Protein kinase A inhibition induces EPAC-dependent acrosomal exocytosis in human sperm

Diana Itzhakov1, Yeshayahu Nitzan2, Haim Breitbart1

To interact with the egg, the spermatozoon must undergo several biochemical and motility modifications in the female reproductive tract, collectively called capacitation. Only capacitated sperm can undergo acrosomal exocytosis, near or on the egg, a process that allows the sperm to penetrate and fertilize the egg. In the present study, we investigated the involvement of cyclic adenosine monophosphate (cAMP)-dependent processes on acrosomal exocytosis. Inhibition of protein kinase A (PKA) at the end of capacitation induced acrosomal exocytosis. This process is cAMP-dependent; however, the addition of relatively high concentration of the membrane-permeable &-bromo-cAMP (8Br-cAMP, 0.1 mmol l−1) analog induced significant inhibition of the acrosomal exocytosis. The induction of acrosomal exocytosis by PKA inhibition was significantly inhibited by an exchange protein directly activated by cAMP (EPAC) ES109 inhibitor. The EPAC selective substrate activated AE at relatively low concentrations (0.02–0.1 mmol l−1), whereas higher concentrations (>5 µmol l−1) were inhibitory to the AE induced by PKA inhibition. Inhibition of PKA revealed about 50% increase in intracellular cAMP levels, conditions under which EPAC can be activated to induce the AE. Induction of AE by activating the acinsegering-protein, gelsolin, which causes F-actin dispersion, was inhibited by the EPAC inhibitor. The AE induced by PKA inhibition was mediated by phospholipase C activity but not by the Ca2+-channel, CatSper. Thus, inhibition of PKA at the end of the capacitation process induced EPAC/phospholipase C-dependent acrosomal exocytosis. EPAC mediates F-actin depolymerization and/or activation of effectors downstream to F-actin breakdown that lead to acrosomal exocytosis.

Asian Journal of Andrology (2019) 21, 337–344; doi: 10.4103/aja.aja_99_18; published online: 18 December 2018

Keywords: acrosomal exocytosis; exchange protein directly activated by cyclic adenosine monophosphate; protein kinase A; sperm

INTRODUCTION

Mammalian spermatozoa must reside in the female reproductive tract for several hours before gaining the ability to fertilize the egg. During this time, the sperm undergoes several biochemical modifications and motility changes, collectively termed capacitation. The capacitated spermatozoon can undergo the acrosome reaction or acrosomal exocytosis (AE), a process that enables it to penetrate and fertilize the egg. The AE is an exocytotic process in which the outer acrosomal membrane fuses with the overlying plasma membrane. AE triggers evoke Ca2+ and H+ efflux via the sperm plasma membrane, as well as Ca2+ mobilization from the acrosome. It is well established that the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) mediates sperm capacitation, including indirect elevation of protein tyrosine phosphorylation, actin polymerization, and hyper-activated motility. Mice lacking the sperm-specific PKA catalytic subunit α2 are sterile, and their sperm cannot develop capacitation-dependent hyper-activated motility.4,5

For many years, it was thought that cAMP/PKA mediates the AE process. In the recent years, it was found that cAMP can mediate cellular processes via a PKA-independent mechanism, which includes the small GTPase Rap1 activated by the guanine nucleotide exchange factor, exchange protein directly activated by cAMP (EPAC).6–8 The best-characterized cAMP targets are PKA, EPAC, and cyclic-nucleotide-gated channels. The testis-specific Na+/H+ exchanger also contains a cAMP binding site.9 In other cell types, it was shown that PKA and EPAC are both involved in exocytic processes by facilitating Ca2+ release from intracellular Ca2+ stores. In mouse sperm, the PKA catalytic subunit α4 is not expressed in the sperm head, suggesting that PKA, in contrast to cAMP10–13 is probably not involved in the mechanism that mediates the AE.10

It was already shown that EPAC mediates human and mouse AE.11,14 In the present study, we showed that inhibition of PKA induces EPAC-dependent AE. Moreover, induction of AE by progesterone, angiotensin II, thapsigargin (TG) or Ca2+-ionophore is also mediated by EPAC suggesting a physiological role of EPAC in the AE mechanism.

MATERIALS AND METHODS

Reagents

A23187, TG, 1-[[6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and 2’-dideoxyadenosine (ddAdo) were purchased from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich Israel Ltd. (Rehovot, Israel) unless otherwise stated.

Sperm preparation

Human semen from fertile men was liquefied by incubation for 30 min at room temperature; then, the semen was loaded on a

1The Mina and Everard Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel; 2Department of Clinical Laboratory Science, Zefat Academic College, Zefat 1320611, Israel.

Correspondence: Dr. H Breitbart (haim.breitbart@biu.ac.il)

Received: 28 August 2018; Accepted: 08 October 2018
40%–80% gradient (PureCepition Lower and Upper Phase Gradient) and centrifuged (5810R, Eppendorf, Hamburg, Germany) for 10 min at 3000 g at room temperature. The lower layer containing the sperm was collected and resuspended twice in Ham's F-10 medium containing 21 mmol l⁻¹ 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 25 mmol l⁻¹ sodium bicarbonate (Cat No. 144-55-8), 0.6% human serum albumin, 7.6 mmol l⁻¹ sodium lactate (Cat No. 312-85-6) washed in Ham's F-10, then centrifuged again, and the sperm allowed to "swim up" after the last wash at 37°C. The motile cells (over 80% motile cells) were collected without the pellet and resuspended in capacitation medium. This procedure allowed motile sperm to be obtained without leukocyte contamination. All experimental protocols were approved and performed according to the relevant guidelines and regulations of the Helsinki Committee of Sheba Hospital, Ramat-Gan, Israel, and informed consent was obtained from all participants.

