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1α,25-Dihydroxyvitamin D_3 Signaling Pathways on Calcium Uptake in 30-Day-Old Rat Sertoli Cells

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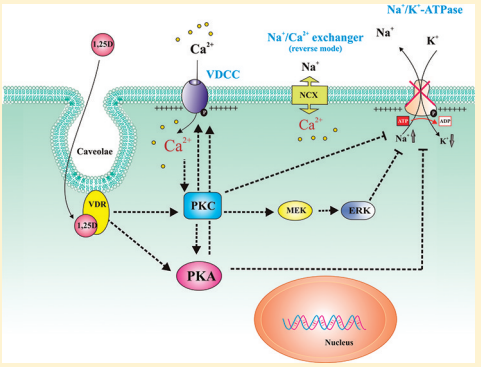
ABSTRACT: 1α,25-Dihydroxyvitamin D_3 ($1,25D_3$) is the active metabolite of vitamin D_3 and the major calcium regulatory hormone in tissues. The aim of this work was to investigate the mechanism of action of $1,25D_3$ on $^{45}Ca^{2+}$ uptake in Sertoli cells from 30-day-old rats. Results showed that 10^{-9} and 10^{-12} M $1,25D_3$ increased the rate of $^{45}Ca^{2+}$ uptake 5 and 15 min after hormone exposure and that $1\alpha,25(OH)_2$ lumisterol$_3$ (JN) produced a similar effect suggesting that $1,25D_3$ action occurs via a putative membrane receptor. The involvement of voltage-dependent calcium channels (VDCC) in $1,25D_3$ action was evidenced by using nifedipine, while the use of Bapta-AM demonstrated that intracellular calcium was not implicated. Moreover, the incubation with ouabain and digoxin increased the rate of $^{45}Ca^{2+}$ uptake, indicating that the effect of $1,25D_3$ may also result from Na^+/K^+-ATPase inhibition. In addition, we demonstrated that the mechanism underlying the hormone action involved extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) activation in a phospholipase C-independent way. Furthermore, a local elevation of the level of cAMP, as demonstrated by incubating cells with dibutyryl cAMP or a phosphodiesterase inhibitor, produced an effect similar to that of $1,25D_3$, and the inhibition of protein kinase A (PKA) nullified the hormone action. In conclusion, the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ uptake in Sertoli cells occurs via VDCC, as well as PKA, PKC, and ERK activation. These protein kinases seem to act by inhibiting Na^+/K^+-ATPase or directly phosphorylating calcium channels. The Na^+/K^+-ATPase inhibition may result in Na^+/Ca^{2+} exchanger activation in reverse mode and consequently induce the uptake of calcium into the cells.



1α,25-Dihydroxyvitamin D_3 ($1,25D_3$) mediates classic, transcription-dependent events and also rapid membrane-initiated signal transduction.[1,2] The seco-steroid hormone, $1,25D_3$, was postulated to act through a membrane-associated receptor to account for the rapid stimulation of calcium uptake in isolated rat intestinal epithelial cells, as well as calcium transport in perfused chick duodenum.[3,4] Binding of $1,25D_3$ to caveolae-associated VDR may result in activation of one or more systems, including phospholipase C (PLC), protein kinase C (PKC), protein kinase A (PKA), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinases like p38 MAPK, G protein-coupled receptors, or phosphatidylinositol 3′-kinase (PI3K). Also, there are a number of possible outcomes, including opening of voltage-dependent calcium or chloride channels.[5−10] In addition, some of these pathways, particularly Raf/MAPK, may integrate rapid responses by cross-talk with the nucleus to modulate gene expression as well as the regulation of voltage-dependent calcium channels (VDCC) and chloride channels.[7]

Especially for the reproductive male system, the wide expression of the $1,25D_3$ receptor and the biological functions were demonstrated by autoradiographic receptor studies on mouse testis.[11] Recently, we have demonstrated that the levels of both mRNA VDR and aromatase expression are higher in 30-day-old rat purified Sertoli cells than in 10- and 20-day-old cells.[12] In addition, it has been described that receptors for $1,25D_3$ are present in the cell cytoplasm and nucleus of male and female reproductive tissues.[13] The presence of $1,25D_3$ receptors in the nucleus of a mouse Sertoli cell line (TM4) and the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ uptake, DNA synthesis, and protein content have been described.[14]

It is well-known that Sertoli cell responses differ substantially according to the testis development, as well as FSH and testosterone action, which are responsible for induction and maintenance of spermatogenesis.[15−17] We have studied the effect and the mechanism of action of FSH, retinol, thyroid hormones, and recently also $1,25D_3$ on plasma membrane ionic systems in rat testis, seminiferous tubules, TM4 cells, and purified Sertoli cells from 3-, 6-, 10-, 11-, 15- and 26-day-old rat testis.[10,18−20] In addition, recently we demonstrated VDR expression in purified Sertoli cells from 30-day-old rats and

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showed that $1,25D_3$ could regulate aromatase gene expression through membrane-initiated mechanisms.[12]

From our previous studies, in whole immature rat testis, the stimulatory effect of $1,25D_3$ was demonstrated through a specific and selective plasma membrane amino acid transport system.[21] 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. Also, recently we demonstrated that a PKA/PKC-dependent $1,25D_3$/VDR non-genotropic pathway leads to Cl^- channel and exocytosis activation in TM4 cells.[10] Furthermore, it was demonstrated that extracellular calcium and VDCCs are necessary to mediate the plasma membrane effects of $1,25D_3$ in immature rat testis.[21]

A number of conformationally restricted analogues locked in the 6-s-*cis* (6C) or 6-s-*trans* (6T) conformation have been used to evaluate the preferred shape of $1,25D_3$ for rapid and genomic responses. The 6C locked forms exhibit a potent agonistic action for rapid response pathways and show activities equivalent to that of conformationally flexible $1,25D_3$, while the 6T locked forms are inactive or less active than $1,25D_3$ in both rapid and genomic actions, as reviewed by Norman.[8] Taking this into account, we aimed to investigate the mechanism of action of $1,25D_3$ on calcium uptake in 30-day-old rat Sertoli cells.

