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The diagram illustrates the Na⁺/Ca²⁺ exchanger (NCX) and its regulation. The NCX is shown as a yellow transporter on the cell membrane, operating in reverse mode, moving Na⁺ out and Ca²⁺ in. It is regulated by PKC and PKA. PKC is activated by Ca²⁺ entering through the voltage-dependent calcium channel (VDCC) and by the VDR (1,25-DHAPo) produced in caveolae. PKA is activated by the VDR. Both PKC and PKA regulate the NCX. The Na⁺/K⁺-ATPase is also shown, moving Na⁺ out and K⁺ in, and is regulated by PKC and PKA. The nucleus is shown at the bottom, containing DNA.

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 1,25D₃ 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 1,25D₃ could regulate aromatase gene expression through membrane-initiated mechanisms.¹²

From our previous studies, in whole immature rat testis, the stimulatory effect of 1,25D₃ 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 Ca²⁺/K⁺ channels on the plasma membrane. Also, recently we demonstrated that a PKA/PKC-dependent 1,25D₃/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 1,25D₃ 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 1,25D₃ 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 1,25D₃, while the 6T locked forms are inactive or less active than 1,25D₃ in both rapid and genomic actions, as reviewed by Norman.⁸ Taking this into account, we aimed to investigate the mechanism of action of 1,25D₃ on calcium uptake in 30-day-old rat Sertoli cells.

EXPERIMENTAL PROCEDURES

Chemicals. 1 α ,25-Dihydroxyvitamin (1,25D₃), 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), bisindolylmaleimide 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-methoxy-pyridimidin-4-yl)imidazole (SB 239063), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059), digoxin, ouabain, theophylline, dibutyl cAMP (di-BucAMP), 1-(6-[(17 β)-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). ⁴⁵CaCl₂ (specific activity of 321 kBq/mg of Ca²⁺) and Optiphas 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 1 α ,25(OH)₂ lumisterol₃ (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 \pm 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/cm² 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% CO₂ 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 MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, and 25 mM NaHCO₃) for 15 min in a Dubnoff metabolic incubator at 32 °C (pH 7.4) and gassed with an O₂/CO₂ mixture (95:5, v/v). After that, the medium was replaced with fresh KRb containing 0.1 μ Ci/mL ⁴⁵Ca²⁺ 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 1,25D₃ 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 μ M; L-type voltage-dependent calcium channel), Bapta-AM (50 μ M; intracellular calcium chelator), ouabain (1 μ M; Na⁺/K⁺-ATPase inhibitor), digoxin (1 μ M; Na⁺/K⁺-ATPase inhibitor), H-89 (10 μ M; PKA inhibitor), theophylline (50 μ M; phosphodiesterase inhibitor), di-BucAMP (500 μ M; cAMP analogue), RO 31-8240 (20 μ M; PKC inhibitor), U-73122 (1 μ M; PLC inhibitor), SB 239063 (10 μ M; p38 MAPK inhibitor), and PD 98059 (10 μ M; MEK inhibitor).^{10,20,21,24–29}

Extracellular ⁴⁵Ca²⁺ from primary Sertoli cells culture was thoroughly washed off in 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄, 10 mM HEPES, 11 mM glucose, and 10 mM LaCl₃ (pH 7.4) for 30 min. The presence of La³⁺, in a washing solution, was found to be essential for the prevention of release of the intracellular ⁴⁵Ca²⁺.³⁰ After La³⁺ cells had been washed, they were homogenized with a 0.5 M NaOH solution, 100 μ L aliquots were placed in scintillation fluid in a LKB rack beta liquid scintillation spectrometer (model LS 6500, Multi-Portose Scintillation Counter, Beckman Coulter, Boston, MA),^{10,20,27} and 50 μ L aliquots were used for protein quantification by the Bradford method.³¹ The results are expressed as picomoles of ⁴⁵Ca²⁺ per microgram of protein or percent of control, which represents an average of 15.95 \pm 0.58 pmol of ⁴⁵Ca²⁺/μg 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 \pm the standard error of the mean (SEM) expressed as picomoles of $^{45}\text{Ca}^{2+}$ 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 1,25D₃ on $^{45}\text{Ca}^{2+}$ Uptake. In this work, we studied the time course and dose–response curve of 1,25D₃ on $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$, the calcium level was additionally monitored for 30 s, 1 min, 5 min, and 15 min without stimuli (control) and in the presence of 1,25D₃ (10^{-13} – 10^{-7} M).

