Endocannabinoid System in Frog and Rodent Testis: Type-1 Cannabinoid Receptor and Fatty Acid Amide Hydrolase Activity in Male Germ Cells

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INTRODUCTION

Endogenous cannabinoids are an emerging class of lipid mediators isolated from brain, peripheral tissues, and reproductive fluids [1, 2]. To date, at least four brain-derived compounds have been identified and designated as endocannabinoids [3]. N-arachidonoyl ethanolamide (anandamide, AEA) is the main endocannabinoid described to date in the testis. It exerts its effects through the activation of G-protein coupled cannabinoid receptors (CNR). However, the activity of AEA in controlling male reproduction is still poorly known. Here we provide direct evidence on the presence of the ‘‘endocannabinoid system,’’ constituted by type-1 cannabinoid receptor (CNR1) and fatty acid amide hydrolase (FAAH), in the frog Rana esculenta testis demonstrating its expression in tubular compartment. In fact, during the annual reproductive cycle, both proteins increase in September, when the appearance of spermatids (SPT) occurs. Immunocytochemistry confirms their localization in germ cells and, in particular, in elongated SPT. Signals are still present in spermatozoa (SPZ), as demonstrated by Western blot analysis. Furthermore, the activation of CNR1 reduces sperm motility. Comparative research, carried out using mouse and rat SPZ, definitely indicates that the endocannabinoid system operates in SPZ of phylogenetically distant species. A conserved physiological role of endocannabinoid system in controlling the inhibition of sperm motility is suggested.

male reproductive tract, sperm, sperm motility and transport, spermatogenesis, testis

ABSTRACT

N-arachidonoyl ethanolamide (anandamide [AEA]) is the main endocannabinoid described to date in the testis. It exerts its effects through the activation of G-protein coupled cannabinoid receptors (CNR). However, the activity of AEA in controlling male reproduction is still poorly known. Here we provide direct evidence on the presence of the ‘‘endocannabinoid system,’’ constituted by type-1 cannabinoid receptor (CNR1) and fatty acid amide hydrolase (FAAH), in the frog Rana esculenta testis demonstrating its expression in tubular compartment. In fact, during the annual reproductive cycle, both proteins increase in September, when the appearance of spermatids (SPT) occurs. Immunocytochemistry confirms their localization in germ cells and, in particular, in elongated SPT. Signals are still present in spermatozoa (SPZ), as demonstrated by Western blot analysis. Furthermore, the activation of CNR1 reduces sperm motility. Comparative research, carried out using mouse and rat SPZ, definitely indicates that the endocannabinoid system operates in SPZ of phylogenetically distant species. A conserved physiological role of endocannabinoid system in controlling the inhibition of sperm motility is suggested.

Introduction

Endogenous cannabinoids are an emerging class of lipid mediators isolated from brain, peripheral tissues, and reproductive fluids [1, 2]. To date, at least four brain-derived compounds have been identified and designated as endocannabinoids [3]. N-arachidonoyl ethanolamide (anandamide, AEA) is the main endocannabinoid described in the testis [4]. It exerts its effects through the activation of G-protein coupled cannabinoid receptors, located on the membrane of target cells [5, 6], mimicking some of the effects played by THC, D9-tetrahydrocannabinol, the active principle isolated from Cannabis sativa [7]. The effect of AEA via CNR depends on its concentration in the extracellular space. This is controlled by a hypothesized membrane transporter (AMT) and by the fatty acid amide hydrolase (FAAH) acting on AEA uptake and hydrolysis, respectively. Endocannabinoids and their receptors, together with AMT and FAAH, constitute the ‘‘endocannabinoid system’’ [5, 8–10].

To date, CNR1 and CNR2 are two different CNR subtypes identified [11, 12], and further spliced variants of CNR1 have been cloned from humans [13, 14]. CNR1 was first isolated in the brain [11], but its expression is now clear in many other tissues, such as peripheral nerves and leukocytes, spleen, testis, uterus, endothelial and muscle cells, as well as placenta and eye [15].