**Sperm capacitation**

Human sperm (1 × 10⁷ cells per ml) were incubated in capacitation media, HAMF-10 at 37°C in 5% CO₂ for 3 h as described previously.¹⁵

**Assessment of sperm acrosomal exocytosis**

Human sperm (1 × 10⁷ cells per ml) were incubated under capacitation conditions for 160 min, and then various compounds as described for each experiment in the figure legends were added for an additional 20 min. The AE inducers for each experiment in the figure legends were added for 1 h. The percentage of acrosome-reacted sperm was determined microscopically (Axio imager Z1, Zeiss, Jena, Germany) using fluorescein isothiocyanate (FITC)-conjugated Pismum sativum agglutinin (PSA). An aliquot of spermatozoa (10⁵ cells per 10 μl) was smeared on a glass slide and allowed to air-dry. The sperm were then fixed with methanol for 15 min at room temperature and washed three times at 5-min intervals. The first and third washes were performed with distilled water (dH₂O), and the second wash with Tris-buffered saline (TBS) (137 mmol l⁻¹ NaCl [Cat No. 7647-14-5], 2.7 mmol l⁻¹ KCl [Cat No. 7447-40-7] and 20 mmol l⁻¹ Tris–HCl, pH 7.6). The slides were air-dried and then incubated in a moist environment with PSA-FITC (50 μmol ml⁻¹ in TBS) for 35 min, then washed twice with dH₂O at 5-min intervals and sealed with ProLong Gold anti-fade reagent (Thermo Fisher Scientific, Waltham, MA, USA). For each treatment, at least 100 cells per slide were evaluated on triplicate slides, using × 400 magnification under an Axio imager Z1 fluorescence microscope. Cells with green staining over the acrosomal cap were considered acrosome intact; those with equatorial green staining or no staining were considered acrosome-reacted. The percent acrosome-reacted cells (5%–10%) at time zero was subtracted from each measurement.

**Intracellular cAMP determination**

The total cAMP concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit from Arbor Assay (Cyclic AMP Direct EIA Kit) according to the manufacturer's instructions. Briefly, sperm (10⁵ cells per ml) were incubated under capacitation conditions. At the end of the incubation, the sperm were lysed, and ELISA was performed according to the manufacturer's protocol. cAMP was determined relative to a standard curve.

**Determination of actin polymerization**

The method was described by us previously.¹⁴ Sperm cells were spread on microscope slides, air-dried, and fixed in 1.75% formaldehyde in TBS for 10 min, then washed once with dH₂O for 5 min, placed in 0.2% Triton X-100 in TBS for 30 min, washed three times at 5-min intervals in TBS and air-dried. Afterward, sperms were incubated in a moist chamber with 4 μmol l⁻¹ phalloidin-FITC in TBS for 60 min, washed four times with dH₂O at 5-min intervals and mounted with ProLong Gold anti-fade reagent. Images were captured on Axiosio imager Z.1 fluorescence microscope at ×400 magnification. Sperm pictures were photographed within 24 h to minimize the loss of fluorescence, at the same exposure. The fluorescence intensity, which indicates the F-actin level, was quantified for whole cells using the "MetaMorp Image J" software (National Institutes of Health Universal Imaging Corp., West Chester, PA, USA) and the background intensity was subtracted. All experiments were carried out in duplicate slides, and at least 50 cells per slide were quantified for fluorescence intensity.

**Statistical analyses**

Data were analyzed for statistical significance by analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) comparison for at least three replicates. Differences of P < 0.05 were considered statistically significant. Sample size appears in the figure legends.

**RESULTS**

The effect of PKA inhibition at the end of sperm capacitation on acrosomal exocytosis

In the present study, we investigated the involvement of cAMP/PKA in human sperm AE. Inhibition of PKA (using H89) at the end of the capacitation process revealed a 3-fold increase in the AE rate (Figure 1). Treatment of capacitated human sperm with TG or progesterone also induced the occurrence of AE and the augmentation of AE by H89, TG, or progesterone was significantly reduced when both soluble and the transmembrane adenylyl-cyclase (AC) were inhibited using 2-Hydroxy-2-methylpropionophenone (2-OH) ddAdo (Figure 1). Interestingly, the induction of the AE by H89 was almost completely blocked by adding a relatively high concentration of the membrane-permeable 8-bromo-cAMP (8Br-cAMP, 0.1 mmol l⁻¹) or by increasing intracellular cAMP using 1-methyl-3-isobutylxanthine (IBMX); this agent inhibits cAMP-phosphodiesterase, the enzyme that degrades cAMP (Figure 1). Similar inhibition was observed when...
the AE was induced by TG or progesterone (Figure 1). TG induces AE by inhibiting the Ca\(^{2+}\)-ATPase (Ca\(^{2+}\) pump) localized in the outer acrosomal membrane, resulting in a decrease of intra-acrosomal Ca\(^{2+}\) leading to the opening of the store-operated-Ca\(^{2+}\)-channel (SOC) of the plasma membrane. Progesterone activates CatSper, which is a sperm-specific Ca\(^{2+}\) channel in the outer acrosomal membrane to release intra-acrosomal Ca\(^{2+}\) leading to the opening of the store-operated-Ca\(^{2+}\)-channel (SOC) of the plasma membrane.

A dose dependence analysis of the effect of 8Br-cAMP on the AE revealed that 5 μmol l\(^{-1}\) and 25 μmol l\(^{-1}\) 8Br-cAMP stimulates AE, whereas higher concentrations showed no effect or even inhibition; nevertheless, all concentrations used inhibited the H89-induced AE (Supplementary Figure 1). The data in Figure 1 and Supplementary Figure 1 indicate that intracellular cAMP mediates the AE induced by PKA inhibition, while relatively high cAMP inhibits this effect. Thus, the different sensitivity to cAMP levels, and the fact that inhibition of PKA caused cAMP-dependent AE suggest that endogenous cAMP levels are probably enhanced when PKA is inhibited. Indeed, we found a 50% increase (from 2.6 to 3.9 pmol per 10\(^{5}\) cells) in cAMP levels in cells treated with H89. Treatment with IBMX, a cAMP-phosphodiesterase (PDE) inhibitor, caused a much higher increase in [cAMP] \(^{-1}\) (up to 5.6 pmol per 10\(^{5}\) cells), which is inhibitory to the AE process as mentioned above.

