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The Presence and Function of Dopamine Type 2 Receptors in Boar Sperm: A Possible Role for Dopamine in Viability, Capacitation, and Modulation of Sperm Motility[1]

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ABSTRACT

Several studies have shown that dopamine and other catecholamines are present in oviduct luminal fluid. We recently reported that dopamine type 2 receptors (DRD2) are present in a wide range of mammalian sperm, suggesting a role for dopaminergic signaling in events such as fertilization, capacitation, and sperm motility. In the present study, we used Western blot analysis to show that boar sperm express DRD2 and that their activation with dopamine (100 nM) has a positive effect on cell viability that can be correlated with AKT/PKB phosphorylation. Bromocriptine (100 nM) and dopamine (100 nM and 10 μM) increased tyrosine phosphorylation during the capacitation period. Immunofluorescence analysis indicated that DRD2 localization is dynamic and depends on the capacitation stage, colocalizing with tyrosine phosphorylated proteins in the acrosome and midpiece region of capacitated boar sperm. This association was confirmed by coimmunoprecipitation analysis. We also showed that bromocriptine (100 nM) and low-concentration dopamine (100 nM and 10 μM) increased total and progressive motility of sperm. However, high concentrations of dopamine (1 mM) decreased tyrosine phosphorylation and motility in in vitro sperm capacitation assays. This can be explained by the presence of the dopamine transporters (DAT, official symbol SLC6A3) in sperm, as demonstrated by Western blot analysis and immunocytochemistry. Taken together, our results support the idea that dopamine may have a fundamental role during sperm capacitation and motility in situ in the female upper reproductive tract.

catecholamines, dopamine, dopamine receptors, dopamine transporter, neurotransmitters, signal transduction, sperm capacitation, sperm motility and transport

INTRODUCTION

Dopamine is a major neurotransmitter within the mammalian central nervous system and has an important role in functions such as cognition, emotion, and motor activity control. Alteration of certain elements of the dopaminergic system is associated with neurological and psychiatric disorders such as Parkinson disease and schizophrenia [1].

Dopamine receptors are seven-transmembrane trimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors, classified into distinct subfamilies based on pharmacological characteristics and sequence homology. Examples are the dopamine type 1 (D1)-like receptors (DRD1 and DRD4) and the D2-like receptors (DRD2, DRD3, and DRD4) [1].

Stimulation of D1-like receptors activates adenylyl cyclase by coupling to stimulatory GTP-binding regulatory protein (G_s protein), increasing cyclic AMP accumulation, which activates cyclic AMP-dependent protein kinase (PKA) [2, 3]. However, stimulation of D2-like receptors showing high affinity for antipsychotic drugs inhibits adenylyl cyclase by coupling to inhibitory GTP-binding regulatory protein ($G_{i/o}$) and decreasing PKA activity [1].

High concentrations of catecholamines have been detected in human semen [4] and in oviductal compartment from human, sow, rabbit, and cow [5–8]. It has also been reported that catecholamine concentrations vary according to the oviductal region and during the different phases of the estrous cycle [5, 8], suggestive of a hypothalamopituitary control system. It is likely that the source of these catecholamines is the catecholaminergic nerve terminal, although dopamine and other catecholamines are present in follicular fluid under physiological conditions [9, 10]. However, expression of tyrosine hydroxylase in equine uterus and cervical epithelia has been reported [11], suggesting that it is potentially available during the first stretch of the sperm journey through the female reproductive tract.

Several types of neurotransmitter receptors have been found in spermatozoa [12], although the role of these receptors is unknown. We recently showed by RT-PCR, Western blot, and immunohystochemistry/cytochemistry that male germ cells and spermatozoa express DRD2 in a conserved manner [13]. This finding indicates that this receptor is a potential target for endogenous dopamine and antipsychotic drugs.

Capacitation is defined as the series of changes that spermatozoa normally undergo during their transit in the female genital tract to reach and bind the zona pellucida for the acrosome reaction and for fertilization of the oocyte [14, 15]. It is a complex and finely tuned process in which membranous, ionic, and metabolic alterations occur at specific times and locations and in which several components of signal transduction pathways are involved [14, 15].

The effect of catecholamines in sperm capacitation has been poorly studied. However, norepinephrine and epinephrine were reported to induce capacitation in mouse [16], hamster [17–19], and bull spermatozoa [20]. Catecholamines were also reported to increase the rates of spontaneous acrosomal reaction [17, 19, 20] and in vitro fertilization [16, 21].

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Our objectives in the present study were to investigate the presence of DRD2 in boar sperm and the effect of specific agonists on sperm survival and on important parameters such as sperm capacitation and motility. We also aimed to show the presence of a catecholaminergic phenotype in spermatozoa that could explain the effects of high catecholamine levels on phosphotyrosine patterns and sperm motility.

MATERIALS AND METHODS

Ethics

All procedures described herein were reviewed and approved by the Autonomous University of Barcelona Animal Care and Use Committee and were performed in accord with the Animal Welfare Law issued by the Catalan government (Generalitat de Catalunya, Spain).

Materials

Antibodies included the following: mouse monoclonal anti-β-tubulin (Sigma, St. Louis, MO); mouse monoclonal anti-phosphotyrosine PY20 (Transduction Laboratories, Lexington, KY); rabbit polyclonal anti-DRD2 (sc-9113) and anti-AKT [PKB] (v-akt murine thymoma viral oncogene homologue 1 [protein kinase B]) antibodies (rabbit anti-phospho-Ser^{473} [sc-7985], rabbit anti-phospho-Thr^{308} [sc-16646], goat anti-AKT, and goat anti-SLC6A3 [sc-7515]) (Santa Cruz Biotechnology, Santa Cruz, CA); and goat anti-mouse, goat anti-rabbit, and rabbit anti-goat horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Inc., Rockford, IL). We obtained MitoTracker Red CM-H_2XRos, as well as Alexa Fluor 488 and 568 conjugated to rabbit and mouse anti-IgG, respectively, from Molecular Probes (Eugene, OR). Dopamine, bromocriptine, and fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) were supplied by Sigma, and other analytical grade reagents were obtained from Merck (Darmstadt, Germany) and BioRad (Hercules, CA).