EXPERIMENTAL PROCEDURES

Chemicals. 1α,25-Dihydroxyvitamin ($1,25D_3$), nifedipine, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (Bapta-AM), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), bisindolylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)maleimide methanesulfonate salt (RO 31-8220), paraformaldehyde, *trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl)imidazole (SB 239063), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059), digoxin, ouabain, theophyline, dibutyryl cAMP (di-BucAMP), 1-(6-{[(17β)-3-methoxyestra-1,3,5[10]trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione (U-73122), Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, penicillin, streptomycin, kanamycin and amphotericin B, Serum Replacement 3, bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I-S), trypsin, soybean trypsin inhibitor, sodium pyruvate, D-glucose, Hepes, and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase-Dispase and bovine serum albumin (BSA) were from Roche Diagnostics (Indianapolis, IN). $^{45}CaCl_2$ (specific activity of 321 kBq/mg of Ca^{2+}) and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Waltham, MA). Polyclonal antibody VDR C-20 (SC 1008) with ultracruz mounting medium with DAPI (SC 24941) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody IgG (goat anti-rabbit) conjugated to Alexa Fluor 488 was from Invitrogen (Eugene, OR). All other chemicals were of analytical grade. The synthetic analogue 1α,25$(OH)_2$ lumisterol$_3$ (JN) was generous gift from A. W. Norman (University of California, Riverside, CA).

Animals. Wistar rats were bred in an animal house and maintained in an air-conditioned room (~21 ± 2 °C) with controlled lighting (12 h−12 h light−dark cycle). Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available ad libitum. All procedures were conducted in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00179).

Primary Culture of Sertoli Cells and Calcium Uptake. Sertoli cells were obtained from 30-day-old Wistar rats. Rats were killed by decapitation, and testes were removed and decapsulated. Sertoli cells were obtained by sequential enzymatic digestion as previously described by Dorrington et al.[22] Sertoli cells were seeded at a concentration of 650000 cells/cm^2 in 24-well culture plates (Falcon, 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, antibiotics (50000 IU/L penicillin, 50 mg/L streptomycin, and 50 mg/L kanamycin), and a fungicide (0.25 mg/L amphotericin B), in a humidified atmosphere of 5% CO_2 and 95% air at 32 °C. Three days after being plated, residual germ cells were removed by a hypotonic treatment using 20 mM Tris-HCl (pH 7.2) for 150 s.[16,23] Cells were washed with PBS, and fresh Ham's F12/DMEM (1:1) medium was added. Five days after being plated, cells were preincubated in Krebs Ringer-bicarbonate buffer (KRb) (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, and 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 32 °C (pH 7.4) and gassed with an O_2/CO_2 mixture (95:5, v/v). After that, the medium was replaced with fresh KRb containing 0.1 μCi/mL $^{45}Ca^{2+}$ over 60 min. For calcium uptake measurements, cells were incubated for a further 30 s, 1 min, 5 min, or 15 min, in the absence (control) or presence of $1,25D_3$ or JN (10^{-13}−10^{-7} M). In some experiments, channel blockers or kinase inhibitors were added during the last 20 min before the hormone was added and maintained during the incubation period (see figure legends). The following drugs were used: nifedipine (100 μM; L-type voltage-dependent calcium channel), Bapta-AM (50 μM; intracellular calcium chelator), ouabain (1 μM; Na^+/K^+-ATPase inhibitor), digoxin (1 μM; Na^+/K^+-ATPase inhibitor), H-89 (10 μM; PKA inhibitor), theophyline (50 μM; phosphodiesterase inhibitor), di-BucAMP (500 μM; cAMP analogue), RO 31-8240 (20 μM; PKC inhibitor), U-73122 (1 μM; PLC inhibitor), SB 239063 (10 μM; p38 MAPK inhibitor), and PD 98059 (10 μM; MEK inhibitor).[10,20,21,24−29]

Extracellular $^{45}Ca^{2+}$ from primary Sertoli cells culture was thoroughly washed off in 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, and 10 mM $LaCl_3$ (pH 7.4) for 30 min. The presence of La^{3+}, in a washing solution, was found to be essential for the prevention of release of the intracellular $^{45}Ca^{2+}$.[30] After La^{3+} cells had been washed, they were homogenized with a 0.5 M NaOH solution, 100 μL aliquots were placed in scintillation fluid in a LKB rack beta liquid scintillation spectrometer (model LS 6500, Multi-Porpose Scintillation Counter, Beckman Coulter, Boston, MA),[10,20,27] and 50 μL aliquots were used for protein quantification by the Bradford method.[31] The results are expressed as picomoles of $^{45}Ca^{2+}$ per microgram of protein or percent of control, which represents an average of 15.95 ± 0.58 pmol of $^{45}Ca^{2+}/\mu$g of protein.