The possible involvement of EPAC in AE induced by PKA inhibition

Since inhibition of PKA (Figure 1) or low concentrations of 8Br-cAMP (Supplementary Figure 1) induced AE, we suggested that cAMP-dependent EPAC might mediate this effect. We, therefore, tested the effect of various concentrations of 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic AMP (8pCPT), a selective activator of EPAC \(^{21}\) on AE. Activation of EPAC by addition of 8pCPT induced AE at relatively low concentrations of 8pCPT (0.02–10 μmol l\(^{-1}\)), whereas at higher concentrations of 8pCPT (25–50 μmol l\(^{-1}\)), the induction was much lower (Figure 2). These results indicate that AE is mediated by EPAC, and relatively high concentrations of EPAC substrate inhibit AE. Increasing intracellular cAMP using IBMX also show significant inhibition (75%) in AE induced by 0.1 μmol l\(^{-1}\) 8pCPT (Figure 2).

To further characterize the role of EPAC in H89-induced AE, we tested the effect of the EPAC-specific inhibitor, ESI09 \(^{22,23}\) on AE induced by H89 and other inducers. As shown in Figure 3, ESI09 inhibited AE induced by 8pCPT, H89, TG, Angiotensin II, progesterone or the

Ca\(^{2+}\)-ionophore A23187, indicating that the AE induced by these six inducers is largely EPAC-dependent.

The involvement of phospholipase C in AE induced by PKA inhibition

It was shown that EPAC-activated Rap GTPase is involved in phospholipase C (PLC) and Ca\(^{2+}\) signaling in the β\(_{2}\)-adrenoreceptor. \(^{24}\) PLC is an important factor involved in the AE mechanism. \(^{25,26}\) It catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to diacylglycerol, which activates protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP\(_3\)), which opens the Ca\(^{2+}\)-channel in the outer-acrosomal membrane to release intra-acrosomal Ca\(^{2+}\), leading to induction of AE in human sperm. \(^{27,28}\) Reduction in intrac-acrosomal Ca\(^{2+}\) by IP\(_3\) leads to SOCC activation \(^{29}\) and indeed, TG-induced AE, which occurs through SOCC activation, was over 90% inhibited by PLC inhibition using U73122 (Figure 3). AE induced by 8pCPT, H89, progesterone, or A23187 were

Figure 2: The effect of 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic (8pCPT) on AE induced by protein kinase A inhibition. After 160 min of incubation (Control), various concentrations of 8pCPT were added and then 50 μmol l\(^{-1}\) N-[12-(p-bromocinnamylamino)ethyl]-5isoquinolinesulfonamide (H89) or 0.5 mmol l\(^{-1}\) 1-methyl-3-isobutylxanthine (IBMX) were added. The values represent the mean ± standard deviation of duplicates from three experiments from three different donors. \(P < 0.05\), \**P < 0.01\), and \***P < 0.001\), significant difference compared to the corresponding control. AE: acrosomal exocytosis.

Figure 3: Inhibitory effect of the phospholipase C inhibitor 1-[6-[[17β]-3-Methoxyestra-1,3,5(10)-tien-17-yl]amino]hexyl]-1H-pyrole-2,5-dione (U73122) on AE. After 160 min of incubation (Control), 1 μmol l\(^{-1}\) U73122 (PLC inhibitor) or 1 μmol l\(^{-1}\) 1-[6-[[17β]-3-Methoxyestra-1,3,5(10)-tien-17-yl]amino]hexyl]-2,5-pyroildinedione (U73343, inactive PLC inhibitor analogue) were added and then the AE inducers \(N\)-[2-(p-bromocinnamylamino)ethyl]-5isoquinolinesulfonamide (H89; 50 μmol l\(^{-1}\)), TG (3 μmol l\(^{-1}\)), progesterone (5 μmol l\(^{-1}\)), 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic (8pCPT; 0.1 μmol l\(^{-1}\)) or antibiotic A23187 Calcium Ionophore (A23187; 10 μmol l\(^{-1}\)) were added. The values represent the mean ± standard deviation of duplicates from three experiments from three different donors. \(P < 0.05\), \**P < 0.01\, and \***P < 0.001\, significant difference compared to the corresponding control. PLC: phospholipase C; AE: acrosomal exocytosis; TG: thapsigargin.

PKA regulates EPAC-dependent acrosomal exocytosis

D Itzhakov et al
Figure 4: Effect of exchange protein directly activated by cAMP inhibitor and N-butanol on AE. After 160 min of incubation (Control), 0.5% butanol (N-butanol) or 10 μmol l \(^{-1}\) EPAC specific inhibitor 09 (ESI09) were added. The AE inducers are: 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic (8pCPT; 0.1 μmol l \(^{-1}\)), N-[2-(p-bromocinnamylamo)ethyl]-5-isouquinolinesulfonamide (H89; 50 μmol l \(^{-1}\)), TG (3 μmol l \(^{-1}\)), angiotensin II (Ang II; 10 nmol l \(^{-1}\)), polyphosphoinositide-binding-peptide (PBP10; 1 μmol l \(^{-1}\)), 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic (8pCPT); 50 μmol l \(^{-1}\)) and antibiotic A23187 calcium ionophore (A23187; 10 μmol l \(^{-1}\)). The values represent the mean ± standard deviation of duplicates from three experiments from three different donors. \(*P < 0.05, **P < 0.01, and ***P < 0.001, significant difference compared to the corresponding control. EPAC: exchange protein directly activated by cAMP; cAMP: cyclic adenosine monophosphate; AE: acrosomal exocytosis; TG: thapsigargin.