1,25D₃ increased the rate of $^{45}\text{Ca}^{2+}$ 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 1,25D₃ increased the rate of $^{45}\text{Ca}^{2+}$ 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 1,25D₃ 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 1,25D₃ on $^{45}\text{Ca}^{2+}$ Uptake. Figure 3 reports an evaluation of the ability of the 6-*s-cis* analogue, by comparison with 1,25D₃, to increase the rate of $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$ uptake compared with that of the control group. Besides the equivalent potency of 10^{-9} M JN compared to 10^{-12} M 1,25D₃ in stimulating $^{45}\text{Ca}^{2+}$ uptake, it also was able to potentiate the 1,25D₃ stimulatory effect on $^{45}\text{Ca}^{2+}$ uptake (Figure 3C).

Influence of Nifedipine, Bapta-AM, Ouabain, and Digoxin on the Stimulatory Effect of 1,25D₃ on $^{45}\text{Ca}^{2+}$ 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 1,25D₃-induced increase in the rate of $^{45}\text{Ca}^{2+}$ uptake was prevented in the presence of this L-type VDCC blocker.

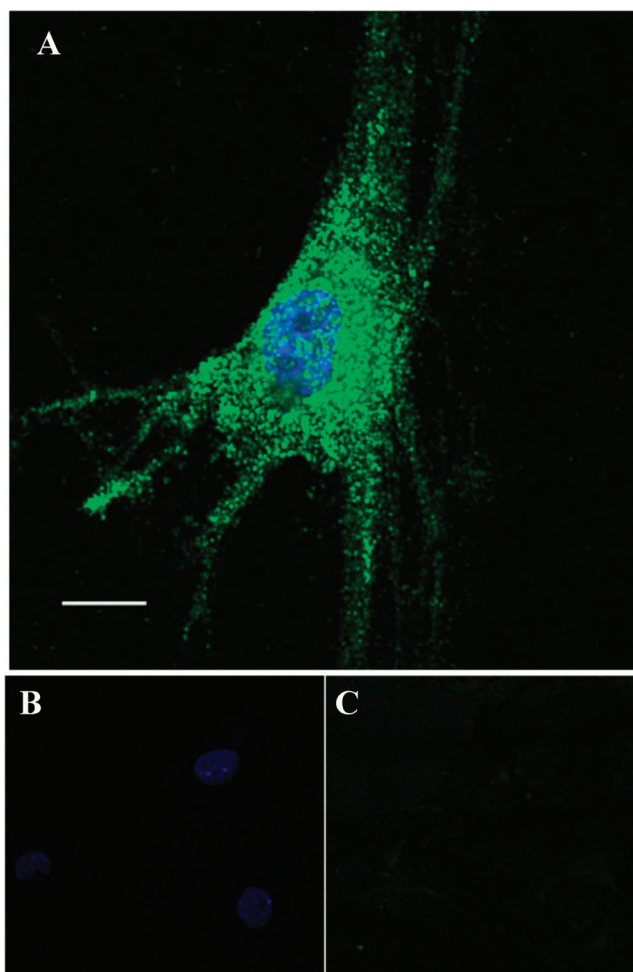


Figure 1. Immunolocalization of VDR in 30-day-old Sertoli cells using confocal microscopy. Chromatin was stained with DAPI (blue), and VDR was revealed by a polyclonal antibody (green). Sertoli cells were fixed and incubated with an anti-VDR antibody (1:500) followed by incubation with a goat anti-rabbit 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 $^{45}\text{Ca}^{2+}$ uptake after incubation with 1,25D₃. Therefore, we investigated whether 1,25D₃ would promote, with the same magnitude, $^{45}\text{Ca}^{2+}$ uptake when intracellular calcium was chelated by Bapta-AM. Results of this analysis indicated that exposure of Sertoli cells to 1,25D₃ for 5 min does not change the maximal rate of $^{45}\text{Ca}^{2+}$ 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 1,25D₃ (Figure 4B). Moreover, we found that ouabain co-incubated with 1,25D₃ did not have a synergistic effect on $^{45}\text{Ca}^{2+}$ uptake (Figure 4B). To improve our understanding of the involvement of Na^+/K^+ -ATPase on the mechanism of action of 1,25D₃, 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 $^{45}\text{Ca}^{2+}$ uptake like 1,25D₃ but also potentiates 1,25D₃-induced $^{45}\text{Ca}^{2+}$ uptake (Figure 4B).

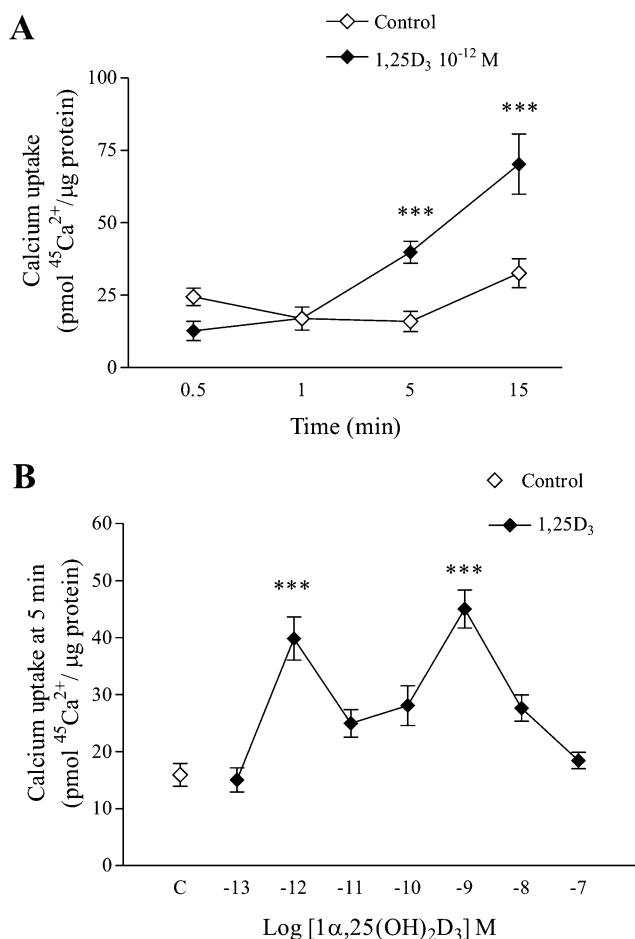


Figure 2. (A) Time course of 1,25D₃ (10⁻¹² M) and (B) dose-response curve of 1,25D₃ on $^{45}\text{Ca}^{2+}$ uptake in 30-day-old rat Sertoli cells. Means ± SEM of three independent experiments. Asterisks denote $p < 0.001$ compared with the control group. The preincubation time was 60 min; the incubation time was 5 min.

Involvement of the PKA Signaling Pathway in the Stimulatory Effect of 1,25D₃ on $^{45}\text{Ca}^{2+}$ Uptake. To study the hypothesis that 1,25D₃-induced $^{45}\text{Ca}^{2+}$ 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 1,25D₃ (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 $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$ uptake. Additionally, both agents, di-BucAMP and theophylline, co-incubated with 1,25D₃ promoted similar rates of $^{45}\text{Ca}^{2+}$ uptake compared with that caused by the 1,25D₃ stimulatory effect.