Immunocytochemical Staining. Sertoli cells obtained from 30-day-old rats were cultured for 5 days as described above. Cells were fixed on microscope slides using 3% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton in PBS at room temperature for 3 min. Unspecific sites were blocked with 3% BSA in PBS for 1 h at room temperature, and slides were incubated overnight with a rabbit anti-VDR polyclonal antibody (1:500 dilution in 3% BSA/PBS)

at 4 °C. After being thoroughly rinsed, the cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500) for 60 min at room temperature, washed with PBS, and mounted with DAPI,[32] with minor modifications. Images were captured with FV10-ASW version 2.0 in an Olympus confocal microscope. Negative controls were incubated in a 3% BSA/PBS mixture without the primary antibody.

Statistical Analysis. The results are means ± the standard error of the mean (SEM) expressed as picomoles of $^{45}Ca^{2+}$ per microgram of protein or percent of control. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by a Bonferroni multiple-comparison test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Localization of VDR in Rat Sertoli Cells. To evaluate the VDR expression at the protein level in 30-day-old rat Sertoli cells, immunofluorescence staining was performed. A strong VDR immunoreactivity was demonstrated in the cytoplasm but was also present in the nucleus of 30-day-old rat Sertoli cell (Figure 1A), whereas the negative control showed an immunonegative reaction (Figure 1B,C).

Time Course and Dose−Response Curve of $1,25D_3$ on $^{45}Ca^{2+}$ Uptake. In this work, we studied the time course and dose−response curve of $1,25D_3$ on $^{45}Ca^{2+}$ uptake in a primary culture of Sertoli cells from 30-day-old rat testis. After calcium equilibration in the Sertoli cells for incubation for 60 min with $^{45}Ca^{2+}$, the calcium level was additionally monitored for 30 s, 1 min, 5 min, and 15 min without stimuli (control) and in the presence of $1,25D_3$ (10^{-13}−10^{-7} M).

$1,25D_3$ increased the rate of $^{45}Ca^{2+}$ uptake after 5 min (150%) and 15 min (115%) exposures, and no effect was observed at 30 s and 1 min in the presence of the hormone (Figure 2A). Figure 2B shows that 10^{-12} and 10^{-9} M $1,25D_3$ increased the rate of $^{45}Ca^{2+}$ uptake (150 and 182%, respectively), yielding high intracellular calcium levels after incubation for 5 min compared with respective control groups. Also, the lower (10^{-13} M) or higher (10^{-8} and 10^{-7} M) concentrations of $1,25D_3$ were not able to change the intracellular calcium concentration significantly after incubation for 5 min.

Effect of Synthetic Agonist JN on the Stimulatory Action of $1,25D_3$ on $^{45}Ca^{2+}$ Uptake. Figure 3 reports an evaluation of the ability of the 6-s-*cis* analogue, by comparison with $1,25D_3$, to increase the rate of $^{45}Ca^{2+}$ uptake into 30-day-old rat Sertoli cells within 5 min. As one can see in Figure 3B, JN was able to modify significantly the intracellular calcium concentrations at 10^{-9} M. This maximal effect triggered by the analogue represents an increase of 60% in the rate of $^{45}Ca^{2+}$ uptake compared with that of the control group. Besides the equivalent potency of 10^{-9} M JN compared to 10^{-12} M $1,25D_3$ in stimulating $^{45}Ca^{2+}$ uptake, it also was able to potentiate the $1,25D_3$ stimulatory effect on $^{45}Ca^{2+}$ uptake (Figure 3C).

Influence of Nifedipine, Bapta-AM, Ouabain, and Digoxin on the Stimulatory Effect of $1,25D_3$ on $^{45}Ca^{2+}$ Uptake. In Figure 4A, it is first demonstrated that nifedipine after incubation for 15 min did not alter the basal calcium cellular equilibrium. However, it was found that a rapid $1,25D_3$-induced increase in the rate of $^{45}Ca^{2+}$ uptake was prevented in the presence of this L-type VDCC blocker.

