

Mechanism of hCG-induced spermiation in the toad *Rhinella arenarum* (Amphibia, Anura)

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ABSTRACT

In *Rhinella arenarum* spermiation occurs as a consequence of LH/FSH increase during the amplexus or by a single dose of hCG, among other gonadotropins. The present study employs an *in vitro* system to study the mechanism of action of hCG in the spermiation of *R. arenarum*. Testicular fragments were incubated for 2 h at 28 °C in the presence or absence of 20 IU hCG with or without different PKA/PKC inhibitors and activators as well as ouabain and amiloride as Na⁺/K⁺ ATPase and transcellular Na⁺ transport inhibitors, respectively. Ouabain did not induce spermiation in absence of hCG and inhibited hCG-induced spermiation in a dose-dependent manner, reaching 90% inhibition with the higher concentration. In contrast, amiloride neither affected spermiation nor steroidogenesis. Activation of PKA with 8Br-cAMP induced spermiation in the absence of hCG while its inhibition with H89 blocked hCG action. On the other hand, PKC inhibition with Bi or STP did not affect hCG-induced spermiation although PKC activation significantly decreased hCG-dependent sperm release. These results suggest that PKC inhibits spermiation but also that the inhibition exerted by the kinase could be blocked by hCG. Taken together, these observations could indicate that PKA is involved in the mechanism of the gonadotropin action, mechanism also requiring the activation of a non-pumping Na⁺/K⁺ ATPase pathway.

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1. Introduction

In vertebrates, testes are organised in two separate compartments, the seminiferous tubules/lobules containing germ cells and Sertoli cells, and the interstitial compartment with Leydig cells and blood vessels, among others. Amphibian testicular organization as well as the interactions between Sertoli cells and germ cells is different on several levels when compared with other vertebrates. Spermatogenesis occurs in a structure called spermatocyst or cyst and the spermiation process includes the breakage of cyst wall and the separation of spermatozoa from Sertoli cells. In anurans, the cyst wall breaks down before spermiation occurs. The open cysts form the wall of the seminiferous lobule, with a layer of Sertoli cells. Spermiation takes place when Sertoli cells take up fluid, resulting in the swelling of the apical cytoplasm containing mature spermatids (Pudney, 1998).

The role of the pituitary in amphibian spermiation was studied by several authors (Galli-Mainini, 1947; Kobayashi et al., 1993; Pozzi and Ceballos, 2000; Pozzi et al., 2006; Cobellis et al., 2008). For instance, during amplexus in *Bufo japonicus*, plasmatic concen-

trations of LH and FSH are increased (Ishii and Itoh, 1992). However, it is difficult to ascertain which of the two gonadotropins is responsible for inducing spermiation. In *Rana catesbeiana*, LH and FSH are also secreted simultaneously, with a higher concentration of plasmatic FSH than LH (Licht et al., 1983). Nevertheless, even if the importance of pituitary gonadotropins for spermiation is accepted (Nagahama, 1986; Parvinen et al., 1986), little is known about the mechanisms whereby gonadotropins induce this process. It has been suggested that 17,20 α -dihydroxy-4-pregnen-3-one (17,20 α DP) is the naturally occurring spermiation-inducing hormone in the testis of the frog *Rana nigromaculata* (Kobayashi et al., 1993). Moreover, *R. esculenta* is a good model to study the steroids biosynthesis dependent spermiation, and it is demonstrated that estrogens and pituitary hormones influence different steps in sperm release as spermatogenesis, spermiation and sperm transport (Cobellis et al., 2008). However, in *Rhinella arenarum* (Pozzi and Ceballos, 2000) as well as in *Rana catesbeiana* and *Leptodactylus ocellatus* (Rosemblit et al., 2006) hCG-induced spermiation does not depend on steroid biosynthesis. In addition, testes from *R. arenarum* do not produce estradiol.