Roles of PKC, PLC, MEK, and p38 MAPK in the Stimulatory Effect of 1,25D₃ on $^{45}\text{Ca}^{2+}$ 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 1,25D₃ on $^{45}\text{Ca}^{2+}$ uptake. However, the presence of a phospholipase C inhibitor, U-73122, did not change $^{45}\text{Ca}^{2+}$ uptake in the control group or in treated cells. Following the pathway of kinases that probably can participate in the mechanism of 1,25D₃ on $^{45}\text{Ca}^{2+}$ uptake; we assayed the cells with SB 239063 that inhibits 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 $^{45}\text{Ca}^{2+}$ uptake but promoted a synergic effect on the stimulatory action of 1,25D₃ on $^{45}\text{Ca}^{2+}$ 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 1,25D₃ on $^{45}\text{Ca}^{2+}$ uptake.

DISCUSSION

In this work, we demonstrate the presence of VDR in Sertoli cells and show that 1,25D₃ 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 1,25D₃ that established that the dose-response curve for transcaltachia is biphasic³³ and also that the hormone can change intracellular calcium concentrations in the pancreatic β-cell line.³⁴ Our findings emphasize the important role of 1,25D₃ in regulating the intracellular calcium concentration via extracellular calcium influx, which plays a role in an early (rapid) downstream signaling activation.

In this study, $^{45}\text{Ca}^{2+}$ uptake was used to demonstrate rapid responses of 30-day-old Sertoli cells to 1,25D₃. First, it was shown that 1,25D₃, 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 1,25D₃ in stimulating calcium uptake and also was able to potentiate the 1,25D₃ 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 1,25D₃ (6T), as already reported for many other systems.^{9,10,12}

The rapid and/or sustained 1,25D₃-stimulated calcium uptake through VDCC seems to be required for physiological responses in Sertoli cells.^{19,21} Following these findings, we demonstrate here that 1,25D₃-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 1,25D₃ treatment can also occur through another kind of channels present in Sertoli cells such as N- and P/Q-type VDCC³⁵ as well as by the transient receptor vanilloid 1 (TRPV1) channels that mediate indirectly the calcium influx in Sertoli cells³⁶ or other TRPV channels that have been reported to mediate 1,25D₃ 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 1,25D₃, was predominantly caused by VDCC channels because storage calcium seemed not to alter the hormone effect. This finding suggests that 1,25D₃ 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 1,25D₃ 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

