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# Promiscuous coupling and involvement of protein kinase C and extracellular signal-regulated kinase 1/2 in the adenosine $A_1$ receptor signalling in mammalian spermatozoa

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ABSTRACT

Mammalian spermatozoa require a maturational event after ejaculation that allows them to acquire the capacity for fertilisation. This process occurs spontaneously during the transit through the female reproductive tract where spermatozoa are in contact with micromolar concentrations of adenosine that might act as a capacitative effector. This study shows that the adenosine $A_1$ receptor agonist, 2-chloro-$N^6$-cyclopentyladenosine, can induce capacitation, i.e., the ability to undergo the acrosome reaction and to become fertile. This receptor, already known to be bound to $G\alpha_{i2}$, is also bound to $G_{q/11}$. These G proteins are functional in the signalling pathway elicited by the $A_1$ receptor and correlate with the multiple intracellular events that follow its activation. The use of protein kinase C isoform inhibitors and MEK inhibitors, resulting in the abolition of the biological response to the selective agonist, indicates the involvement of protein kinase C and MEK in its signalling. In agonist-treated spermatozoa an extracellular calcium influx, involvement of $\alpha$ and $\gamma$ PKC isoforms and transient phosphorylation of ERK1/2 have been observed. Our results, besides showing that adenosine $A_1$ receptor prompts mammalian spermatozoa to undergo the acrosome reaction hence supporting a role for adenosine as agent for fertilisation, show that 2-chloro-$N^6$-cyclopentyladenosine triggers signalling mechanisms that involve both $G\alpha_{i2}$ and $G_{q/11}$, extracellular calcium influx, modulation of classical $Ca^{2+}$-dependent PCK isoforms and up-regulation of the ERK1/2 phosphorylation.



## 1. Introduction

Adenosine is an important signalling molecule that elicits a large number of biological effects via four G protein-coupled proteins denoted $A_1$, $A_{2A}$, $A_{2B}$, $A_3$ receptors (ARs) [1]. The four adenosine receptors couple via G proteins to an intricate network of signalling pathways that enables the endogenous modulator adenosine to induce a variety of responses in cells [2].

A multiplicity of G proteins coupling has been documented for a variety of GPCRs and the simultaneous functional coupling with unrelated G proteins provides a mechanism for the activation of multiple intracellular effectors by a single receptor [3]. It is known that adenosine $A_1$Rs couple to

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pertussis toxin (PTX) sensitive G proteins ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, and $G_o$) and stimulates numerous intracellular signalling events [4–6]. Experimental results obtained with cultured smooth muscle cells [4] and astrocytes [7], chick embryo ventricular myocytes [6], cardiac myocytes [8,9], transfected CHO [10] and COS-7 cells [11] link the adenosine $A_1$ receptors ($A_1R$) to phospholipase C (PLC), protein kinase C(PKC), and extracellular-regulated kinase (ERK)1/2 activation and suggest that $G_{i/o}$ βγ-subunit might be involved either in the direct coupling of $A_1R$ to PLC or in the augmentation of $G_{q/11}$-coupled receptor response [12]. Changes in second messengers suggest that this receptor is able to adopt agonist-specific conformations that lead to the differential activation of $G_i$, $G_s$ and $G_q$ [13].

Adenosine $A_1$ receptors, present in mammalian spermatozoa and mainly localised at the acrosomal domain, the equatorial segment, and the middle piece [14], are coupled to a PTX sensitive $G_{\alpha i2}$ protein and cyclopentyladenosine (CPA), a specific adenosine $A_1R$ agonist, activates PLC with subsequent $IP_3$ generation and release of intracellular calcium [15]. Studies with adenosine $A_1R$ knockout mice show that the receptor must be functional for optimal sperm activation and in vivo fertility [16]. Mammalian ejaculated spermatozoa are infertile and two sequential activation processes, namely capacitation and acrosome reaction, are needed to successfully transit through the female reproductive tract and fertilize an egg. Spermatozoa, undergoing a series of biochemical and ultrastructural changes as responses to changes in the surrounding environment [17,18], presumably possess receptors that sense environmental signals and translate them into cellular responses that are necessary to acquire the ability to fertilize an egg. During the transit in the female genital tract, spermatozoa are in contact with adenosine at micromolar concentrations [19,20] hence, the proposal that the nucleosides might represent one of the multiple capacitative effectors. Capacitation is mainly regulated by increase in intracellular cAMP and increase in PKA activity that mediate protein tyrosine phosphorylation, an event regarded as a capacitation marker [15,21–23].

The capacitative effect of adenosine $A_1R$ selective agonist is not related to receptor-mediated cAMP increase since this receptor is coupled with the inhibitory subtype $G_{\alpha i2}$ that leads to the inhibition of adenylyl cyclase. However, this receptor stimulates phospholipase C (PLC) and can increase $IP_3$ levels [15] that, in turn, acting on specific $IP_3$ receptors, stimulates intracellular calcium release, an event strongly related to capacitation [24]. $IP_3$ receptors are present in mammalian spermatozoa [25–27]. Components of the ERK pathway are also present in spermatozoa and involved in capacitation [28,29] and acrosome reaction [30] but sperm receptors responsible for activation of the ERK pathway during capacitation and acrosome reaction have not yet been identified.

It is suggested that GPCRs can be functionally coupled to multiple G proteins of different classes and that such promiscuous coupling is probably critical in modulating the efficacy and the potency of cell signalling [31]. This study investigates whether a promiscuous G protein coupling to adenosine $A_1R$ could explain the multiple intracellular signalling events elicited by its activation. Assuming sperm capacitation as the biological response to the agonist, we were able of clarifying some aspects, i.e., cellular calcium influx, involvement of PKC and ERK1/2, of the signalling triggered by the selective $A_1$ agonist chloro-$N^6$-cyclopentyladenosine (CCPA) in mammalian spermatozoa.

## 2. Materials and methods

### 2.1. Materials

All reagents used in the study were from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Bisindolylmaleimide V and U0124 were from Calbiochem (La Jolla, CA).

### 2.2. Medium

BWW medium consisted of 95 mM NaCl, 44 μM sodium lactate, 25 mM $NaHCO_3$, 20 mM Hepes, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 0.27 mM sodium pyruvate, 5 U/ml penicillin, 5 μg/ml streptomycin, pH 7.4 and 0.3% BSA, unless otherwise indicated.