Sperm Preparation and Culture Conditions

Boar semen was obtained from eight healthy and mature animals (2–3 years old) from a commercial farm (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain). Samples were diluted to a concentration of 2×10^7 in a commercial extender for refrigerated semen (MR-A Extender; Kubus, S.A., Majadahonda, Spain) and distributed in 100-ml commercial artificial insemination receptacles that were sent to the laboratory of the Universitat Autònoma de Barcelona. All doses were kept at 16°C for approximately 90 min. Samples (six doses) were centrifuged at $600 \times g$ for 10 min at 16°C, and the pellets were washed three times in bovine serum albumin (BSA)-free medium. Spermatozoa were then resuspended in capacitating media [22–24]. All doses were mixed and pooled and then equally distributed into several tubes and incubated in the presence or absence of the agonist for 0–4 h at 38.5°C in cell incubators at 5% atmospheric CO_2. Ligands used for treatment were bromocriptine (100 nM) (a DRD2-specific agonist) and several concentrations of dopamine (100 nm, 10 μM, and 1 mM), and one set of tubes (without agonist) was used as a control. The capacitating media consisted of the following: 20 mM Hepes, 112 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 5 mM glucose, 21.7 mM L-lactate, 1 mM sodium piruvate, 29.7 μM $CaCl_2$, 36 mM $NaHCO_3$, and 0.6% BSA (pH 7.4, 230 mOsm).

Evaluation of the Achievement of Capacitation Status

Achievement of the capacitation status after incubation with the capacitating medium was assessed. This was performed after the following procedures on spermatozoa obtained at different incubation times:

Viability assessment (percentage). This percentage was assessed using eosin-nigrosin staining according to the technique by Bamba [25]. In this procedure, viable spermatozoa were observed as white on a pinkish-purple background, whereas nonviable sperm were stained a pinkish-red color and were located in specific areas or in the whole sperm cell. Viability percentages were established from a count of 200–300 spermatozoa in each sample.

Sperm acrosomal integrity assessment. The structural status of sperm acrosomes was assessed by FITC-PSA stain [26, 27]. In this staining, sperm aliquots were fixed and permeabilized for at least 30 min at 4°C in 100% methanol to allow entry of PSA. Permeabilized spermatozoa dried onto slides were then covered with a droplet of FITC-PSA (100 mg/ml) in PBS for 10 min. After repeated washing of the slide in bidistilled water, the spermatozoa were analyzed using epifluorescence (Zeiss Axioplan 2 microscope; Carl Zeiss Gmbh, Jena, Germany). In each case, sperm head scoring was performed in pairs to a count of up to 200 spermatozoa (100 on each slide), and results were analyzed in triplicate. Intense acrosomal staining was indicative of an intact acrosome. Spermatozoa showing any acrosome alteration had much fainter fluorescence in the sperm head or no demonstrable fluorescence.

Sperm motility assessment using a computer-assisted analysis system. The computer-aided sperm motility analysis (CASA) was performed using a commercial system (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). In this system, sperm samples were previously warmed at 37°C for 5 min in a water bath, and 5-μL aliquots of these samples were then placed on a warmed (37°C) slide and covered with a 22-mm^2 coverslip. Our CASA system was based on the analysis of 25 consecutive digitalized photographic images obtained from a single field at 200× magnification on a dark field. These 25 consecutive photographs were taken during a time lapse of 1 sec, implying an image-capturing velocity of one photograph taken every 40 milliseconds. Two to three separate fields were taken for each sample.

Sperm motility descriptors obtained after the CASA analysis included the following: curvilinear velocity (VCL) (the mean path velocity of the sperm head along its actual trajectory in micrometers per second), linear velocity (VSL) (the mean path velocity of the sperm head along a straight line from its first to its last position in micrometers per second), the mean velocity (VAP) (the mean velocity of the sperm head along its average trajectory in micrometers per second), linearity coefficient percentage ([VSL/VCL] × 100), straightness coefficient (STR) percentage ([VSL/VAP] × 100), wobble coefficient percentage ([VAP/VCL] × 100), the mean amplitude of lateral head displacement (the amplitude of lateral head displacement in relation to the VCL path and the VAP path in micrometers), and frequency of head displacement (the frequency with which the actual sperm trajectory crosses the average path trajectory in hertz).

After these parameters were obtained, total motility was defined as the percentage of spermatozoa showing a VAP exceeding 10 μm/sec. Progressive motility was defined as the percentage of spermatozoa showing an STR exceeding 45%.

Sperm motility data obtained from the CASA analysis were processed using the SAS statistical package (SAS Institute Inc., Cary, NC) [28]. Normality of data distributions was assessed using Shapiro-Wilks test, which is included in the UNIVARIATE procedure. This analysis includes the assumptions that all the data included in the same experimental group were congruent and that there were no significant differences among sperm of the same experimental group obtained in three independent experiments. Two-way ANOVA was performed using PROC GLM, followed by Tukey-Kramer test (TUKEY option of the LSMEANS statement) for multiple comparisons at $P < 0.05$. A total of 2996 spermatozoa, including all groups, were analyzed using this protocol. Statistical analyses were performed in a manner such that each experimental group included only those sperm that were analyzed in its specific experimental group.

Assessment of the overall tyrosine phosphorylation status. The tyrosine phosphorylation status of spermatozoa was evaluated using Western blot analysis after SDS-PAGE. This technique was applied as follows:

Step 1 comprised protein extraction from the boar sperm. Sperm proteins were isolated essentially according to the technique by Baker et al. [29], with some modifications. Specifically, aliquots (5×10^6 sperm) were taken before and during incubation at different times and treatments. Cellular suspensions were centrifuged ($600 \times g$ for 3 min) and then solubilized with 2% w/v SDS, 0.375 M Tris, pH 6.8, 10% sucrose, 10 mM PMSF, 10 mg/ml of leupeptin, 100 mM sodium orthovanadatum, and 1 mM NaF and heated at 100°C for 5 min. The homogenate was centrifuged at $20\,000 \times g$ for 10 min; the supernatant was retained and stored at −80 °C until used. Protein concentration was determined using the method by Bradford [30] with a commercial kit (BioRad).

Step 2 comprised SDS-PAGE and Western blotting. Samples were boiled for 1 min at 100°C in buffer containing 2% (v/v) 2-mercaptoethanol. The SDS-PAGE was conducted on 10 μg per line of proteins using 10% or 12% polyacrylamide gels at 100-mA constant current per gel. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Perkin Elmer, Boston, MA) at 400-mA constant current for 1 h. Membranes were blocked for 1 h at room temperature with Tris-buffered saline (TBS) (0.02 M Tris, pH 7.6, 0.15 M NaCl) containing 5% (w/v) BSA. Membranes were incubated at room temperature overnight with primary antibodies diluted in TBS containing 4% (w/v) BSA and 0.01% Tween-20. Antibody dilutions used were 1:2000 for anti-PY20 and anti-β-tubulin and 1:500 for anti-DRD2, anti-phospho-AKT-Ser^{473}, anti-phospho-AKT-Thr^{308}, anti-AKT-total, and anti-SLC6A3. After incubation, the membranes were washed four times for 5 min with TBS containing 0.03% Tween-20 and were incubated for 1 h at room temperature with horseradish peroxidase-conjugated second antibody at a concentration of 1:5000 in TBS containing 4% (w/v) BSA and 0.01% Tween-20. The membranes were washed as already described, and protein detection was performed using an ECL kit (Amersham Biosciences, Arlington Heights, IL).