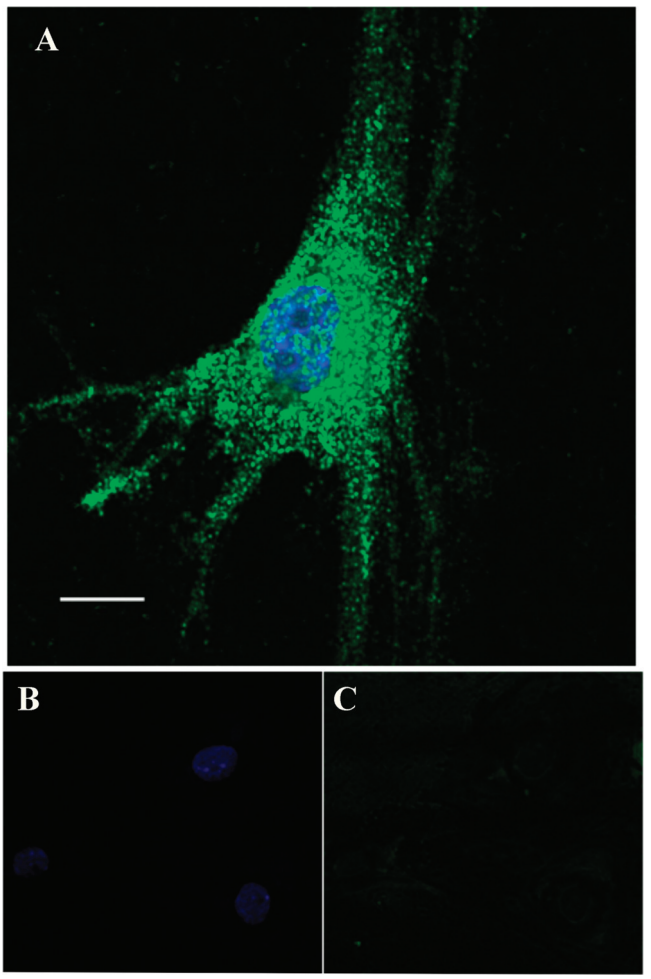

Figure 1. Immunolocalization of VDR in 30-day-old Sertoli cells using confocal microscopy. Chromatin was stained with DAPI (blue), and VDR was revealed by a polyclonal antibody (green). Sertoli cells were fixed and incubated with an anti-VDR antibody (1:500) followed by incubation with a goat anti-rabbit secondary antibody as described in Experimental Procedures. Strong immunofluorescence was observed using FITC coloration in the cytoplasm of Sertoli cells (A). The negative control slide showed immunonegative reaction in Sertoli cells (B). The scale bar is 10 μm.

We sought to determine if the intracellular calcium could influence rapid $^{45}Ca^{2+}$ uptake after incubation with $1,25D_3$. Therefore, we investigated whether $1,25D_3$ would promote, with the same magnitude, $^{45}Ca^{2+}$ uptake when intracellular calcium was chelated by Bapta-AM. Results of this analysis indicated that exposure of Sertoli cells to $1,25D_3$ for 5 min does not change the maximal rate of $^{45}Ca^{2+}$ uptake even when intracellular calcium was depleted (Figure 4A). In addition, we have verified that the inactivation of Na^+/K^+-ATPase by ouabain immediately increased the intracellular level of calcium similar to that observed for $1,25D_3$ (Figure 4B). Moreover, we found that ouabain co-incubated with $1,25D_3$ did not have a synergistic effect on $^{45}Ca^{2+}$ uptake (Figure 4B). To improve our understanding of the involvement of Na^+/K^+-ATPase on the mechanism of action of $1,25D_3$, we also used digoxin, another steroidal glucoside with affinity for Na^+/K^+-ATPase.[29] Under our experimental conditions, we observed that digoxin not only promotes activation on $^{45}Ca^{2+}$ uptake like $1,25D_3$ but also potentiates $1,25D_3$-induced $^{45}Ca^{2+}$ uptake (Figure 4B).

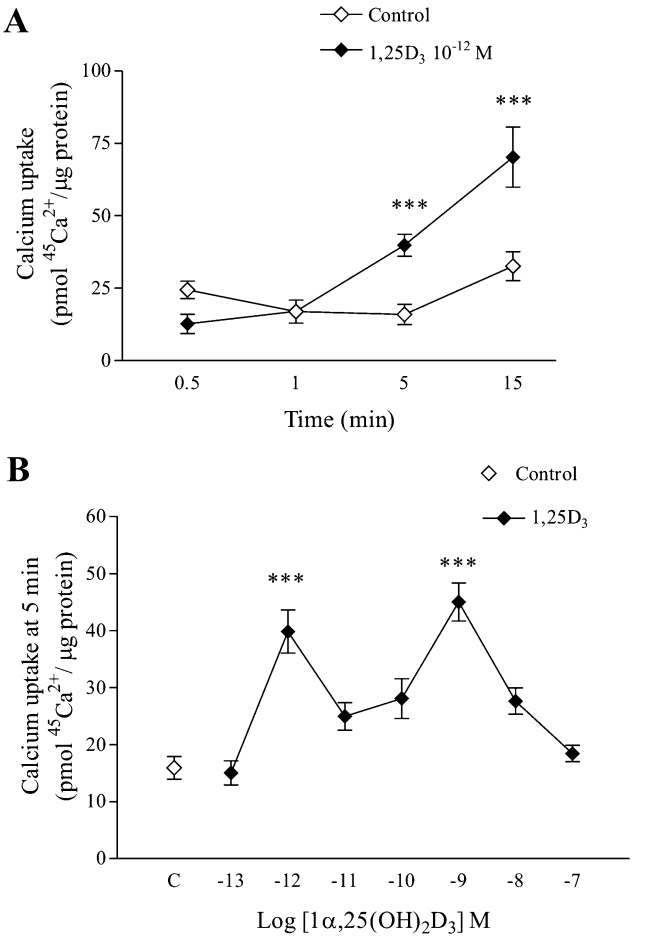

Figure 2. (A) Time course of $1,25D_3$ (10^{-12} M) and (B) dose–response curve of $1,25D_3$ on $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. Means ± SEM of three independent experiments. Asterisks denote $p < 0.001$ compared with the control group. The preincubation time was 60 min; the incubation time was 5 min.

Involvement of the PKA Signaling Pathway in the Stimulatory Effect of $1,25D_3$ on $^{45}Ca^{2+}$ Uptake. To study the hypothesis that $1,25D_3$-induced $^{45}Ca^{2+}$ uptake may involve the PKA pathway, the incubation was conducted in the presence of PKA inhibitor H-89, phosphodiesterase inhibitor theophylline, and cAMP agonist di-BucAMP with or without $1,25D_3$ (10^{-12} M). As shown in Figure 5, the presence of the PKA inhibitor caused a complete inhibition of the stimulatory action of the hormone on $^{45}Ca^{2+}$ uptake without changing the basal rate of uptake. Moreover, the local elevation of the level of cAMP produced by the accumulation of the synthetic nucleotide increased the rate of $^{45}Ca^{2+}$ uptake. Additionally, both agents, di-BucAMP and theophylline, co-incubated with $1,25D_3$ promoted similar rates of $^{45}Ca^{2+}$ uptake compared with that caused by the $1,25D_3$ stimulatory effect.