### 2.3. Mice

Adenosine $A_1R$ knockout and wild type C57BL/6 mice were a gift of Dr. Johansson (Karolinska Institutet, Stockholm, Se). All the experiments were conducted using protocols following EUR directives, approved by the Institutional Animal Care and Use Committee of Perugia University.

### 2.4. Sperm collection and assessment of capacitation

Murine epididymal spermatozoa were extracted from adult mice (8–14 weeks) and Zonae Pellucida (ZP) were prepared from homogenised ovaries of 22-day-old virgin female as described [16]. The ability of the spermatozoa to respond to ZP and undergo the acrosome reaction was utilised as an assay for capacitation since the ZP-induced acrosome reaction occurs only in capacitated spermatozoa. $10 \times 10^6$ sperm, incubated for the indicated times in BWW at 37 °C in 5% $CO_2$/95% humidified air, were assessed for the capacitated state following incubation of the sperm with 2 ZP/μL for 30 min.

Human semen with normal sperm characteristics according to World Health Organisation criteria (vol. ~2 ml, concentration $>20 \times 10^6$ cells/ml; motility >50%, normal morphology >15%) was collected by masturbation from healthy donors. Approval for the donation of human semen samples was obtained from the University of Perugia human ethics committee. After liquefaction at 37 °C, motile spermatozoa were selected and suspended in BWW medium as described [15]. Sperm were assayed for capacitation by induction of AR with L-α-lysophosphatidylcholine (LPC) at 100 μg/ml. This concentration of LPC was previously shown to induce the AR in capacitated sperm while having no effects on uncapacitated sperm. Prior to drying and staining, randomly selected slides containing ~$10^5$ cells were examined to verify sperm motility and viability. The acrosomal status of the sperm was determined on air-dried sperm smears by Coomassie blue staining. At least 200 cells were scored with an Axioplan Zeiss microscope (Gottingen, Germany). The

percentage of capacitation was assessed by subtracting spontaneous acrosome reaction % (SAR) to LPC-induced acrosome reaction % (LPC-IAR) in humans and to ZP-induced acrosome reaction % (ZP-IAR) in mice, respectively, at each indicated time. In experiments performed to determine the effect of specific $A_1$ agonist on the capacitative process, BSA was substituted with chloro-$N^6$-cyclopentyladenosine (CCPA, Tocris Bioscience, Bristol, UK). The $A_1$ agonist and antagonist were always used in the presence of 0.2 U/ml adenosine deaminase (ADA) because of the role of adenosine $A_1$R as an ADA-binding protein [32]. Inhibitors of kinases, added to spermatozoa 15 min prior to CCPA addition, did not cause a decrease in sperm motility and viability over the incubation period.

### 2.5. *In vitro fertilisation*

Males of proven fertility (3–5-month-old), caged alone at least 3 days before IVF, were killed by cervical dislocation. The cauda epididymis and a portion of the vas deferens of each mouse were removed, quickly placed into a culture dish containing 2 ml of BWW-no BSA and transferred into another dish with 200 μl of BWW medium either in the presence of 0.3% BSA or in the presence of 10 nM CCPA, as capacitative agents. Four incisions were made in the cauda epididymis to allow spermatozoa to swim out. After capacitation, tissue fragments were discarded and the spermatozoa suspensions were collected in BWW and adjusted to $16 \times 10^6$ cells/ml. C57/B16 female mice, 22-day-old, were induced to superovulate by i.p. injection of 5IU pregnant mare's serum gonadotrophin (PMSG) followed by 5IU human corionic gonadotropin (hCG) 48 h later. Animals were killed by cervical dislocation 13 h after hCG injection. Oviducts were placed in BWW and the cumulus–oocyte complexes (COCs) were recovered by gentle dissection of the oviducts. After brief digestion with 1 mg/ml hyaluronidase (2 min at 37 °C), the oocytes ($15 \pm 5$) were added to the fertilisation droplet, which contained capacitated spermatozoa ($4 \times 10^5$ cells). After 4 h of incubation at 37 °C in 5% $CO_2$, oocytes were washed twice in 50 μl BWW under oil, transferred to droplets of the same medium, and returned to the incubator. Fertilisation was assessed by recording the number of two-cell embryos 24 h after insemination observed at 400× magnification using an inverted microscope with the specimen table maintained at 37 °C.

### 2.6. *Western blotting*

After the indicated treatments, samples solubilised with electrophoresis sample buffer supplemented with phosphatase inhibitors (100 μM sodium vanadate, 20 mM β-glycerolphosphate, 5 mM sodium fluoride, and 10 nM okadaic acid), were loaded on a 12% SDS-PAGE, and transferred to a nitrocellulose membrane. mAb anti-phosphorylated ERK1/2 (P-ERK 1/2) (1:1000 in 5% BSA) was from Cell Signalling Technology (Beverly, MA); anti-ERK 2 (1:500 in 5% skimmed milk) was from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins were visualised with anti-rabbit IgG or anti-mouse IgG (1:2500), horseradish peroxidase-conjugated using the ECL detection kit (Amersham Biosciences, Little Chalfont, UK). Intensities of the bands were normalised with ERK2 to determine phosphorylation fold-induction.

### 2.7. *Immunoprecipitation procedure*

Human sperm ($700 \times 10^6$ cells), washed in PBS at $800 \times g$ for 10 min at room temperature, was suspended in cold 50 mM Tris–HCl, pH 7.4 containing 0.5% digitonin, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and protease inhibitor cocktail. After sonication (Virsonic 50; Virtis Co., Gardiner, NY) on ice with 15 bursts of 10 s (10-s intervals) and extraction for 60 min at 4 °C, the supernatant was obtained by ultracentrifugation at $105{,}000 \times g$ (1 h at 4 °C). The preparation was assayed with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Richmond, CA) for protein quantitation. Aliquots of the detergent extracts (~400 μg protein) were incubated with the antibodies (anti-adenosine $A_1$receptor antibody, anti-$G_{\alpha i2}$, and anti-$G_{q/11}$ at 12 μg/ml, Santa Cruz Biotechnology), previously coupled to protein A Sepharose CL4B by an overnight incubation at 4 °C in constant rotation. Nonspecific immunoprecipitation was assessed by using the same amount of rabbit nonimmune IgG. The mixture was incubated for 3 h at 4 °C with constant rotation. Immunoprecipitates were washed twice with 0.5% digitonin, 0.5% CHAPS in Tris–HCl 50 mM, NaCl 140 mM, $NaN_3$ 0.025% (TSA buffer), twice with 0.1% digitonin, 0.1% CHAPS in TSA buffer, and once with TSA buffer alone. Pelleted samples were then dissolved in SDS-PAGE sample buffer, boiled for 3 min, and centrifuged at $12{,}000 \times g$ for 2 min. The supernatant was analysed by SDS-PAGE at 15%, transferred to a nitrocellulose membrane and immunoblotted with mouse anti-$G_{\alpha i2}$ (1:100), rabbit anti-$G_{q/11}$ (1:100) and goat anti-adenosine $A_1$ receptor (1:100) (Santa Cruz Biotechnology). Proteins were visualised with anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG (1:2500), horseradish peroxidase-conjugated using the ECL detection kit (Amersham Biosciences).