In *R. arenarum*, it was demonstrated that spermiation is more efficiently induced by human FSH than for human LH and hCG (Pozzi et al., 2006). Moreover, in the toad, human recombinant FSH could elicit spermiation by acting directly on Sertoli cells since it was demonstrated that iodinated hrFSH binds to a population-cell

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resembling Sertoli cells (Pozzi et al., 2001). In other amphibian species as well, it has been proposed that mammalian FSH mainly exerts its actions on Sertoli cells (Ji et al., 1995; Ito and Abé, 1999; Yamamoto et al., 2001; Yazawa et al., 2001). In *R. arenarum*, it was also suggested that both spermiation and androgen secretion be mediated by different receptors in Sertoli and Leydig cells, respectively (Pozzi et al., 2006).

Several years ago, Burgos and Vitale-Calpe (1967a,b) suggested that during toad spermiation there is a swelling of the Sertoli cells terminal cytoplasmic processes, resulting in the evagination of the lacunae that housed spermatids, thereby shedding them into the lumen of the lobule. Authors also demonstrated that gonadotropins induced a significant increment in testicular water and sodium content as well as a decrease in the activity of Na^+/K^+ ATPase, suggesting that the gonadotropin-elicited inhibition of the sodium pump could be involved in the mechanism of gonadotropin-induced spermiation.

Na^+/K^+ ATPase is an integral membrane protein found in most mammalian cells. It was initially discovered as an energy transducing ion pump that transports 3 Na^+ out and 2 K^+ into the cell by hydrolysis of one molecule of ATP (Kaplan, 2002). Because of the importance of the Na^+/K^+ ATPase in many cellular processes, its regulation has been acutely studied. In vertebrates, there are a lot of evidences about the direct and indirect regulation of the ATPase by protein-kinases like PKA and PKC, but until now this regulation seems to be tissue-specific and depending of the isoform and the organism. Interestingly, several evidences have demonstrated the existence of a non-canonical action of the Na^+/K^+ ATPase which does not involve pumping ions. This non-canonical function depends on the interaction of the Na^+/K^+ ATPase with various proteins including lipid kinases, membrane transporters, channels, and cellular receptors (Tian et al., 2006). Apparently, Ouabain binds with the same affinity to the non-pumping Na^+/K^+ ATPase and triggers various phosphorylation cascades and second messengers. In this situation, Ouabain could act as a Na^+/K^+ ATPase activator and not as an inhibitor (Liang et al., 2007).

It has been established that hCG stimulates progesterone production in rat granulose cells and increases granulose cells cAMP content. However, this process is diminished by Digoxin and Digitoxin but not by Ouabain (Na^+/K^+ ATPase inhibitor), suggesting that the synthesis of progesterone has a Na^+/K^+ ATPase independent mechanism (Chen et al., 2001). Digoxin inhibits the synthesis of testosterone by a mechanism involving a decrease in both activity of the cytochrome P450scc cyclic AMP production and cytochrome P450scc activity (Lin et al., 1998).

In addition, a model for a junctional protein complex between Sertoli cell and elongating spermatids has been described in mammals, suggesting that the phosphorylation of these proteins by several kinases is a central control of mammalian spermiation (Chapin et al., 2001). This complex involves multiple proteins such as integrins, catenins, actin and vinculin, and a large variety of kinases, having structural and functional functions (Chapin et al., 2001).

Taking into account the investigation of Russo and Burgos (1969) about the role of the sodium pump in the spermiation and its regulation by PKA and PKC as well as the proposal in mammals that these kinases are involved in spermiation, the main purpose of this paper is to analyze the effect of Ouabain as Na^+/K^+ ATPase inhibitor in the process of spermiation and to study the effects of PKA and PKC on hCG-induced spermiation and steroids biosynthesis.

2. Materials and methods

2.1. Materials

All radioinert steroids, ouabain, phorbol-12-myristate-13-acetate and 8Br-cAMP were from Sigma-Aldrich Inc. (St. Louis, MO)

and [^3H]testosterone (3418.8 GBq/mmol) were from NEN (Boston, MA). Staurosporine (STP), bisindolylmaleimide I (Bi), phorbol-12-myristate-13-acetate (PMA) and H89 were from Calbiochem (Merck, Darmstadt, Germany). Human chorionic gonadotropin (hCG) was from Elea Laboratory (Buenos Aires, Argentina). All other chemicals were of reagent grade.