Roles of PKC, PLC, MEK, and p38 MAPK in the Stimulatory Effect of $1,25D_3$ on $^{45}Ca^{2+}$ Uptake. As shown in Figure 6A, we verified that inhibition of endogenous PKC activity by RO 31-8220 abolished the stimulatory effect of 10^{-12} M $1,25D_3$ on $^{45}Ca^{2+}$ uptake. However, the presence of a phospholipase C inhibitor, U-73122, did not change $^{45}Ca^{2+}$ uptake in the control group or in treated cells. Following the pathway of kinases that probably can participate in the mechanism of $1,25D_3$ on $^{45}Ca^{2+}$ uptake; we assayed the cells with SB 239063 that inhibis its established targets, p38α and p38β or PD 98059, an inhibitor of the mitogen-activated protein kinase (MAPK) cascade. Figure 6B shows that incubation with the p38 MAPK inhibitor did not alter significantly the basal rate of $^{45}Ca^{2+}$ uptake but promoted a synergic effect on the stimulatory action of $1,25D_3$ on $^{45}Ca^{2+}$ uptake. On the other hand, PD 98059, which inhibits ERK1 and ERK2 activation by the dual MAPK kinase MEK, significantly suppressed the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ uptake.

DISCUSSION

In this work, we demonstrate the presence of VDR in Sertoli cells and show that $1,25D_3$ induces an increase in the intracellular levels of calcium in 30-day-old Sertoli cells exhibiting a biphasic dose–response profile. These results are consistent with previous studies with $1,25D_3$ that established that the dose–response curve for transcaltachia is biphasic[33] and also that the hormone can change intracellular calcium concentrations in the pancreatic β-cell line.[34] Our findings emphasize the important role of $1,25D_3$ in regulating the intracellular calcium concentration via extracellular calcium influx, which plays a role in an early (rapid) downstream signaling activation.

In this study, $^{45}Ca^{2+}$ uptake was used to demonstrate rapid responses of 30-day-old Sertoli cells to $1,25D_3$. First, it was shown that $1,25D_3$, a natural agonist, stimulates calcium uptake in a very short-term period in these cells. After that, it was found that conformationally restricted 6C analogue JN was equivalent in potency to $1,25D_3$ in stimulating calcium uptake and also was able to potentiate the $1,25D_3$ stimulatory effect. These results point to the fact that the functional vitamin D-related receptor involved in rapid responses has different binding sites requirements for JN (6C) and $1,25D_3$ (6T), as already reported for many other systems.[9,10,12]

The rapid and/or sustained $1,25D_3$-stimulated calcium uptake through VDCC seems to be required for physiological responses in Sertoli cells.[19,21] Following these findings, we demonstrate here that $1,25D_3$-induced calcium uptake, within minutes, was directly and mostly related to VDCC.

On the other hand, we cannot discard the possibility that calcium uptake observed with $1,25D_3$ treatment can also occur through another kind of channels present in Sertoli cells such as N- and P:Q-type VDCC[35] as well as by the transient receptor vanilloid 1 (TRPV1) channels that mediate indirectly the calcium influx in Sertoli cells[36] or other TRPV channels that have been reported to mediate $1,25D_3$ calcium influx in intestinal epithelium.[37] Indeed, our data reinforce the idea that the rapid response activation in 30-day-old Sertoli cells, elicited by $1,25D_3$, was predominantly caused by VDCC channels because storage calcium seemed not to alter the hormone effect. This finding suggests that $1,25D_3$ serves as a chemical intermediate linking the tight regulation of intracellular calcium concentration with critical protein targets to start and/or sustain many physiological stimuli in testis and Sertoli cells.[10,12,14,38]

As demonstrated previously by Menegaz et al.,[21] $1,25D_3$ plays a role in plasma membrane Na^+-dependent amino acid transport system A. System A is dependent on an intracellular Na^+ gradient and is linked strictly to the Na^+/K^+-ATPase.[19,39] We hypothesized a possible link between calcium uptake and steroid-binding membrane Na^+/K^+-ATPase, because ouabain and digoxin are well-known as specific and effective inhibitors of the Na^+ pump.[40,41] Particularly interesting is the reciprocal