### 2.8. *Kinetworks™ KPSS.11 phosphoprotein analysis*

Total cell lysates were prepared according to Kinexus protocol (Kinexus Bioinformatic Corporation, British Columbia, Canada). Briefly, control human spermatozoa (BWW alone) and CCPA-treated human spermatozoa were incubated for 3.5 h and, after washing with ice-cold PBS, were suspended in lysis buffer (20 mM Tris, 20 mM β-glycerophosphate, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM sodium vanadate, 0.5% Nonidet P-40, and 1 mM dithiothreitol) supplemented with 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 μg/ml pepstatin A, and sonicated for 15 s. Cell debris was removed by centrifugation at $100{,}000 \times g$ for 30 min at 4 °C. Protein concentration was determined with protein assay kit (Bio-Rad, Hercules, CA). The screen required 500 μg of protein. Samples were analysed by Kinetworks™ KPSS.11 Phosphoprotein Screen. The Kinetworks™ analysis involves resolution of a single lysate sample by SDS-PAGE, and subsequent immunoblotting with panels of up to three primary antibodies reacting with a distinct subset of at least 36 known phosphorylated cell signalling proteins of distinct molecular masses per channel in a 20-lane Immunetics multiblotter. The antibody mixtures were carefully selected to avoid overlapping cross-reactivity with target proteins. After further incubation with a mixture of relevant horseradish peroxidase-conjugated secondary antibodies

(Santa Cruz Biotechnology), the blots were developed using ECL Plus reagent (Amersham Biosciences), and the Trace Quantity units were arbitrary based on the intensity of ECL fluorescence detection for target immunoreactive proteins recorded with a Fluor-S MultiImager and quantified using Quantity One software (Bio-Rad, Hercules, CA). Kinexus reports show normalised CPM, i.e., the trace quantity of the band corrected to a scan time of 60 s and normalised to correct for differences in protein amounts. Results of this screening were provided in the form of Western blots and tables showing the quantitative analysis of these blots by Kinexus that performed all the normalisation and statistical analysis on the data. Assuming control values as 100%, results were given as difference between treated and control samples. When the level of the phosphorylated forms was unchanged, results were indicated as zero. More detailed information and protocols of the Kinetworks™ analysis can be found at the Kinexus Bioinformatics Corp. website (http://www.kinex-us.ca).

### 2.9. Protein kinase C activity

Determination of Protein kinase C activity was performed with StressXpress® PKC kinase activity assay kit (Stressgen Bioreagents, Ann Arbor, MI) following the manufacturer's instructions. Briefly, human spermatozoa were suspended in BWW either in the presence or in the absence of $Ca^{2+}$ and incubated in the presence of 10 nM CCPA for 5 min. $Ca^{2+}$-free BWW was prepared by replacing $CaCl_2$ by equimolar amounts of $MgCl_2$ and by adding 2 mM EGTA. Lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF) and 10 μg/ml leupeptin and aprotinin) was then added to the suspension, allowed to stand for 10 min on ice and sonicated. After centrifugation at 15,000 × *g* for 15 min, the cytosolic fraction was used to determine PKC activity. Protein concentration was determined with protein assay kit (Bio-Rad, Hercules, CA).

### 2.10. Measurement of intracellular calcium in sperm

The intracellular free calcium concentration ($[Ca^{2+}]_i$) was assessed using the fluorescent $Ca^{2+}$ indicator Fura-2AM. Spermatozoa were loaded with 2 μM Fura-2AM (20 min, 37 °C), washed in BWW either in the presence or in the absence of $Ca^{2+}$ (300 × *g*, 5 min) and resuspended at a concentration of $2 \times 10^6$ sperm/ml. $Ca^{2+}$-free BWW was prepared by replacing $CaCl_2$ by equimolar amounts of $MgCl_2$ and by adding 2 mM EGTA. The $[Ca^{2+}]_i$ was determined by loading 3 ml of sperm into a prewarmed cuvette, and fluorescence intensity recorded using a Perkin-Elmer LS50B fluorescence spectrofluorimeter (Wellesley, MA) following excitation at 340 and 380 nm, and emission at 510 nm, and calculating the fluorescence ratio (*F*). The $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where $K_d$ = 224 nM. $F_{max}$ and $F_{min}$ were recorded at the end of each incubation. $F_{max}$ was determined after the addition of 1% Triton X-100 made up in BWW containing 2 mM $CaCl_2$, and $F_{min}$ was determined after addition of 5 mM EGTA (pH 10) to the cuvette.

### 2.11. Statistical analysis

Data were analysed by variance analysis (ANOVA, two-tails, paired values) followed by the protected least-significant difference test. A difference was considered to be statistically significant with $p < 0.05$.