Indirect Immunofluorescence Analysis

Indirect immunofluorescence was used to detect DRD2 and phosphoproteins at different intervals during the capacitating incubation. Aliquots of spermatozoa suspension were obtained (with or without treatment), spread onto slides, and fixed in 4% paraformaldehyde. The slides were blocked for 60 min at room temperature in 5% BSA and 0.3% Triton X-1000 PBS (pH 7.4). Slides were washed in PBS and incubated overnight at 4°C in a humidified camera with the following antibodies: anti-DRD2 (1:50), anti-PY20 (1:200), anti-phospho-AKT-Ser^{473} (1:200), anti-phospho-AKT-Thr^{308} (1:200), or anti-SLC6A3 (1:200). As negative controls for DRD2 immunoreaction, the slides were incubated with antibodies preabsorbed with the synthetic peptide that included the immunogenic region used to produce the antibody. Specifically, primary antibody was preincubated overnight at 48°C with a 10-fold molar excess of the synthetic peptide (Alpha Diagnostic International Inc., San Antonio, TX), and the preabsorbed antibody was used for immunofluorescent analysis. Spermatozoa were washed three times with PBS (pH 7.4) and were incubated for 60 min at 4°C with anti-rabbit IgG-Alexa Fluor 488 and/or anti-mouse IgG-Alexa Fluor 568 (both in 1:500 dilution). Finally, the slides were washed three times as already described and mounted. Stained cells were visualized and evaluated using confocal laser scanning fluorescence microscopy (Fluoview FV1000; Olympus, Tokyo, Japan). The images obtained were processed using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

Immunoprecipitation Analysis

Sperm samples treated with or without dopamine (100 nM) were washed twice in ice-cold PBS (pH 7.4), 1 mM PMSF, and 1 mM Na_2Vo_4. After centrifugation at 600 × *g*, the pellet was homogenized in lysis buffer with 150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM edetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM PMSF, 10 mg/ml of leupeptin, and 1 mM orthovanadatum. The lysate was precleared with protein A-sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) for 60 min at 4°C. For immunoprecipitation, equal amounts of protein (200 μg of total cell lysate) were incubated overnight at 4°C with 2 μg of rabbit polyclonal antibody anti-DRD2, followed by the addition of protein A-sepharose beads and incubated for a further 2 h at 4°C. Bound immune complexes were washed three times with lysis buffer containing protease and phosphatase inhibitors and detergents. The first supernatant fraction was stored for further analysis, and the pellets were solubilized in sample buffer. The supernatant and pellet proteins were separated using SDS-PAGE, transferred to a PVDF membrane (Millipore Corporation, Billerica, MA), and immunoblotted with PY20 mouse anti-phosphotyrosine monoclonal antibodies (1:2000).

Statistical Analysis

The viability and densitometric data reported herein are expressed as the mean (± SEM) of 10 and four independent samples, respectively. Statistical analyses were performed using Student *t*-test with Bonferroni correction for viability and variance analysis (ANOVA) and using Dunnett posttest for Western blotting densitometrics; we used GraphPad (GraphPad Software, Inc., San Diego, CA) for both analyses, and differences were considered significant and highly significant at $P < 0.05$ and $P < 0.01$, respectively.

RESULTS

Boar Spermatozoa Express DRD2, and Cellular Localization of These Receptors Depends on Capacitation State

The expression of DRD2 in rat, mouse, bovine, and human spermatozoa has been recently documented [13]. To confirm the presence of DRD2 in boar spermatozoa, we showed that Western blot analysis of extracted proteins demonstrated a single immunoreactive band of the expected molecular size (~50 kDa) (Fig. 1A). Moreover, we confirmed the specificity of the antibody by using proteins extracted from rat and mice brain. As negative controls, preabsorbed antibodies were used to probe Western blots (data not shown). Immunofluorescence analysis revealed that DRD2 in fresh spermatozoa is detectable in the flagellar region, mainly in the midpiece (Fig. 1C). After 2-h incubation in capacitating medium, DRD2 showed strong immunoreactivity in the acrosomal region (Fig. 1D, arrowheads). No changes in immunoreactivity were observed in the principal piece compared with that in fresh spermatozoa. The specificity of the reaction was confirmed in experiments that showed an absence of immunoreaction when preabsorbed antibodies were used (Fig. 1B).

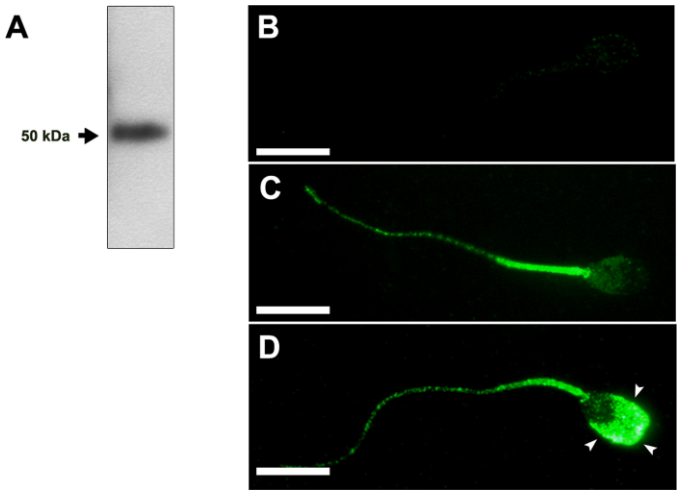

FIG. 1. Presence of DRD2 in boar sperm cells. **A**) Immunoblotting analysis of sperm extracts probed with polyclonal antibody against DRD2. Protein extracts (50 μg) were subjected to SDS-PAGE (12%), followed by immunoblotting. Note the specific band of the expected size (48–50 kDa). **B**–**D**) Indirect immunofluorescent localization of DRD2 in boar sperm. **B**) Negative control was incubated with preabsorbed antibody, and spermatozoa were capacitated at 0 h (**C**) and at 2 h (**D**). Both are representative images obtained from three independent determinations. After 2-h incubation in capacitating medium, DRD2 showed strong immunoreactivity in the acrosomal region (arrowheads). Bar = 10 μm.

