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Research paper

1α,25(OH)$_2$-vitamin D$_3$ stimulates rapid plasma membrane calcium influx via MAPK activation in immature rat Sertoli cells

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ABSTRACT

It was characterized that the rapid response to 1α,25(OH)$_2$-vitamin D$_3$ (1,25D$_3$) on $^{45}Ca^{2+}$ influx in rat Sertoli cells was mediated by voltage-dependent Ca^{2+} channels (VDCCs), PKC, ERK1/2 and p38 MAPK pathways. In primary culture of 10 day-old rat Sertoli cells as well as in the whole testis, the time-course of $^{45}Ca^{2+}$ influx did not change significantly in basal conditions. However, 1,25D$_3$ showed stimulatory effect on $^{45}Ca^{2+}$ influx from 10^{-15} to 10^{-8} M after 60 s of incubation. The maximum effect was around 140% at 10^{-12} M on purified Sertoli cells showing a steady state on $^{45}Ca^{2+}$ influx between 10^{-11} and 10^{-9} M. Under this experimental condition, 1,25D$_3$ stimulated $^{45}Ca^{2+}$ influx from 73% to 106% and no effect was observed at 10^{-16}, 10^{-8} and 10^{-7} M in whole testis. VDCC activities are mandatory for a full and complete stimulatory effect of 1,25D$_3$ in these approaches. K^+ and Cl^- channels also are strongly involved in this rapid response coordinated by 1,25D$_3$. The participation of some selected kinases, points to PKC and ERK1/2 upstream activity to p38 MAPK activation suggesting an intracellular cross-talk between rapid $^{45}Ca^{2+}$ influx and nuclear events. In addition, the comparative effect of microtubule disassembles and ClC-3 channel blocker on $^{45}Ca^{2+}$ influx provides tivates p38 s and that and/or the secretory activity of Sertoli cell.

1. Introduction

The steroid hormone 1α,25(OH)$_2$-vitamin D$_3$ (1,25D$_3$) activates multiple signaling pathways in its target cells [1,2]. The vitamin D receptor (VDR), a member of the superfamily of nuclear receptors exerts 1,25D$_3$-dependent responses in the nucleus as a ligand-activated transcription factor [3]. In addition to these relatively slow (hours to days) genomic effects, 1,25D$_3$ generates rapid responses (seconds to minutes) including Ca^{2+} uptake. The first clear demonstration of 1,25D$_3$-induced nongenomic response emanated from the *ex vivo* study in perfused chick intestine, was transcaltachia (the rapid hormonal stimulation of intestinal Ca^{2+} absorption). In that study, it was observed that the transfer of $^{45}Ca^{2+}$ present in chick intestine to the circulatory system was stimulated within 4–5 min after addition of physiological concentrations of 1,25D$_3$ in the celiac artery [4]. Numerous rapid responses mediated by 1,25D$_3$ have been reported, including modulation of voltage-dependent Ca^{2+} and Cl^- channels [1,5], exocytosis of bone material [6], and modulatory effect on protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) [2,5,7].

There is accumulating evidence that vitamin D and VDR are important in reproductive tract. Successful fertility rates are significantly decreased in vitamin D deficient male rats [8,9]. 1,25D$_3$ effects on cellular Ca^{2+} homeostasis have been reported in a variety of cell types as chicken intestinal mucosa [10], and osteoblastic cells [6]. It has been described that VDR are present in the cytoplasm and nucleus of male and female reproductive tissues [11]. In addition, the wide expression of VDR as well as the biological functions was demonstrated by autoradiographic receptor studies on mouse testis [12]. The presence of VDR in the nucleus of a mouse Sertoli cell line and the stimulatory effect of 1,25D$_3$ on $^{45}Ca^{2+}$ influx, DNA synthesis and protein content have been shown [13].

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In previous studies in whole immature rat testis, we demonstrated the stimulatory effect of $1,25D_3$ through a specific and selective plasma membrane amino acid transport system [14]. These results pointed to both genomic effects, which can be triggered by PKA, and to rapid responses involving Ca^{2+}/K^+ channels on the plasma membrane. Furthermore, it was demonstrated that extracellular Ca^{2+} as well as voltage-dependent Ca^{2+} channels (VDCCs) are necessary to mediate plasma membrane effect of $1,25D_3$ [14], similar to results observed for thyroxin in immature rat testis [15]. Recently, we demonstrated for the first time $1,25D_3$ potentiation of Cl^- currents required for exocytosis, and identified protein kinase signaling underlying ion channel modulation by this hormone in TM4 cells [5]. So, the present study was conducted to analyze the rapid response and underlying the mechanism of action of $1,25D_3$ targeting the $^{45}Ca^{2+}$ influx in purified Sertoli cell cultures and whole testis from 10 day-old rats.

2. Materials and methods

2.1. Chemicals

1α,25(OH)$_2$-vitamin D_3 ($1,25D_3$), verapamil, flunarizine, nifedipine, 9-anthracene carboxylic acid (9-AC), 4, 4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), tolbutamide, apamine, KT-5720, H-89, RO 31-8220, SB 23,9063, PD 98059, and colchicine were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, penicillin, streptomycin, kanamycin and amphotericin B were from Sigma Chemical Company (St. Louis, MO, USA). Collagenase–dispase and bovine serum albumin (BSA) were from Roche Diagnostics (Mannheim, Germany). Serum Remplacement 3, bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I–S), trypsine, soybean trypsin inhibitor, sodium pyruvate, D-glucose, Hepes, sodium bicarbonate were purchased from Sigma Chemical Company (St. Louis, MO, USA). [^{45}Ca]$CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}) and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Boston, USA). The antibodies p44/42 MAP Kinase (anti-ERK1/2), phospho-p44/42 MAP Kinase (Thr202/Tyr204) were obtained from Cell Signaling Technology (Boston, MA, USA) and peroxidase conjugated anti-rabbit IgG from GE Healthcare (Amersham, Buckinghamshire, UK). The Immobilon™ Western chemiluminescent HRP substrate was obtained from Millipore. All other chemicals were of analytical grade.