## 3. Results

### 3.1. Time-course of capacitation in the presence of CCPA and N0840

We have shown that the selective adenosine $A_1$R agonist $N^6$-cyclopentyladenosine has a capacitative effect on human spermatozoa [15] and that murine spermatozoa, lacking the adenosine $A_1$ receptor, are less responsive to capacitating effectors implying that adenosine $A_1$Rs are involved in the efficiency of the process [16]. To confirm the involvement of the receptor in the sperm activation process that is mandatory to sperm fertility, we analysed the time course of capacitation either in the presence of the selective adenosine $A_1$ receptor agonist CCPA ($K_d$ = 0.8 nM) or in the presence of the selective adenosine $A_1$ receptor antagonist N0840 ($K_d$ = 10 nM) in murine (Fig. 1A) and human (Fig. 1B) spermatozoa. In the

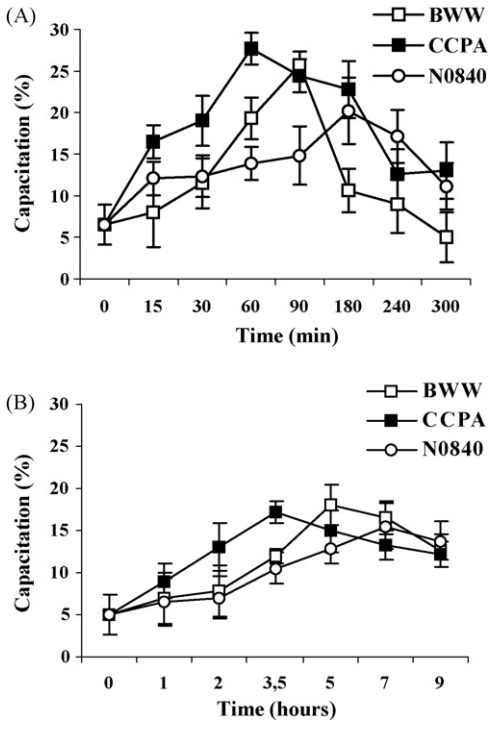



**Fig. 1 – Effect of CCPA and N0840 on capacitation. Spermatozoa, prepared as described in Section 2, were incubated with BWW, BWW-no BSA either with 10 nM CCPA or 100 nM N0840 and withdrawn at the indicated times. Acrosome-reacted cells were determined with Coomassie blue staining. (A) Murine spermatozoa and (B) human spermatozoa. Results represent the mean ± S.D. of *n* = 7 independent experiments performed in triplicate and 200 sperm counted/sample.**

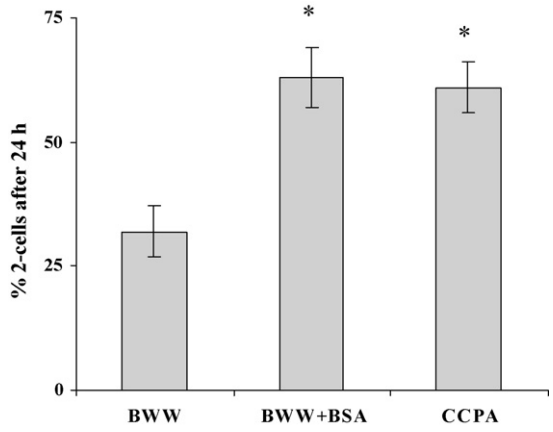



**Fig. 2 – In vitro fertilisation using CCPA-capacitated spermatozoa. Four hundred thousand spermatozoa, capacitated in BWW-no BSA (negative control), BWW (positive control), and in BWW-no BSA-CCPA, were added to each fertilisation drop containing 15 ± 5 oocytes and incubated for 4 h in BWW. Fertilisation was assessed by recording the number of two-cell embryos 24 h after fertilisation. The spermatozoa used for IVF were derived from different mice of proved fertility. Data given as mean ± S.D. of $n = 3$ independent experiments, are reported as % of 2-cells after 24 h assuming the initial number of oocytes (15 ± 5) as 100%. $^{*}p < 0.05$ vs. negative control (BWW-no BSA).**

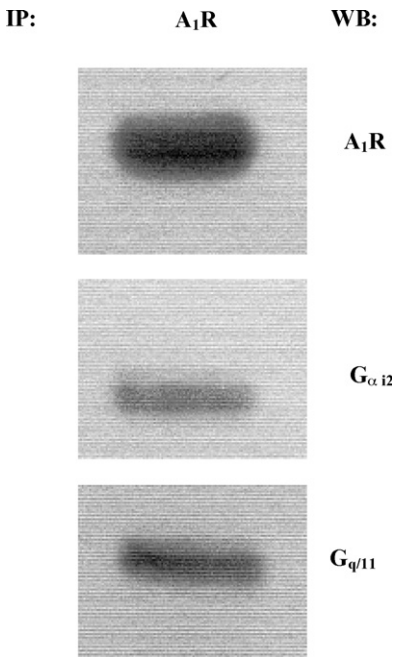



**Fig. 3 – Co-localisation of $G_{\alpha i2}$ and $G_{q/11}$ detergent-extracted sperm proteins (~400 μg) incubated with the anti-$A_1$R antibody (12 μg/ml), previously coupled to protein A Sepharose CL4B, were analysed by SDS-PAGE at 15%. The immunoprecipitate was then transferred to a nitrocellulose membrane and incubated with anti-adenosine $A_1$R (positive control), anti-$G_{\alpha i2}$ and $G_{q/11}$ antibodies. A representative experiment, repeated three times with similar results, is shown. IP: sample immunoprecipitated with the anti-adenosine $A_1$R. WB: sample immunoblotted with anti-adenosine $A_1$R, anti-$G_{\alpha i2}$ and anti-$G_{q/11}$.**

presence of 10 nM CCPA, the maximal degree of capacitation was obtained after 60 min incubation in mice and after 3.5 h incubation in humans whereas in the presence of 100 nM N0840 the capacitative process was delayed and the maximal degree of capacitation was reached after 180 min incubation in mice and after 7 h incubation in humans.

### 3.2. *In vitro fertilisation*

To further support the role of adenosine $A_1$R agonist as capacitative agent, we performed in vitro ferilisation (IVF) experiments in the presence of 10 nM CCPA and results were compared to control tests where murine spermatozoa were incubated in BWW either in the presence (positive control) or in the absence (negative control) of 0.3% BSA (Fig. 2). The addition of BSA caused an increase in the number of 2-cell embryos compared to BWW alone. The substitution of BSA with CCPA resulted in a similar number of 2-cell embryos confirming the role of the agonist in the acquisition of the fertilisation capacity.