Effect of Dopamine and Bromocriptine on Tyrosine Phosphorylation During Capacitation

To clarify the effect of bromocriptine and several concentrations of dopamine on boar sperm capacitation, the level of tyrosine phosphorylation was measured by Western blot analysis (Fig. 2). Spermatozoa incubated with bromocriptine showed an increase in the time course-induced tyrosine phosphorylation. This effect was observed in proteins of approximate molecular sizes of 180, 100, 60, 40/50, and 25 kDa. The concentration-response of dopamine revealed a biphasic effect on tyrosine phosphorylation. Thus, 100 nM and 10 μM dopamine increased the phosphorylation signal, mainly in proteins with a molecular mass between ~40 and ~58 kDa. In contrast, spermatozoa incubated with 1 mM dopamine showed a marked decrease in tyrosine phosphorylation except for phosphoproteins in the range between ~30 and ~45 kDa.

Localization and Association of DRD2 and Tyrosine Phosphorylated Proteins During Capacitation

To identify the subcellular localization of DRD2 in relation to phosphotyrosine immunoreaction, we performed a double immunofluorescence in noncapacitated and capacitated spermatozoa (Fig. 3A). Fresh spermatozoa showed immunoreactivity to PY20 antibody exclusively in the subequatorial region of the head (Fig. 3A, arrowheads), which was unrelated to immunostained DRD2 (Fig. 3A, arrows). However, the capacitated spermatozoa showed a strong phosphotyrosine immunoreaction in the acrosomal region and the anterior region of the midpiece (Fig. 3A). Remarkably, both DRD2 and PY20 had strong colocalization patterns in this region and in the anterior portion of the flagella (Fig. 3A). No immunoreaction

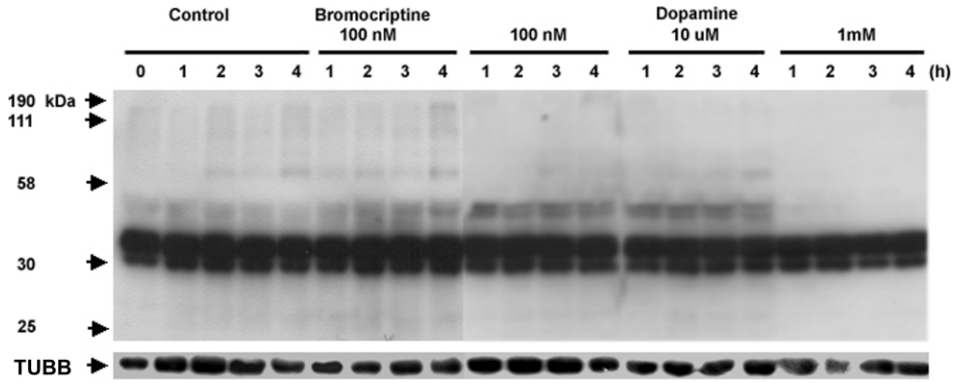

FIG. 2. Concentration-response effect of dopamine and bromocriptine on tyrosine phosphorylation during capacitation. Western blot analysis of proteins isolated from boar sperm incubated in capacitating medium at 38.5°C for the indicated times (0, 1, 2, 3, and 4 h). Sperm protein extracts (20 μg) were subjected to SDS-PAGE (10%) and were probed with PY20 anti-phosphotyrosine antibody. β-Tubulin (TUBB) was used as an internal control. The figure shows a representative Western blot from three independent replicates. uM, micromolar.

was observed when spermatozoa were probed in the absence of primary antibodies (data not shown). Considering that DRD2 is a target of serine and threonine and not tyrosine phosphorylation, we investigated if the colocalization pattern between DRD2 and tyrosine phosphorylated proteins is related to the presence of a complex. Coimmunoprecipitation analysis indicated that DRD2 is associated with tyrosine phosphorylated proteins in the range of 60–100 kDa after 2–4 h of incubation in the capacitating medium (Fig. 3B). This effect was apparently independent of dopamine receptor activation (Fig. 3B).

