacitated human sperm. Aliquots of sperm (200 µl, 6×10^6/ml) were preincubated with 100 µM S-PKI α 5-24, control peptide S-CaNBP, PKI α 5-24, or H_2O for 5 min prior to the addition of P_4 (3.18 µM) or solvent (0.1% DMSO). P_4 or solvent control samples were then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality. *Inhibitory effect of S-PKI α 5-24 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of S-CaNBP control (P_4+ and P_4−).

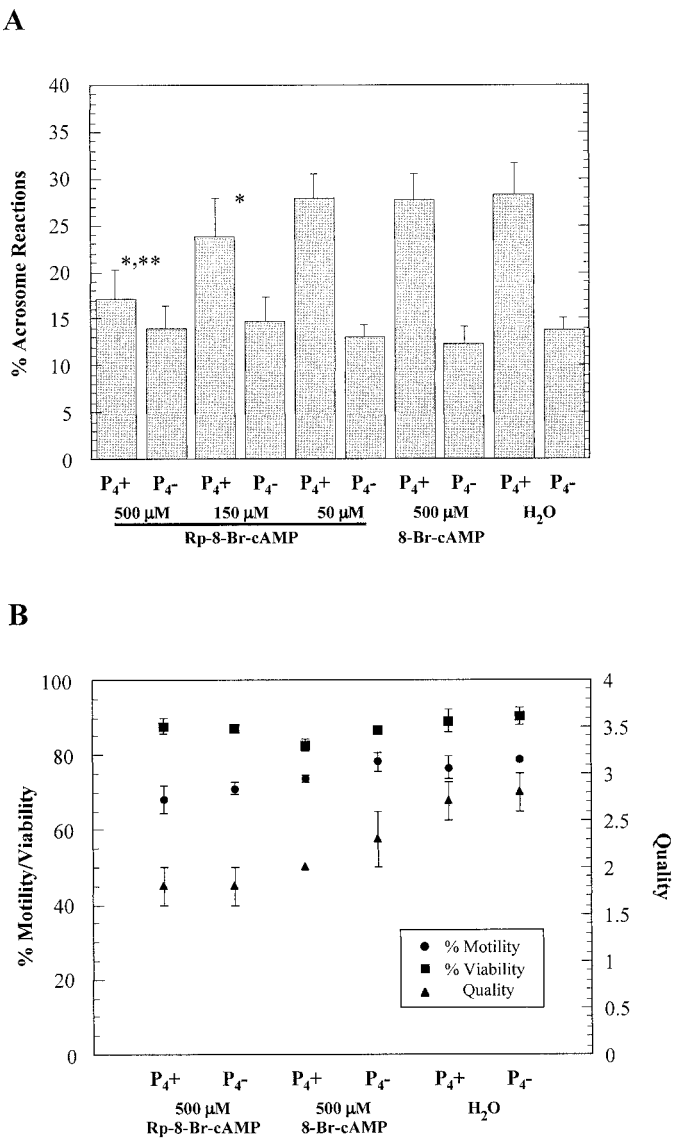

FIG. 3. Effect of Rp-8-Br-cAMP and P_4 on capacitated human sperm. Capacitated sperm were pooled, spun at $300 \times g$ for 12 min, recounted, resuspended in wash medium (3 mg/ml BSA), realiquoted at 200 µl/tube (6×10^6 sperm/ml), and allowed to equilibrate for 30 min in the incubator. Aliquots were then preincubated with Rp-8-Br-cAMP, 8-Br-cAMP, or H_2O for 5 min prior to the addition of P_4 (3.18 µM) or solvent (0.1% DMSO). P_4 or solvent control samples were then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality. *Inhibitory effect of Rp-8-Br-cAMP (P_4+ and P_4−) significantly different ($P < 0.05$) from that of 8-Br-cAMP control (P_4+ and P_4−). **Inhibitory effect of Rp-8-Br-cAMP significantly different ($P < 0.05$) from that of H_2O control (P_4+ and P_4−).