2.2. Animals

Reproductive male toads of *R. arenarum* were collected near Buenos Aires City during all year long. Animals were maintained with free access to water and fed with crickets, diet being supplemented with liver. For tissue preparation, animals were anaesthetized with MS222 in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of The Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina. Testes were rapidly excised; placed in ice-cold saline; and fat bodies, mesorchia, and Bidder's organ were removed.

2.3. Incubation system

Both testes from individual toads were cut with scissors into homogenous small pieces weighing approximately 20 mg each. Slices were individually transferred to culture plates containing 1 ml Krebs–Ringer–glucose–Hepes solution (KRGH), pH 7.4 and pre-incubated for 30 min in order to remove unbound spermatozoa. After pre-incubation, media were replaced with KRGH and submitted to different treatments as described below. Incubations were performed during 2 h at 28 °C (Pozzi and Ceballos, 2000). Within each experiment both testes from each toad received all the treatments (for statistical analysis see below).

2.4. Effect of different activators and inhibitors

Testicular slices were incubated with or without 20 UI of hCG according to Pozzi and Ceballos (2000) (Elea, Argentina) in the presence or absence of Ouabain (0.01, 0.1, 1 mM) as a Na^+/K^+ ATPase inhibitor; amiloride (1, 10, 100 μM) as inhibitor of transcellular Na^+ transport; STP (0.1, 1, 10, 100 nM) and Bi (1, 10, 100 nM) as PKC inhibitors; PMA (1, 10 μM) as PKC activator; H89 (0.01, 0.1, 1 μM) as a PKA inhibitor; 8Br-cAMP (0.1, 0.5, 1 mM) as PKA activator.

2.5. Spermiation assay

After incubation, 100 μl medium was separated to evaluate spermiation by counting spermatozoa with a Neubauer chamber. Results were expressed as the number of spermatozoa/ml incubation medium.

2.6. Testosterone measurement

The effect of hCG and Ouabain on steroid biosynthesis was analyzed by assaying testosterone by radioimmunoassay in incubation media. Testosterone antibody was from The Colorado State University (Co, USA) and was employed in a dilution of 1:125,000. The buffer employed was 10 mM PBS, 1% gelatin, 20 mM EDTA, pH 7.4. Dilutions were 1:4 for tissue and 1:5 for incubation media. The cross-reactivity of testosterone antibody with dihydrotestosterone was 15%. The sensitivity of the assay was 10 pg/ml. Steroids were assayed in triplicate. Intra and inter-assay coefficients of variation were under 10%, respectively. Charcoal–Dextran method was used to separate bound and free hormones. Radioactivity in the supernatant (bound) was determined by liquid scintillation

counter. Scintillation counting was carried out with Wallac 1409 DSA equipment (Wallac Co., Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co., Turku, Finland). Androgen production was expressed as media contents per ml.

2.7. Statistical analysis

Results were expressed as means \pm SE and analyzed and compared by using a randomized replicated block ANOVA test and a *posteriori* contrast by using Tuckey' test (Steel and Torrie, 1980). A randomized replicated block ANOVA test – instead of a randomized block ANOVA – was chosen because in each experiment both testes correspond to the same animal. That test considers the toad as the independent unit or block, and each testis as two replicates within the block. The signification level in all tests was 5% or 1%.

3. Results

In order to determine if the spermiation process is related to the activity of the Na^+/K^+ ATPase, fragment of testes were treated with different concentrations of Ouabain, a well-known inhibitor of the sodium pump. Fig. 1A shows that spermiation was not induced by inhibiting the Na^+/K^+ ATPase in the absence of hCG. However, Ouabain inhibited in a dose-dependent manner hCG-induced spermiation, reaching a 90% inhibition with the higher dose employed (Fig. 1A). Nevertheless, neither basal nor hCG-stimulated testosterone production was modified by the presence of Ouabain (Fig. 1B), suggesting a specific role for the Na^+/K^+ ATPase in hCG-elicited spermiation but not in steroidogenesis. In addition, amiloride – an inhibitor of transcellular Na^+ transport – neither affected spermiation nor steroidogenesis (Fig. 2A and B).