Figure 5: The actin fluorescence intensity. Human sperm (1 × 10⁷ cells per ml) were incubated in Ham’s F-10 capacitation medium for 160 min (Control). Next, 10 μmol l \(^{-1}\) EPAC specific inhibitor 09 (ESI09) was added for an additional 20 min. Then TG (3 μmol l \(^{-1}\)), polyphosphoinositide-binding-peptide (PBP10; 1 μmol l \(^{-1}\)), N-[2-(p-bromocinnamylamo)ethyl]-5-isouquinolinesulfonamide (H89; 50 μmol l \(^{-1}\)), 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic (8pCPT; 0.1 μmol l \(^{-1}\)) were added for an additional 1 h. The values represent the mean ± standard deviation of duplicates from two experiments from two different donors. (a) F-actin fluorescence units. (b) Fluorescence microscope figures. Scale bars = 5 μm. EPAC: exchange protein directly activated by cAMP; cAMP: cyclic adenosine monophosphate; TG: thapsigargin.

Interestingly, the addition of TG to cells treated first with H89 or PBP10, there is 68% or 80% stimulation of AE, respectively (Supplementary Table 1).

The involvement of CatSper in AE induced by PKA inhibition

The sperm-specific Ca²⁺-channel CatSper is essential for the fertilizing ability of sperm. Since AE depends on Ca²⁺ influx into the sperm, we tested whether CatSper mediates AE induced by EPAC activation and by other inducers using the CatSper inhibitor, (1S,2S)-2-(2-(N-(3-Benzimidazol-2-yl)propyl)-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropoxycarbonyl dihydrochloride hydrate (NNC 55-0396). To find the optimal concentration of NNC, we tested its effect on AE-induced by progesterone, known to activate CatSper. We found that 1 μmol l \(^{-1}\) NNC caused complete inhibition of progesterone-induced AE. AE induced by TG was almost completely inhibited by NNC, while the AE induced by H89, 8pCPT or A23187 was only slightly reduced (Figure 6). The absence of an effect of NNC on Ca²⁺-ionophore A23187-induced AE, conditions under which the inhibition of Ca²⁺-channels is bypassed, indicates the specificity of NNC.

DISCUSSION

In the present study, we investigated the involvement of CAMP/PKA in human sperm AE. Treatment of capacitated human sperm with the PKA inhibitor H89, TG, or progesterone induced the occurrence of AE which was significantly reduced when both soluble and the transmembrane AC were inhibited indicating that CAMP-mediated this AE. However, the induction of the AE by H89, TG, or progesterone was significantly inhibited by adding a relatively high concentration of the membrane-permeable 8Br-cAMP (0.1 mmol l \(^{-1}\)) or by increasing intracellular cAMP using IBMX. In human sperm, progesterone activates Rap1 in the acrosomal region in a CAMP-dependent manner, and Rap1 activates PLC, leading to Ca²⁺ release from the acrosome.
PKA regulates EPAC-dependent acrosomal exocytosis

D Itzhakov et al

Figure 6: The effect of voltage-dependent calcium channel of sperm - CatSper inhibition on AE. After 160 min of incubation (Control), 1 µmol l⁻¹ CatSper inhibitor (1S,2S)-2-(2-(N-[(3-Benzimidazol-2-yl)(propyl)-N-methylaminoethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride hydrate (NCC 55-0396) were added and then the AE inducers N-[2-(p-Bromocinnamylamino)ethyl]-5isoquinolinesulfonamide (H89; 50 µmol l⁻¹), TG (3 µmol l⁻¹), progesterone (5 µmol l⁻¹), 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic (8pCPT; 0.1 µmol l⁻¹), or antibiotic A23187 calcium ionophore (A23187; 10 µmol l⁻¹) were added. The values represent the mean ± standard deviation of duplicates from three experiments from three different donors. *P < 0.05, **P < 0.01, and ***P < 0.001, significant difference compared to the corresponding control. AE: acrosomal exocytosis; TG: thapsigargin.

...causes also reduction of intra-acrosomal Ca²⁺ leading to the opening of SOCC. Since intracellular concentrations of cAMP are well regulated by its synthesis and degradation systems, it is reasonable to assume that elevated [cAMP] inhibits this Ca²⁺ mobilization process. Since the activation of AE by the PKA inhibitor H89 is also inhibited by 8Br-cAMP, it is possible that the AE induced by H89 also requires Ca²⁺ mobilization. This point is further discussed below.

The reduction in the AE induced by PKA inhibition by the AC inhibitors indicates that a cAMP-dependent-PKA-independent mechanism mediates this effect. Moreover, the significant reduction of AE induced by PKA inhibition by relatively low concentration of 8Br-cAMP (25 µmol l⁻¹), further supports such a cAMP-dependent-PKA-independent mechanism. A dose-dependence analysis of the effect of 8Br-cAMP on the AE revealed that 25 µmol l⁻¹ 8Br-cAMP stimulates AE, whereas higher concentrations showed no effect or even inhibition; nevertheless, all concentrations used inhibited the H89-induced AE. Thus, the AE induced by PKA inhibition is highly sensitive to increased cAMP concentrations. The data indicate that intracellular cAMP mediates the AE induced by PKA inhibition, while relatively high cAMP inhibits this effect. Thus, the different sensitivity to cAMP levels, and the fact that inhibition of PKA caused cAMP-dependent AE suggest that endogenous cAMP levels are probably enhanced when PKA is inhibited. Indeed, we found a 50% increase in [cAMP] levels in cells treated with H89. Treatment with IBMX caused a much higher increase in [cAMP] (up to 5.6 pmol per 10⁶ cells), which is inhibitory to the AE process as mentioned above. Thus, 3.9 pmol cAMP per 10⁶ cells is the suitable concentration for AE induction; however, 5.6 pmol cAMP per 10⁶ cells is inhibitory to the AE process. The strong effects of small changes in cAMP levels emphasize the importance of tight regulation of intracellular cAMP levels by the sperm cell. The enhanced effect of PKA-inhibition on [cAMP] is probably due to the fact the inhibition of PKA might decrease binding of cAMP to PKA, resulting in an increase in free cAMP in the cells.