S-Ht31 and the P_4-Initiated AR

Ht31 is an AKAP that has been cloned and sequenced from a human thyroid cDNA library [42]. The domain in Ht31 that interacts with regulatory subunit II (RII) is an amphipathic helix-binding motif [42, 43]. Ht-31 peptides based on this motif have been shown to disrupt the binding of several different AKAPs to PKA [44, 45]. Stearated Ht-31 is a cell-permeable version of this peptide. In a previous study, S-Ht31 was shown to powerfully inhibit the motility of uncapacitated sperm from many species [35]. We were interested in discovering how this peptide would affect the P_4-initiated AR as well as the quality and percentage motility and viability of capacitated and uncapacitated human sperm. Unexpectedly, S-Ht31 caused a significant stimulation of the P_4-initiated AR in capacitated sperm (Fig. 4A). When expressed as percentage stimulation of the P_4-initiated AR versus that with DMSO, S-Ht-31 at 10 µM, 3 µM, and 1 µM S-Ht-31 significantly stimulated the AR by 83.2 ± 12.4% ($P < 0.0004$), 83.4 ± 28.4% ($P < 0.0002$), and 57.9 ± 19.3% ($P < 0.0038$), respectively, versus inactive control peptide (10 µM S-Ht-31-P) and 64.1 ± 12.0% ($P < 0.0004$), 59.9 ± 15.6% ($P < 0.0002$), and 45.2 ± 10.1% ($P < 0.0038$), respectively, versus H_2O. There was no significant inhibition by 0.3 µM S-Ht-31. Interestingly, in the absence of P_4, 10 µM S-Ht31 stimulated the AR 21.2 percentage points above its control (10 µM S-Ht31-P). It is

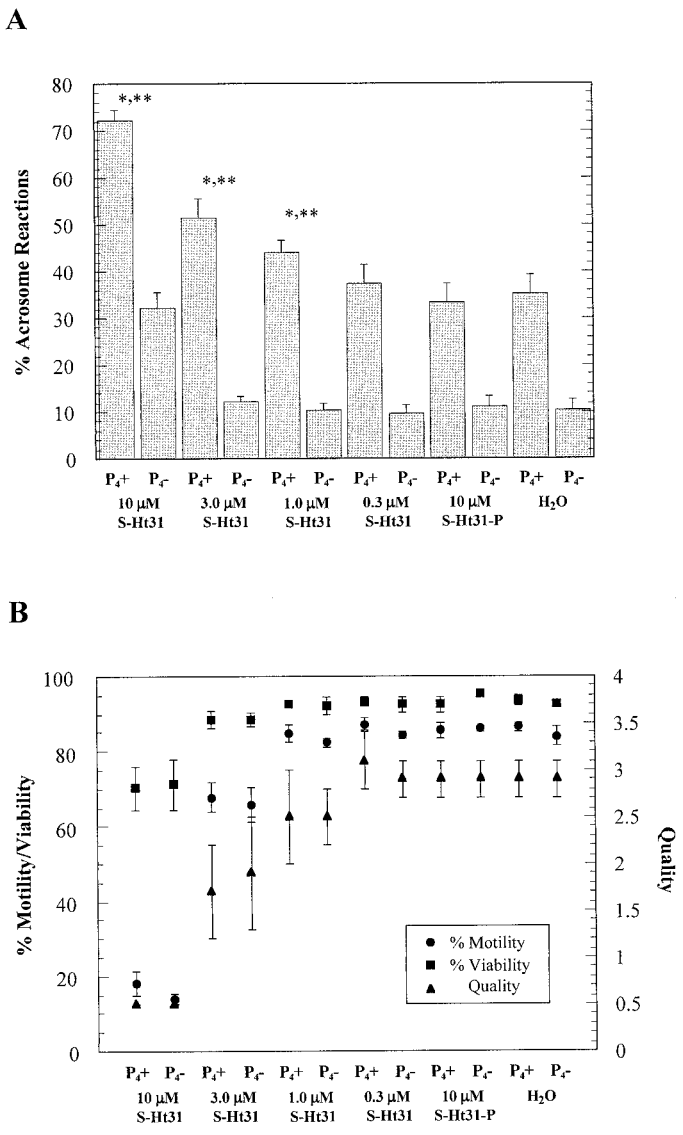

FIG. 4. Effect of S-Ht31 and P_4 on capacitated human sperm. Aliquots of sperm (200 μl, 6×10^6/ml) were preincubated with S-Ht31, inhibitory control peptide S-Ht31-P, or H_2O for 5 min prior to the addition of P_4 (3.18 μM) or solvent (0.1% DMSO). P_4 or solvent control was then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality. *Stimulatory effect of S-Ht31 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of S-Ht31-P control (P_4+ and P_4−). **Stimulatory effect of S-Ht-31 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of H_2O control (P_4+ and P_4−).