2.2. Animals

Wistar rats were bred in animal house and maintained in an air-conditioned room (about 21 °C) with controlled lighting (12 h/12 h light/dark cycle). The suckling rats were kept with their mothers until euthanasia. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00023).

2.3. Primary Sertoli cell culture and $^{45}Ca^{2+}$ influx

Sertoli cells were obtained from 10 day-old Wistar rats. Rats were killed by decapitation, testes were removed and decapsulated. Sertoli cells were obtained by sequential enzymatic digestion as previously described by Dorrington et al. [16]. The cells were seeded at the concentration of 200 000 cells/cm^2 in 24 wells Falcon culture plates (Deutscher, Brummath, France) and cultured for 72 h in Ham's F12/DMEM (1:1) medium supplemented with serum replacement 3, 2.2 g/L sodium bicarbonate and antibiotics (50,000 IU/L penicillin, 50 mg/L streptomycin, 50 mg/L kanamycin), fungicide (0.25 mg/L amphotericin B), in a humidified atmosphere of 5% CO_2:95% air at 34 °C. Three days after plating, residual germ cells were removed by a brief hypotonic treatment using 20 mM Tris–HCl (pH 7.2). After 2.5 min of incubation the hypotonic solution was removed, the cells were washed with PBS (PAN, Dutscher, Brumath, France) and fresh medium Ham's F12/DMEM (1:1) was added. This method, which is based on the differential response of Sertoli and germ cells to osmolarity changes, is highly efficient in removing germ cells and in obtaining pure Sertoli cell cultures. The hypotonic treatment did not alter the morphology, functional activities and FSH responsiveness of Sertoli cells [17]. On day 5 after plating cells were pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 34 °C, (pH 7.4) and gassed with O_2:CO_2 (95:5; v/v). After that, the medium was changed by fresh KRb containing 0.1 μCi/mL $^{45}Ca^{2+}$ during 60 min. For Ca^{2+} influx measurements, cells were incubated for a further 30, 60, 150, 300 or 600 s in the absence (control) or presence of $1,25D_3$ (10^{-13} to 10^{-7} M) [14,15]. In some experiments channel blockers or kinase inhibitors were added during the last 20 min before the hormone addition and maintained during all the incubation period (see figures). The following drugs were used: Flunarizine (1 μM), nifedipine (100 μM), verapamil (100 μM), 9-AC (1 μM), DIDS (200 μM), tolbutamide (100 μM), apamine (0.1 μM), H-89 (10 μM), KT-5720 (1 μM), RO 31-8220 (20 μM), SB 23,9063 (10 μM), PD 98059 (10 μM), and colchicine (10 μM) [14,15,18–20]. The results are expressed as pmol $^{45}Ca^{2+}$/μg protein or % of control which represents an average of 204.55 ± 18.26 pmol $^{45}Ca^{2+}$/μg protein.

2.4. $^{45}Ca^{2+}$ influx in whole testis

One gonad (alternately left and right) from 10 day-old rats was used as treated and the contralateral one as the control. The testes were weighed, decapsulated and pre-incubated in KRb buffer as described for Sertoli cells. After that, the testes were transferred to another series of wells with fresh KRb containing 0.1 μCi/mL $^{45}Ca^{2+}$ during 60 min. Finally, $1,25D_3$ was included in this KRb containing $^{45}Ca^{2+}$ and the tissues were incubated with different doses of $1,25D_3$ (10^{-16} to 10^{-7} M) for 60 s. In some experiments ionic channel blockers were added during the last 20 min before the hormone addition and maintained during all the incubation period as described above [15,19,20].

Extracellular $^{45}Ca^{2+}$ from either primary Sertoli cell culture or whole testis was thoroughly washed off in 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM Glucose, 10 mM $LaCl_3$ solution, pH 7.4 for 30 min. The presence of La^{3+}, in washing solution, was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$ [21]. After La^{3+} cells or tissue washing, they were removed to screw cap tubes containing 1 mL of distilled water. They were frozen at −20 °C and afterwards boiled for 10 min; 100 μL aliquots of tissue medium were placed in scintillation fluid and in a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Porpose Scintillation Counter-Beckman Coulter, Boston, USA) [20]. The results are expressed as pmol $^{45}Ca^{2+}$/μg protein or % of control which represents an average 14.50 ± 1.0 pmol $^{45}Ca^{2+}$/mg protein.

2.5. Western blot analysis

Sertoli cells were incubated with/without 10^{-10} M $1,25D_3$ for 1 min at 34 °C in the KRb. After hormone treatment, the cells were rapidly homogenized in 100 μL of a lysis solution containing 2 mM

EDTA, 50 mM Tris–HCl, pH 6.8, 4% (w/v) and protein concentration was determined. For electrophoresis analysis, samples were dissolved in 20% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations (40 μg) were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemli [22] and transferred to nitrocellulose membranes for 60 min at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 120 min of incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (TTBS), and then incubated overnight at 4 °C with anti-ERK1/2 and anti-phospho ERK1/2 (Thr202/Tyr204) diluted 1:1000 in TBS containing 5% albumin. The blot was then washed twice for 5 min with TTBS and incubated for 120 min in TBS with 5% albumin containing anti-rabbit IgG 1:1000. The blot was washed twice again for 5 min with TTBS and twice for 5 min with TBS. The blot was then developed using the Immobilon™ Western chemiluminescence HRP substrate kit [23]. Autoradiograms and immunoblots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

2.6. Statistical analysis

The results are means ± S.E.M. expressed as pmol $^{45}Ca^{2+}$/μg of protein, pmol $^{45}Ca^{2+}$/mg of tissue or % of control. The total protein was measured according to the Bradford method [24]. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Bonferroni multiple comparison test. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Basal time-course and dose–response curve of 1,25D$_3$ on $^{45}Ca^{2+}$ influx

After the Ca^{2+} equilibrium in the Sertoli cells and in the testis for 60 min of incubation, Ca^{2+} measurement was additionally monitored for 30, 60, 150, 300 and 600 s without stimuli. Fig. 1A and B, show that no significant changes on $^{45}Ca^{2+}$ influx was observed neither in Sertoli cells nor in the testis during this time-course studied.