### 3.3. *Co-localisation of adenosine $A_1$R, $G_{\alpha i2}$ and $G_{q/11}$*

We have shown that adenosine $A_1$ receptor is coupled with $G_{\alpha i2}$ subunit and its activation causes an increase in $IP_3$ generation that is inhibited by U73122, a PLC inhibitor [15]. To determine whether mammalian spermatozoa adenosine $A_1$ receptor is also coupled with $G_{q/11}$, known to be responsible for PLC activation, we performed immunoprecipitation experiments. Detergent-extracted sperm proteins were immunoprecipitated with anti-adenosine $A_1$R antibody and immunodetected with anti-adenosine $A_1$R (positive control), anti-$G_{\alpha i2}$ and -$G_{q/11}$ antibodies. The adenosine $A_1$ receptor co-precipitated with $G_{\alpha i2}$ and $G_{q/11}$ indicating that this receptor is coupled with the two G proteins (Fig. 3). Samples, immunoprecipitated either with anti-$G_{\alpha i2}$ or with anti-$G_{q/11}$ antibodies, and then blotted with anti-adenosine $A_1$R and/or anti-$G_{\alpha i2}$ and -$G_{q/11}$ antibodies, confirmed the dual coupling (data not shown). Samples in which specific antibody was substituted with non-immune rabbit IgG were used as negative control.

### 3.4. *Effect of inhibitors on CCPA-induced capacitation*

To assess the involvement of the two G proteins in the response to the selective adenosine $A_1$ receptor agonist CCPA, we analysed the biological response of human spermatozoa in the presence of PTX, a $G_i$ inhibitor, and 4-hydroxynonenal (HNE), a $G_{q/11}$ inhibitor [33], added to sperm suspension. Several data from the literature link adenosine $A_1$ receptor to PLC, PKC and ERK1/2 activation [4,6–9,12,34,35] and one of the signalling pathways triggered by ARs in most cells involve mitogen activated protein kinases (MAPK) [2,34,36]. To verify the involvement of PKC and MAPK in the sperm adenosine $A_1$ receptor signalling, we analysed the effects of these protein

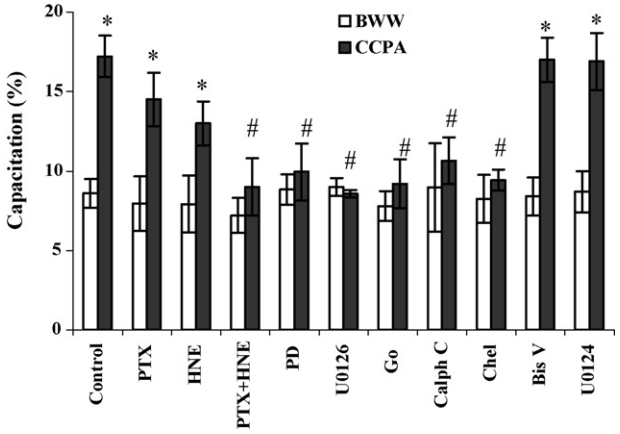
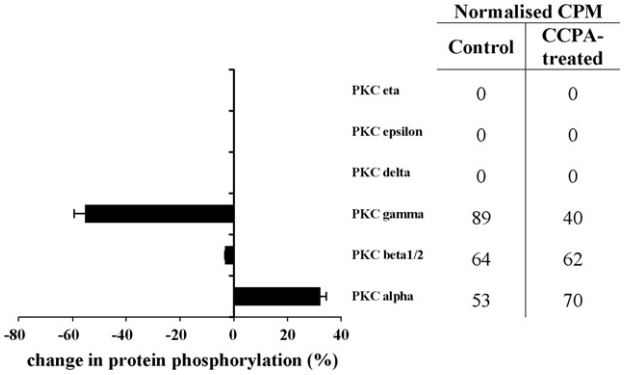



**Fig. 4 – Effect of inhibitors on CCPA-induced capacitation. Human spermatozoa were incubated at 37 °C in BWW (white bars) or in BWW-no BSA with CCPA (black bars). Inhibitors: $G_i$ proteins, 100 ng/ml PTX; $G_{q/11}$ proteins, 20 μM HNE; MEK, 30 μM PD98059 (PD), and 300 nM U0126; PKC, 10 nM Gö6976 (Go), 150 nM calphostin C (Calph C), and 3 μM chelerytrine (Chel), 0.3 μM U0124, inactive analogue of U0126, 25 μM Bisindolylmaleimide V (Bis V), negative control compound for PKC inhibitors; were added 15 min prior to CCPA addition. After 3.5 h of incubation, spermatozoa were washed and capacitation was measured by the LPC-induced acrosome reaction. The acrosomal status was evaluated as described in Section 2. Values as mean ± S.D. ($n = 8$). *$p < 0.05$ vs. respective control spermatozoa in BWW medium; #$p < 0.05$ vs. control spermatozoa in BWW-CCPA medium.**




| | Normalised CPM | |
|---|---|---|
| | Control | CCPA-treated |
| PKC eta | 0 | 0 |
| PKC epsilon | 0 | 0 |
| PKC delta | 0 | 0 |
| PKC gamma | 89 | 40 |
| PKC beta1/2 | 64 | 62 |
| PKC alpha | 53 | 70 |

**Fig. 5 – Kinetworks™ KPSS-11.0 phosphoprotein analysis in whole cell lysates from human spermatozoa. Spermatozoa were incubated at 37 °C in BWW (control) or in BWW-no BSA with 10 nM CCPA for 3.5 h, lysed, and KPSS-11.0 Kinetworks™ protein screens were performed. Graphical representation of CCPA-induced change in protein phosphorylation is shown. Control values were assumed as 100% and results given as difference (mean ± S.D., $n = 3$) between treated and control samples. When the level of the phosphorylated forms was unchanged, results were indicated as zero. Normalised CPM values (arbitrarily based on ECL florescence intensity values), representative of one experiment repeated three times with similar results, are shown in table format at the side of the graph.**

kinase inhibitors, i.e., MEK inhibitors (30 μM PD98059, and 300 nM U0126), and PKC inhibitors (10 nM Gö6976, 150 nM calphostin C, and 3 μM chelerytrine) on the biological response to the receptor activation. After 15 min incubation, CCPA was added and incubated for 3.5 h to allow capacitation. Spermatozoa were then washed from the inhibitors and capacitation measured by LPC-induced acrosome reaction. The treatment with PTX and HNE caused a small decrease in the percentages of capacitated cells, whereas the treatment with their combination determined a significant reduction in the number of capacitated cells, implying the concomitant involvement of the two G proteins in the response. The treatment with protein kinase inhibitors prevented CCPA-induced sperm capacitation suggesting the involvement of MEK and PKC isoforms in the biological response of the spermatozoa to adenosine $A_1R$ activation. Inactive analogues of PKC and ERK1/2 inhibitors, i.e., bisindolylmaleimide V and U0124, did not modify the number of capacitated cells (Fig. 4).