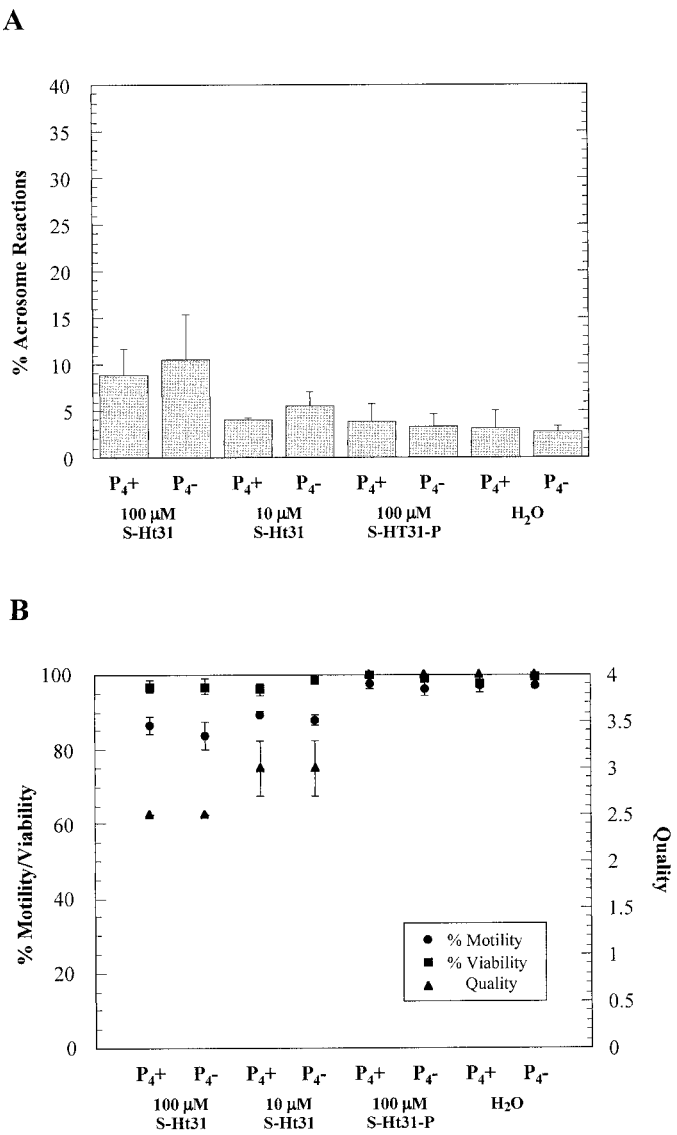

FIG. 5. Effect of S-Ht31 and P_4 on uncapacitated human sperm. Aliquots of sperm (200 μl, 6×10^6/ml) were preincubated with S-Ht31, inhibitory control peptide S-Ht31-P, or H_2O for 5 min prior to the addition of P_4 (3.18 μM) or solvent (0.1% DMSO). P_4 or solvent control was then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality.

important to note that at this same concentration, S-Ht31 caused a drop in percentage viability of 24.4 percentage points as compared to S-Ht31-P. In capacitated sperm, S-Ht31 dramatically reduced percentage motility and quality (Fig. 4B).

The effect of high concentrations of S-Ht-31 on the P_4-initiated AR in uncapacitated sperm can be seen in Figure 5A. The nonsignificant effect of S-Ht31 on the P_4-initiated AR at these concentrations is in clear contrast to the effect of S-Ht31 on capacitated sperm. S-Ht31 had little effect on percentage motility and viability in uncapacitated sperm, but it did cause a perceptible and significant reduction in the quality of the motility (Fig. 5B).