As illustrated in Fig. 1C, 1,25D$_3$ stimulates $^{45}Ca^{2+}$ influx from 10^{-12} to 10^{-8} M after 60 s of incubation. It was observed that the maximum stimulatory effect of 1,25D$_3$ was around 140% at 10^{-12} M, compared with control group. An initial peak rise followed by a sustained rise from 10^{-12} to 10^{-9} M was evident and significant decrease on $^{45}Ca^{2+}$ influx between 10^{-12} and 10^{-8} M was observed.

The effect of 1,25D$_3$ (from 10^{-16} to 10^{-7} M) on $^{45}Ca^{2+}$ influx was also studied in whole testis after 60 s of incubation. The stimulatory action was observed from 10^{-15} to 10^{-9} M with a peak rise of Ca^{2+} at 10^{-15} M and a sustained effect until 10^{-9} M of 1,25D$_3$. No stimulatory effect was observed at 10^{-16}, 10^{-8} and 10^{-7} M. Although the stimulatory effect profile of 1,25D$_3$ on $^{45}Ca^{2+}$ influx was similar, in terms of percentage, the average of 1,25D$_3$ stimulatory effect on

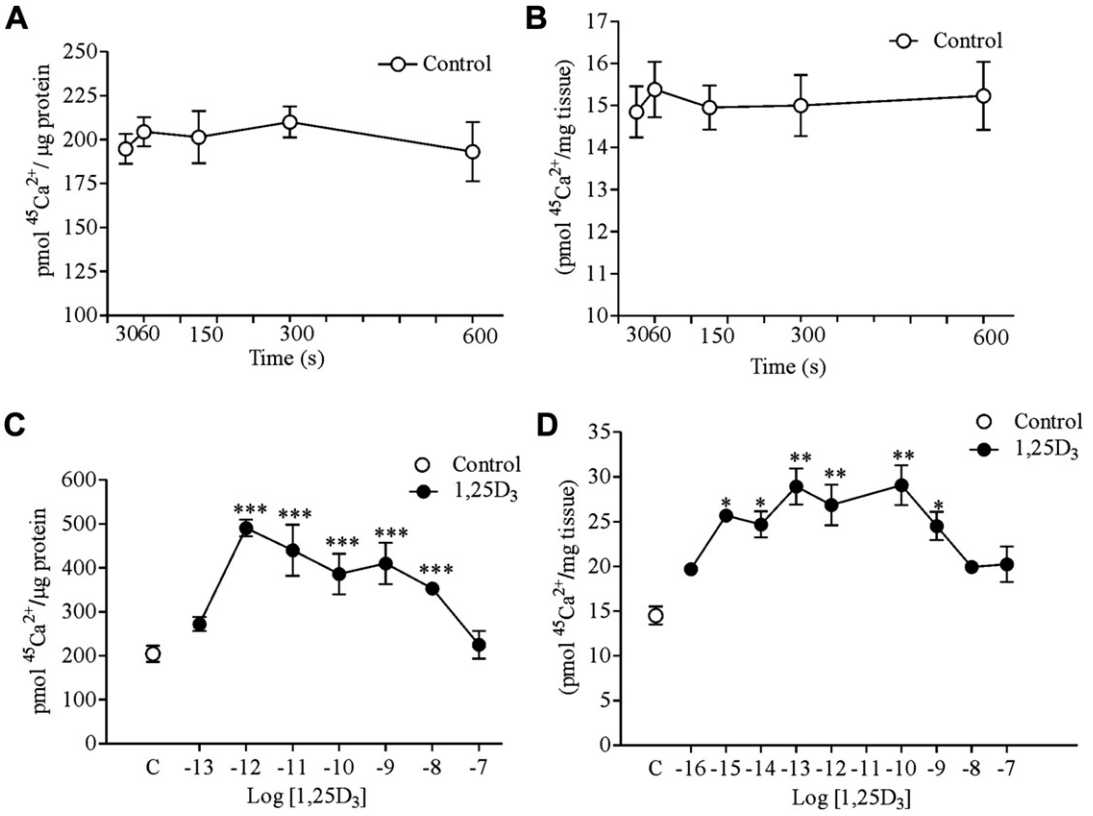

Fig. 1. Basal $^{45}Ca^{2+}$ influx in Sertoli cells (A) and testis (B). Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. After that the groups were incubated for an additional time 30, 60, 150, 300 and 600 s, in the same solution. Dose–response curve of 1,25D$_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells (C) and testis (D). Pre-incubation: 60 min. After that Sertoli cells or testis were incubated with or without different concentration of 1,25D$_3$ for 60 s in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. ***$P < 0.001$, **$p < 0.01$ and *$p < 0.05$ compared with control group.

$^{45}Ca^{2+}$ influx was around 110% in Sertoli cells and 90% in whole testis (Fig. 1C and D).

3.2. Involvement of voltage-dependent Ca^{2+} channels in the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx

Although we have reported the involvement of extracellular Ca^{2+} in the mechanism of action of 1,25D_3 in amino acid accumulation in immature rat testis [14], in the present study, we also used $^{45}Ca^{2+}$ influx as a tool to demonstrate the direct effect of 1,25D_3 and rapid response of Sertoli cells and testis to this hormone.

Fig. 2A shows the involvement of two Ca^{2+} channel subtypes in the stimulatory action of 1,25D_3 on $^{45}Ca^{2+}$ influx in Sertoli cells. We first demonstrated that the blockers themselves, at the concentrations used, did not alter the basal Ca^{2+} cellular equilibrium. It was observed that when Sertoli cells were incubated for 25 min with 100 μM nifedipine or 1 μM flunarizine, L- and T-type VDCC blockers respectively, the effect of the hormone was partially prevented. To better characterize the high-voltage Ca^{2+} currents involvement in the 1,25D_3 stimulatory effect on $^{45}Ca^{2+}$ influx, we used another specific L-VDDC blocker, verapamil, which completely abolished the 1,25D_3 effect.