Current knowledge of CNR-mediated effects is concentrated mainly on the brain system. Anyway, it is now well established that endocannabinoids/cannabinoids influence reproductive function [16, 17]. THC has been reported to account for the majority of the reproductive consequences of marijuana use. In females, inhibition of ovulation has been described [18]. More recently, it has been shown that AEA levels seem to be critical in regulating the window of blastocyst implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state [19–21]. In males, cannabinoids inhibit spermatogenesis and reduce the weight of reproductive organs as well as production, motility, and viability of sperm cells [22]. Finally, they regulate testosterone production by Leydig cells both in vivo and in vitro [23] and cause impotency in male rat [24]. However, few data exist to establish the role of endocannabinoids in the male physiology of reproduction.

To give insight into the role of the endocannabinoid system in germ cell physiology, we took advantage of the anuran amphibian Rana esculenta as an animal model to explore expression and localization of CNR1 and FAAH proteins in the testis during the annual sexual cycle. Several features can be ascribed to this experimental model. Spermatogenesis occurs in cysts consisting of Sertoli cells enveloping cluster of germ cells at a synchronous stage [25]. During the annual sexual cycle, new cysts monthly appear and progress throughout spermatogenesis stages depending on endocrine and local events [26]. This conveys the considerable advantage to have in each period of the year a defined and well-known population of germ cysts. As a consequence, the possibility of studying monthly different stages of germ cells, appearing progressively during the annual sexual cycle, represents a powerful tool to easily delineate the molecular signaling implicated in the control of spermatogenesis [27–31].

An ancient phylogenetic association of endocannabinoid signaling with reproductive physiology has recently been indicated by the demonstration of orthologues CNR and FAAH in ovary and testis of the invertebrate Ciona intestinalis [32]. The phylogenetically more recent presence of CNR1 in mammalian testis [33] and sperm [34, 35] confirms the
putative ancient association between the endocannabinoid system and reproduction. Indeed, besides acrosome reaction in sea urchin, AEA signaling has been proposed to regulate capacitation in boar [35] and human [36] sperm (hyper-activated motility and ability to undergo physiological acrosome reaction with zona pellucida of egg). Consistently, boar SPZ have been shown to produce their own AEA [35] and to possess vanilloid receptor, FAAH, CNR2, and CNR1, the last also evidenced in sea urchin [36] and human sperm [34]. These findings have led to the suggestion that germ cells synthesize FAAH and CNR1. To date, FAAH has been demonstrated in isolated immature Sertoli cells [37] and CNR1 in Leydig cells [33]. Interestingly, during the preparation of this manuscript, a study on mouse testis has appeared [38] showing CNR1 in Leydig cells and also in luminal epithelia of seminiferous tubules (from spermatagonia to spermatocyte). With this knowledge we sought to investigate 1) the comparative expression of CNR1 and FAAH in frog, mouse and rat SPZ, 2) their activity in frog SPZ, and 3) the localization of FAAH and CNR1 in *R. esculenta* testis during spermatogenesis.

We provide evidence that both CNR1 and FAAH proteins are present in frog testicular germinal compartment and isolated SPZ. Furthermore, comparative analysis, carried out on frog, mouse, and rat, strongly demonstrates that the endocannabinoid network is highly conserved during evolution to regulate SPZ function.

**MATERIALS AND METHODS**

**Animals**

Except in August and October, male frogs, *R. esculenta* (n = 36), were monthly captured near Napoli (Italy) and divided in three groups. Each group (n = 12) was processed separately. Animals were killed under anesthesia with MS222 (Sigma-Aldrich Corp., St. Louis, MO), and testes were removed and immediately processed for histological analysis (n = 4), immunocytochemistry (n = 6), or protein extract preparations (n = 14). Sprague-Dawley rats and CD1 mice (Charles River Laboratories, Lecco, Italy) were also used. Experiments have been approved by the Italian Ministry of Education and the Italian Ministry of Health.

