ORIGINAL

Ivermectin as an inhibitor of the plasma membrane and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases in rat *vas deferens*

Ivermectina como um inibidor das Ca^{2+}-ATPases de membrana plasmática e de retículo sarco/endoplasmático no ducto deferente de rato

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ABSTRACT

Objective

The present work investigated the effect of ivermectin on Ca^{2+} content and on the Ca^{2+}-ATPase activity (represented by the plasma membrane Ca^{2+}-ATPase and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase present in rat *vas deferens*.

Methods

The assays were carried out using ultracentrifuged homogenate preparations from rat *vas deferens* in the presence or absence of the 12-kDa FK506-binding protein-Ca^{2+} release channel complex. Measures of Ca^{2+} content and Ca^{2+} ATPase activity were then carried out in function of different concentrations of ivermectin.

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Results

The data show that ivermectin (10 μM) reduces the sarcoplasmic reticulum Ca^{2+} content in FK506-binding protein (+) and FK506-binding protein (-) fractions of ultracentrifuged homogenate from rat *vas deferens* (inhibition of 50% and 40%, respectively, $p<0.05$) and inhibits both the activities of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase and plasma membrane Ca^{2+}-ATPases pumps (33% and 16%, respectively, $p<0.05$).

Conclusion

These data suggest that ivermectin effects Ca^{2+} handling in the rat vas deferens, indicating that this drug could alter the contractility of this smooth muscle. Therefore, ivermectin could be an interesting pharmacological tool to alter the physiological function of *vas deferens* and to manipulate the fertility status of male rats.

Indexing terms: Calcium. Ivermectin. Rats.

RESUMO

Objetivo

O presente trabalho investigou o efeito da ivermectina no conteúdo de Ca^{2+} e na atividade Ca^{2+}-ATPásica (representada pela Ca^{2+}-ATPase de membrana plasmática e pela Ca^{2+}-ATPase de retículo sarco/endoplasmático presente no ducto deferente de rato.

Métodos

Os ensaios foram realizados por meio de preparações de homogeneizado ultracentrifugado de duto deferente de rato na presença ou ausência do complexo proteína de ligação ao FK506 de 12 kDa-canal liberador de Ca^{2+}. Após esse procedimento, avaliações do conteúdo de Ca^{2+} e da atividade Ca^{2+}-ATPásica foram realizadas em função de diferentes concentrações de ivermectina.

Resultados

Os dados mostram que a ivermectina (10 μM) reduz o conteúdo de cálcio no retículo sarcoplasmático de frações FK506-binding protein (+) e FK506-binding protein (-) de homogeneizado ultracentrifugado de duto deferente de rato (50% e 40% de inibição, respectivamente, $p<0,05$) e inibe as atividades das enzimas Ca^{2+}-ATPase de retículo sarco/endoplasmático e Ca^{2+}-ATPase de membrana plasmática (33% e 16%, respectivamente, $p<0,05$).

Conclusão

Os dados sugerem que a ivermectina afeta a mobilização de cálcio no duto deferente de rato, o que indica que esse fármaco pode alterar a contratilidade desse músculo liso. Dessa forma, ivermectina pode ser ferramenta farmacológica interessante para alterar a função fisiológica do duto deferente e manipular o estado de fertilidade de ratos machos.

Termos de indexação: *Cálcio. Ivermectina. Ratos.*

INTRODUCTION

Ca^{2+} homeostasis appears to play a crucial role in the functioning of *vas deferens*, a thick walled tube of smooth muscle that acts as a conduit for the movement of sperm from the epidydimis to the urethra. Several studies corroborate with this hypothesis, such as α-adrenoceptor knockout mice, with failure in the Ca^{2+} mobilization, which show reduced contractility and sperm number in the *vas*

deferens and ejaculation dysfunction[1]. The use of tamsulosin and alfuzosin, α-adrenergic blockers which block Ca^{2+} signals, have been reported to produce ejaculatory dysfunctions including anejaculation or retrograde ejaculation, due to differential pharmacological actions at the level of the *vas deferens*[2-4]. Moreover, deficiency in the P2X purinoceptor (P2X1, a protein that in humans is encoded by the P2X1 gene) results in a 90% decrease in male fertility through a reduction of sperm in the ejaculate associated with a decrease in neurogenic *vas deferens* contraction[5]. Finally, our group recently demonstrated that chronic malnutrition affected several components related to intracellular Ca^{2+} handling in *vas deferens*, reducing the haploid cell count in this organ and decreasing the reproductive capacity of the male adult Wistar rats[6].