Since inhibition of PKA or low concentrations of 8Br-cAMP-induced AE, we suggested that cAMP-dependent-PKA might mediate this effect. It was shown previously that EPAC mediates AE in human sperm. Activation of EPAC by addition of 8pCPT induced AE at relatively low concentrations of 8pCPT (0.1–10 µmol l⁻¹), whereas at higher concentrations (25–50 µmol l⁻¹ 8pCPT), the induction was much lower. These results indicate that AE is mediated by EPAC, and relatively high concentrations of EPAC substrate inhibit AE. The effect of 0.1–1.0 µmol l⁻¹ 8pCPT on AE is similar to the effect of H89, whereas higher concentrations of 8pCPT (5–50 µmol l⁻¹) inhibit the induced effect of H89 on AE. Thus, at relatively high concentrations, 8pCPT inhibits AE induced by low 8pCPT or by H89. Moreover, AE induced by a low concentration of 8pCPT is inhibited by treatment with IBMX, suggesting that EPAC or its downstream effectors are inhibited by relatively high concentrations of intracellular cAMP.

To further prove the role of EPAC in H89-induced AE, we tested the effect of the EPAC-specific inhibitor, ESI09. On AE. ESI09 inhibited AE induced by 8pCPT, H89, TG, Angiotensin II, progesterone or the Ca²⁺-ionophore A23187, indicating that the AE induced by these six inducers is largely EPAC-dependent. Thus, AE induced by PKA inhibition is mediated by EPAC; however, relatively high concentrations of EPAC substrate inhibit this activity. This finding is further supported by the inhibitory effect of high cAMP concentration on AE induced by PKA inhibition or by low concentrations of 8pCPT using IBMX.

The cAMP-dependent changes in EPAC dynamics are induced by allosteric rather than simple binding effects. These authors also show that EPAC contains multiple clusters of residues for which dynamics is either quenched or enhanced by cAMP. Thus, it is possible that at relatively high concentrations of cAMP, EPAC activity is reduced by allosteric effects, and when EPAC binds ESI09, this inhibitory effect is partially reversed, and its activity is rescued. Das et al. also showed that Gly-238 in EPAC is highly sensitive to cAMP although not to the antagonist Rp-cAMP, suggesting an allosteric role at Gly-238. These findings suggest differences in the affinities of cAMP and ESI09 to different regions of EPAC.

It has been suggested that the regulatory unit of EPAC2 contains high (B-site) and low (A-site) affinity sites for cAMP. Binding of cAMP to the B-site activates EPAC, whereas it is possible that binding to the A-site inhibits EPAC activity. This idea might explain why a relatively high level of cellular cAMP is inhibitory to EPAC-dependent AE. ESI09 is a competitive inhibitor of EPAC, which competes with cAMP binding. When low concentrations of 8pCPT (0.1 µmol l⁻¹) were used to activate EPAC-dependent AE, ESI09 caused almost complete inhibition of AE. However, when 50 µmol l⁻¹ 8pCPT was used, which itself caused very slight stimulation of AE, a two-fold increase in AE was observed in the presence of ESI09, and an even greater increase in AE induced by ESI09 was seen when both 50 µmol l⁻¹ 8pCPT and H89 were present in the reaction mixture. We suggest that in the presence of 50 µmol l⁻¹ 8pCPT, the A-site of EPAC (the inhibitory site) is occupied by the cAMP analogue, and therefore no stimulation of AE is observed. The addition of ESI09 competes with cAMP for binding to this site, inducing its release and resulting in EPAC reactivation. These dual effects of ESI09, support our suggestion of high affinity activating, and low-affinity inhibitory sites for cAMP binding in EPAC. In conclusion, to achieve optimal EPAC-dependent AE, intracellular cAMP levels must be well regulated by its production and degradation systems in the cell.

Two isoforms of EPAC are present in sperm cells, EPAC1 and EPAC2, both are inhibited by ESI09. Here, we show that treatment with N-butanol inhibited AE induced by 8pCPT, H89, TG, or A23187 indicating that EPAC1 is the dominant isoform involved in AE. The fact that AE induced by TG is only slightly inhibited by N-butanol (31%) suggests that AE-induced by TG is probably...
mediated by EPAC2 as well. Although N-butanol inhibits actin polymerization and increases spontaneous-AE when present during sperm capacitation, it does not cause any increase in the AE when added at the end of the capacitation, probably because of its inhibitory effect on EPAC1.

PLC is an important factor involved in the AE mechanism.29,30 TG-induced AE, which occurs through SOCC activation, was over 90% inhibited by PLC inhibition using U73122. AE induced by 8pCPT, H89, progesterone, or A23187 were also almost completely blocked by PLC inhibition. These data demonstrated that AE, whether induced by activation of EPAC using 8pCPT, or by inhibition of PKA, activation of progesterone-R, or Ca2+-ionophore is mediated by PLC. Thus, under these various conditions by which EPAC mediates AE, we see that PLC is also involved. The inhibition of 8pCPT-induced AE by the PLC inhibitor U73122 suggests that EPAC might activate PLC. Indeed, it was shown that the human sperm PLCγ isoform is activated by EPAC-activated Rap1 and is required for Ca2+ mobilization from the acrosome.14 The fact that AE induced by the Ca2+-ionophore A23187 depends on EPAC and PLC indicates that high influx of Ca2+ is not sufficient to induce AE and that Ca2+ mobilization from the acrosome is necessary for AE to occur. This conclusion was supported by other studies working with permeabilized human sperm.13,14

To examine the relationship between EPAC and actin in sperm, we looked for EPAC involvement in AE induced by activation of gelsolin using PBP10, a peptide containing the PIP2-binding domain of gelsolin. Treatment with PBP10 release gelsolin from F-actin, leading to its activation and F-actin dispersal, resulting in AE.15,16 It was seen that AE induced by PBP10 is almost completely blocked by 0.1 mmol l−1 8Br-cAMP or 50 µmol l−1 8pCPT or the EPAC inhibitor ES109, suggesting that EPAC mediates F-actin breakdown and/or the AE process downstream of F-actin dispersion. It was shown that TG, PBP10, H89, or 8pCPT induce F-actin breakdown and this breakdown is significantly inhibited by the EPAC inhibitor ES109. These data clearly indicate that EPAC mediates F-actin depolymerization.