Incubation with H89, a widely used PKA inhibitor, resulted in a significant decrease in gonadotropin-induced spermiation, with a 70% inhibition at the higher concentration used (Fig. 3A). On the other hand, no effect in the absence of hCG was obtained (data not shown). Additionally, treatment with 8Br-cAMP induced spermiation in the absence of hCG but did not increase the effect of the gonadotropin (Fig. 3B). Taken together, the inhibition of spermiation by H89 and the induction of sperm release by 8Br-cAMP suggest that the activation of PKA could be involved in the spermiation evoked by hCG. In contrast, incubation with Bi or STP, two well-known inhibitors of PKC, did not alter hCG-induced spermiation (Fig. 4A and B, respectively). Furthermore, treatments with PMA, a PKC activator, did not induce spermiation in absence of the gonadotropin but high concentrations of PMA significantly decreased hCG-dependent spermiation (Fig. 5). These results allow the proposal that the inhibition of PKC is necessary for hCG action.

In addition, to corroborate the participation of PKA in hCG-dependent spermiation and its relationship with the Na^+/K^+ ATPase, testes were incubated with the higher doses of 8Br-cAMP in the presence of different concentrations of Ouabain. As can be seen in Fig. 6, Ouabain also decreased spermiation induced by 8Br-cAMP.

4. Discussion

In the toad *R. arenarum*, it has been suggested that hCG induces a decrease in the activity of the Na^+/K^+ ATPase of Sertoli cells, this decrease being part of the mechanism of hCG-induced spermiation (Russo and Burgos, 1969). Therefore, if this hypothesis was certain upon inhibiting selectively the pump the process of spermiation could be activated. In the experimental conditions of this work, Ouabain, in all the concentrations employed, was not able to induce spermiation in the absence of hCG. These results have at least

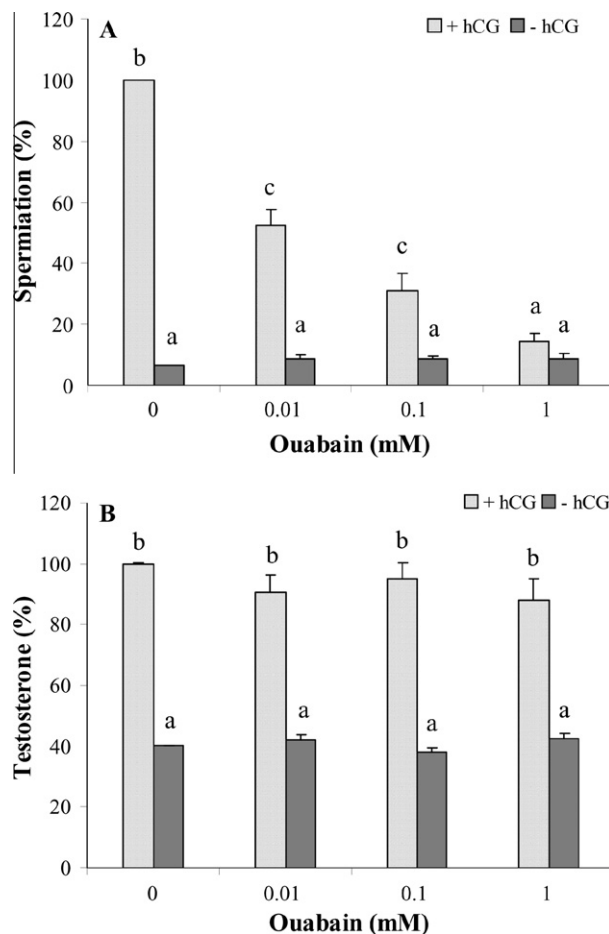


Fig. 1. Effect of different concentrations of Ouabain on: (a) Basal and hCG-induced spermiation. (b) Basal and hCG-induced testosterone biosynthesis. Testis fragments were incubated with or without 20 IU hCG at 28 °C during 2 h in KRGH medium, pH 7.4. Results were referred to 20 IU hCG (100%) and expressed as the mean of 8 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $7.7 \times 10^6 \pm 0.7 \times 10^5$. Testosterone concentration in hCG-treated testes: 5.70 ± 0.28 ng/ml.

three different explanations: (1) the Na^+/K^+ ATPase is not involved in spermiation; (2) the inhibition of this pump is necessary but not sufficient condition for inducing spermiation; and (3) Na^+/K^+ ATPase participates in the process but in a different way.