Several proteins are involved to ensure finely tuned Ca^{2+} mobilization in *vas deferens*, including: (1) intracellular Calcium Release Channels (CRC), responsible for Ca^{2+} efflux from intracellular Ca^{2+} stores: Ryanodine Receptor (RyR1, RyR2, RyR3 isoforms) and Inositol Trisphosphate Receptor (IP_3R1, IP_3R2, IP_3R3 isoforms); (2) the Plasma Membrane and Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPases (PMCA and SERCA)[7-11]. Both the efflux and the influx of Ca^{2+} from intracellular stocks are well regulated by the 12 kDa FK506 Binding Protein (FKBP12), an immunophilin located in the cytoplasm of cells, that is the pharmacological receptor for the immunosuppressant drugs FK506 (tacrolimus) and rapamycin[12]. The disruption of the tightly bound FKBP12-CRC complex by FK506 or rapamycin, or the disruption of the weak bound mechanically, shifts the channels to a subconductance state increasing Ca^{2+} leakage through RyR or IP_3R[13].

Ivermectin is an anti-helmintic and a member of the avermectin class[14,15]. In the range of 30 μM, this macrocyclic lactone is known to inhibit SERCA1 and SERCA2b pumps, but it directly activates the CRC at low concentrations[16,17]. These actions impair Ca^{2+} uptake or increase Ca^{2+} release from Sarcoplasmic/Endoplasmic Reticulum (SR/ER) resulting in the reduction of Ca^{2+} content in this Ca^{2+} store. As a consequence, these actions improve cytosolic calcium levels that may promote a reduction of contractility efficiency of muscle tissues as a consequence of the emptying of SR[16,17].

The molecular evidence for Ca^{2+} homeostasis on rat *vas deferens* function has been demonstrated previously: (1) subcellular fractions obtained from rat vas deferens homogenate contain vesicles of SR origin presenting SERCA pumps, RyR and IP_3R[18]; (2) the isoform of the SERCA pump expressed in rat *vas deferens* is probably SERCA2b[11,19]; and (3) treatment at 37°C and pH 7.4 is sufficient to dissociate the FKBP12-CRC complex in rat *vas deferens*[12]. The aim of this work was to demonstrate the effect of ivermectin on Ca^{2+}-ATPase activity and Ca^{2+} content of SR vesicles of rat *vas deferens* in the presence or in the absence of FKBP12–CRC complex.

METHODS

All experimental procedures involving the animals were approved by the Committee for Ethics in Animal Experimentation of the *Universidade Federal do Rio de Janeiro*, and were carried out in accordance with the Committee's guidelines (DFBCICB074-10/16 Protocol).

Reagents and solutions

Ivermectin was provided by Calbiochem® (CN Biosciences, Inc, Darmstadt, Germany). Reagents and antibodies were purchased from the Sigma Chemical Co. Rainbow Molecular Weight Markers were provided by GE Healthcare (Buckinghamshire, United Kingdom) and peroxidase-conjugated secondary antibodies were purchased from Promega Corporation (Madison, WI). $^{45}Ca^{2+}$ and [g-^{32}P]ATP were purchased from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Buckinghamshire, United Kingdom).