### 3.5. *Kinexus KPSS.11 analysis*

To determine which of the PKC isoforms are involved in the response to the activation of adenosine $A_1R$ by CCPA, we used Kinexus KPSS.11 immunoblot analysis of phosphorylated protein kinases focusing on conventional and atypical PKC isoforms by comparing control (untreated) and CCPA-treated spermatozoa after 3.5 h of incubation (Fig. 5). PKC α, β and γ were the only phosphorylated isoforms detected in control samples and, whereas PKC β was found to be unchanged in CCPA-treated spermatozoa, α and γ isoforms underwent modifications. PKC γ was reduced by 60% and PKC α was increased by 32% suggesting that the response to the adenosine $A_1R$ agonist proceeds through the activation of α and the concomitant deactivation of γ isoforms.

### 3.6. *CCPA-induced $[Ca^{2+}]_i$ oscillations in spermatozoa*

We have already shown that adenosine $A_1R$ affects calcium levels of mammalian spermatozoa and that the response to its selective agonist proceeds via activation of PLC with a subsequent increase in $IP_3$ levels [15]. $IP_3$ receptors are present in spermatozoa [25–27] and an increase of intracellular calcium is suggested to be closely related to capacitation [24]. Therefore, we evaluated the impact of extracellular calcium ($Ca^{2+}$) on murine sperm CCPA-induced capacitation. Cells were incubated in BWW medium with or without $Ca^{2+}$ and the $[Ca^{2+}]_i$ measured by fluorescence using Fura-2AM as indicator (Fig. 6). The addition of CCPA caused, within 50 s, a two-fold rise of $[Ca^{2+}]_i$ as compared to basal level (210 ± 20 nM). This response was prevented by incubation in the BWW-no $Ca^{+2}$ medium ($CaCl_2$ substituted by equimolar amount of $MgCl_2$) but the subsequent addition of 2 mM $Ca^{2+}$ was followed by similar rise of $[Ca^{2+}]_i$. The presence of 2 mM EGTA before addition of CCPA abolished the agonist-induced effect (data not shown). No modifications in $[Ca^{2+}]_i$ were observed in adenosine $A_1R$ knockout murine spermatozoa treated with CCPA in BWW medium. These results show that

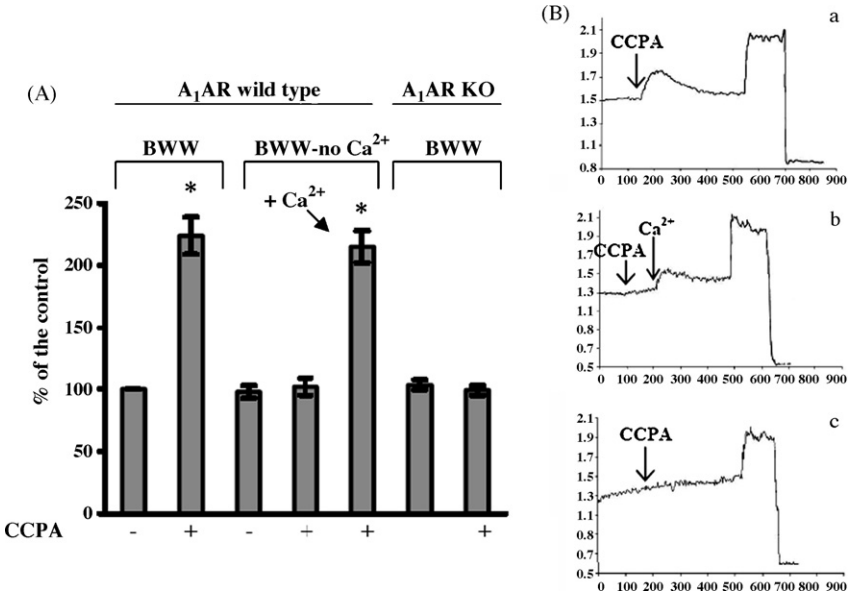



**Fig. 6 – Relationship between intracellular and extracellular calcium. Murine spermatozoa were incubated in either BWW or BWW-no $Ca^{2+}$ as indicated. After loading with 2 μM FURA 2AM, cells were centrifuged, washed and the $[Ca^{2+}]_i$ was determined as described in Section 2. (A) Data from three experiments performed in duplicate are given as mean ± S.D. ($n = 5$). $^*p < 0.05$ vs. control spermatozoa (BWW), (B) A representative experiment of adenosine $A_1R$ wild type sperm suspended in BWW (a), BWW-no $Ca^{2+}$ (b), and adenosine $A_1R$ knockout murine spermatozoa (c), repeated three times with similar results, is shown.**

CCPA, by activating adenosine $A_1R$, causes the entry of calcium from the extracellular medium.

### 3.7. *CCPA-induced PKC activation*

To verify that the activation of adenosine $A_1R$ by CCPA increases PKC activity and that the increase is dependent on calcium influx, we evaluated the effect of CCPA on sperm PKC activity either in the presence or in the absence of external calcium (Fig. 7). Determinations, performed in BWW, showed an increase in sperm PKC activity after 5 min incubation with CCPA whereas PKC activity was not modified in the absence of external calcium.

### 3.8. *Time course study of ERK1/2 phosphorylation during CCPA-induced capacitation*

To investigate whether CCPA induces ERK1/2 phosphorylation in mammalian spermatozoa, we analysed the phosphorylation levels of ERK1/2 in the early phases that follow the activation of the adenosine $A_1R$. The analysis was performed on whole-cell extracts from human spermatozoa (Fig. 8A), adenosine $A_1R$ wild type (Fig. 8B), and adenosine $A_1R$ knockout (Fig. 8C) murine spermatozoa treated with CCPA. Fold-induction of phosphorylated ERK1/2 increased with time of incubation after the $A_1R$ agonist addition. In human spermatozoa ERK1/2 phosphorylation was significantly different from the corresponding control at 2–5 min and then, during the observed time, i.e., 60 min, fold-induction of phosphorylated ERK1/2 equaled the respective controls. In murine wild type spermatozoa ERK1/2 phosphorylation was significantly different from the corresponding control at 30–45 s whereas in adenosine $A_1R$ knockout murine spermatozoa the incubation in the presence of CCPA did not cause any variations of the phosphorylation ratio during the observation time.