With the purpose to test those possibilities, testes were incubated with hCG in the presence of several concentrations of Ouabain. Unexpectedly, the incubation with Ouabain caused a significant decrease in the spermiation induced by the gonadotropin when compared with the control without the inhibitor. These results put forward a fundamental role of Na^+/K^+ ATPase in the mechanism of action of hCG as its blockade causes the inhibition of spermiation, by suppressing the effect of hCG. In consequence, it is possible to rule out the proposal that the sodium pump is not involved in spermiation.

Regarding the role of the Na^+/K^+ ATPase in the stimulation of steroidogenesis no results exist at the present that support or exclude its participation. The fact that Ouabain affects neither basal nor hCG-stimulated steroidogenesis indicates that Na^+/K^+ ATPase is not involved in the mechanism employed by the gonadotropin to increase the biosynthesis of testosterone. Since Ouabain does not inhibit the effect of hCG in steroidogenesis it is possible to presume that spermiation and steroidogenesis are regulated by different mechanisms. These conclusions differ from the hypothesis of Kobayashi et al. (1993) for *R. nigromaculata*. These authors

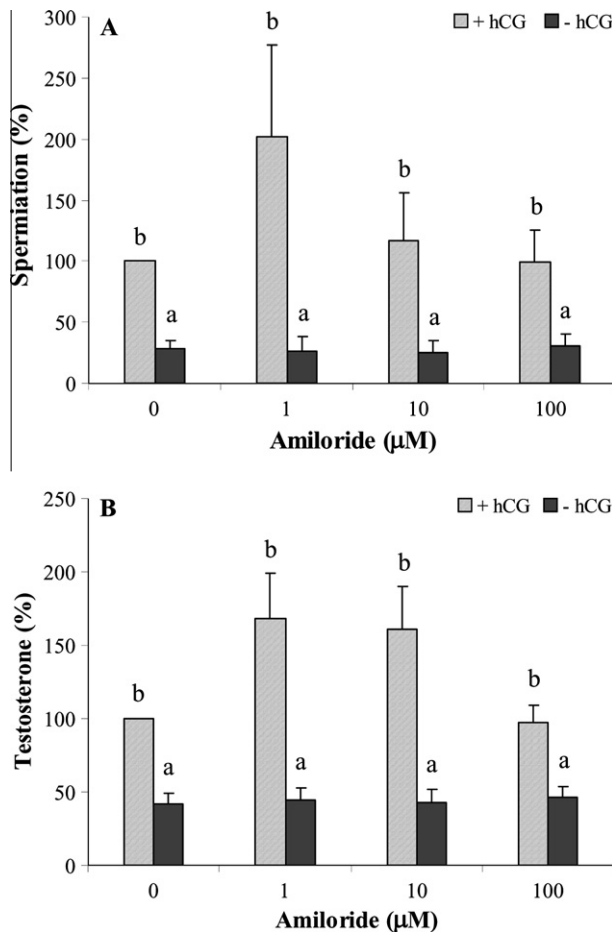


Fig. 2. Effect of different concentrations of Amiloride on: (a) Basal and hCG-induced spermiation. (b) Basal and hCG-induced testosterone biosynthesis. Testis fragments were incubated as described in Fig. 1. Results were referred to 20 IU hCG (100%) and expressed as the mean of 7 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $3.7 \times 10^6 \pm 4.4 \times 10^5$. Testosterone concentration in hCG-treated testes: 4.76 ± 0.95 ng/ml.