Ultracentrifuged homogenate preparation

Preparation of membranes was carried out as described previously[6,12,18]. Briefly, rat *vasa deferentia* were removed and immersed in cold

Tyrode´s solution containing 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$ plus 0.36 mM NaH_2PO_4 (pH 7.4), 5.55 mM glucose, 1.77 mM $CaCl_2$ and 0.40 mM $MgCl_2$. The tissue was dissected, homogenized and the crude homogenate was centrifuged at 108,000 g for one hour to obtain the whole homogenate fraction. The pellets were resuspended in Tris-HCl-buffered 0.25 M sucrose solution (pH 7.4) and separated into two portions. The first portion was used as the control or FKBP(+) and the second portion was incubated at 37°C for 30 minutes in pH and ionic conditions favorable for FKBP12 dissociation from sarcoplasmic reticulum (250 mM sucrose, 5.5 mM Tris, 0.2 mM PMSF, 2 mg/mL antipain and 5 mg/mL aprotinine, pH 7.4) followed by ultracentrifugation at 108,000 g [treated fraction or FKBP(-)][18,20,21]. The protein content was determined using the Lowry method[22].

Measurement of $^{45}Ca^{2+}$ uptake

Ca^{2+} uptake experiments were performed at 37°C using 50 mM HEPES-Tris buffer (pH 7.4), 10 mM NaN_3, 0.3 mM EGTA, 5 mM ATP-Na_2, 4 mM $MgCl_2$ and traces of $^{45}CaCl_2$ (specific activity: H" 1.5´10^9 Bq/mmol). The experiments were performed in the presence of 10 µM free Ca^{2+} concentration, according to Fabiato & Fabiato[23], in the absence or in the presence of different concentrations of ivermectin dissolved in Dimethyl Sulfoxide (DMSO) (0.008-0.8%; controls). The reaction was started by the addition of rat *vas deferens* preparations to the medium and stopped two hours later by rapid filtration; the filters containing the vesicles were washed twice with 20 mL of a cold solution (2 mM $LaNO_3 \cdot 6H_2O$, 20 mM MOPS and 100 mM KCl, pH 7.0, 4 °C). The filters were dried and immersed in a scintillation cocktail (0.02% 1,4-bis (5-phenyloxazol-2-yl) - POPOP - benzene in toluene) and the radioactivity was counted[6,12]. The specific Ca^{2+} uptake was calculated by subtracting the uptake measured in the absence of 5 mM ATP-Na_2 (blanks) from the total uptake[6,12].

Measurement of Ca^{2+}-ATPase activity

The rat *vas deferens* preparations were incubated for two hours at 37°C under the same experimental conditions used in the Ca^{2+} uptake assays in the absence or in the presence of 3 mM thapsigargin, a specific inhibitor of SERCA pumps[24-26]. Thapsigargin-sensitive Ca^{2+}-ATPase activity was calculated by the subtraction of Ca^{2+}-ATPase activity measured in the presence of thapsigargin from Ca^{2+}-ATPase activity measured in the absence of thapsigargin (total Ca^{2+}-ATPase activity). Experiments were stopped by the addition of 1 mL of a cold mixture containing 26 g/mL of charcoal in 0.1 N HCl[6,12]. The tubes were centrifuged and the supernatant was added to the filters. These filters were dried and immersed in scintillation cocktail (0.01% POPOP and 0.4% PPO dissolved in toluene) and the radioactivity was counted by means of liquid scintillation. The Ca^{2+}-ATPase activity was determined by measuring the $[^{32}P]P_i$ released from [γ-^{32}P]ATP (specific activity: H" 1.5´10^{10} Bq/mmol) hydrolysis followed by centrifugation[6,12,27]. The total specific activity of Ca^{2+}-ATPase was in the range of 2.4-5.3 µmolPi/mg in the FKBP(+) fraction using 10 µM of free Ca^{2+}.

Statistical analysis

Data are presented as the means ± standard error mean of at least three experiments. Statistical comparisons were determined by using one-way (Analysis of Variance [Anova]), followed by Bonferroni's test, and significance was set at $p<0.05$.

RESULTS

In order to investigate the effect of ivermectin on the Ca^{2+} content of SR vesicles present in FKBP(+) and FKBP(-) fractions from rat *vas deferens*, the $^{45}Ca^{2+}$ uptake was measured in the presence of different concentrations of ivermectin (0.1-10 µM). Figure 1 shows that 10 mM ivermectin (concentration usually used to inhibit SERCA1 and SERCA2b pumps and to

directly activate CRCs) significantly reduces the Ca^{2+} content of vesicles in the FKBP(+) and FKBP(-) fractions (inhibition of ~50% and ~40%, respectively). In these assays, different concentrations of DMSO were used as controls (100%) for the different concentrations of ivermectin, since this solvent alone does not significantly modify the Ca^{2+} content of the vesicles (data not shown).