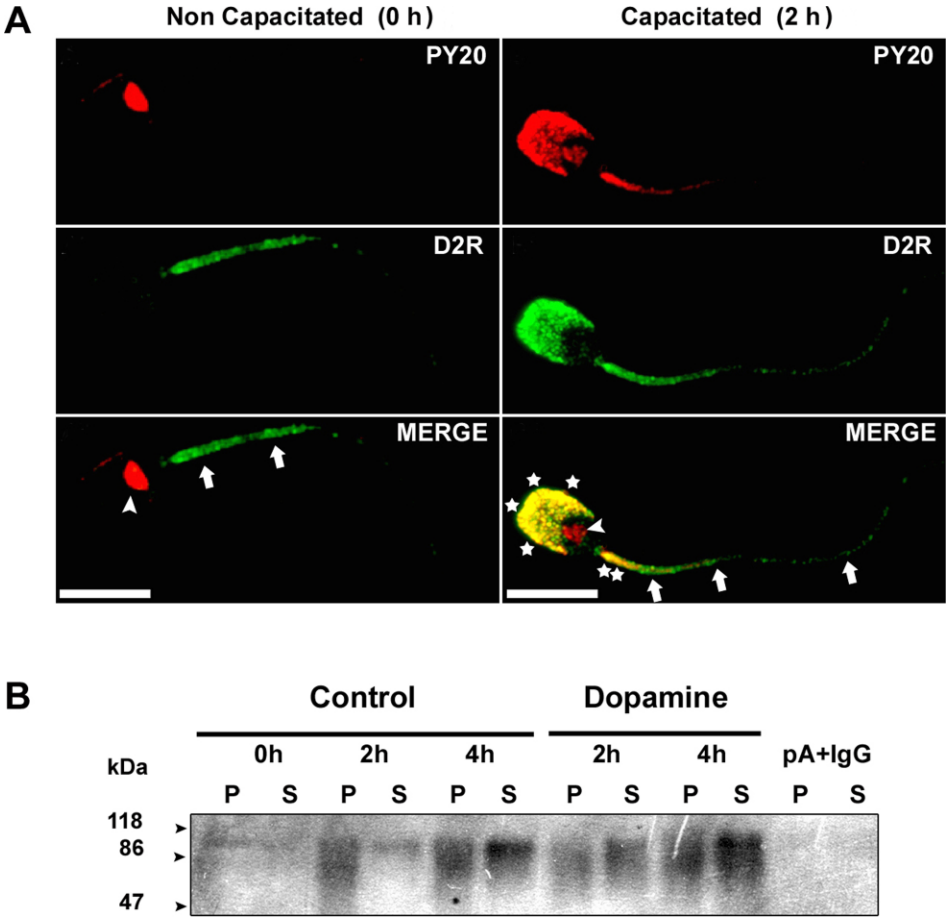

FIG. 3. Localization and association dynamics of DRD2 and phosphotyrosine proteins in noncapacitated and capacitated sperm. **A**) Double immunolocalization of DRD2 (D2R) and tyrosine phosphorylated proteins during capacitation. For immunofluorescence, noncapacitated and capacitated spermatozoa were spread onto coated slides and after 2 h were reacted with DRD2 antibody (rabbit) and PY20 (mouse) and visualized by confocal microscopy using anti-rabbit IgG-Alexa Fluor 488 (DRD2) and anti-mouse IgG-Alexa Fluor 568 (PY20). Note the strong appearance and colocalization of DRD2 and PY20 in the acrosome and midpiece after 2 h of capacitation (stars). Absence of colocalization is observed in fresh noncapacitated sperm and some discrete regions of capacitated sperm (arrowheads and arrows). No differences in the localization pattern were observed in capacitated sperm incubated with 100 nM dopamine (data not shown). Bar = 10 μm. **B**) Coimmunoprecipitation of DRD2 and tyrosine phosphorylated proteins during sperm capacitation. Total proteins isolated from boar spermatozoa were immunoprecipitated with rabbit polyclonal anti-DRD2 antibody and protein A-sepharose and resolved by SDS-PAGE. They were then transferred to PVDF membranes and probed with mouse monoclonal anti-phosphotyrosine (PY20) antibodies, followed by incubation with a secondary antibody coupled to peroxidase and analyzed using the ECL system. Pellet (P) and supernatant (S) fractions of the immunoprecipitation are shown for each time and treatment. There was no reaction when protein A-sepharose with IgG (rabbit anti DRD2) was loaded as a control. pA+IgG, protein A-sepharose with IgG.

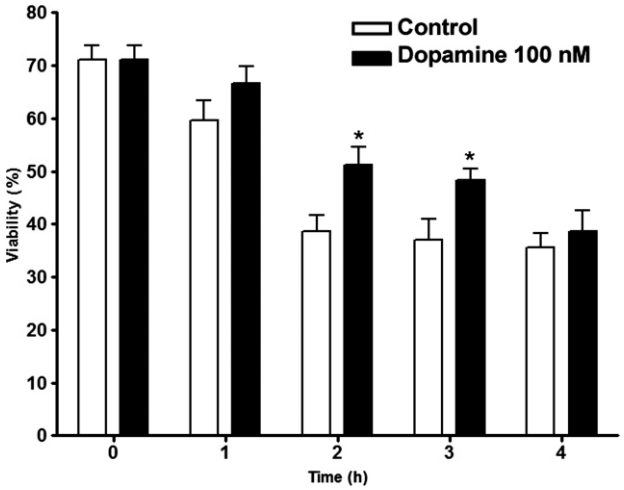

FIG. 4. Effect of 100 nM dopamine on sperm viability during capacitation. Viability was determined by eosin-nigrosin staining immediately after the capacitation experiment in the absence and presence of 100 nM dopamine at the indicated times. Each bar represents the mean ± SEM of 10 experiments. A minimum of 100 spermatozoa were counted for each assay. *Significant differences between treatment with and without dopamine at the respective time ($P < 0.01$, Student *t*-test).

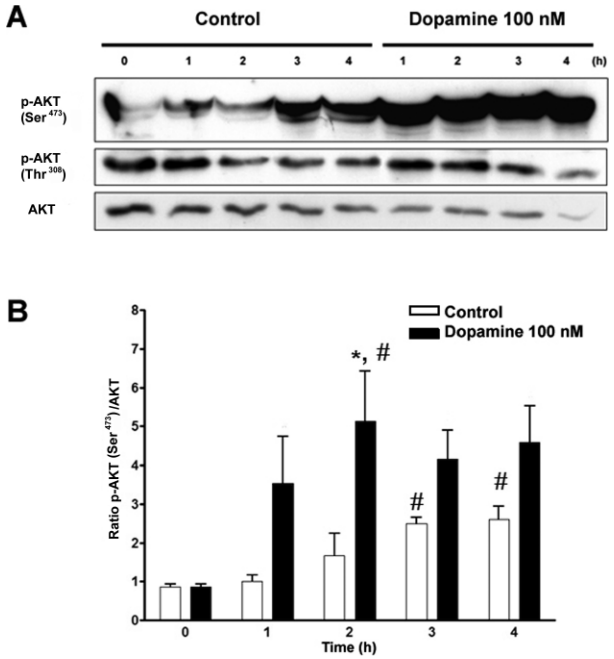

FIG. 5. Effect of dopamine (100 nM) on AKT activation state during sperm capacitation. Western blot of proteins isolated from boar sperm and incubated at 38.5°C for the indicated times (0, 1, 2, 3 and 4 h) in capacitating medium in the presence or absence of dopamine (100 nM). **A**) Protein extracts (20 μg) were subjected to SDS-PAGE (10%) and were probed with phospho-AKT (Ser^{473}), phospho-AKT (Thr^{308}), and AKT antibody by immunoblot analysis. The panel shows a representative Western blot from four independent replicates. **B**) Densitometry analysis of normalized data with total AKT (only for phospho-AKT [Ser^{473}]). Each bar represents the mean ± SEM of four independent experiments. *Significant difference ($P < 0.05$, one-way ANOVA-Dunnett posttest) compared with control treatment. #Significant difference ($P < 0.05$, one-way ANOVA-Dunnett posttest) relative to the starting time.

Dopamine Increases Sperm Viability and Phosphorylation of AKT (Ser^{473}) During Capacitation

Next, we investigated the effect of dopamine on cellular viability during achievement of the in vitro capacitation using the eosin-nigrosin staining procedure [25]. Our results showed that treatment with 100 nM dopamine significantly ($P < 0.01$) decreased or shifted the apparition of dead spermatozoa (positive to staining with eosin-nigrosin) at 2 and 3 h of capacitation compared with the respective controls. No other significant effects were observed (Fig. 4).

The classic protein kinase implicated in survival or viability in several cellular types is phosphoinositide 3-kinase (PIK3), and the best-characterized downstream effector of the PIK3 pathway is AKT/PKB. To test whether AKT was related to the achievement of the in vitro sperm capacitation and viability, we analyzed phosphorylation of the two AKT-specific phosphorylation residues (Ser^{473} and Thr^{308}) in a time course capacitation experiment (Fig. 5A). Our results showed that in noncapacitated spermatozoa (0 h) AKT was phosphorylated at both specific phosphorylation sites. Following this, the AKT phosphorylation signal increased, mainly in the Ser^{473} residue, throughout the capacitation period. Treatment with dopamine (100 nM), however, induced early, strong, and sustained phosphorylation of AKT at the Ser^{473} residue. Densitometric analysis (Fig. 5B) revealed that dopamine significantly ($P < 0.05$) increased these phosphorylation signals at 2 h ($P < 0.05$). The effect observed in AKT clearly correlated with the membrane integrity assessment using eosin-nigrosin staining. No differences were observed in relation to phospho-Thr^{308} (data not shown).