suggested that the activation of steroidogenesis is a prerequisite for hCG-elicited spermiation. Moreover, reinforce the idea previously proposed by Pozzi and Ceballos (2000) that in *R. arenarum* hCG induces spermiation and steroidogenesis by acting on different cell types or at least by different mechanisms. Studies carried out in our laboratory showed that in *R. arenarum* CNK (inhibitor of the enzyme 3β -hydroxysteroid dehydrogenase/isomerase) did not block the spermiation induced by hCG (Pozzi and Ceballos, 2000). In this work, it was also shown that aminoglutethimide (inhibitor of the Cyp450scc) failed to block the induction of the spermiation by hCG, even though steroidogenesis was completely suppressed. Besides, in *R. arenarum* it was also suggested that hCG could elicit spermiation by acting directly on Sertoli cells (Pozzi et al., 2001).

In addition, in other species such *R. catesbeiana* and *L. ocellatus* (Rosemblit et al., 2006) it was also proposed that both processes – spermiation and steroidogenesis – are under independent regulation, hCG-induced spermiation being not dependent on steroid biosynthesis in Leydig cells.

As mentioned under Introduction, the regulation of the Na^+/K^+ ATPase in vertebrates involves several kinases. In *R. arenarum*, the inhibition of PKA activity by H89 blocked the effect of hCG, signifying that this gonadotropin – at least in the experimental conditions of this work – or LH/FSH during the amplexus could induce spermiation through the activation of that kinase. On the other

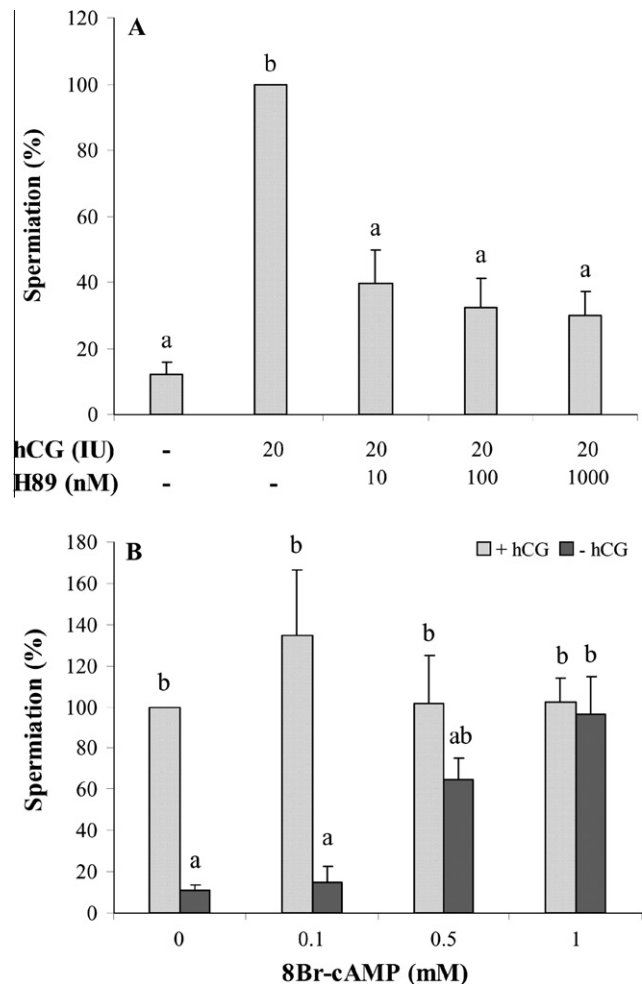


Fig. 3. (a) Effect of H89 on hCG-induced spermiation. (b) Effect of 8Br-cAMP on basal and hCG-induced spermiation. Testis fragments were incubated as described in Fig. 1. Results were referred to 20 IU hCG (100%) and expressed as the mean of 8 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $3.9 \times 10^6 \pm 9.4 \times 10^5$ and $4.1 \times 10^6 \pm 1.5 \times 10^6$, respectively.

hand, treatment with 8Br-cAMP, an activator of PKA, induced spermiation in the absence of hCG, confirming that PKA participates in the mechanism of the gonadotropin. These results are in agreement with previous reports in the same species showing that human recombinant FSH and hCG elicited an increase of cAMP in Sertoli cells (Pozzi et al., 2001). This assumption is reinforced by evidences indicating the presence of specific sites for PKA phosphorylation in the Na^+/K^+ ATPase of another bufonid, *B. marinus* (Beguin et al., 1994), allowing the proposal that hCG activates PKA which consecutively could turn on Na^+/K^+ ATPase inducing spermiation. The fact that treatments with 8Br-cAMP increased spermatozoa release but it did not prevent the effects of ouabain suggests that the inhibitory effect of ouabain on spermiation is associated with a post-cyclic AMP pathway.