We showed that AE induced by TG, H89, or 8pCPT is mediated by PLC activity. In our previous study in human sperm, we showed that hydrolysis of PIP2 by PLC leads to gelsolin activation and F-actin breakdown. Moreover, F-actin breakdown induced by PBP10 is independent of PLC activity.15 It was suggested elsewhere that EPAC mediates PLC activation,14 which might be the mechanism by which EPAC leads to gelsolin activation. The fact that EPAC mediates PLC-independent F-actin breakdown induced by PBP10 indicate that EPAC has a direct effect on F-actin depolymerization in addition to its indirect effect due to PLC activation.

It is possible that other mechanisms not involving PLC are likely to mediate the effect of EPAC on the AE. It has been suggested that EPAC can activate the fusion component soluble-NSF-attachment protein receptor (SNARE) known to mediate the AE.40,41 SNARE is physically associated with F-actin leading to granule exocytosis in platelets.42 In MIN6 cells, F-actin negatively regulates insulin exocytosis via binding to the SNARE protein, syntaxin 4.43 Furthermore, in this insulin secretion system, gelsolin forms a complex with syntaxin 4 under basal conditions, which is relieved on stimulus-induced Ca2+ influx to activate gelsolin and induce its dissociation from syntaxin 4 to facilitate insulin exocytosis.44,45 Thus, it seems that syntaxin 4 activity is regulated by its binding to F-actin and gelsolin. To allow the outer acrosomal and the plasma membranes to fuse, the F-actin network produced between the two membranes during capacitation should be dispersed.46 Activation of gelsolin in sperm causes F-actin dispersion which would activate SNARE and other fusion proteins leading to AE.47,48 A working model suggested for the mechanism of AE indicates that EPAC might affect the AE through two mechanisms: one is EPAC-RAP-PLC-IP3 leading to Ca2+ release from the acrosome, and the second is EPAC-Rab3A-SNARE-AE.14,42 Since F-actin dispersion and AE induced by PBP10 is independent of PLC activity, we suggest that the inhibition of PBP10-induced AE by the EPAC inhibitor ES109 is due to inhibition of the EPAC-Rab3-SNARE-AE cascade.

TG treatment further stimulates the AE in the presence of PBP10 or H89. These suggest that intracellular Ca2+ mobilization induced by TG is an important step for achieving AE, even under conditions whereby F-actin is already dispersed. The fact that AE induced by TG is inhibited by ES109 and by U73122 suggest that EPAC involve in PLC-dependent Ca2+ mobilization which is supported by previous study.11

The sperm-specific Ca2+-channel CatSper is essential for the fertilizing ability of sperm.44 Since AE depends upon Ca2+ influx into the sperm, we tested whether CatSper mediates AE induced by EPAC activation and by other inducers using the CatSper inhibitor, NNC 55-0396. AE induced by TG was almost completely inhibited by NNC, while the AE induced by H89, 8pCPT, or A23187 was only slightly reduced. We suggested before that AE induced by H89 is mediated by EPAC. Here we showed that AE induced by H89 or by direct activation of EPAC using 8pCPT are not affected by CatSper inhibition. These data further support our model that inhibition of PKA activates EPAC leading to AE. Moreover, these data suggest that AE induced by EPAC is probably not mediated by Ca2+ influx into the cell via CatSper but rather by Ca2+ mobilization from the acrosome as a result of PLC activation by EPAC-activated GTPase, as described above. This conclusion is supported by other studies in human sperm, which show that EPAC activation induces PLC-dependent Ca2+ mobilization.14

The fact that AE-induced by progesterone is mediated by EPAC/PLC indicates that Ca2+ mobilization from the acrosome is a necessary step for AE even under conditions by which CatSper is activated. Moreover, the fact that AE induced by EPAC is probably not mediated by Ca2+ influx into the cell via CatSper but rather by Ca2+ mobilization from the acrosome as a result of PLC activation by EPAC-activated GTPase, as described above. This conclusion is supported by our findings.

**CONCLUSIONS**

In summary, the data presented here indicate that the inhibition of PKA results in an increase in intracellular cAMP and activation of EPAC-dependent AE. A further increase in CAMP levels is inhibitory for this process. We showed that low concentrations of the selective EPAC substrate added to the cells stimulate AE, whereas high concentrations show no effect on AE, and inhibit AE induced by PKA inhibition. It is well known that cellular levels of CAMP are precisely regulated by its production and decomposing systems. In this study, we provide one of the reasons why such regulation is important. Moreover, this study...
PKA regulates EPAC-dependent acrosomal exocytosis
D Itzhakov et al

provides data regarding the involvement of cAMP, PKA, and EPAC in the AE mechanism.

AUTHOR CONTRIBUTIONS
DI performed the experiments, helped to write the manuscript, and performed the statistical analysis. YN made critical notes and helped to analyze the data. HB wrote the manuscript, interpreted the data, and supervised DI in her master studies. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

ACKNOWLEDGMENTS
We would like to thank Dr. Gili Band and Dr. Nir Etzioni from Sheba Hospital, Ramat-Gan, Israel, for their help in obtaining sperm cells. We thank Dr. Maya Finkelstein from Wolfson Hospital, Holon, Israel, for helping in F-actin fluorescence studies.