S-PKI and S-Ht31 and the P_4-Initiated AR

In order to discover whether the stimulation by S-Ht31 of the P_4-initiated AR was at least in part due to the actions of PKA, 30 μM S-PKI α 5-24 or peptide control was added to capacitated sperm 5 min prior to the addition of 3 μM S-Ht31 or its inactive control S-Ht31-P. Figure 6A shows the expected inhibition by PKI α 5-24 of the P_4-initiated AR, in addition to the significant inhibition by PKI α 5-24 of the S-Ht31-stimulated P_4-initiated AR ($n = 5$). Expressed as percentage inhibition of the P_4 alone- or S-Ht31 plus P_4-initiated AR versus DMSO, S-PKI α 5-24 inhibited the former stimulation by 82.3 ± 5.9% ($P < 0.002$) and the latter stimulation by 65.4 ± 3.9% ($P < 0.001$). As expected, the percentages motility and quality were significantly reduced by treatments with S-Ht31 (Fig. 6B). In contrast, the percentage viability was unaffected by all of the treatments. In separate experiments, S-PKI α 5-24 had

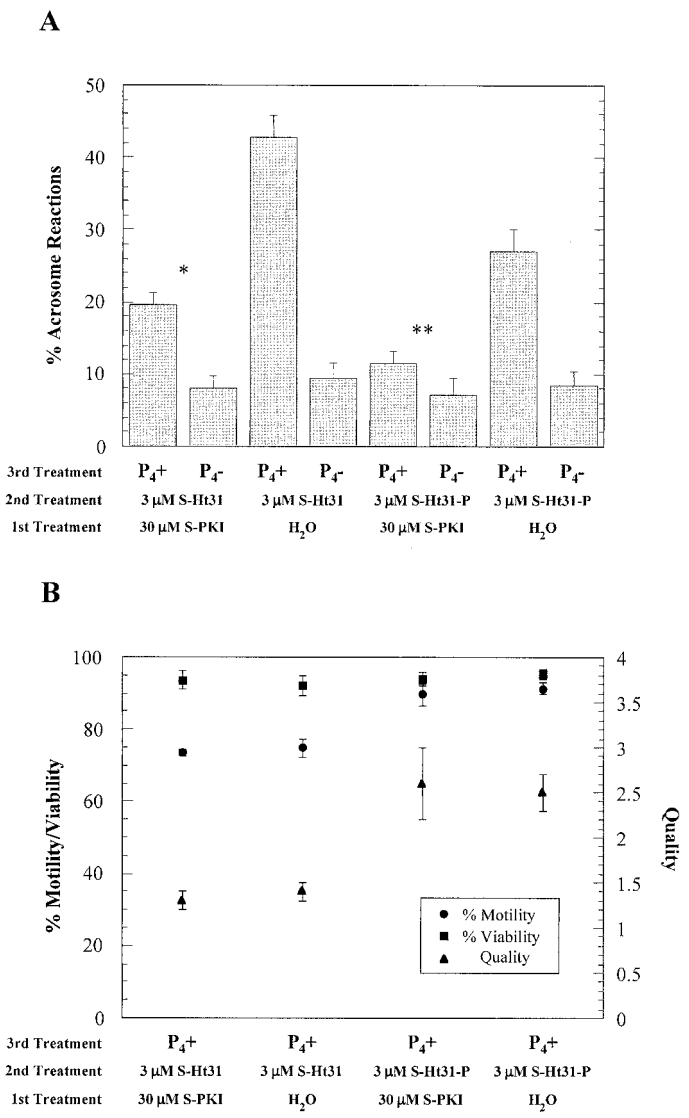

FIG. 6. Effect of S-PKI α 5-24 and S-Ht31 on capacitated human sperm stimulated with P_4. Aliquots of sperm (200 μl, 6 × 10⁶/ml) were preincubated with S-PKI α 5-24 or control peptide S-CaNBP prior to the addition of S-Ht31 or its control peptide S-Ht31-P. Five minutes after the addition of S-Ht31 or control, P_4 (3.18 μM) or solvent (0.1% DMSO) was added. P_4 or solvent control was then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality. *S-PKI α 5-24 and S-Ht31 (P_4+ and P_4−) treatment significantly different ($P < 0.05$) from treatment with H_2O and S-Ht31 (P_4+ and P_4−). **S-PKI α 5-24 and S-Ht31-P (P_4+ and P_4−) treatment significantly different ($P < 0.05$) from treatment with H_2O and S-Ht31-P (P_4+ and P_4−).