Considering our previous data of nongenomic actions of 1,25D_3 in intact whole testis [14], we take advantage of this approach to compare the profile of $^{45}Ca^{2+}$ influx stimulated by 1,25D_3 with that in isolated Sertoli cells. As showed in Fig. 2B, the efficacy of the L- and T-VDCC blockers (without disturbing the basal Ca^{2+} balance), was similar to that observed in isolated purified Sertoli cells strengthening therefore, the view that 1,25D_3 was able to activate these channels in the testis.

3.3. Involvement of K^+ channels in the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx

It is well know that Ca^{2+} currents can be influenced by other ions as demonstrated by the presence of Ca^{2+}-dependent K^+ channels and Ca^{2+}-dependent Cl^- channels [5,25,26]. Moreover, K^+ is one of the ionic components engaged to keep the basal electrical characteristics of the cells. In the present study, we demonstrated that both 0.1 μM apamine (specific of Ca^{2+}-dependent K^+ channel blocker) and 100 μM tolbutamide (ATP-dependent K^+ channel blocker) prevented the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx both in cultured Sertoli cells and in whole testis (Fig. 3A and B).

3.4. Involvement of Cl^- channels in the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx

The independent movement of either K^+ efflux or Cl^- influx in cells can produce hyperpolarization that changes completely the activity of the voltage-dependent channels in the plasma membrane. Taking this into account, we investigated the involvement of Ca^{2+}-activated Cl^- currents on the effect of 1,25D_3 on $^{45}Ca^{2+}$ influx by using 1 μM of 9-AC. The stimulatory action of the hormone was prevented by this blocker, suggesting that the activity of this type of channel might be part of the regulatory mechanism of 1,25D_3 effects initiated on the plasma membrane. Also, we analyzed the involvement of voltage-dependent Cl^- channels in isolated Sertoli cells and in the whole testis. The selective voltage-dependent Cl^- channels blocker (DIDS) was able to partially inhibit (55%) the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx (Fig. 4A and B).

3.5. Involvement of PKC and PKA in the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx

As shown in Fig. 5A, treatment of Sertoli cells with RO 31-8220 (20 μM), a PKC inhibitor, did not have any effect on control group; however, it significantly abolished the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx. We also found that incubation of Sertoli cells with PKA inhibitors KT-5720 and H-89, selective and potent PKA blockers did change neither basal $^{45}Ca^{2+}$ influx nor stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx (Fig. 5B).

3.6. Involvement of MEK/MAPK pathways in the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx

There are increasing evidence that 1,25D_3 stimulates nongenomic effects associated with Ca^{2+}-activated and kinase signaling pathways. In this context, in Sertoli cells, we performed the $^{45}Ca^{2+}$ influx assay in the presence of PD 98059, a inhibitor of mitogen-activated protein kinase (MAPK) cascade, or SB 23,9063 that inhibits its established targets p38α and p38β, as already demonstrated [2,15,18,27]. Fig. 6A shows that the presence of 10 μM PD 98059 significantly diminished the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx. However, p38 MAK inhibitor (10 μM) completely blocked $^{45}Ca^{2+}$ influx stimulated by 1,25D_3 (Fig. 6B), evidencing a kinase-dependent mechanism.

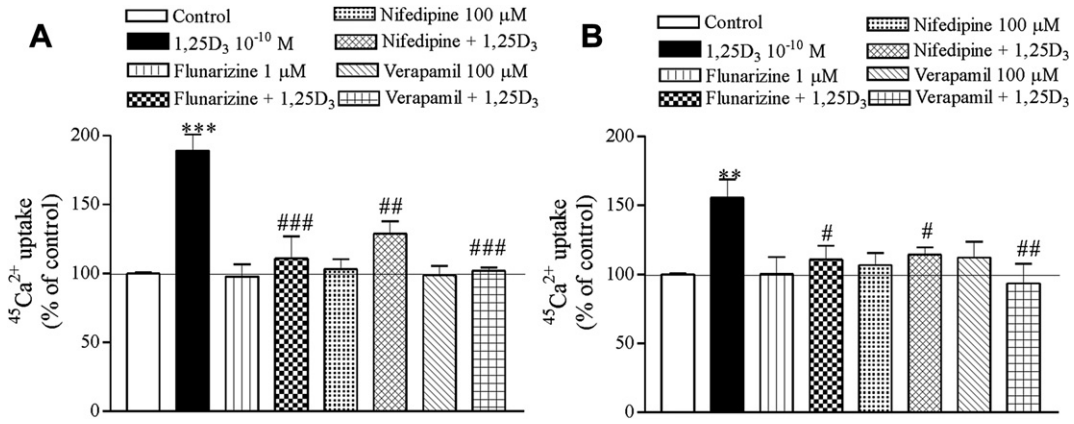

Fig. 2. Involvement of voltage-dependent Ca^{2+} channels (T and L types) on the stimulatory effect of 1,25D_3 on $^{45}Ca^{2+}$ influx in Sertoli cells (A) and testis (B). Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of 1,25D_3 and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. ***$P < 0.001$ and **$p < 0.01$ compared with control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with 1,25D_3 group.