**Materials**

Propidium iodide, protease inhibitors, and anandamide (AEA) were purchased from Sigma-Aldrich Corp. (Milan, Italy). The N-(4-hydroxyphenyl) arachidonylamine (AM404), an analogue of AEA that potentiates the activity of endogenous AEA-inhibiting FAAH [39], were purchased from Cayman Chemical. The selective CNR1 antagonist SR141716A [40] was produced by Sanofi Research (Montpellier, France).

**Tissue Protein Preparation**

Tissues (rat brain, mouse and frog testis) were homogenized in fivefold (w/v) excess lysis buffer (25 mM Hepes pH 7.9, 1 mM EDTA, 6 mM MgCl2) in the presence of protease inhibitors (4 μg/ml of leupeptin, aprotinin, pepstatin A and chymostatin and 5 μg/ml of TPCK). Homogenate was centrifuged for 15 min at 800 x g. Supernatants were collected, and pellets were rehomogenized with lysis buffer as described previously. Protein concentration of the combined supernatants, consisting of soluble proteins and much of the membrane fractions, was determined using the Lowry method [41].

**Western Blot Analysis**

Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter (Amersham Pharmacia Biotech) at 280 mA for 2.5 h at 4°C in order to evaluate CNR1 and FAAH immunoreactivity. Filters were treated with blocking solution (5% nonfat powdered milk, 0.25% Tween-20 in Tris-buffered saline, TBS, pH 7.6) for 3 h to prevent nonspecific binding and then incubated with the primary antibody (anti-CNR1 Ab and anti-FAAH Ab diluted 1:1000; anti-MAPK1 Ab diluted 1:500) in PBS 3% nonfat powdered milk solution overnight at 4°C on an orbital shaker. Filters were washed in TBS-0.25% Tween20, incubated with 1:1000 horseradish peroxidase-conjugated IgG (DAKO Corp.) in TBS-1% normal swine serum (NSS, DAKO) and then washed three times in TBS-0.25% Tween20. The immune complexes were detected using the ECL-Western blotting detection system (Amersham Pharmacia Biotech) following the manufacturer’s instructions. Specificity of the reactions was tested through competition studies using the antibody previously preabsorbed for 18 h at 4°C on orbital shaker with a large excess (2 μg) of the corresponding antigen. The membranes, stripped at 60°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 7.6), were reprobed with MAPK1 antibody (sc-154, Santa Cruz Biotechnology, Inc.) to quantify protein content.

**Immunocytochemistry**

Testes were removed and fixed overnight in paraformaldehyde 4% in phosphate buffer (PB) 0.1 M, pH 7.4, cryoprotected, embedded in Kel-F medium (Bio-Optica, Milan, Italy), and frozen in liquid isopentane. Sections (12 μm thick), obtained with a cryostat, were mounted on 3-aminopropyltriethoxysilan (TESPA)-coated slides and stored at −20°C until use.

In order to analyze the distribution of the CNR1 and FAAH antigens, sections were incubated with primary antibody (dilution 1:800 in 0.01 M PBS, 0.1% Triton-X100) overnight at 4°C. Immunoreactivity was revealed by avidin-biotin complex (ABC) system and H2O2/DAAB (3,3′-diaminobenzidine-tetraydrochloride) as substrate/chemogen. To check the specificity of the immunoreactions, controls were treated with antibodies preabsorbed for 18 h at 4°C on an orbital shaker with a large excess (2 μg) of the corresponding antigen. Furthermore, specificity was confirmed by omitting primary antibody.

Sections were observed under a light microscope (Zeiss Axioskop) and then photographed. Images were processed with the software Adobe PhotoShop 7.0 (Adobe Systems Incorporated, Mountain View, CA).