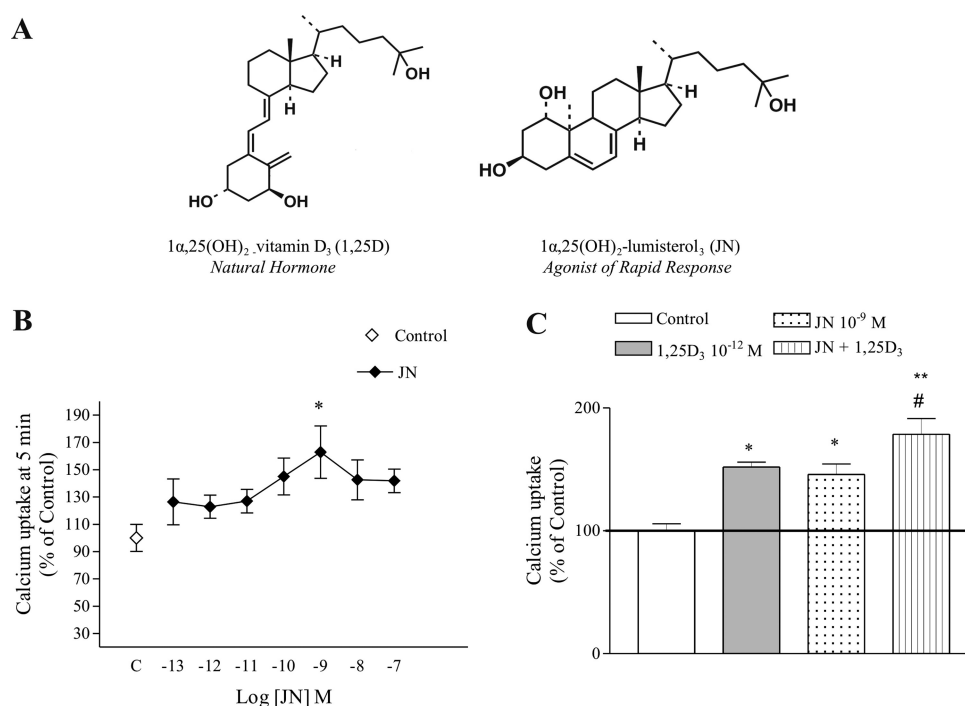


Figure 3. Effect of synthetic agonist $1\alpha,25(\text{OH})_2$ lumisterol $_3$ (JN) on $^{45}\text{Ca}^{2+}$ uptake. (A) Chemical structures of $1,25\text{D}_3$ and its rapid response analogue. (B) Dose-response curve of JN on $^{45}\text{Ca}^{2+}$ uptake in 30-day-old rat Sertoli cells. (C) Effect of JN (10^{-9} M) on the stimulatory effect of $1,25\text{D}_3$ (10^{-12} M). Means \pm 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 $1,25\text{D}_3$ group. The preincubation time was 60 min; the incubation time was 5 min.

regulation of Na^+/K^+ -ATPase and the $\text{Na}^+/\text{Ca}^{2+}$ 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 $1,25\text{D}_3$ genomic and nongenomic responses in a variety of cell systems.^{12,21} Handling in vitro cAMP levels using theophylline or including a cAMP agonist (dibutyl 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 dibutyl cAMP suggests the participation of cAMP. In addition, the co-incubation of $1,25\text{D}_3$ with theophylline or $1,25\text{D}_3$ with dibutyl 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 $1,25\text{D}_3$.

One of the nongenomic events related to $1,25\text{D}_3$ that has a weak relationship with cyclic AMP/PKA is calcium influx.⁴⁴ We recently demonstrated in TM4 cells that the binding of $1,25\text{D}_3$ 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 $1,25\text{D}_3$.

In particular in PLC activity- and calcium-dependent PKC isoforms, $1,25\text{D}_3$ induced changes in phosphoinositide turnover, PKC translocation, and intracellular calcium levels, as revised by Fleet.⁶ Results of our analyses indicated that $1,25\text{D}_3$ 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 $1,25\text{D}_3$.¹⁰

Binding of $1,25\text{D}_3$ 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 $1,25\text{D}_3$ 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 $1,25\text{D}_3$ corroborates previous data showing $1,25\text{D}_3$ modulates myoblast proliferation,⁴⁷ vimentin phosphorylation,²⁸ and calcium uptake in 30-day-old rat testis.⁴⁸

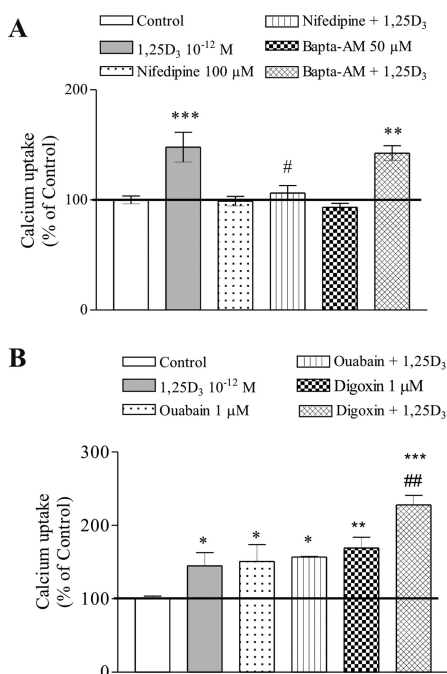


Figure 4. Involvement of voltage-dependent calcium channel (VDCC), intracellular calcium, and Na⁺/K⁺ATPase in ⁴⁵Ca²⁺ uptake in 30-day-old rat Sertoli cells. Nifedipine (100 μM) and Bapta-AM (50 μM) (A), and ouabain (1 μM) and digoxin (1 μM) (B), were added in the last 15 min of the preincubation and 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 1,25D₃, one number sign denotes *p* < 0.05 and two number signs denote *p* < 0.01 compared with the 1,25D₃ group.