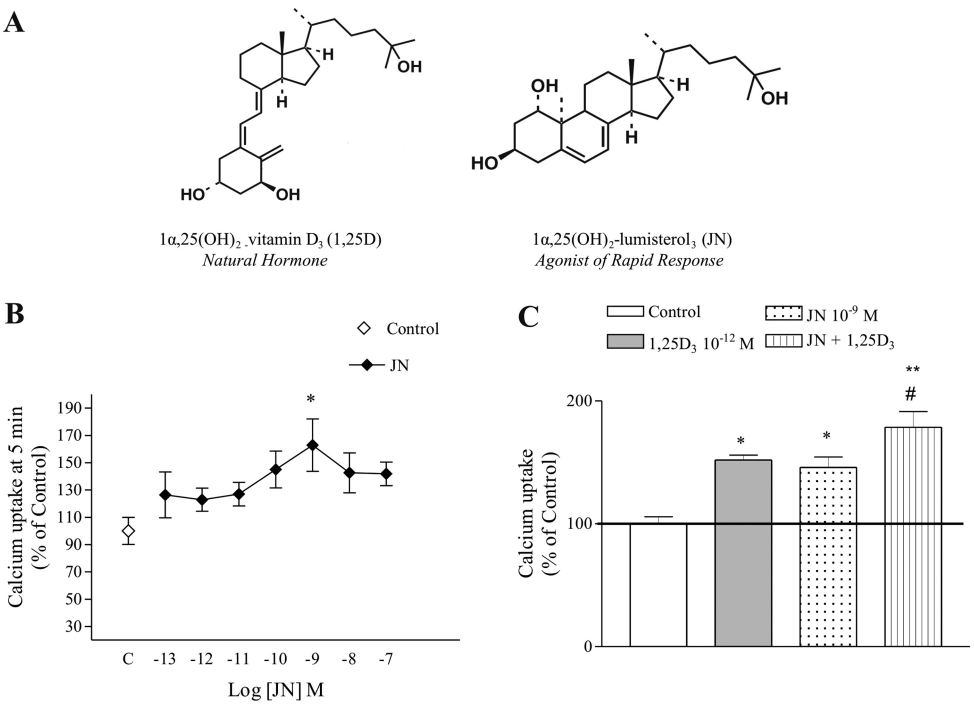

Figure 3. Effect of synthetic agonist 1α,25(OH)$_2$ lumisterol$_3$ (JN) on $^{45}Ca^{2+}$ uptake. (A) Chemical structures of 1,25D$_3$ and its rapid response analogue. (B) Dose–response curve of JN on $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. (C) Effect of JN (10^{-9} M) on the stimulatory effect of 1,25D$_3$ (10^{-12} M). Means ± SEM of three independent experiments. Compared with the control group, one asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.01$. The number sign denotes $p < 0.05$ compared with the 1,25D$_3$ group. The preincubation time was 60 min; the incubation time was 5 min.

regulation of Na^+/K^+-ATPase and the Na^+/Ca^{2+} exchanger expressed in a number of pathophysiological situations and during development.[42] It has been postulated that Na^+/K^+ ATPase inhibition, and consequently Na^+ accumulation, evokes membrane depolarization and VDCC opening. Na^+ accumulation can also activate the Na^+/Ca^{2+} exchanger in a reverse mode where Ca^{2+} is imported and Na^+ removed from the cell and both transporters contribute to cytosolic calcium accumulation.[43] All these conditions can give rise to a significant alteration of cellular behavior as exocytosis and/or nuclear transcription, which in turn may be a way to regulate cell metabolism in actively secreting cells.

It has been shown previously that cAMP/PKA acts as a mediator of both 1,25D$_3$ genomic and nongenomic responses in a variety of cell systems.[12,21] Handling in vitro cAMP levels using theophylline or including a cAMP agonist (dibutyryl cAMP) normally is able to keep the cAMP concentration close to the basal levels. Under this experimental condition, the slight calcium uptake induced by theophylline and also by dibutyryl cAMP suggests the participation of cAMP. In addition, the co-incubation of 1,25D$_3$ with theophylline or 1,25D$_3$ with dibutyryl cAMP did not potentiate the hormone stimulatory effect. Taken together, these findings corroborate the involvement of the cAMP/PKA signaling pathway in the mechanism of action of 1,25D$_3$.

One of the nongenomic events related to 1,25D$_3$ that has a weak relationship with cyclic AMP/PKA is calcium influx.[44] We recently demonstrated in TM4 cells that the binding of 1,25D$_3$ in a memVDR stimulates cAMP production and activation of PKA to phosphorylate the channel, culminating in secretory activity.[10] In view of the evidence involving PKA in the regulation of calcium uptake, we can refer to results reported by Massheimer and de Boland,[45] who also described that calcium influx through L-VDCC occurred with a parallel increase in adenylate cyclase activity and membrane protein phosphorylation in soleus muscle from a vitamin D-deficient chick incubated for a short term in the presence of 1,25D$_3$.

In particular in PLC activity- and calcium-dependent PKC isoforms, 1,25D$_3$ induced changes in phosphoinositide turnover, PKC translocation, and intracellular calcium levels, as revised by Fleet.[6] Results of our analyses indicated that 1,25D$_3$ directly and rapidly induces a significant increase in the rate of calcium uptake that seemed to be mediated by PKC and, apparently, independent of PLC activity. These results reinforce the involvement of atypical PKC isoforms because RO 31-8220 is able to block all PKC isoforms.[46] In addition, in our previous work, we demonstrated that preincubation of TM4 cells with the PKC activator (PMA) induced a significant increase in the outward Cl^- currents. On the other hand, we found an inhibition of endogenous PKC activity by Go6983 that reduced control currents by 50% and abolished any further potentiation of the Cl^- currents by 1,25D$_3$.[10]

Binding of 1,25D$_3$ to the caveolae-associated VDR may result in activation of signal transduction pathways through PLC, PKC, G-protein, MAPK, PLA$_2$, PI3K, and Ras/MAPK that are promptly able to modulate ionic channels and/or enzyme activities and to evoke a faster and efficient gene expression.[8] We have used inhibitors of protein kinases to gain further insights into the mechanism of action of 1,25D$_3$ on calcium uptake in 30-day-old Sertoli cells. As shown in this work, PD 98059 prevented the activation of the classical MAPK cascade in this cell-based assay. The involvement of the ERK signaling pathway in calcium uptake triggered by 1,25D$_3$ corroborates previous data showing 1,25D$_3$ modulates myoblast proliferation,[47] vimentin phosphorylation,[28] and calcium uptake in 30-day-old rat testis.[48]