The role of PKC was taken under consideration due to its capacity to directly phosphorylate Na^+/K^+ ATPase even if its action may vary depending on the tissue and the organism (Beguin et al., 1994; Feschenko and Sweadner, 1994; Han et al., 2006). That spermiation is not significantly modified by the inhibition of PKC confirms that this kinase is not implied in spermiation or at least, in agreement with the proposal of Feschenko and Sweadner (1994) in mammals, the action of PKC alone could not be enough to regulate spermiation. Another possibility is that PKC was inhibiting sperm release; its effect being suppressed by hCG. Results of this

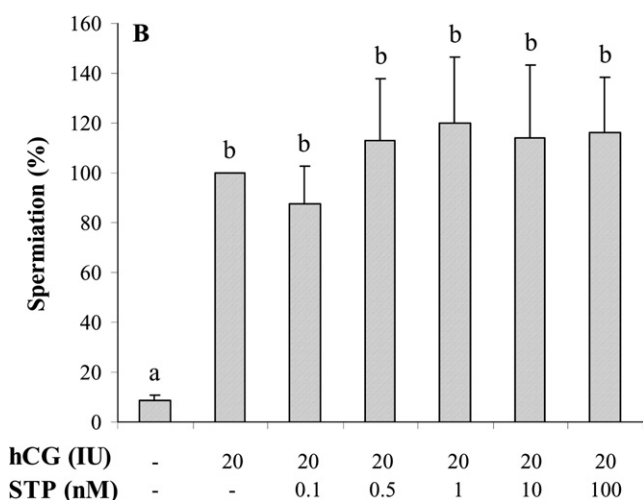
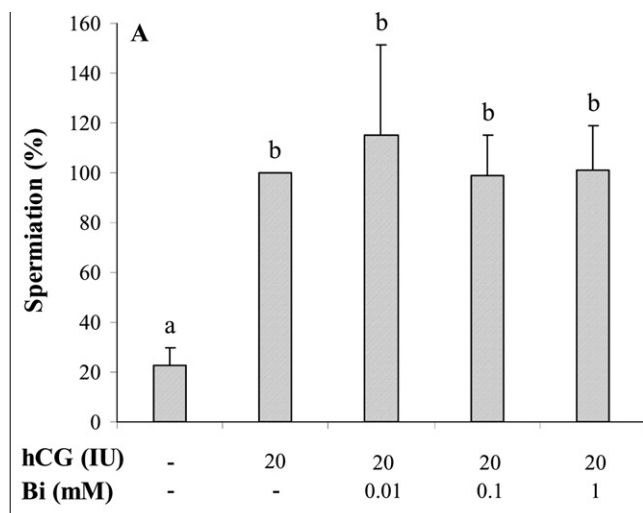


Fig. 4. Effect of PKC inhibitors on basal and hCG-induced spermiation: (a) Treatment with Bi. (b) Treatment with STP. Testis fragments were incubated as described in Fig. 1. Results were referred to 20 IU hCG (100%) and expressed as the mean of 9 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $4.5 \times 10^6 \pm 1.1 \times 10^6$ and $1.9 \times 10^6 \pm 1.2 \times 10^5$ for Bi and STP, respectively.

paper support this hypothesis since the incubation with Bi or STP in the presence of hCG did not affect hCG-induced spermiation but PMA inhibited the effect of the gonadotropin.