**Frog SPZ Collection and Treatments**

Male frogs (n = 6) were stimulated with homologous hypophysis homogenate to induce spermatogenesis as previously described [42, 43]. Spermatozoa were collected in their fluid from the cloacae, filtered, and analyzed under a light microscope to exclude contamination by other cell types. *AEa* treatment. Fresh SPZ samples were diluted 1:5 in amphibian Krebs ringer bicarbonate buffer (KRB), divided in four aliquots, and treated as follows for 15 min: 1 ml control, 1 ml treated with 1 μM AEA, 1 ml treated with 1 μM AEA in combination with 10 μM CNR1 antagonist (SR141716A), 1 ml treated with 10 μM SR141716A alone. After incubation, viability and motility were evaluated. Thereafter, AEA-treated SPZ sample was pulled down by centrifugation (1000 × g) washed three times with KRB to remove AEA and once again analyzed for viability and motility.

*AM404* treatment. Fresh SPZ samples were incubated with or without 1 μM FAAH inhibitor (AM404) for 15 min. After treatment, sperm viability and motility were evaluated.

**Cloacal fluid dilution.** We have also evaluated the effects of cloacal fluid dilution on sperm motility. A fresh SPZ sample was divided in aliquots and diluted 1:5 and 1:50 with KRB. An undiluted SPZ sample was used as control group. All samples were analyzed to evaluate sperm motility.

**SR141716A** treatment. Fresh SPZ samples were incubated in presence or absence of the selective CNR1 antagonist SR141716A [40] at different concentrations (1.0, 5.0, 10 μM) for 15 min. After treatment, sperm motility was evaluated.

**Evaluation of Viability and Motility of Frog SPZ**

SPZ viability was evaluated using propidium iodide and flow cytometric analysis and plotted as the percentage of viable/total SPZ. SPZ motility was evaluated under a light microscope (magnification 20×) using hemocytometer and expressed as the percentage of motile/total SPZ.

**Protein Extraction from Mouse and Rat SPZ**

Mouse and rat SPZ were collected in phosphate-buffered saline (PBS) pH 7.4 by cutting epididymis in small pieces. SPZ samples were filtered, examined under a light microscope to exclude contamination by other cell types, and centrifuged at 1000 × g for 15 min at 4°C. The packed cells were subjected to lysis in triple-detergent buffer (PBS pH 7.4 containing 0.02% sodium azide; 0.1% SDS; 1% Nonidet P-40; 0.5% sodium deoxycholate; 4 μg/ml of leupeptin, aprotinin, pepstatin A, and chymostatin; and 5 μg/ml of TPCK) and then submitted three times to sonication for 5 min, each time at 25 mW. Protein concentration was evaluated by the Lowry method [40].
Antibodies

We used two polyclonal antibodies directed against C-terminus (residues 401–473) or N-terminus (first 77 residues) of rat CNR1 antigen. Specificity of the antibodies was already extensively investigated in several species [44–47] and here checked again by preincubation with an excess amount (2 μg) of the cognate peptide.

Anti-FAAH (Alexis Biochemicals, Lausen, Switzerland) and anti-MAPK1 (Santa Cruz Biotechnology) were commercial antibodies. Specificity of FAAH immunoreactivity was verified using mouse testis as positive control tissue [37] and by preincubation with excess amount (2 μg) of the cognate peptide.

Data Presentation and Statistics

Western blot signals were quantified by densitometry analysis carried out using GELDOC1,00-UV system (Biorad, Hercules, CA). CNR1 and FAAH levels were plotted as quantitative densitometry analysis of signals corrected on the basis of invariable MAPK1 content [48]. Values were expressed as optical density (OD) units.

Student t-test and ANOVA followed by Duncan test for multigroup comparison were carried out, where appropriate, to evaluate the significance of differences. Data were expressed as the mean ± SEM from at least three independent experiments.