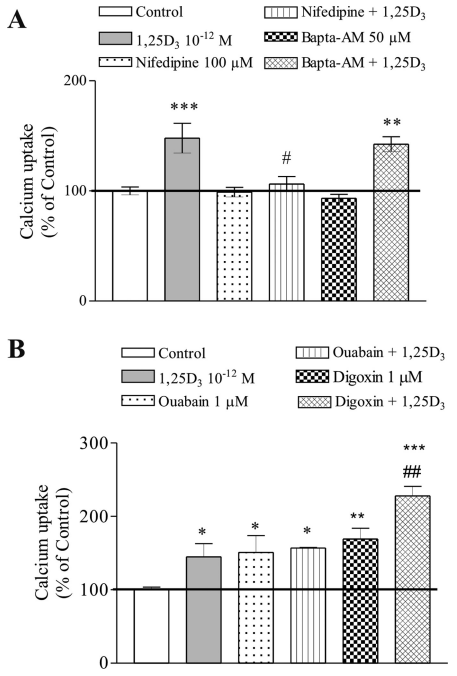

Figure 4. Involvement of voltage-dependent calcium channel (VDCC), intracellular calcium, and Na^+/K^+ATPase in $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. Nifedipine (100 μM) and Bapta-AM (50 μM) (A), and ouabain (1 μM) and digoxin (1 μM) (B), were added in the last 15 min of the preincubation and during the incubation time. Means ± SEM of three independent experiments. Compared with the control group, one asterisk denotes $p < 0.05$, two asterisks denote $p < 0.01$, and three asterisks denote $p < 0.001$. For combinations of inhibitors with $1,25D_3$, one number sign denotes $p < 0.05$ and two number signs denote $p < 0.01$ compared with the $1,25D_3$ group.

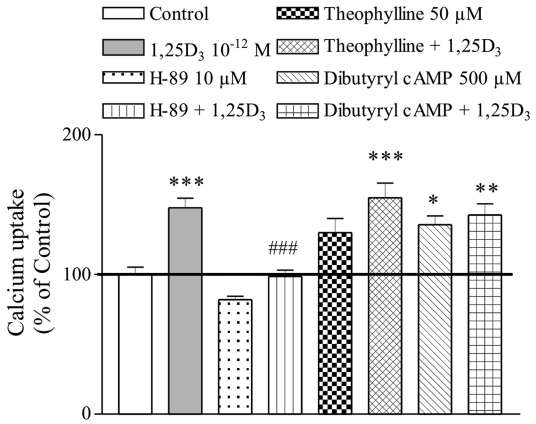

Figure 5. Involvement of the PKA signaling pathway in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ uptake. Effect of H-89 (10 μM), theophylline (50 μM), and dibutyryl cAMP (500 μM) in the stimulatory effect of $1,25D_3$ (10^{-12} M) on $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. Means ± SEM of three independent experiments. Compared with the control group, one asterisk denotes $p < 0.05$, two asterisks denote $p < 0.01$, and three asterisks denote $p < 0.001$. For combinations of inhibitors or analogues with $1,25D_3$, three number signs denote $p < 0.001$ compared with the $1,25D_3$ group. The preincubation time was 60 min; the incubation time was 5 min.

On the other hand, the synergistic effect of $1,25D_3$ on calcium uptake was observed when the specific inhibitor of p38 MAPK was co-incubated with the hormone. The upstream activators of p38 MAPK are numerous, and various isoforms

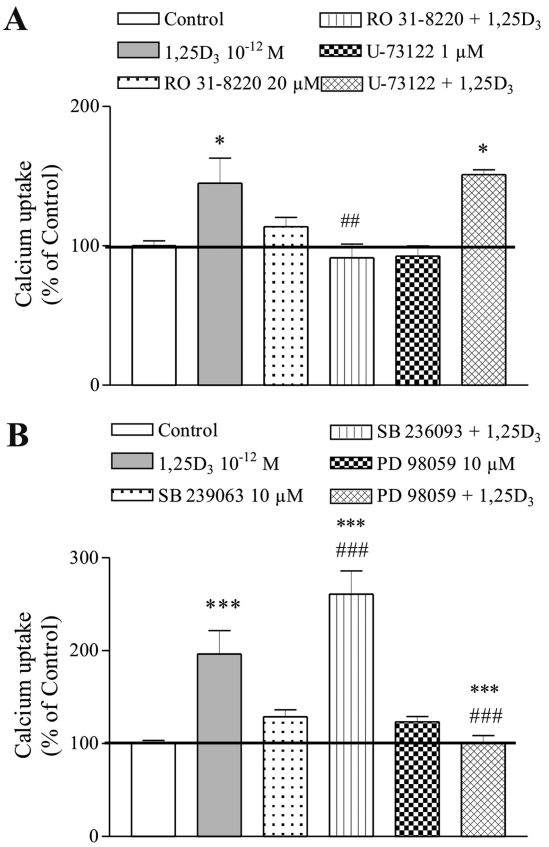

Figure 6. Role of PKC, PLC, ERK, and p38 MAPK in the stimulatory effect of $1,25D_3$ on $^{45}Ca^{2+}$ uptake. (A) Effect of RO 31-8220 (20 μM) and U-73122 (1 μM) in the stimulatory effect of $1,25D_3$ (10^{-12} M) on $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. (B) Effect of PD 98059 (10 μM) and SB 239063 (10 μM) in the stimulatory effect of $1,25D_3$ (10^{-12} M) on $^{45}Ca^{2+}$ uptake in 30-day-old rat Sertoli cells. Means ± SEM of three independent experiments. Compared with the control group, one asterisk denotes $p < 0.05$ and three asterisks denote $p < 0.001$. For combinations of inhibitors with $1,25D_3$, two number signs denote $p < 0.01$ and three number signs denote $p < 0.001$ compared with the $1,25D_3$ group. The preincubation time was 60 min; the incubation time was 5 min.