The Ca^{2+}-ATPase activity assays were performed to investigate the effect of ivermectin in different types of Ca^{2+} pumps present in the FKBP(+) fraction from rat *vas deferens*. Figure 2 shows that ivermectin inhibits about 30% of the total Ca^{2+}-ATPase activity related to the sum of the activities of PMCA and SERCA pumps in this fraction. However, to discriminate if ivermectin inhibits PMCA or SERCA pumps activities, Ca^{2+}-ATPase activity was measured in the absence and in the presence of 3 µM Thapsigargin (Tg), a specific inhibitor of SERCA pumps[24-26] in the FKBP(+) fraction. Figure 2 shows that 30 mM ivermectin inhibits the activity of both PMCA (15.8±6.3%) and SERCA (32.7±15.0%) pumps present in the FKBP(+) fraction in different proportions.

DISCUSSION

Ca^{2+} homeostasis seems to play a crucial role in the function of *vas deferens*, a thick walled tube of smooth muscle that functions as a conduit for the movement of sperm from the epididymis to the urethra. Several molecular components were demonstrated in this organ[6,12,18]. Changes in membrane proteins such as Ca^{2+} pumps and Ca^{2+} channels localized in the plasma membrane or in the membrane of intracellular organelles disturb the dynamic equilibrium of Ca^{2+}, impair cellular activities and, consequently, tissue function[28]. In the present study, we demonstrated that ivermectin, a macrocyclic lactone, inhibits SERCA and PMCA pumps and reduces $^{45}Ca^{2+}$ uptake, probably by activation of calcium release channels. These Ca^{2+} disturbances could be involved in the reduction of male fertility.

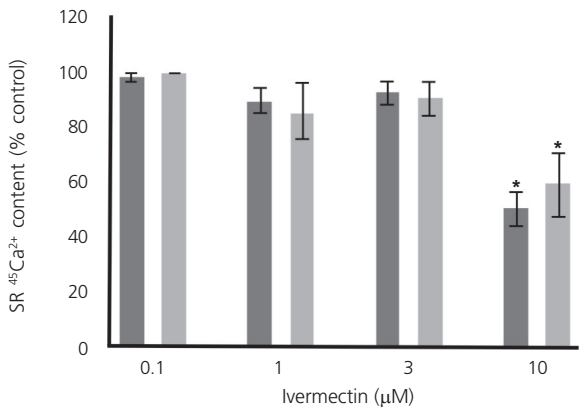

Figure 1. Percentage of ATP-dependent Sarcoplasmatic (SR) Ca^{2+} uptake in FK506-binding protein (+) (grey bars) and FK506-binding protein (-) (white bars) in rat *vas deferens* ultracentrifuged homogenate in the presence of increased concentrations of ivermectin (0.1-10 µM).

Note: *****Statistically different from control with dimethyl sulfoxide (100%, represented for the dotted line; $p<0.05$). Values are means ± standard error mean of four experiments carried out in triplicate.

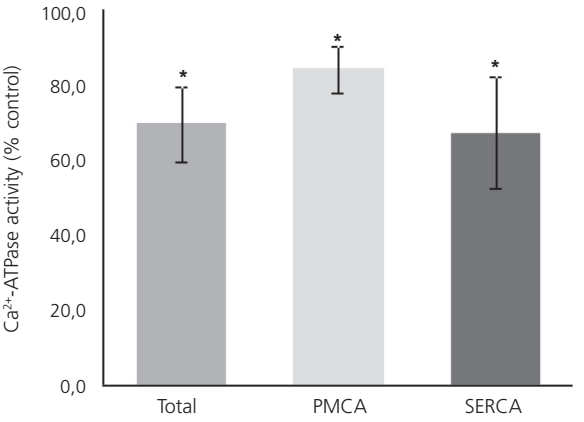

Figure 2.Percentage of total Ca^{2+}-ATPase (grey bar), Plasma Membrane Ca^{2+}-ATPases (PMCA) or thapsigargin-resistant (white bar) and Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPases (SERCA) or thapsigargin-sensitive (striped bar) activity in the FK506-binding protein (+) fraction of rat vas deferens ultracentrifuged homogenate in the presence of 30 mM ivermectin in relation to the control (6% dimethyl sulfoxide).