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

REFERENCES
1. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, et al. Capacitation of mouse spermatozoa: I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995; 121: 1129–37.
2. Brener E, Rubinstein S, Cohen G, Shiterkani K, Rivlin J, et al. Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. J Biol Reprod 2003; 68: 837–45.
3. Shahar S, Wiser A, Ickowicz D, Lubart R, Shulman A, et al. Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyper-activated motility. Hum Reprod 2011; 26: 2274–82.
4. Morgan DJ, Weisenhaus M, Shum S, Su T, Zheng R, et al. Tissue-specific PKA inhibition using a chemical genetic approach and its application to studies on sperm capacitation. Proc Natl Acad Sci U S A 2008; 105: 20740–5.
5. Nolan MA, Babcock DF, Wennemuth G, Brown W, Burton KA, et al. Sperm-specific protein kinase A catalytic subunit CalpHα2 orchestrates CAMP signaling for male fertility. Proc Natl Acad Sci U S A 2004; 101: 13483–8.
6. Bos JL. Epac proteins: multi-purpose cAMP targets. Trends Biochem Sci 2006; 31: 680–6.
7. Cheng X, Ji Z, Tsalkova T, Mei F, Epac and PKA: A tale of two intracellular CAMP receptors. Acta Biochim Biophys Sin (Shanghai) 2008; 40: 651–62.
8. Gloorich M, Bos JL. Epac: defining a new mechanism for CAMP action. Annu Rev Pharmacol Toxicol 2010; 50: 355–75.
9. Wang D, Hu J, Bobulevska IA, Quill TA, McLeroy P, et al. Sperm-specific Na+/H+ exchanger (SNHHE) is critical for expression and in vivo bioactive regulation of the soluble adenyl cyclase (sAC). Proc Natl Acad Sci U S A 2007; 104: 9325–30.
10. Wertheimer E, Krupf D, de la Vega-Beltran JL, Sanchez-Cardenas C, Navarrete F, et al. Compartimentalization of distinct CAMP signaling pathways in mammalian sperm. J Biol Chem 2013; 288: 35307–20.
11. Leclerc P, Kopf G, Mouse sperm adenyl cyclase: general properties and regulation by the zona pellucida. J Biol Reprod 1995; 52: 1227–33.
12. Breitbart H, Spurzin B. The biochemistry of the acrosome reaction. Mol Human Rep 1997; 3: 195–202.
13. Branham MT, Mayorga LS, Tomes CN. Calcium-induced acrosomal exocytosis requires the small G proteins Rap1 and Rab3. Nat Cell Biol 2002; 4: 901–6.
14. Chen H, Ding C, Wild C, Liu H, Wang T, et al. Efficient synthesis of ESI-09, a novel non-cyclic nucleotide EPAC antagonist. Tetrahedron Lett 2013; 54: 1546–9.
15. Zhu Y, Chen H, Boulton S, Mei F, Ye N, et al. Biochemical and pharmacological characterizations of ESI-09 based EPAC inhibitors: defining the ESI-09 “therapeutic window”. Sci Rep 2015; 5: 9344.
16. Sehrawat S, Cullere X, Patel S, Italiano J Jr, Mayadas TN. Role of Epac1, an exchange factor for Rap GTPases, in endothelial microtubule dynamics and barrier function. Mol Biol Cell 2008; 19: 1261–70.
17. Jeong HW, Li Z, Brown MD, Sacks DB. IGQAP1 binds Rap1 and modulates its activity. J Biol Chem 2007; 282: 20752–62.
18. Consonni S, Gloerich M, Spanjaard E, Bos JL. CAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. Proc Natl Acad Sci U S A 2012; 109: 3814–9.
19. Han S, Huh J, Kim W, Jeong S, Min so, et al. Phospholipase D2 activates HIF-1α/VEGF pathway via phosphatidic acid. Exp Mol Med 2014; 46: e126.
20. Schmidt M, Ewelin S, Weenink PA, van Dorp F, Rehmaha H, et al. A new phospholipase-C-calcium signaling pathway mediated by cyclic AMP and a Rap GTPase. Nat Cell Biol 2001; 3: 1020–4.
21. Etkowitz N, Tiros H, Chazan R, Jaldet Y, Daniel L, et al. Bovine sperm acrosome reaction induced by G-protein-coupled receptor agonists is mediated by epidermal growth factor receptor transactivation. Dev Biol 2009; 334: 447–57.
22. Breitbart H, Finkelstein M. Regulation of sperm capacitation and the acrosome reaction by PIP3, and actin modulation. Asian J Androl 2015; 17: 597–600.
23. Ruege MC, Luccchesi O, Bousa MA, Tomes CN, Epac, and Rab3 act in concert to mobilize calcium from sperm’s acrosome during exocytosis. Cell Commun Signal 2014; 12: 43.
24. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, et al. A family of calcium-binding proteins that directly activate Rap1. Science 1998; 282: 2275–9.
25. Finkelstein M, Megnaghi B, Ickowicz D, Breitbart H. Regulation of sperm motility by PIP3, and actin polymerization. Dev Biol 2013; 381: 62–72.
26. Ren D, Navarro B, Perez G, Jackson AC, Hsu S, et al. A sperm ion channel required for sperm motility and male fertility. Nature 2001; 413: 603–9.
27. Lucchesi O, Ruege MC, Bousa MA, Quevedo MF, Tomes CN. The signaling module cAMP/Epac/Rap1/PLC-mediated/IP3 mobilizes acrosomal calcium during sperm exocytosis. Biochim Biophys Acta 2016; 1863: 544–61.
28. Das R, Mazzab-Jafari MT, Chowdhury S, SidDas S, Selvaratnam RN, et al. Entropy-driven CAMP-dependent allosteric control of inhibitory interactions in exchange proteins directly activated by CAMP. J Biol Chem 2008; 283: 19691–703.
29. de Roeij J, Rehmaha H, van Triest M, Cool RH, Wittinghofer A, et al. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem 2000; 275: 20829–39.
30. Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, et al. A novel EPAC-specific cAMP inhibitor suppresses pancreatic cancer cell migration and invasion. Mol Pharmacol 2013; 83: 122–8.
31. Shabaty O, Breitbart H. CaMKII stimulates spontaneous acrosomal exocytosis in sperm through induction of actin polymerization. Dev Biol 2016; 415: 64–74.
32. De Blas GA, Roggero CM, Tomes CN, Mayorga LS. Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biol 2005; 3: e323.
33. Rodriguez F, Zaletti MN, Mayorga LS, Tomes CN. MuclinB1 controls SNARE protein complex assembly during human sperm acrosomal exocytosis. J Biol Chem 2012; 287: 43825–39.
34. Woronowicz K, Diks JR, Rozenvany N, Dowal L, Blair PS, et al. The platelet actin cytoskeleton associates with SNAREs and participates in alpha-granule secretion. Biochemistry 2010; 49: 4533–42.
35. Jewell JL, Lu W, Oh E, Wang Z, Thurmond DC. Filamentous actin regulates insulin exocytosis through direct interaction with syntaxin 4. J Biol Chem 2008; 283: 10716–26.
36. Kalwat MA, Wiseman DA, Luo W, Wang Z, Thurmond DC. Gelsolin associates with the N terminus of syntaxin 4 to regulate insulin granule exocytosis. Mol Endocrinol 2012; 26: 128–41.
37. Ramos LS, Zippin JH, Kamenetsky M, Buck J, Levin LR. Glucose and GLP-1 stimulate HA-1-mediated acrosomal exocytosis in sperm. J Biol Chem 2008; 283: 20882–9.
38. Enserink JM, Christensen AE, de Roeij J, van Triest M, Schwede F, et al. A novel Epac-specific CAMP analogue demonstrates independent regulation of Rap1 and Rab3. Nat Cell Biol 2002; 4: 901–6.
39. De Blas GA, Golden N, Breiker C, Kashikar ND, Weyand I, et al. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature 2011; 471: 382–6.
40. Strunk T, Goodwin N, Breiker C, Kashikar ND, Weyand I, et al. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature 2011; 471: 382–6.
PKA regulates EPAC-dependent acrosomal exocytosis