can be activated by different pathways. The initiators Rho/Rac GTPases can lead to the activation of various MKKKs, and then following a cascade of phosphorylation by a MKK, p38 MAPK activates a number of MSKs, MAPK-interacting kinases (MNKs), MAPK-activated kinases (MKs), and a number of transcription factors and regulates plasma membrane ionic channels such as N-type VDCC.[49−51]

In the testis, all p38 MAPK isoforms, except p38γ, have been identified, and the native form of p38 MAPK can be found in Sertoli cells and elongated spermatids.[51] We previously demonstrated p38 MAPK activation in hypothyroid immature rat testis.[52] Under that condition, among the three major classical subfamilies of MAPKs (ERK1/2, JNK, and p38 MAPK), p38 MAPK was the only one that showed high phosphorylation levels, indicating its role in the testis to the plenitude cytoskeleton function. In this work, the effect of $1,25D_3$ was potentiated by p38 MAPK inactivation, suggesting a different pathway regulating $1,25D_3$-stimulated calcium influx. In that situation, $1,25D_3$ could be inhibiting p38 MAPK, or simply, this inhibition could be result of cross talk between

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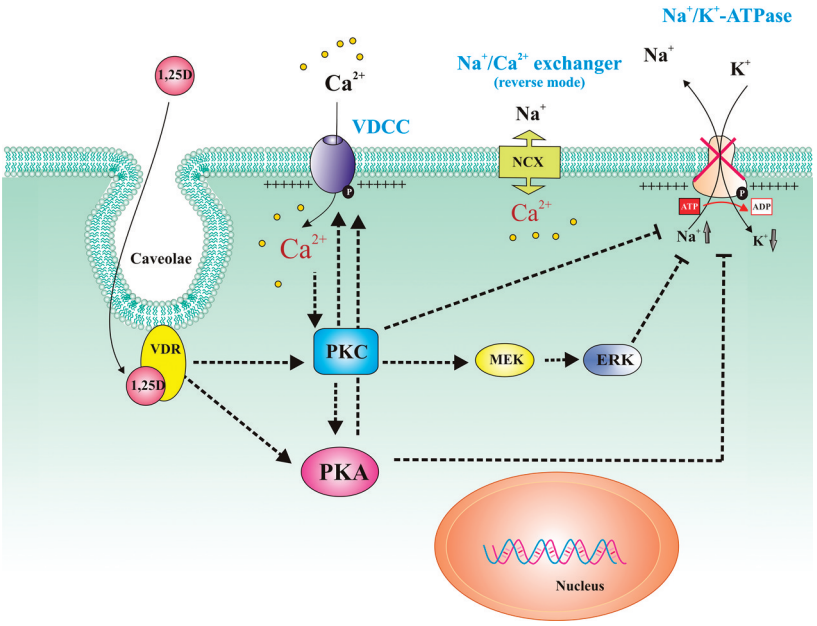

Figure 7. Schematic representation of 1α,25-dihydroxyvitamin D_3-induced signaling pathways mediating calcium uptake in rat Sertoli cells. $1,25D_3$ binds to the membrane-associated VDR and stimulates a variety of signaling pathways such as PKA and PKC. These kinases phosphorylate VDCC, which promote the entry of extracellular calcium into the cells. Calcium uptake might activate calcium messenger systems, such as PKC that phosphorylates and activates MEK-ERK. Also, PKA and PKC could phosphorylate and inhibit Na^+/K^+ ATPase activity with subsequent Na^+ accumulation, which could lead to membrane depolarization and VDCC opening. Also, the accumulation of Na^+ can result in Na^+/Ca^{2+} exchanger activation in a reverse mode where Ca^{2+} is imported and Na^+ is removed from the cell. The dotted arrows indicate the pathways probably involved in the mechanism of action of $1,25D_3$.

ERK and p38 MAPK where the p38 inhibition could lead to ERK activation as suggested by others.[53]

In conclusion, our results demonstrate that the stimulatory action of $1,25D_3$ on $^{45}Ca^{2+}$ uptake in Sertoli cells occurs by VDCC, as well as PKA, PKC, and ERK activation. These protein kinases seem to act either by inhibiting the Na^+/K^+ pump or by directly phosphorylating the calcium channels. The inhibition of the Na^+/K^+ pump by $1,25D_3$ might result in Na^+/Ca^{2+} exchanger activation in reverse mode and consequently accumulation of calcium in the cells (Figure 7).

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ABBREVIATIONS

$1,25D_3$, 1α,25-dihydroxyvitamin D_3; VDCC, voltage-dependent calcium channel; JN, 1α,25$(OH)_2$ lumisterol$_3$; VDR, vitamin D receptor; KRb, Krebs Ringer-bicarbonate buffer; di-BucAMP, dibutyryl cyclic AMP; NCX, Na^+/Ca^{2+} exchanger.

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