RESULTS

Analysis of Endocannabinoid System in Frog Testis

Presence of CNR1 was investigated in *R. esculenta* testes collected during the annual reproductive cycle. Rat brain was used as positive control.

Figure 1 shows Western blot analysis. In both brain and testis extracts, a 66-kDa band, as expected, was seen (panel A). The use of the preabsorbed antiserum fails to detect the signals, demonstrating specificity of the reaction in both extracts (panel B). To quantify CNR1 content during the annual reproductive cycle, frog testis lysates were collected monthly and analyzed. Western blot shows a 66-kDa signal present throughout the year (Fig. 2A) with significant seasonal variations. Quantitative densitometry analysis of signals indicates a significantly higher expression of CNR1 in September (*P* < 0.05) as compared with other months (Fig. 2B). Seasonal characteristics of frog testicular histology are well known [25] and are shown in Figure 2C to reveal the new spermatogenic stages that appear in September, when CNR1 increases.

FAAH protein content was then investigated by Western blotting. Since FAAH was previously detected in *Ciona* testis [32], in boar SPZ [35], and also in membranes isolated from postnatal mouse Sertoli cells [37], mouse testis was used as positive control. Comparative analysis, carried out in frog and mouse testis, indicates the presence of a single immunoreactive band with a molecular weight of 55 kDa, as expected (Fig. 3A). Absence of signal, obtained using the preabsorbed antibody, demonstrates specificity of the immunoreactivity (Fig. 3B). To quantify FAAH protein content during the annual reproductive cycle, frog testis lysates were analyzed. A 55-kDa signal was always detected (Fig. 4A). A significantly higher expression was evaluated in the September–February period (Fig. 4B) as compared with other months (*P* < 0.05).
To further validate the previously mentioned results, CNR1 and FAAH were localized during the annual sexual cycle in testes of animals collected during the mitotic, meiotic, and postmeiotic phase by immunocytochemistry. Histological preparations, using hematoxylin-eosin stain, confirm that testes from the September–October period are rich in postmeiotic germ cells differentiating into SPZ (Fig. 5A). Immunocytochemistry demonstrates a clear and strong immunopositivity for both CNR1 (Fig. 5B) and FAAH (Fig. 5C) in these cells. During the other periods of the year, and in particular when mitotic (spermatogonia [SPG]) and postmitotic (spermatocytes [SPC]) stages appear (Fig. 5, D–I), a weak CNR1 immunopositivity was detected in SPG (Fig. 5E) and SPC (Fig. 5H); weak FAAH immunopositivity was detected in SPZ (Fig. 5F) and SPC (Fig. 5I). No immunopositivity was detected in the interstitial compartment. Specificity of the signals was tested through the use of preabsorbed antibodies (Fig. 5, L and M).

**Effects of Endocannabinoids on Viability and Motility of Frog SPZ**

Evaluation of the effects of AEA on the percentage of viable and motile SPZ has been carried out through in vitro treatment (Fig. 6, A and B). We have found that 1 μM AEA strongly reduces the number of motile SPZ ($P < 0.01$) as compared with control group. The inhibitory effect was largely antagonized by 10 μM SR141716A (Fig. 6B). A slight significant increase of motile SPZ, as compared with controls, was observed treating samples with SR141716A alone ($P < 0.05$). Finally, motility was restored to control values removing AEA by washing. Control of viability indicated that cell death does not occur in nonmotile SPZ.

AEA is present in human seminal plasma [2]. Thus, through an indirect approach, we have evaluated the presence of endocannabinoids in the fluid that transports frog SPZ to the cloaca. When fresh SPZ samples were directly incubated in presence or absence of FAAH inhibitor (AM404) (Fig. 7), we have observed that, similarly to AEA, AM404-treated SPZ preserve their viability and that the percentage of motile SPZ was reduced ($P < 0.01$). Furthermore, increasing dilutions of the cloacal fluid (Fig. 8A) induces a linear significant increase of percentage of motile SPZ compared to control group ($P < 0.01$). Moreover, the selective CNR1 antagonist SR141716A (Fig. 8B) significantly increases the percentage of motile SPZ in undiluted samples at either 5 or at 10 μM ($P < 0.01$) in a dose-dependent manner.