Differential Subcellular Localization of Phospho-AKT According to Specific Phosphorylation Site During Capacitation

To visualize and localize the two phosphorylation sites for AKT in spermatozoa, immunofluorescence for AKT (phospho-Ser^{473} and phospho-Thr^{308}) during capacitation was performed. Our results showed that phospho-AKT was localized in different regions according to the specific phosphorylation sites (Fig. 6). At the beginning, the phospho-Thr^{308}-AKT is mainly localized in the head of spermatozoa, specifically in the acrosomal and subequatorial region, and shows increased immunoreactivity in the midpiece (arrowhead) throughout capacitation (2 and 4 h). In contrast, staining of phospho-Ser^{473}-AKT was restricted to the principal piece and the midpiece anterior region, colocalizing with phospho-Thr^{308}-AKT (Fig. 6, arrows). In addition, in fresh spermatozoa AKT was phosphorylated (Ser^{473} and/or Thr^{308}) in the head (acrosome and subequatorial regions), neck, midpiece, and principal piece. Following incubation (2–4 h) in the capacitation media, spermatozoa showed an increased intensity of phosphorylated AKT in the mitochondrial region, which was accompanied by loss of phosphorylation in the acrosomal region (Fig. 6).

Effects of DRD2 Agonists on Sperm Motility During Incubation in Capacitating Medium

Incubation with different concentrations of dopamine and with bromocriptine (100 nM) induced very few changes in the incubation time-dependent evolution of motion parameters in sperm incubated in the capacitation medium (data not shown). However, some important effects were seen in the time-dependent evolution of the percentage of total motility. As summarized in Table 1, incubation in the capacitating medium induced a progressive decrease in the percentage of total motility of control samples from 57.8% ± 0.3% at Time 0 to 31.0% ± 0.3% after 4-h incubation. Addition of lower

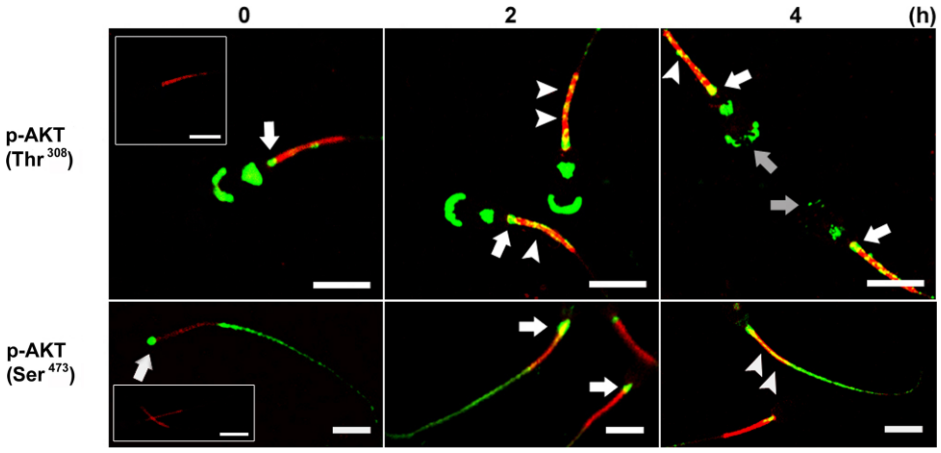

FIG. 6. Subcellular localization of phospho-AKT during sperm capacitation. Confocal immunofluorescent localization of phospho-AKT (Ser^{473}) and phospho-AKT (Thr^{308}) under capacitation conditions (0, 2, and 4 h). Spermatozoa were prestained with MitoTracker Red CM-H2XRos (red), and both phospho-AKTs (rabbit) were detected with secondary antibody IgG-Alexa Fluor 488 (green). Note that colocalization of both activated states was limited to discrete points on the anterior region (arrows in the neck) of the midpiece (arrowheads), as well as the loss of the immunoreactive pattern of AKT (Thr^{308}) in the acrosome region at 4 h of incubation (grey arrows). The inset at Time 0 corresponds to a negative control in the absence of primary antibody for both immunoreaction analyses. Bars = 10 μm.

dopamine concentrations and incubation in the presence of bromocriptine (100 nM) had only small and isolated significant effects on the time-dependent evolution of total motility. Sperm incubation in the capacitation medium in the presence of dopamine (1 mM) induced a significant ($P < 0.05$) further decrease in the percentages of total and progressive motility from 57.8% ± 0.3% and 47.1% ± 0.2%, respectively, at Time 0 to 25.5% ± 0.3% and 19.9% ± 0.3%, respectively, after 4-h incubation (Tables 1 and 2).

Boar Spermatozoa Have a Catecholaminergic Phenotype

To explain the effects of high concentrations of dopamine and other catecholamines on tyrosine phosphorylation and sperm motility, the presence of the dopamine transporter (DAT, officially known as solute carrier family 6 [neurotransmitter transporter, dopamine], member 3, SLC6A3) in boar spermatozoa was evaluated using Western blot analysis and indirect immunolocalization. Western blot analysis with polyclonal antibodies revealed a major immunoreactive band migrating with an apparent molecular weight of 75–80 kDa (Fig. 7A). Immunolocalization analysis showed strong SLC6A3 immunostaining on the membrane in the principal piece of the flagella (Fig. 7, B and C). SLC6A3 immunoreactivity was confirmed in human, horse, and mouse spermatozoa (data not shown), suggestive of conserved expression and function in these cell types.

DISCUSSION

The first evidence suggesting the presence of DRD2 in spermatozoa was a radiobinding study [31] with ^{3}H-spiperone in rabbit sperm. However, dopamine and other catecholamines have been detected in several compartments of the sperm's journey through the female reproductive tract at the time of fertilization, specifically in seminal fluids and oviduct [4, 7]. We recently demonstrated that rat male germ cells and spermatozoa from rat, mouse, bull, and human express DRD2 [13]. Using immunocytochemistry and Western blot analysis, we show herein that DRD2 is present in boar sperm, confirming that its presence is highly conserved in mammalian sperm. Moreover, DRD2 localization is dynamic and depends on the capacitation status (Fig. 1, C and D).

Protein phosphorylation and dephosphorylation are universal biochemical mechanisms used in the control of protein function. Changes in tyrosine phosphorylation following changes in the levels of cAMP and activation of PKA have been proposed as the key mechanisms regulating sperm functions such as the initiation and regulation of motility and the regulation of sperm capacitation and the acrosome reaction [32–34].