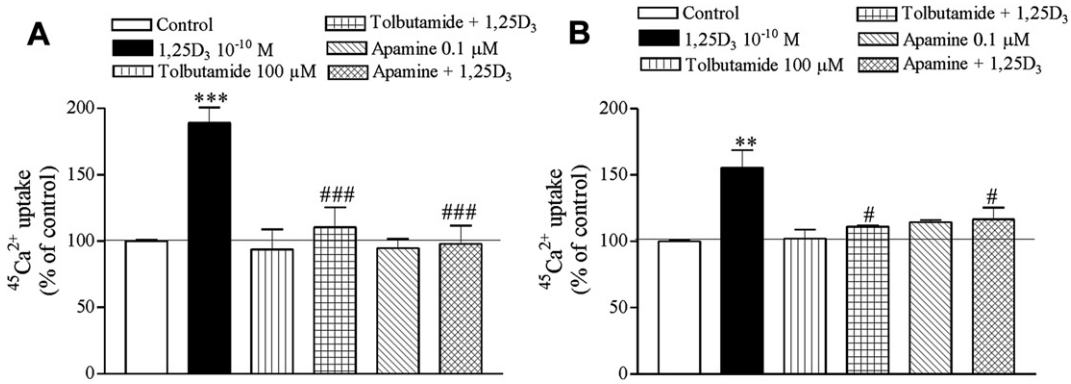

Fig. 3. Involvement of K^+ channels on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells (A) and testis (B). Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of $1,25D_3$ and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{***}P < 0.001$ and $^{**}p < 0.01$ compared with control group; $\#p < 0.05$ and $\#\#\#p < 0.001$ compared with $1,25D_3$ group.

3.7. Effect of $1,25D_3$ on total and phospho ERK1/2 levels in Sertoli cells

Considering that our findings support the involvement of ERK MAPK on $1,25D_3$ action in 10 day-old rat Sertoli cells, the effect of $1,25D_3$ on total and phospho ERK1/2 levels was investigated. Results showed that $1,25D_3$ did not alter the total immunocontent of ERK1/2 after 1 min incubation (Fig. 7A). On the other hand, the hormone induced a rapid phosphorylation and activation of ERK1/2 in Sertoli cell cultures (Fig. 7B). The Coomassie blue stained gel was presented as a protein loading control (Fig. 7C).

3.8. Involvement of microtubules integrity and ClC-3 Cl^- channel activity in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx

The contribution of microtubules integrity to the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx was verified by using 10 μM colchicine, a network microtubule disruptor. Fig. 8 shows that the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx diminished significantly in the presence of colchicine. In addition, in this figure, we compared the effect of DIDS, a specific blocker of voltage-dependent Cl^- channels, and colchicine in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx. Both agents were able to disturb the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx suggesting that, at least in part, as much microtubules network as ClC-3 channel activity (both involved on cellular secretion), participate on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells.

4. Discussion

The evidence of Sertoli cells as primary site for $1,25D_3$ receptor binding in mouse testis was reported by Schleicher et al. [12]. Also, it has been reported that $1,25D_3$ binds to cytosolic and nuclear fractions of whole rat testis and its various cellular components [11].

The effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx was very rapid, occurring in seconds, a time lag non-compatible with the classical scheme for a nuclear receptor action, confirming a plasma membrane effect of $1,25D_3$ and a rapid response in Sertoli cells, as previously reported by Menegaz et al. [14]. Our data are in agreement with Akerstrom & Walters [13] who demonstrated that $1,25D_3$ induced a dose-dependent rapid uptake of $^{45}Ca^{2+}$ within 5 min in a mouse Sertoli cell line. Also, the rate of $^{45}Ca^{2+}$ influx is in line with those reported for retinol stimulatory effect on $^{45}Ca^{2+}$ influx in isolated Sertoli cells [28], as well as to testosterone in whole testis and/or Sertoli cells [29,30].

In general, intracellular Ca^{2+} is tightly regulated by multiple Ca^{2+} channels, pumps, exchangers and buffers. Ca^{2+} influx can be regulated, at least, by three major mechanisms: VDCCs, second messenger-mediated channels and/or receptor-mediated channels [31–33].

Considering our previous evidence that nongenomic actions of $1,25D_3$ deeply involve extracellular Ca^{2+} in immature rat testis [14], we then analyzed the mechanism of Ca^{2+} influx, by investigating L- and T-type VDCCs, which were described to be present in the plasma membrane of rat Sertoli cells and in the testis [34]. Indeed,

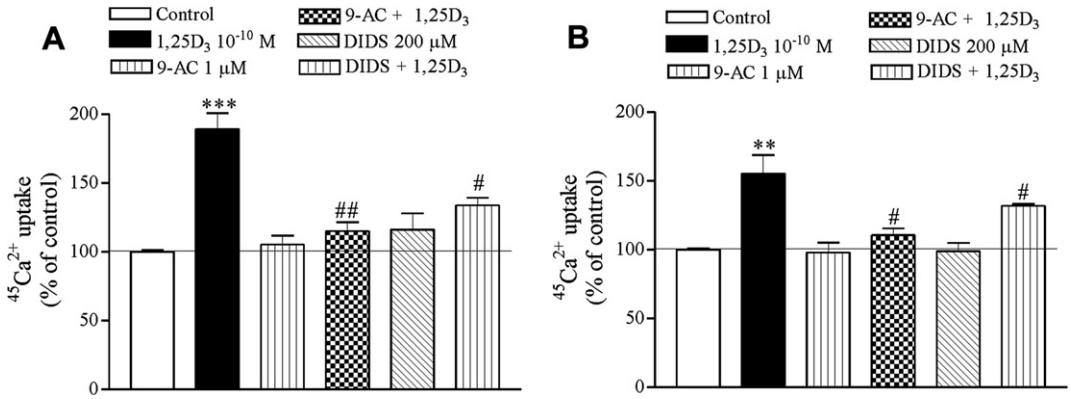

Fig. 4. Involvement of Cl^- channels on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells (A) and testis (B). Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of $1,25D_3$ and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{***}P < 0.001$ and $^{**}p < 0.01$ compared with control group; $\#p < 0.05$ and $\#\#p < 0.01$ compared with $1,25D_3$ group.