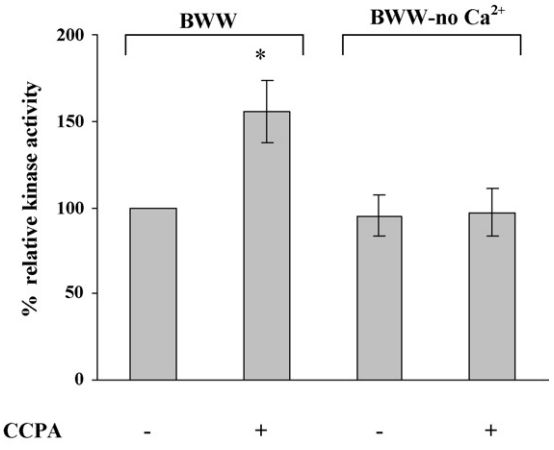



**Fig. 7 – Effect of CCPA on PKC activity. Human spermatozoa, resuspended in either BWW or BWW-no $Ca^{2+}$ as indicated, were incubated for 5 min with or without 10 nM CCPA. Cells were then lysed as described in Section 2 and 10 μg cytosolic fraction used to determine PKC activity. Results, calculated as relative kinase activity, i.e., (Abs 450 $nm_{(sample)}$ − Abs 450 $nm_{(blank)}$)/quantity of crude protein used per assay, are given as mean ± S.D. ($n = 3$) assuming the control (BWW alone) as 100%. $^*p < 0.05$ vs. control.**

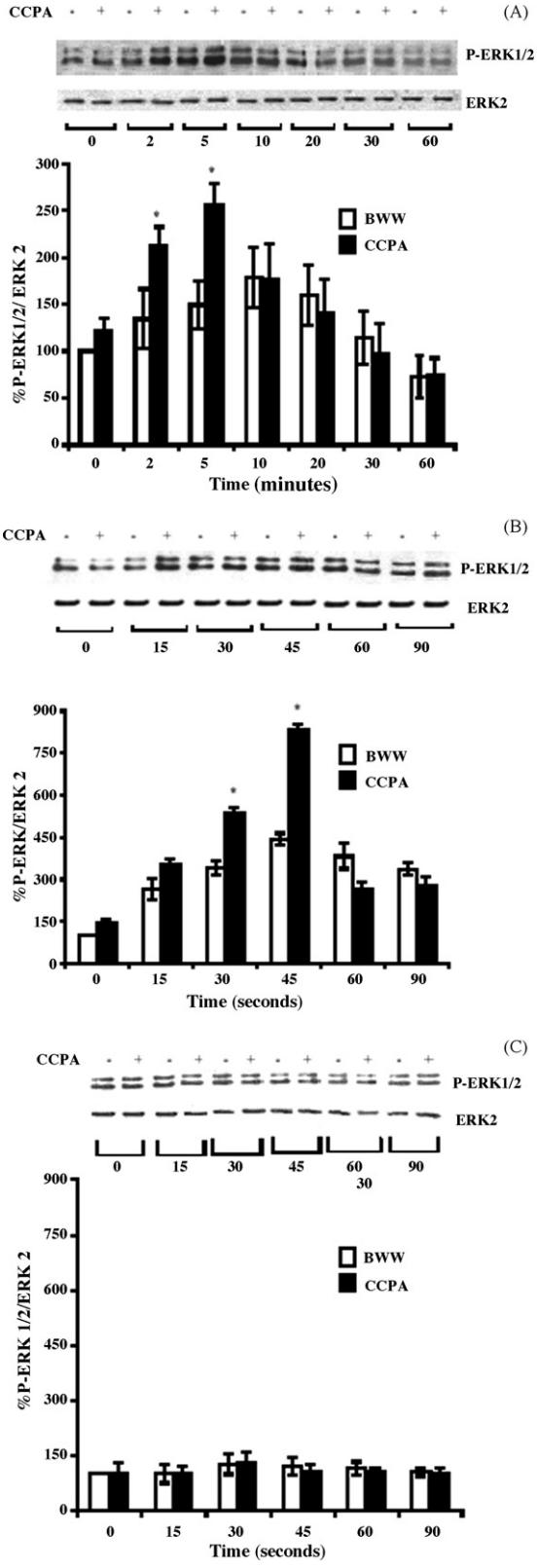



**Fig. 8 – Time course study of ERK1/2 phosphorylation during CCPA-induced capacitation. Human and murine spermatozoa were incubated at 37 °C in BWW (white bars) or in BWW-no BSA with CCPA (black bars). Aliquots were withdrawn at the indicated times. Proteins were electrophoresed, electrotransferred and immunoblotted using the anti-P-ERK1/2 and ERK2 antibodies. The level of phosphorylation was estimated by densitometry analysis and normalised with ERK2. P-ERK1/2/ERK2 ratio of control spermatozoa (BWW medium) at zero time was used as 100%. (A) Human spermatozoa; (B) adenosine $A_1$R wild type murine spermatozoa and (C) adenosine $A_1$R knockout murine spermatozoa. A representative experiment, repeated five times with similar results, is shown. Densitometry results are given as mean ± S.D. ($n = 5$). $^*p < 0.05$ vs. corresponding control spermatozoa (BWW medium).**

### 3.9. *Involvement of G proteins and PKC in ERK1/2 phosphorylation*

To better define the involvement of the two G proteins in the response to the selective $A_1$ agonist CCPA, we analysed the phosphorylated ERK1/2 levels of human spermatozoa in the presence of the two inhibitors, i.e., PTX and HNE. Moreover, since several data from the literature have already shown the existence of a PKC/ERK 1/2 signalling pathway in proliferating cells [37,38], to assess whether PKC was responsible for the ERK1/2 phosphorylation in highly differentiated cells, i.e., spermatozoa, we also determined phosphorylated ERK1/2 levels in the presence of the PKC inhibitors that reduced the biological response (Fig. 9). ERK1/2 phosphorylation, determined 5 min after CCPA addition, was significantly decreased in the presence of all the tested inhibitors supporting the dependence of ERK1/2 phosphorylation either on the two G proteins or on PKC. Identical results, i.e., lack of increases in phosphorylated ERK1/2 levels, were obtained with murine spermatozoa observed at 45 s (data not shown). Note that the combination of PTX and HNE, chelerytrine and Gö6976 decreased ERK1/2 phosphorylation confirming the role of the two G proteins and PKC in the MEK cascade [37,38].