**Analysis of Endocannabinoid System in Frog and Rodent SPZ**

The endogenous cannabinoid system was investigated in isolated SPZ. Comparative analysis was carried out using frog, mouse, and rat SPZ collected from cloacae or epididymis. Total lysates were analyzed by Western blot using anti-CNR1 and anti-FAAH antibodies. Figure 9 shows the presence of CNR1 (panel A) and FAAH (panel B) in all extracts. In particular, two CNR1 signals of 63 and 66 kDa were observed. Absence of immunoreactivity with preabsorbed antisera indicates the specificity of the signals (panels C and D).

**DISCUSSION**

Evidence suggests that endocannabinoid signaling acts in the male reproductive tract. Rat testis is able to synthesize AEA [4], the main endogenous cannabinoid, which is also detected in human seminal plasma [49]. In mouse, the presence of CNR1 has been demonstrated in Leydig [33] and very recently, during the preparation of this manuscript, has also been described in germ cells from SPG to SPZ [38]. Consistently, human [34], boar [35], and sea urchin [36] SPZ have been shown to possess CNR1. Expression and activity of FAAH have been observed in isolated immature Sertoli cells [37] and boar SPZ [35] suggesting that, in addition to CNR1, FAAH may be synthesized by germ cells during spermatogenesis.

To give insight into the role exerted by the endocannabinoid system in the testis, we have investigated the presence of CNR1 and FAAH during the annual reproductive cycle of *R. esculenta*. As shown in previous studies, the frog model appears to be useful in this respect [31]. We provide evidence that both CNR1 and FAAH are present in germ cells. Comparative analysis, carried out also using mouse and rat models, extends results demonstrating the presence of CNR1 and FAAH in nonmammalian and mammalian SPZ [34–36, 50, 51]. Therefore, we show that a complete endocannabinoid system operates in SPZ of phylogenetically distant vertebrate species. Furthermore, similarly to the presence of AEA in human seminal plasma regulating hyperactivated motility of sperm [2, 36, 49], we provide indirect evidence that cloacal fluid contains endocannabinoids, and we also indicate that the endocannabinoid system is involved in controlling frog SPZ motility.

During the annual reproductive cycle, CNR1 was detected by Western blot from January until December. Quantitative densitometry analysis of signals reveals a significantly higher expression in September than in other months. RT-PCR study also shows, in frog testis, a high CNR1-mRNA level in...
September [52]. Taking into account the temporal progression of spermatogenesis in this animal model [25], it seems reasonable to correlate the increase of CNR1 to the appearance of SPT that occurs in September–October [25]. A clear and strong immunolocalization of CNR1 in elongated SPT during this period confirms our hypothesis. Interestingly, in the other months the weak immunolocalization of CNR1 in SPG and SPC validates the weak CNR1 signal detected all year-round by Western blot. While the localization of CNR1 in germ cells agrees with results reported in mouse [38], its absence in the interstitial compartment may suggest phylogenetic differences among species. Further research will be necessary to clarify this point.

The profile of FAAH partially mirrors that of CNR1. Immunocytochemistry clearly detects FAAH in elongated SPT and SPC. A weak immunopositivity was detected also in SPC. Taken together, our results suggest that CNR1 and FAAH are present in germ cells. This confirms [38] and adds new insights into the endocannabinoid system in the testis. Indeed, we show, for the first time, that, in addition to immature Sertoli cells [37], presence of FAAH also occurs in germ cells other than SPT [35]. The high expression of FAAH during September–February indirectly suggests that, in this period, low intratesticular endocannabinoid levels exist. In mice, chronic administration of cannabinoids disrupts mature SPZ. Indeed, SPZ collected from cauda epididymis shows severe abnormalities in morphology [53, 54] suggesting that high cannabinoid levels might damage SPT maturation phase or SPZ. Immunodetection of CNR1 demonstrates that cannabinoids are able to act on either SPT or SPZ. Thus, the increase of FAAH during the September–February period may act to decrease testicular endocannabinoid levels to protect SPT-SPZ maturation.