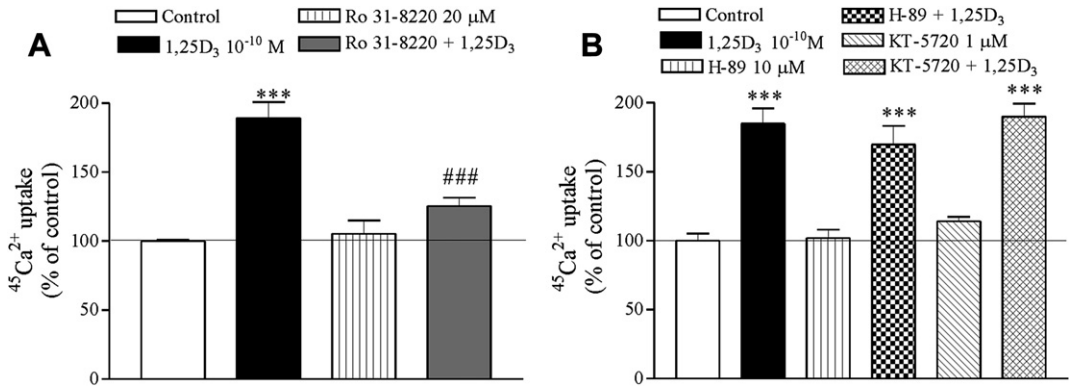

Fig. 5. Involvement of PKC (A) and PKA (B) on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells. Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of $1,25D_3$ and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{***}P < 0.001$ compared with control group; $^{###}p < 0.001$ compared with $1,25D_3$ group.

our data demonstrate that Sertoli cells and whole testis treated with L- or T-type VDCC blockers (flunarizine, nifedipine or verapamil), lead to an effective blockage of $1,25D_3$-induced Ca^{2+} influxes. Therefore, we conclude that the rapid Ca^{2+} influx elicited by $1,25D_3$ in Sertoli cell and in the testis occurs predominantly through VDCCs. However, as deeply discussed by Friel and Chiel [33], due to the complexity of cellular Ca^{2+} signaling, it has been difficult to predict how pharmacological perturbations or natural stimuli handling calcium flux and how it reflect with ultimate effects of calcium on cell permeability, depolarizing stimulus, protein phosphorylation, gene regulation and cellular secretion. Our data corroborate the physiological importance of extracellular Ca^{2+} to $1,25D_3$ action on plasma membrane [14], and to thyroxin action, as recently demonstrated in testis [15].

K^+ channels are a ubiquitous family of membrane proteins that play critical roles in electrolyte transport, cell volume regulation and also along with the coordinated activity of other ion channels and membrane transporters. These channels are also essential for fluid secretion. Besides, K^+ channels play an essential role to maintain an adequate electrical driving force for Ca^{2+} entry [35].

We demonstrated the participation of ATP-dependent K^+ channels and Ca^{2+}-dependent K^+ currents in studies involving plasma membrane events and nongenomic responses to thyroid hormones [15,19,36,37], as well as to $1,25D_3$ in immature rat testis [14]. In the present work, the functional integrity of K^+ currents was essential to the hormone effect, since the blockage of K^+ channels by tolbutamide and apamine abolished the stimulatory action of $1,25D_3$ on Ca^{2+} influx. In agreement with this, several reports sustained the role of K^+ channels and Ca^{2+} influx associated with the regulation of cellular secretion [25,38].

Modulation of voltage-dependent Ca^{2+} conductance by changing Cl^- concentration was described in the testis [26]. We recently showed nongenomic potentiation of $1,25D_3$ on outwardly rectifying; DIDS-sensitive Cl^- currents coupled to exocytosis in mouse TM4 Sertoli cells [5]. In this context, 9-AC, an aromatic compound that has been widely used as an anion probe to study Ca^{2+}-activated Cl^- currents as well as DIDS, a stilbene derivative which blocks ClC-3 outwardly rectifying Cl^- channels [39,40], were used to study the influence of these both types of ionic currents in the stimulatory effect of $1,25D_3$ on Ca^{2+} influx. The total blockage of the hormone effect on Ca^{2+} influx by 9-AC highly suggest the modulatory role of Cl^- currents on Ca^{2+} influx in purified Sertoli cells and in whole testis. However, although the presence of ClC-2, ClC-3, ClC-4, ClC-5, ClC-6 and ClC-7 members of the family of voltage-dependent Cl^- channels were previously described to be expressed in primary culture rat Sertoli cells [41] and in mouse TM4 Sertoli cells [5,42], they seemed not to be the main Cl^- channels currents to modulate Ca^{2+} influx in Sertoli cells and testis from 10 day-old rat, in our experimental conditions.

We investigated the signaling events underlying the effect of $1,25D_3$ on Ca^{2+} influx keeping in mind the role of PKC and PKA commonly described either by phosphorylation of ionic channels or as a target cross-talk proteins to mediate extracellular signals to the nucleus [7,14,41,43]. It has been reported that $1,25D_3$ activation of

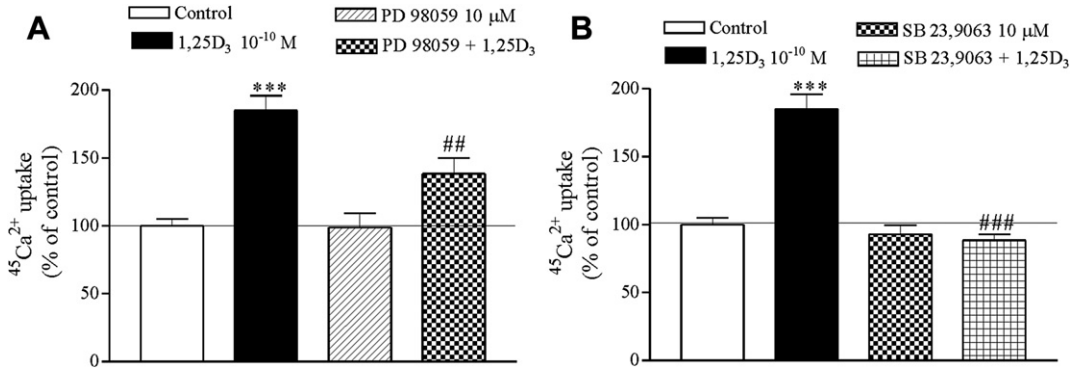

Fig. 6. Involvement of MEK (A) and p38 MAPK (B) on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells. Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of $1,25D_3$ and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{***}P < 0.001$ compared with control group; $^{##}p < 0.01$ and $^{###}p < 0.001$ compared with $1,25D_3$ group.