Our evidence indicates that dopamine (100 nM) has an effect on sperm capacitation, specifically on tyrosine phosphorylation (Fig. 2), but not on the acrosomal reaction (data not shown). Bromocriptine (100 nM), a specific agonist of DRD2, clearly induced tyrosine phosphorylation of a wide range of molecular weights without affecting the total and progressive motility in relation to control treatment (Tables 1 and 2). However, dopamine concentration-response assays suggested that high dopamine concentrations (1 mM) strongly decreased the tyrosine phosphorylation pattern and sperm motility by a transporter-mediated mechanism for catecholamine uptake. These changes in relation to controls were detected from 3 h of treatment for total motility and from the first hour of incubation for progressive motility. Thus, the inhibitory effects on tyrosine phosphorylation and motility may be mediated by an excess of dopamine oxidation products. Dopamine is known to be toxic in vitro [35, 36] and in vivo [37, 38]. In neurodegenerative disorders, the degeneration of catecholaminergic neurons has been attributed to increased cytosolic levels of dopamine and its oxidized metabolites [37–40].

TABLE 1. Comparative kinetic analysis of total sperm motility during capacitation in the presence of dopamine and bromocriptine.[a]

Time (h)	Control	Bromocriptine (100 nM)	Dopamine (100 nM)	Dopamine (10 μM)	Dopamine (1 mM)
0	57.81 ± 0.30^{b,c}	57.81 ± 0.30^{b,c}	57.81 ± 0.30^{b,c}	57.81 ± 0.30^{b,c}	57.81 ± 0.30^{b,c}
1	40.27 ± 0.27^{b,d}	37.90 ± 0.30^{e,d}	41.19 ± 0.29^{b,d}	37.14 ± 0.28^{c,d}	34.74 ± 0.32^{f,d}
2	27.06 ± 0.46^{b,g}	42.95 ± 0.27^{e,g}	34.79 ± 0.36^{f,g}	36.13 ± 0.35^{f,d}	26.91 ± 0.36^{b,g}
3	36.06 ± 0.27^{b,h}	29.90 ± 0.33^{e,h}	28.57 ± 0.31^{e,h}	33.21 ± 0.43^{b,g}	21.88 ± 0.35^{f,h}
4	31.00 ± 0.34^{b,i}	31.10 ± 0.30^{b,h}	31.51 ± 0.25^{b,i}	28.79 ± 0.33^{b,h}	25.45 ± 0.30^{e,g}

[a] Results are given as percentages of the total number of spermatozoa that demonstrated a VCL >20 μm/sec. Values are expressed as mean ± SEM of three different experiments.

[b–i] Different superscript letters indicate significant differences between treatments and treatment times ($P < 0.05$).

TABLE 2. Comparative kinetic analysis of sperm progressive motility during sperm capacitation in the presence of dopamine and bromocriptine.[a]

Time (h)	Control	Bromocriptine (100 nM)	Dopamine (100 nM)	Dopamine (10 μM)	Dopamine (1 mM)
0	47.13 ± 0.22[b,c]	47.13 ± 0.22[b,c]	47.13 ± 0.22[b,c]	47.13 ± 0.22[b,c]	47.13 ± 0.22[b,c]
1	32.18 ± 0.25[b,d]	28.69 ± 0.28[e,d]	33.83 ± 0.27[b,d]	29.02 ± 0.27[e,d]	28.96 ± 0.30[e,d]
2	29.09 ± 0.43[b,f]	35.26 ± 0.26[e,f]	31.30 ± 0.34[b,f]	30.10 ± 0.33[b,d]	19.12 ± 0.34[g,f]
3	30.44 ± 0.25[b,f]	22.02 ± 0.32[e,h]	24.05 ± 0.29[e,h]	25.21 ± 0.40[e,f]	17.74 ± 0.33[g,f]
4	28.00 ± 0.32[b,h]	28.68 ± 0.28[b,d]	30.16 ± 0.24[b,f]	25.08 ± 0.31[e,f]	19.87 ± 0.28[i,f]

[a] Results are given as percentages of the total number of spermatozoa that demonstrated a STR >45%. Values are expressed as mean ± SEM of three different experiments.
[b–i] Different superscript letters indicate significant differences between treatments and treatment times ($P < 0.05$).

The activation of DRD2 is classically associated with a decrease in cAMP levels [41]. However, recent studies associate DRD2 with $G_{\alpha s}$ protein when the cell is coexpressing other G protein-coupled receptors, specifically the cannabinoid receptor CB1 [42] present in human [43] and boar [44] spermatozoa. In heterologous expression systems, it has been shown that the molecular mechanism in the versatile cAMP-signaling pathway involves a dynamic hetero-oligomeric interaction [45]. Modulation of the interaction of the two receptors could affect cAMP levels before and after sperm capacitation begins in a manner that may be independent of the presence of progesterone. Future studies are needed to clarify this point because both dopamine and the endocannabinoid anandamide have been detected in the oviduct [5–8, 46].

Results of previous studies suggested that catecholamines have a role in the later stages of sperm capacitation. Specifically, epinephrine (0.5–50 μM), norepinephrine (50 μM), and isoproterenol (20 μM) increase the number of hamster sperm with hyperactivated whiplash motility [17]. These agonists do not increase the percentage of motile cells but increase the proportion of spontaneous acrosomal reactions, likely associated with toxicity. At physiological catecholamine concentrations (40–80 nM), norepinephrine but not epinephrine increased lysophosphatidylcholine-induced acrosome reactions of bull sperm and increased the proportion of spontaneous acrosomal reactions in cells capacitated for 2 h in the presence of heparin. Curiously, norepinephrine induced sperm capacitation at 3.1 μM and inhibited sperm capacitation at 3.1 mM [20]. Other findings suggest that catecholamines inhibit the soluble form of testis adenylate cyclase and propose that the high-concentration effect may be mediated by cytosolic catechols [47].

Recently, pharmacological evidence has demonstrated that norepinephrine and epinephrine increase the speed of flagellar beat by a nonreceptor-mediated mechanism [48]. The mechanism by which catecholamines induce these effects in sperm is unknown. One explanation could be that catecholamine agonists (<100 μM) might act as inhibitors of phosphodiesterase to increase accumulation of the cAMP that is produced by basal soluble adenylyl cyclase activity, as suggested by Goren and Rosen [49]. If that is the case, catecholamines would be incorporated into the cytosol by sodium- and chloride-dependent transport mechanisms. Together with our results, this strongly suggests the presence of at least one of the following monoamine transporters: SLC6A3, norepinephrine transporter (NET, officially known as solute carrier family 6 [neurotransmitter transporter, glycine], member 5, SLC6A5), or serotonin transporter (SERT, officially known as solute carrier family 6 [neurotransmitter transporter, serotonin], member 4, SLC6A4). However, none of these has been described in sperm, to our knowledge. Studies [50–54] have evaluated radiobinding and the effects of SLC6A3 and SLC6A5 inhibitors such as cocaine and amphetamine in testis or spermatozoa. We show herein by Western blot (Fig. 7A) and immunocytochemical analyses (Fig. 7, B and C) that the dopamine transporter is present in boar spermatozoa. To our knowledge, this is the first evidence showing that sperm cells have a catecholaminergic phenotype and that sperm are a sensitive cellular target for cocaine, amphetamine, and antidepressive and antipsychotic drugs. SLC6A3 immunoreactivity seen in other mammalian spermatozoa was confirmed in human, mouse, bull, and horse, suggestive of conserved expression and function of SLC6A3 in these cells (Supplemental Figure 1 available at www.biolreprod.org).