AEA signaling has been proposed to regulate sperm functions required for fertilization [35, 36], and consistently SPZ have recently been shown to express CNR1 [34–36]. Nevertheless, the molecular basis of the involvement of endocannabinoid system in controlling sperm function and male fertility remains unclear. We demonstrate that CNR1 and FAAH are present in frog, mouse, and rat SPZ, confirming recent results obtained in boar [35]. While in boar the correlation between the endocannabinoid system and the ability of sperm cells to react to zona pellucida has been demonstrated, in frog we have investigated SPZ motility. We show that AEA-treated frog SPZ lose motility. This effect was counteracted by the selective CNR1 antagonist SR141716A, and it is also reverted by washing, demonstrating that AEA, through CNR1 activity, reduces the percentage of motile SPZ and that this was not due to toxicity, damage, or death of cells.

FIG. 5. Sections of *R. esculenta* testis collected in September (A–C), March (D–F), and June (G–I) analyzed by hematoxylin-eosin stain (A, D, G) as well as by immunocytochemistry for CNR1 (B, E, H) or FAAH (C, F, I) presence. Controls of immunoreactions (L, M) were carried out during the annual reproductive cycle using sections randomly chosen. (September sections are represented here since all spermatogenic stages are present.) Stars indicate the interstitial compartment. SPG, Spermatogonia; SPC, spermatocytes; SPT, spermatids; SPZ, spermatozoa. Results are representative of at least three separate assays. Original magnification ×400.
Indeed, apart from previously described results and the control of viability carried out to validate our data, it should be considered that we have used 1 M of AEA in the present study, while the toxicity reported for human sperm is 10 M [34]. Whether other processes leading to fertilization are affected in frog requires further investigation.

The presence of a functional cannabinoid receptor implies the presence of an endogenous ligand. To reveal endocannabinoids into cloacal fluid, an indirect approach has been used. In fact, AM404 inhibits FAAH activity [39], and AM404-treated SPZ lose motility, indicating that endocannabinoids are present in the cloacal fluid. Therefore, we conclude that endocannabinoid concentration in the cloacal fluid may be fundamental in determining the percentage of motile SPZ. As a
consequence, it could act to keep the SPZ quiescent until dilution in the aquatic environment during mating. In fact, linear dilution of the cloacal fluid enhances sperm motility, demonstrating that cloacal fluid substances control the percentage of motile SPZ depending on their concentrations. Treatment of SPZ with increasing concentrations of SR141716A unequivocally confers this control to endocannabinoids through CNR1 activation. Curiously, SPZ contain two CNR1 forms. This led us to hypothesize that posttranslational modifications of CNR1 could act to control the molecular pathway associated with SPZ activity. Further studies are in progress in this respect. Where endocannabinoids present in the cloacal fluid come from is not indicated by the present data, but it should be emphasized that boar SPZ produce AEA to regulate their own function [35].

In conclusion, we report data on the presence of CNR1 and FAAH proteins in germ cells and isolated SPZ of *R. esculenta*. Comparative analysis, carried out in frog, mouse, and rat, extends previous results on CNR1 localization in vertebrates and invertebrates. Furthermore, we demonstrate also that the biochemical machinery to hydrolyze endocannabinoids is highly conserved in phylogenetically distant species. In particular, in frog, the endocannabinoid system in SPZ is associated to their motility. Our data add strong support to the role played by endocannabinoids in the control of male fertility, and, consequently, this may be taken into consideration to develop new pharmacological approaches to treat male infertility.

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REFERENCES