Note: *****Statistically different from control with dimethyl sulfoxide (100%, represented for the dotted line; $p<0.05$). Values are means ± standard error mean of eight experiments carried out in triplicate.

It has been demonstrated that FKBP12 is physiologically associated with CRC from rat *vas deferens*[6,12]. It seems that FKBP12 stabilizes the CRC closed conformation, but when FKBP12 is stripped off the RyR, it becomes leaky resulting in a diminished net Ca^{2+} accumulation into RyR-gated stores by subconductance states of this channel[12]. According to Carmody *et al*.[20], the immunophylin FKBP12 presents different degrees of association among CRC in distinct tissues. The dissociation of FKBP12 from RyR1 in skeletal muscle requires the presence of the immunosuppressants, FK506 and rapamycin, macrocyclic lactones that bind FKBP12. However, FKBP12 was found to dissociate from both brain and cardiac microsomes presenting other RyR isoforms and IP_3R by a simple high speed centrifugation under favorable ionic conditions, pH and temperature[12,20,21], as is the case with the rat *vas deferens* ultracentrifuged homogenate[12].

Our data also show that the FKBP12-CRC complex dissociation does not modify the effect of ivermectin (10 μM) in rat *vas deferens* (Figure 1) as it has been found that ivermectin can also directly activate CRCs to reduce the SR Ca^{2+} content[16]. It was not possible to evaluate the effect of 30 mM ivermectin due to the fact that the unspecific signal (blank: measured in the absence of ATP) was greater than the specific signal (total: measured in the presence of ATP), which may indicate that this macrocyclic lactone can be in its limit of solubility. With 100 μM of ivermectin, it was not possible to dilute this drug in the reaction medium.

As 10 μM ivermectin inhibited Ca^{2+} accumulation in FKBP(+) (50%) and FKBP(-) (40%), Ca^{2+}-ATPase activity was measured in order to investigate if ivermectin could inhibit PMCA or SERCA pumps using a saturating concentration of thapsigargin (3 μM), a specific inhibitor of SERCA pumps (Figure 2)[24-26]. The percentage contribution of each type of Ca^{2+} pump (PMCA or SERCA) for the total Ca^{2+}-ATPase activity measured is different according to the enzymatic preparation used. Probably, the maximum percentage of inhibition of each pump by ivermectin depends on their contributions. This explains why the sum of the effects of ivermectin on PMCA and SERCA is different from 30.3±10.0% (the inhibitory effect of ivermectin on the total Ca^{2+}-ATPase activity). Bilmen and coworkers[17] reported that ivermectin inhibits the skeletal muscle sarcoplasmic reticulum and brain microsomal endoplasmic reticulum Ca^{2+}-ATPase (SERCA1 and SERCA2b isoforms, respectively), but the effect of ivermectin on plasma membrane Ca^{2+}-ATPase or on another types of thapsigargin resistant-Ca^{2+} pump has not been described[29].

In conclusion, the findings of this work indicate that ivermectin reduces the Ca^{2+} content of SR vesicles by inhibiting the activity of the Ca^{2+} pumps (SERCA and PMCA), and because of this action, ivermectin may be used to manipulate Ca^{2+} handling in rat *vas deferens*. In this context, ivermectin could be an important pharmacological tool to investigate possible alterations of Ca^{2+} mobilization processes as a cause of male infertility.

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CONTRIBUTORS

H Muzi-FIlho conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. DRA Souza performed the experiments. CBV Scaramello conceived and designed the experiments. VMN Cunha conceived and designed the experiments, analyzed the data and wrote the manuscript.

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