Taken all these results into account, it could be proposed that hCG have two mechanisms of action by acting in two different cell types: (1) In Leydig cells steroidogenesis could be activated by a Na^+/K^+ ATPase-independent mechanism, and (2) in Sertoli cells, spermiation being elicited by a mechanism involving the Na^+/K^+ ATPase and PKA but not an amiloride-sensitive sodium channel. However, even if the present study demonstrates that the Na^+/K^+ ATPase is involved in the regulation of spermiation, the mechanism by which this protein induces sperm release seems to be different from the classical pumping activity. Similarly, the role of Na^+/K^+ ATPase in bull sperm capacitation is related to the non-canonical action of this protein (Thundathil et al., 2006; Newton et al., 2010). In bulls' spermatozoa, the binding of ouabain to Na^+/K^+ ATPase inhibits motility and induces tyrosine phosphorylation but did not increase intracellular calcium levels in spermatozoa (Thundathil et al., 2006).

However, in the present study is not possible to exclude the role of PKA in the phosphorylation of proteins involved in the interaction of Sertoli cell-spermatozoa. As previously described, the

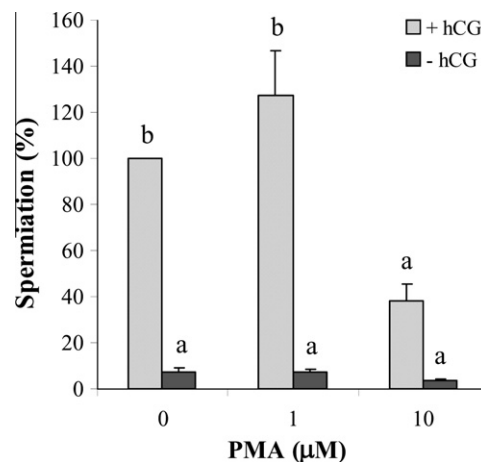


Fig. 5. Effect of PMA on basal and hCG-induced spermiation. Testis fragments were incubated as described in Fig. 1. Results were referred to 20 IU hCG (100%) and expressed as the mean of 5 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $4.1 \times 10^6 \pm 6.5 \times 10^5$.

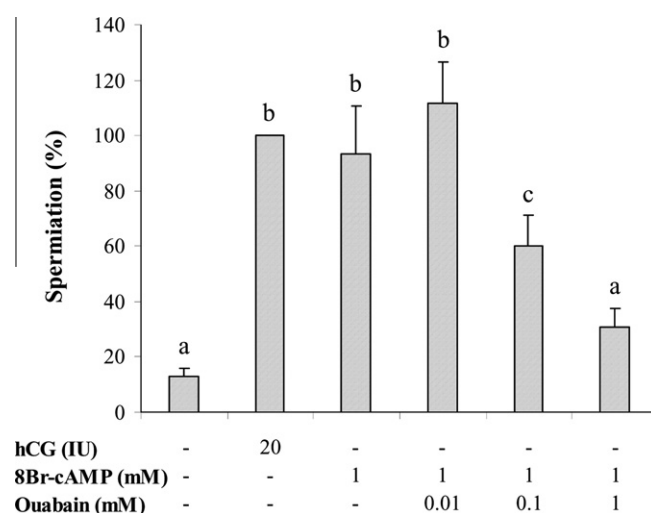


Fig. 6. Effect of 8Br-cAMP and Ouabain on basal and hCG-induced spermiation. Testis fragments were incubated as described in Fig. 1. Results were referred to 20 IU hCG (100%) and expressed as the mean of 6 independent experiments \pm SE. Different letters mean significant differences with $p < 0.01$. Number of spermatozoa per ml obtained after hCG treatment: $3.5 \times 10^6 \pm 6.9 \times 10^5$.

phosphorylation of these proteins by kinases is a central control of mammalian spermiation (Chapin et al., 2001).

In summary, the present study demonstrates that in *R. arenarum* both Na^+/K^+ ATPase and PKA mediate the action of hCG while the activation of PKC would have an opposite effect on the signalling pathway of the gonadotropin. In addition, even Na^+/K^+ ATPase is involved in hCG-induced spermiation, amiloride does not affect gonadotropin action suggesting that more experiments are necessary to define the pool of Na^+/K^+ ATPase involved in spermiation.

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