In neurons, the mechanism by which dopamine or its analogues are neurotoxic is linked to dopamine uptake into the neuron and subsequent interaction with mitochondrial structures, or extracellularly by oxidizing membrane lipids [55]. Several processes may be involved in the toxic effect of intracellular dopamine in the neuron, including induction of oxidative stress [56, 38], apoptosis [57–59], and interference with mitochondrial respiration [60]. In sperm cells, it is likely that these effects are related to the effect of a high concentration of dopamine on tyrosine phosphorylation and motility. Further studies are needed to confirm and specify SLC6A3 functionality in spermatozoa.

How does dopamine increase cell viability at nanomolar concentrations? DRD2 activation induced survival in several cell models via the AKT pathway [61–64]. Our findings show that dopamine (100 nM) increases the number of viable spermatozoa at 2 h of capacitation and increases AKT phosphorylation at Ser^{473}. The correlation observed between viability and AKT phosphorylation suggests that dopamine may have an important role in viability at early stages of the capacitation process and in motility.

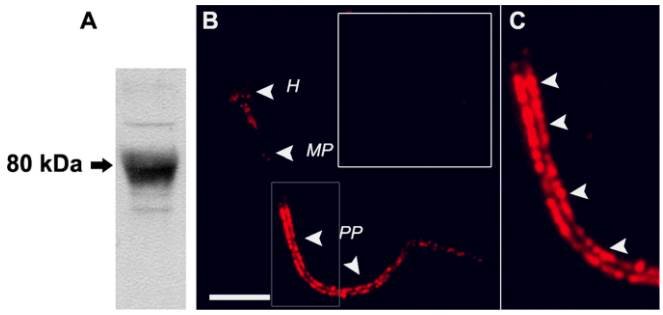

FIG. 7. Presence of dopamine transporter (SLC6A3) in boar sperm. **A**) Immunoblotting analysis of boar sperm extracts probed with polyclonal antibody against dopamine transporter. Protein extracts (50 μg) were subjected to SDS-PAGE (10%), followed by immunoblotting. Note the specific band of the expected size (75–80 kDa). **B**) Indirect immunofluorescent SLC6A3 localization in boar sperm. The inset in B corresponds to a negative control in the absence of primary antibody. **C**) High magnification of a region of the principal piece shown in **B**. H, head; MP, midpiece; PP, principal piece; arrowheads, location of the positive signal; bar = 10 μm.

AKT is classically described as a prosurvival serine/threonine kinase that is activated in response to trophic factors and is generally associated with a protective role, with upstream transactivation of PIK3 (PI3K) after DRD2 activation and AKT phosphorylation [61, 63]. Inhibitors of PIK3 enhanced motility in human [65] and boar [66] spermatozoa. However, the AKT inhibitor IV (Calbiochem, San Diego, CA) absolutely and instantly suppressed motility but not tyrosine phosphorylation (data not shown). We show herein that dopamine (100 nM) increases viability, AKT phosphorylation, and total and progressive motility. This suggests that AKT, but not PIK3, is necessary for total and progressive motility and that likely in spermatozoa AKT is phosphorylated by a PIK3-independent activation mechanism, similar to that described for striatal neurons [62]. A transactivation mechanism between DRD2 and epidermal growth factor receptor (EGFR) has been proposed [63] that would involve participation of a protein-signaling complex harnessing DRD2, EGFR, and c-src (v-src sarcoma [Schmidt-Ruppin A-2] viral oncogene homologue [avian]) kinase [64]. Using coimmunoprecipitation assays, we demonstrated that DRD2 increases its association with unknown tyrosine phosphorylated proteins during the capacitation period (Fig. 3B) independent of DRD2 activation, as the coimmunoreactivity pattern (pellet fraction) did not show differences between treatments with and without dopamine. In addition, DRD2 and tyrosine phosphorylated proteins have a dynamic pattern of localization. In noncapacitated sperm cells, DRD2 is localized in the flagella, whereas tyrosine phosphorylated proteins are mainly circumscribed to the postequatorial area of the sperm head. However, DRD2 is preserved in the flagella and strongly increases immunoreactivity in the acrosomal region of sperm heads during capacitation. Phosphotyrosine proteins increase in the midpiece and the acrosomal region, colocalizing with DRD2 (Fig. 3A). A similar change in the localization pattern of tyrosine phosphorylated proteins was described previously in pig spermatozoa incubated in noncapacitation and capacitation media [23]. The increase in DRD2 immunoreactivity in the acrosomal region can be explained by the exposure of DRD2 epitopes in spermatozoa undergoing the capacitation process, characterized by loss of cholesterol and by changes in the architecture of the plasma membrane [67]. However, we cannot rule out de novo translation of proteins, possibly by association of sperm mRNAs with the mitochondrial translation machinery [68]. Coimmunoprecipitation and immunocytochemical analyses suggest the presence of a signaling complex that contains DRD2 and tyrosine phosphorylation target proteins. Further studies are required to clarify this possibility, as well as the role of EGFR in mammalian sperm [69, 70]. The AKT activation (phosphorylation) mechanism in spermatozoa is unknown, and its molecular effectors could be involved in sperm viability and motility. The staining pattern of phospho-AKT obtained by immunocytochemical analysis was present in several sperm compartments, suggesting a possible function in acrosome reaction, energetic control, and motility.

We conclude that the effect of dopaminergic agonists on sperm function occurs at two levels. First, bromocriptine (100 nM) and dopamine (<10 μM) increase viability, tyrosine phosphorylation, and motility by DRD2 activation. Second, high concentrations of dopamine (1 mM) decrease tyrosine phosphorylation and motility by a mechanism involving dopamine uptake by a dopamine transporter, by cytosolic accumulation of dopamine, or by enzymatic oxidation or auto-oxidation of dopamine.

In summary, dopamine seems to act as a physiological modulator of viability, capacitation, and sperm motility. Thus, dopamine may be a physiological modulator involved in the control of in vivo fertilization. Furthermore, the presence of a dopaminergic system in spermatozoa (SLC6A3 and DRD2) may help to explain reproductive disorders linked to addictive syndromes such as drug (cocaine or amphetamine) addiction, as well as for antipsychotic drug use.

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