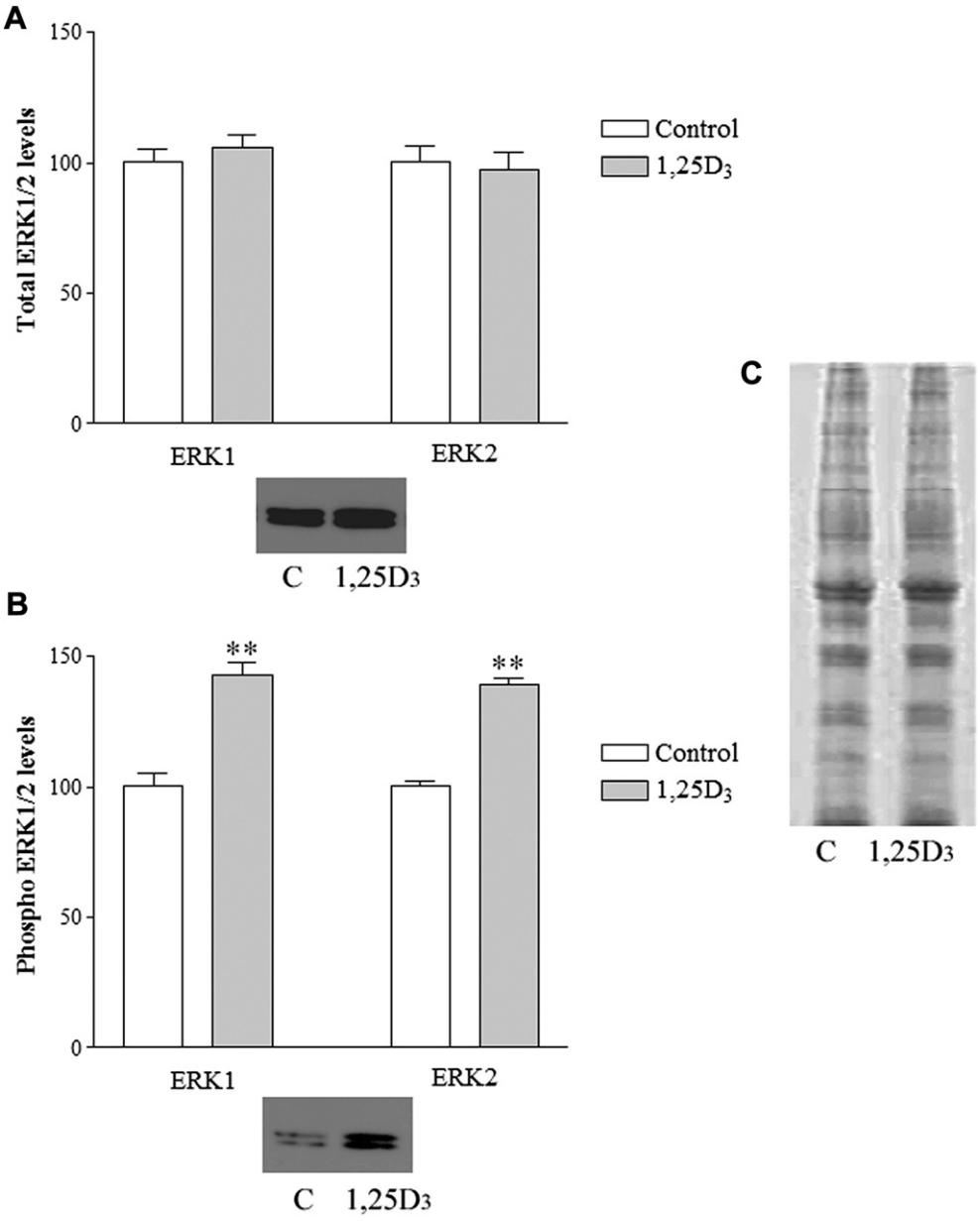

Fig. 7. Effect of $1,25D_3$ on total ERK1/2 (A) and phosphorylated ERK1/2 (B). Cells were incubated for 1 min with/without the hormone. Cells were lysed and prepared to immunoblotting analysis. The Coomassie blue stained gel is showed as a representative protein loading control (C). The total and phospho ERK1/2 levels were measured, as described in methods section. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{**}P < 0.01$ compared with control group.

PKC may cause PKA activation via a cross-talk mechanism with the cAMP pathway to mediate $1,25D_3$-dependent Ca^{2+} uptake in the skeletal muscle [44]. Herein, we demonstrated the involvement of PKC in $1,25D_3$ nongenomic modulation of Ca^{2+} currents that is in agreement with results obtained in other tissues [45,46]. Also, consistent with these findings, the results of our previous report [14] evidenced differential mechanism of action to $1,25D_3$ targeting the amino acid accumulation, supporting that $1,25D_3$ can non-genomically alter distinct signal-transducting pathways.

It has been described that the conformationally flexible $1,25D_3$ can interact with the VDR localized in the cell nucleus to generate genomic responses or in caveolae of the plasma membrane to generate rapid responses [47,48]. Binding of $1,25D_3$ to the caveolae-associated VDR may result in the activation of one or more systems. There are a number of possible outcomes including opening of cells voltage-dependent Ca^{2+} or Cl^- channels or generation of second messengers. Some of these second messengers, particularly Raf/MAPK, may be involved in cross-talk with the nucleus to modulate gene expression [49]. We found a significant inhibition of Ca^{2+} influx when PD 98059, an inhibitor of MAPK cascade was co-incubated with $1,25D_3$. In addition, we detected a significant increase on thymidine DNA incorporation at 5, 15 and 60 min, corroborating the high nuclear activity of Sertoli cells at this age (data not shown). Recently, we have clearly demonstrated that $1,25D_3$ is concerned in the aromatase expression regulation in immature rat Sertoli cells involving a putative membrane VDR associated with a nongenomic pathway and PKA activation [50]. These are in agreement with some reports showing the partial involvement of MEK (ERK1/2) on rapid signal transduction of $1,25D_3$ in intestine [49], NB4 cells and osteoblasts [51,52].