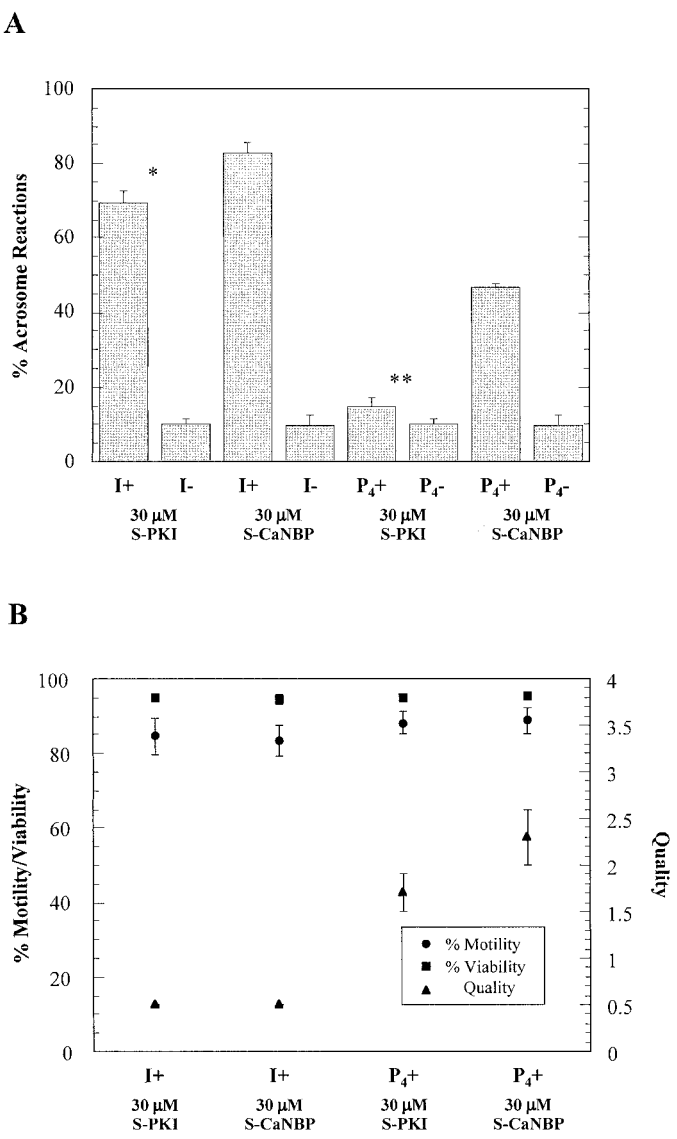

FIG. 7. Effect of S-PKI α 5-24 and ionomycin, P_4, or solvent on capacitated human sperm. Aliquots (200 μl) of sperm (6 × 10⁶/ml) were preincubated with S-PKI α 5-24 or inhibitory control peptide S-CaNBP for 5 min prior to the addition of 50 μM ionomycin (I), P_4 (3.18 μM), or solvent (0.1% DMSO). Ionomycin, P_4, or solvent control samples were then incubated for 5 min more prior to motility and viability assays or fixation and AR assay. The data represent mean ± SEM of **A**) percentage ARs or **B**) percentage motility, percentage viability, or quality. *Inhibitory effect of S-PKI α 5-24 (I+ and I−) significantly different ($P < 0.05$) from that of S-CaNBP control (I+ and I−). **Inhibitory effect of S-PKI α 5-24 (P_4+ and P_4−) significantly different ($P < 0.05$) from that of S-CaNBP control (P_4+ and P_4−).

no effect on the S-Ht31 stimulation of the P_4-initiated AR when added at the same time (data not shown).

S-PKI and the Ionomycin-Initiated AR

The data shown in Figure 7A indicate that S-PKI α 5-24 was able to inhibit the AR initiated by the Ca^{2+} ionophore ionomycin (18.5 ± 2.0% vs. the S-CaNBP control, $P < 0.037$). Ionomycin dramatically reduced sperm quality but had no effect on the percentage motility and viability (Fig. 7B).