## 4. Discussion

Our results provide the first demonstration of the promiscuous coupling of the adenosine $A_1$ receptor to $G_{\alpha i2}$ and $G_{q/11}$ in mammalian spermatozoa and show that $G_{\alpha i2}$ and $G_{q/11}$ are functional and synergistic in the signal transduction elicited by the adenosine $A_1$R activation. The selective agonist CCPA induces the first process of sperm activation that leads to sperm fertility via a pathway that involves calcium influx, activation of classical calcium-dependent PKC isoforms and ERK1/2 phosphorylation.

At present the molecular basis of capacitation, i.e., the ensemble of cellular modifications that allow mammalian sperm to bind to the oocyte zona pellucida and undergo the acrosome reaction [17,18], is only partially known. Capacitation, regarded as a signal transduction receptor-mediated phenomenon, is an emerging concept [39–43] and our data, showing that the stimulation of adenosine $A_1$R triggers a cascade of signalling events that leads to sperm fertility, support this concept. The constant presence of adenosine $A_1$ receptors on spermatozoa suggested a functional role, later identified, either in human or in murine species, as capacitative [15,16]. Murine spermatozoa, lacking the adenosine $A_1$R,

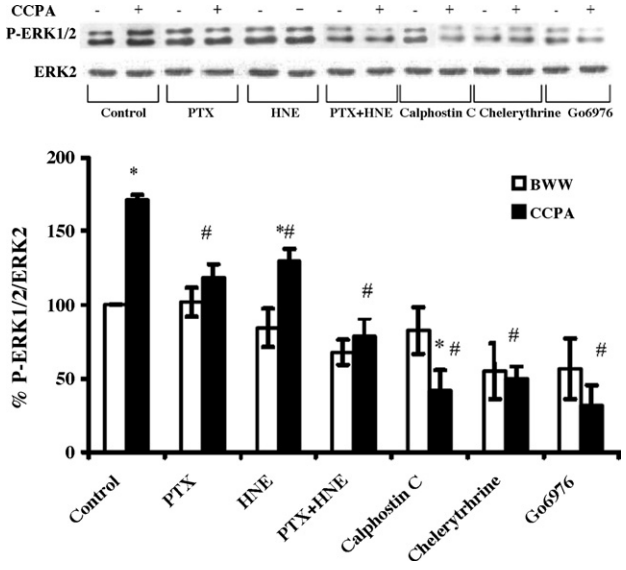



**Fig. 9 – G proteins- and PKC-dependent phosphorylation of ERK1/2. Human spermatozoa were incubated in BWW-no BSA for 15 min with inhibitors of G proteins ($G_i$ proteins, 100 ng/ml PTX; $G_{q/11}$ proteins, 20 μM HNE) and inhibitors of PKC (10 nM Gö6976, 150 nM calphostin C, and 3 μM chelerytrine), and then 0.3% BSA (white bars) or 10 nM CCPA (black bars) were added for 5 min. Sperm proteins were immunoblotted as described in Section 2. Intensities of the bands were normalised with ERK2 and densitometry results are given as mean ± S.D. ($n = 5$). P-ERK1/2/ERK2 ratio of control spermatozoa (BWW medium alone) at 5 min was used as 100%. A representative experiment, repeated five times with similar results, is shown. *$p < 0.05$ vs. respective control spermatozoa in BWW medium; #$p < 0.05$ vs control spermatozoa in BWW-CCPA medium.**

showed a delayed capacitation indicating that the adenosine $A_1$ receptor modulates the kinetic of the process without affecting the number of capacitated cells [16]. Although pharmacological tools have to be used critically since kinase inhibitors may be non-specific [44] and appear to act on non-kinase sites [45] and, in an attempt to elucidate the mechanism of adenosine $A_1$ receptor activation, it should be taken into account that some PKC inhibitors may also act as adenosine receptor antagonists [45], our results strongly suggest the involvement of Protein kinase C family in the signalling. PKC family consists of at least 10 subtypes that can be classified into three subgroups based on their $Ca^{2+}$-dependence, i.e., classical, novel and atypical PKC. Kinexus analysis of the phosphorylation levels of PKC isoforms showed that the biological response to CCPA is associated with a marked activation of α and concomitant deactivation of γ isoforms. It has been reported that PKC α is involved in the regulation of cell permeability and in the parallel process of actin cytoskeleton reorganisation [46,47]. Therefore, its activation during the capacitative process, which is known to involve cell permeability changes [48], seems to be plausible. Data in the literature on the role of PKC γ [49] report that PKC γ phosphorylates Gβ which enhances the potency of Gβγ to stimulate adenylyl cyclase II (ACII) activity. Adenosine $A_1$R is known to be an inhibitory receptor that decreases the cAMP level. Therefore, CCPA signalling does not require activation of adenylyl cyclase and our finding, showing deactivation of PKC γ after the selective agonist treatment, is consistent with its role. MEK inhibitors, U0126 and PD98059, block the activation of downstream ERK1/2. Specifically, PD98059 inhibits the phosphorylation of MEK by Raf, while U0126 inhibits the activated phospho-MEK to phosphorylate ERK1/2 [50]. We found that PD98059 and U0126 decrease the percentages of capacitated cells suggesting that MEK is activated via adenosine $A_1$ receptor using Raf-MEK pathway. Our time course study performed with human and murine spermatozoa shows that CCPA leads to an increase in ERK1/2 phosphorylation compared to the corresponding controls at 5 min and 45 s, respectively. In adenosine $A_1$ receptor knockout murine spermatozoa, incubated either in the presence or in the absence of CCPA, no modifications of the ratio between phosphorylated/unphosphorylated ERK is observed.

In conclusion, we have shown that in mammalian spermatozoa the adenosine $A_1$ receptor is coupled with $G_{\alpha i2}$ and $G_{q/11}$ and that calcium influx, classic PKC, and MEK are involved in the biological response and in the transient activation of ERK1/2. Our data confirm the physiological role of adenosine and shed light on the mechanism involved in sperm activation/fertility via adenosine $A_1$ receptor stimulation.

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