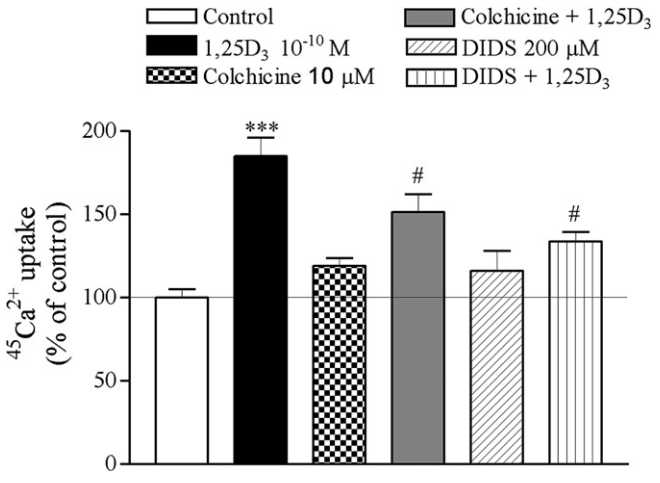

Fig. 8. Effect of microtubules integrity and ClC-3 channel activity on the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx in Sertoli cells. Pre-incubation time: 60 min in the presence of $^{45}Ca^{2+}$ 0.1 μCi/mL. Drugs were added 25 min before the incubation time. Incubation: 60 s in the presence of $1,25D_3$ and $^{45}Ca^{2+}$ 0.1 μCi/mL. Values are means ± S.E.M for three independent experiments carried out in quadruplicate. $^{***}P < 0.001$ compared with control group; $\#p < 0.05$ compared with $1,25D_3$ group.

Several studies suggest that steroid hormones and related compounds can activate members of the MAPKs, including ERK1/2 and p38 MAPK in different rapid responses linked or not to nuclear activation [2]. Pardo et al. [7] reported that p38 MAPK stimulation by $1,25D_3$ involves Ca^{2+}, c-Src and PKA as upstream regulators to c-Fos induction in intestinal cells. Also, p38 MAPK family has been involved in classic genomic events in HL60 and THP-1 cell lines [52].

p38 MAPK downstream effectors consist in a cascade of kinases and transcription factors [40,53]. The presence of SB 23,9063 provided strong evidence that Ca^{2+} and PKC are upstream modulators of ERK1/2 in the activation of p38 MAPK by $1,25D_3$ in Sertoli cells. In agreement with our results, extracellular Ca^{2+} has been shown to modulate p38 MAPK and CREB [41,54]. Furthermore, it has also been reported that depending on the stimulus and of the cell type, p38 activation has been shown to be dependent [55,56], or independent of PKC [8,57]. Taking together these results indicate that more than one signal contributes to the $1,25D_3$ mechanism of action, depending on the tissue. In this study we found that PKC and p38 MAPK are stimulated by $1,25D_3$ to generate a rapid response of Sertoli cells connecting plasma membrane effect with genomic activity and supporting a role for PKC in the upstream activity to ERK1/2 and p38 MAPK activation. In addition, the stimulatory effect of $1,25D_3$ also on ERK1/2 phosphorylation corroborates an intracellular cross-talk to connect the rapid Ca^{2+} influx to nuclear activity demonstrating that there is more than one mode of $1,25D_3$ action targeting Ca^{2+} channels, suggesting multiples sites of possible regulation.

In order to verify the contribution of microtubules assembly in Sertoli cell in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx, we co-incubated the hormone with a microtubules disruptor, colchicine. We showed that microtubule network has a significant contribution on intracellular substances or vesicles traffic on Sertoli cells as already demonstrated to plasma membrane amino acid transport in immature rat testis, in the presence of colchicine [34,58].

The involvement of Cl^- channels activities in bone material secretion has been reported [1,6]. In addition, ClC-3 channel, which is known to be involved in exocytosis, is present at the membrane of secretory vesicles. Also, recently it was reported by our group that Cl^- channels (ClC-3), highly expressed in mouse Sertoli cells line, are found in the organelles membrane as well as on the plasma membrane and participates on Sertoli cell secretory activities [5]. So, we compared the effect of a specific blocker of ClC-3 in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ influx demonstrated in the Fig. 4A and B, with the colchicine effect to suggest the Ca^{2+} contribution on intracellular vesicles trafficking in Sertoli cells. Taking in account these findings, studies are underway (confocal microscopy and electrophysiology) to clarify the involvement of Ca^{2+} influx and the activation of ClC-3 channels to mediate a rapid response of Sertoli cells to secretory activities regulated by $1,25D_3$.

5. Conclusion

In conclusion, these results suggest a possible interaction of $1,25D_3$ with the plasma membrane of Sertoli cells which might open K^+_{ATP}, $K^+_{Ca^{2+}}$ and Cl^- channels "hyperpolarizing" the cells. This hyperpolarization could induce an opening of VDCCs, Ca^{2+} influx and "depolarization". The local Ca^{2+} transient activates PKC that may regulate plasma membrane ionic channel activities (Ca^{2+} and/or Ca^{2+}-dependent Cl^- channels). Also, PKC has a putative central role as upstream regulator of MEK and ERK1/2 to p38 MAPK activation. p38 MAPK can modulate cellular responses through the activation of phosphorylation of other kinases, cytoplasmic and membrane proteins and/or activation of specific transcription factors altering gene transcription. Concomitantly, a specific signal that links Ca^{2+} influx to microtubules movement and ClC-3 activity can produce some of the effects commonly associated with local Ca^{2+} increase, as for example, secretory activity.

Acknowledgments

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