DISCUSSION

Involvement of PKA in the P_4-Initiated AR

The evidence supporting the participation of a specific signal transduction pathway or signal transduction pathway component in a biological response is often multifactorial. In the case of the P_4-initiated AR, several lines of evidence point to the participation of PKA. First, the enzyme is present in mammalian sperm and has the proper cellular location to be involved in the AR. Several isoforms of the PKA regulatory subunit—RIα, RIβ, and RIIα—have been demonstrated by Western blotting of human sperm preparations [24]. Immunocytochemical studies of bovine sperm

revealed that the RI subunits (α and β) were found primarily in the acrosomal region, whereas the RII (α) subunit was localized exclusively to the tail [24]. Other studies have detected RI subunits (subclass not identified) in the anterior region of the mouse sperm head and have shown that PKA activity increases during capacitation [23]. Secondly, P_4 has been shown to stimulate a Ca^{2+}-dependent increase in cAMP in human sperm (although it is unclear to what extent the sperm in this study were capacitated) [46]. Thirdly, incubation of human sperm in a medium containing a low concentration of bicarbonate sharply reduced the ability of P_4 to initiate the AR, but this inhibition could be largely reversed with dibutyryl cAMP (dbcAMP) [47]. Given that bicarbonate stimulates mammalian adenylate cyclases and that dbcAMP is a membrane-permeable cAMP analogue (reviewed in [47]), those results suggested a role for cAMP and therefore for PKA in the P_4-initiated AR. Finally, as demonstrated in the present study, highly specific inhibitors of PKA significantly inhibit the P_4-initiated AR.

An obvious limitation in the use of inhibitors is their specificity and sensitivity. Fortunately, we were able to use two specific inhibitors of PKA: PKI and Rp-8-Br-cAMP. PKI α- and PKI α-based peptides have been shown to be both specific and potent [37]. It was interesting to observe in our study that the stearate moiety on PKI α 5-24 was essential to PKI's ability to inhibit the P_4-initiated AR. At 100 μM, PKI α 5-24 had virtually no effect, whereas S-PKI α 5-24 dramatically inhibited the P_4-initiated AR. As might be expected, given the established role of PKA in sperm motility [24, 48, 49], the highest concentration of S-PKI α 5-24 (100 μM) also caused a significant inhibition of sperm percentage motility and quality.

Rp-8-Br-cAMP, like PKI α, is a specific inhibitor of PKA [41] but lacks the potency of PKI α. Consequently, Rp-8-Br-cAMP was used at nearly 50 times the levels of S-PKI α 5-24 in order to obtain comparable inhibition of the P_4-initiated AR. As with S-PKI α 5-24, the highest level of Rp-8-Br-cAMP (500 μM) inhibited both the percentage motility and quality of the sperm.

The use of 8-Br-cAMP in our study served two purposes. On one hand we used it as a noninhibitory control for Rp-8-Br-cAMP, but we also employed it to test whether a high level of a potent, cell-permeable cAMP analogue could stimulate the AR in the absence of P_4. In our study, 500 μM 8-Br-cAMP had no ability to stimulate the AR with or without P_4. These results agree with those of Sabeur and Meizel [47], which showed that dbcAMP (0.1 μM to 1 mM) could not, at either a low bicarbonate concentration (1 mM) or at the concentration of bicarbonate used in the present study (25 mM), initiate the human sperm AR in the absence of P_4.

Those and the present results with cAMP analogues and PKI are in contrast to the findings of a previous study by De Jonge and coworkers [50]. In that study, 1 mM 8-Br-cAMP, as well as 1 mM dbcAMP, caused a large stimulation of the AR in capacitated human sperm, and the stimulation of the AR by dbcAMP was inhibitable by 100 μM PKI (from a crude extract of rabbit muscle). It is possible that the cAMP levels in the sperm in the present study were sufficiently high under the capacitation conditions used that the addition of 500 μM 8-Br-cAMP did not further stimulate PKA and was not in itself sufficient to stimulate the AR. The ability of 100 μM PKI to inhibit the AR stimulation by dbcAMP in the study by De Jonge et al. is interesting as it suggests that the sperm utilized in their study were capable of internalizing the highly positively charged full-length rabbit skeletal muscle PKI α isoforms (molecular weight range ~8000 to 11 000 [51]). Perhaps the contrasting results between the past and present studies from this laboratory and the study by De Jonge and coworkers are due to differences between physiologically distinct sperm populations. For example, in the latter studies, sperm were capacitated in the absence of exogenous metabolites.