D Itzhakov et al

48. Tomes CN, Michaut M, De Blas G, Visconti P, Matti U, et al. SNARE complex assembly is required for human sperm acrosome reaction. Dev Biol 2002; 243: 326–38.
49. Escoffier J, Boisseau S, Serres C, Chen CC, Kim D, et al. Expression, localization and functions in acrosome reaction and sperm motility of Ca\(_{\text{3.1}}\) and Ca\(_{\text{3.2}}\) channels in sperm cells: an evaluation from Ca\(_{\text{3.1}}\) and Ca\(_{\text{3.2}}\) deficient mice. J Cell Physiol 2007; 212: 753–63.
50. Cohen R, Buttte DE, Asano A, Mukai C, Nelson JL, et al. Lipid modulation of calcium flux through Ca\(_{\text{2.3}}\) regulates acrosome exocytosis and fertilization. Dev Cell 2014; 28: 310–21.
51. Stival C, Ritagliati C, Xu X, Gervasi MG, Luque GM, et al. Disruption of protein kinase A localization induces acrosomal exocytosis in capacitated mouse sperm. J Biol Chem 2018; 293: 9435–47.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

©The Author(s)(2018)
Supplementary Figure 1: Dose response of the effect of 8-bromo-cAMP on AE induced by PKA inhibition. After 160 min incubation of (Control), 8-Bromo-cAMP (8Br-cAMP) was added for an additional 20 min at the concentrations indicated and then 50 μmol l⁻¹ N-[2-(p-Bromocinnamylamino)ethyl]-5isoquinolinesulfonamide (H89) was added for 1 h. The values represent the mean ± s.d. of duplicates from three experiments from three different donors. "P < 0.01, significant difference compared to the corresponding control; ""P < 0.001, significant difference compared to the corresponding control. cAMP: cyclic adenosine monophosphate; AE: acrosomal exocytosis; PKA: protein kinase A; s.d.: standard deviation.

Supplementary Table 1: The effect of thapsigargin on acrosomal exocytosis in the presence of N-[2-(p-Bromocinnamylamino)ethyl]-5isoquinolinesulfonamide (H89) or polyphosphoinositide-binding-peptide

| Treatment | TG (AE cells [%]) | Cont (AE cells [%]) |
|-----------|------------------|-------------------|
| Cont      | 40.0±5.00""      | 9.00±1.00         |
| H89       | 47.0±2.65'       | 28.0±2.00'        |
| PBP10     | 41.6±2.90'       | 23.3±2.30         |

The values AE cells (%) represent the mean±s.d. of duplicates from three experiments from three different donors. "P<0.05, significant difference compared to the corresponding control; ""P<0.01, significant difference compared to the corresponding control. TG: thapsigargin; AE: acrosomal exocytosis; PBP10: polyphosphoinositide-binding-peptide; s.d.: standard deviation; Cont: Control

Supplementary Figure 2: Effect of elevated 8-bromo-cAMP on AE induced by Gelsolin activation. After 160 min of incubation (Control), 50 μmol l⁻¹ 8-(4-chlorophenylthio)-2′,5′-cyclic (8pCPT), 0.1 mmol l⁻¹ 8-bromo-cAMP (γ8Br(cAMP), or 10 μM EPAC specific inhibitor 09 (ESI09) were added for an additional 20 min. Then, 1 μmol l⁻¹ PBP10 was added for 1 h. The values represent the mean ± s.d. of duplicates from three experiments from three different donors. "P < 0.01, significant difference compared to the corresponding control; ""P < 0.001, significant difference compared to the corresponding control. cAMP: cyclic adenosine monophosphate; AE: acrosomal exocytosis; PBP10: polyphosphoinositide-binding-peptide; s.d.: standard deviation.