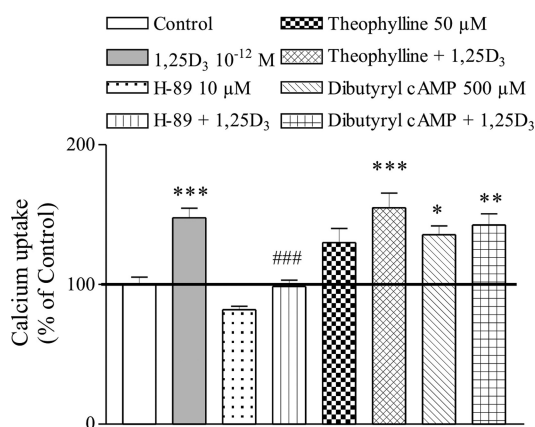


Figure 5. Involvement of the PKA signaling pathway in the stimulatory effect of 1,25D₃ on ⁴⁵Ca²⁺ uptake. Effect of H-89 (10 μM), theophylline (50 μM), and dibutyl cAMP (500 μM) in the stimulatory effect of 1,25D₃ (10⁻¹² M) on ⁴⁵Ca²⁺ uptake in 30-day-old rat Sertoli cells. Means ± SEM of three independent 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 1,25D₃, three number signs denote *p* < 0.001 compared with the 1,25D₃ group. The preincubation time was 60 min; the incubation time was 5 min.

On the other hand, the synergistic effect of 1,25D₃ 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

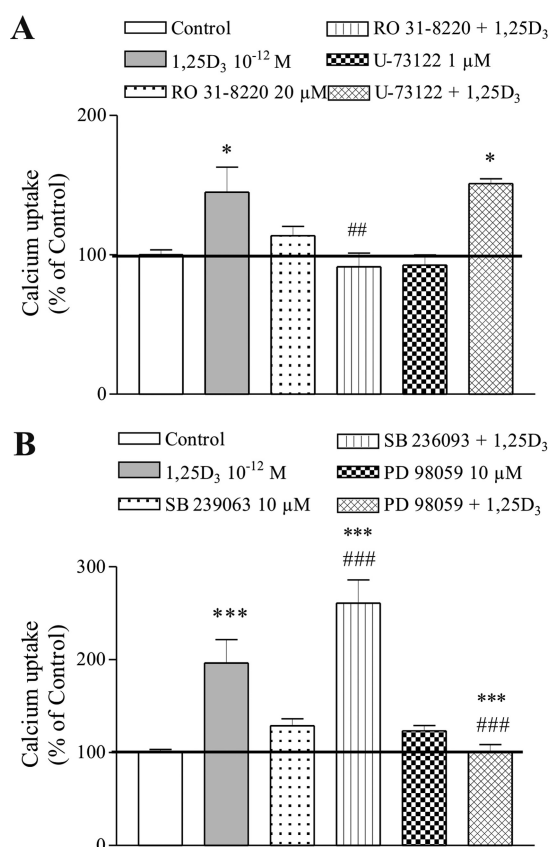


Figure 6. Role of PKC, PLC, ERK, and p38 MAPK in the stimulatory effect of 1,25D₃ on ⁴⁵Ca²⁺ uptake. (A) Effect of RO 31-8220 (20 μM) and U-73122 (1 μM) in the stimulatory effect of 1,25D₃ (10⁻¹² M) on ⁴⁵Ca²⁺ uptake in 30-day-old rat Sertoli cells. (B) Effect of PD 98059 (10 μM) and SB 239063 (10 μM) in the stimulatory effect of 1,25D₃ (10⁻¹² M) on ⁴⁵Ca²⁺ 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 1,25D₃, two number signs denote *p* < 0.01 and three number signs denote *p* < 0.001 compared with the 1,25D₃ 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 plentitude cytoskeleton function. In this work, the effect of 1,25D₃ was potentiated by p38 MAPK inactivation, suggesting a different pathway regulating 1,25D₃-stimulated calcium influx. In that situation, 1,25D₃ could be inhibiting p38 MAPK, or simply, this inhibition could be result of cross talk between

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