Finally, in order to test whether PKA has a role in the AR after extracellular Ca^{2+} entry, we examined the ability of S-PKI α 5-24 to inhibit the ionomycin-initiated AR. S-PKI α 5-24 was able to inhibit the ionomycin-initiated AR by only 18.5% as compared to an inhibition of the P_4-initiated AR by 86.8%. This result suggests that PKA may have a more important role prior to the influx of extracellular Ca^{2+} (an upstream effect) than after this influx (a downstream effect).

Involvement of AKAPs in the P_4-Initiated AR

The first AKAPs to be described in somatic cells were found to bind the RII but not the RI subunit [25]. Recently a second type of AKAPs, D-AKAPs, have been shown to bind both RI and RII [52, 53]. The predominant AKAP in the sperm of many species, including humans, is AKAP110, which is found in the acrosomal region and tail of sperm [22]. Two other AKAPs have been found in sperm: AKAP82, localized to the fibrous sheath of the tail [23], and AKAP 84, localized to the mitochondria in the tail [54]. Given that the RI subunit is primarily localized in the head and the RII subunit is exclusively confined to the tail [24], AKAP110 could still play a role in regulating PKA in the sperm head if it were a D-AKAP. Evidence supporting this possibility comes from studies showing that AKAP110 binds the RIα subunit (Carr, unpublished results).

The stearated AKAP peptide used in this study, Ht31, consists of the RII binding motif sequence found in the human thyroid AKAP Ht31. Previous studies with Ht31 peptides have shown that these peptides disrupt the RII-AKAP interaction [42, 43, 45]. Typically, the effects of Ht31 peptides are similar to the effects of PKI on PKA, namely, the diminution of a PKA-mediated response [25]. Surprisingly, in the present work, the actions of S-Ht31 and S-PKI α 5-24 were opposite with respect to the P_4-initiated AR. Preincubation with S-Ht31 caused a concentration-dependent increase in the P_4-initiated AR, in contrast to a concentration-dependent decrease with S-PKI α 5-24 preincubation. The greatest stimulation with S-Ht31 was seen at 10 μM in capacitated sperm, but it was also strong at 3 μM. At 10 μM, however, the sperm percentage viability dropped by 24.4 percentage points. It is possible that the greater stimulation at 10 μM S-Ht31 versus 3 μM S-Ht31 in both the P_4-initiated AR and the solvent control is due to the effect of cell death on the AR as opposed to a specific effect of S-Ht31. The inhibitory effect of S-Ht31 on percentage motility and quality of capacitated sperm is consistent with the previously published results with uncapacitated bovine sperm, but less than expected with uncapacitated human sperm [35].

In order to test whether PKA is involved in the S-Ht31 stimulation of the P_4-initiated AR, we preincubated sperm with S-PKI α 5-24 prior to the addition of S-Ht31. The addition of P_4 with the inactive control peptide S-Ht31-P stimulated the AR 18.5 percentage points above that with

the DMSO control as compared to 33.3 percentage points with S-Ht31. S-PKI α 5-24 inhibits the former stimulation by 15.5% percentage points (a 82.3% inhibition) and the latter stimulation by 23.3 percentage points (a 65.4% inhibition). Since the inhibition by S-PKI α 5-24 of the P_4-initiated AR with S-Ht31 is 50% greater (23.3/15.5) than the inhibition by S-PKI α 5-24 of the P_4-initiated AR with S-Ht31-P, it is likely that a significant portion of the S-Ht31 stimulation of the P_4-initiated AR involves the catalytic subunit of PKA.

Possible Roles of PKA in the P_4-Initiated AR

Given these findings, how might PKA be involved in the P_4-initiated AR? As stated earlier, P_4 induces an increase in cAMP levels that is dependent on extracellular Ca^{2+}. Once activated by cAMP, how is PKA involved in the P_4-initiated-AR? Spungin and Breitbart [55] reported that cAMP could stimulate the release of Ca^{2+} from isolated bovine sperm acrosomes. In addition, this Ca^{2+} release was at least partially inhibitable by the PKA inhibitor H89. Detection of acrosomal inositol 1,4,5-trisphosphate receptors in several species is consistent with the presence of such acrosomal Ca^{2+} stores [56]. Release of intracellular Ca^2 in mammalian sperm, including that of humans, leads to AR initiation, probably by stimulation of Ca^{2+} influx [56–58]. Phosphorylation via PKA might stimulate such Ca^{2+} release from acrosomal stores during the P_4-initiated AR. If such an endoplasmic reticulum-like Ca^{2+}-ATPase Ca^{2+} pump exists in the human sperm acrosome, it may play a role in the P_4-initiated AR, and its phosphorylation via PKA might control its activity.

If both RIα and RIβ are found in the head of human sperm as they are in bovine sperm, then it is possible that two or more populations of PKA are important for the P_4-initiated AR, perhaps with roles both upstream and downstream of Ca^{2+} entry. One such upstream role might be in the activation of the sperm $GABA_A$ receptor/Cl^- channel. In human sperm the $GABA_A$ receptor/Cl^- channel appears to regulate Ca^{2+} entry, since the $GABA_A$ receptor/Cl^- channel blocker, picrotoxin, diminishes the P_4-initiated Ca^{2+} influx and eliminates the P_4-mediated Ca^{2+} wave in the human sperm head [59]. PKA is known to potentiate GABA-mediated currents in neuronal $GABA_A$ receptors [60, 61], and may play as similar role in sperm.

If voltage-operated Ca^{2+} channels are involved in the P_4-initiated AR (reviewed in [13]), PKA might also directly modulate their activity as it does some types of voltage-operated Ca^{2+} channels in several different somatic cells [62].

The fact that the ionomycin-initiated AR was inhibited by S-PKI α, although to a much lesser extent than the P_4-initiated AR, suggests another possible role of PKA in one or more events downstream of Ca^{2+} entry.

Possible Roles of AKAPs in the P_4-Initiated AR

The mechanism by which S-Ht31 stimulates the P_4-initiated AR is not known. Typically, Ht-31 peptides inhibit PKA-regulated processes. This inhibition most likely occurs by displacing the kinase from its substrates (i.e., disruption of PKA compartmentalization) [25]. It is possible in sperm that Ht31 peptides stimulate PKA-regulated processes by allowing the kinase to dissociate away from inhibitory regulators such as protein phosphatases or phosphodiesterases. It is well established that the phosphatase calcineurin is found tethered to AKAP79 [63]. Whether other phosphatases or phosphodiesterases are associated with AKAP 110 remains to be seen.

In contrast to the usual role of AKAPs, which is to bring into association PKA with its substrates, it is conceivable that AKAPs in the sperm head act to prevent the association of PKA with substrates, thus reducing the probability of spontaneous ARs. In this model, AKAPs would sequester PKA until appropriate signal transduction steps take place during the initiation of the AR. In the present work, addition of S-Ht31 to sperm would allow PKA to dissociate from the AKAPs and phosphorylate key substrates involved in the AR. The fact that S-Ht31 cannot itself initiate the AR may be explained by the requirement for the involvement of additional signal transduction pathways (e.g., PKC or PTK) or steps in the P_4-initiated AR.

It also may be possible, given that AKAP84 is localized to the mitochondria in the sperm tail [54], that S-Ht31 causes a disruption in mitochondrial Ca^{2+} regulation leading to an increase in cytosolic Ca^{2+} in the sperm head. This increase in cytosolic Ca^{2+} might then stimulate the AR by increasing cAMP levels, or stimulate the AR by other more direct mechanisms.

In conclusion, the inhibitory effects of the specific PKA inhibitors S-PKI α 5-24 and Rp-8-Br-cAMP strongly suggest, for the first time, the involvement of this kinase in the signal transduction cascade leading to the P_4-initiated AR. S-Ht31, a cell-permeable peptide shown to disrupt RII and AKAP binding, produces a strong stimulation of the P_4-initiated AR. The inhibition of this effect by S-PKI α 5-24 supports a role for PKA in S-Ht31 stimulation. Given these results and the presence of AKAP110 in the sperm head, the participation of AKAPs in the P_4-initiated AR is likely (e.g., via compartmentalization, coordinating regulation, and/or sequestration of PKA). Finally, the ionomycin-initiated AR is only partially inhibited by S-PKI α 5-24, suggesting that PKA may have a role both upstream and downstream of ionophore-mediated Ca^{2+} influx.

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