



1 α ,25(OH) $_2$ -Vitamin D $_3$ stimulation of secretion via chloride channel activation in Sertoli cells[☆]

Danusa Menegaz^{a,b}, Antonio Barrientos-Duran^a, Andrew Kline^a, Fatima R.M.B. Silva^b, Anthony W. Norman^a, Mathew T. Mizwicki^a, Laura P. Zanello^{a,*}

^a Department of Biochemistry, University of California, 900 University Ave., Riverside, CA 92521, USA

^b Department of Biochemistry, Federal University of Santa Catarina, Florianopolis, Brazil

ARTICLE INFO

Article history:

Received 4 September 2009

Received in revised form

22 December 2009

Accepted 22 January 2010

Keywords:

1 α ,25(OH) $_2$ -Vitamin D $_3$

Chloride channels

Secretion

Sertoli cells

ABSTRACT

Sertoli cell secretory activities are highly dependent on ion channel functions and critical to spermatogenesis. The steroid hormone 1 α ,25(OH) $_2$ -vitamin D $_3$ (1,25(OH) $_2$ -D $_3$) stimulates exocytosis in different cell systems by activating a nongenotropic vitamin D receptor (VDR). Here, we described 1,25(OH) $_2$ -D $_3$ stimulation of secretion via Cl⁻ channel activation in the mouse immature Sertoli cell line TM4. 1,25(OH) $_2$ -D $_3$ potentiation of chloride currents was dependent on hormone concentration, and correlated with a significant increase in whole-cell capacitance within 20–40 min. In addition, Cl⁻ currents were potentiated by the nongenomic VDR agonist 1 α ,25(OH) $_2$ lumisterol D $_3$ (JN), while 1,25(OH) $_2$ -D $_3$ potentiation of channels was suppressed by nongenomic VDR antagonist 1 β ,25(OH) $_2$ -vitamin D $_3$ (HL). Treatment of TM4 cells with PKC and PKA activators PMA and forskolin respectively, increased Cl⁻ currents significantly, while PKC and PKA inhibitors Go6983 and H-89, respectively, abolished 1,25(OH) $_2$ -D $_3$ stimulation of Cl⁻ currents, suggesting phosphorylation pathways in 1,25(OH) $_2$ -D $_3$ mediated channel responses. RT-PCR demonstrated the expression of outwardly rectifying ClC-3 channels in TM4 cells. Taken together, our results demonstrate a PKA/PKC-dependent 1,25(OH) $_2$ -D $_3$ /VDR nongenotropic pathway leading to Cl⁻ channel and exocytosis activation in Sertoli cells. We conclude that 1,25(OH) $_2$ -D $_3$ appears to be a modulator of male reproductive functions at least in part by stimulating Sertoli cell secretory functions.

© 2010 Published by Elsevier Ltd.

1. Introduction

Hormonally active 1 α ,25(OH) $_2$ -vitamin D $_3$ (1,25(OH) $_2$ -D $_3$) generates biological responses in multiple tissues via genomic and nongenomic mechanisms that involve binding to a vitamin D receptor (VDR) [1–5]. On one hand, the 1,25(OH) $_2$ -D $_3$ /VDR complex functions in the cell's nucleus as a transcription factor for the modulation of the expression of target genes that control cell proliferation and differentiation among other cellular processes. On the other hand, 1,25(OH) $_2$ -D $_3$ interaction with a nongenotropic VDR activates plasma membrane functions by means of rapid modulation of signaling pathways involved in ion channel activation and cytoplasmic calcium signals [6–14]. Vitamin D $_3$ analogs known to exert nongenotropic effects in a variety of cell systems include the agonist 1 α ,25(OH) $_2$ lumisterol D $_3$ (JN) and antagonist 1 β ,25(OH) $_2$ -vitamin D $_3$ (HL). Among the most recently described nongenomic

effects of the steroid 1,25(OH) $_2$ -D $_3$ are those involving the flow of ions across the cell membrane as part of activation of secretory processes [15–17]. In particular, 1,25(OH) $_2$ -D $_3$ -sensitive voltage-gated chloride channels have been shown to play a crucial role in exocytosis in bone cells [17]. Voltage-gated Cl⁻ channels function as an electric shunt that couples to H⁺-ATPase-driven loading of secretory vesicles, and are therefore crucial for the onset of exocytosis.

Secretory activities of Sertoli cells are critical to spermatogenesis [18]. Sertoli cells express a variety of ion channels involved in cellular secretory functions [19–22]. The hormonal regulation of fluid secretion by Sertoli cells is important in male reproduction, and involves multiple signaling pathways including second messengers and modulation of ion channel activities [23,24]. It has been known for some time that vitamin D plays an essential role in reproductive functions. Vitamin D deficiency causes gonadal insufficiency in rats [25]. At the cellular level, 1,25(OH) $_2$ -D $_3$ triggers short-term physiological events including Ca²⁺ uptake activation [26], and PKA-dependent amino acid accumulation that involves Ca²⁺ and K⁺ channels present in Sertoli cell plasma membrane [27].

Sertoli cells of different species express a VDR; however, the molecular mechanisms of 1,25(OH) $_2$ -D $_3$ actions on these cells remain unclear [28–32]. Here, we used patch-clamp electrophysiology to investigate 1,25(OH) $_2$ -D $_3$ modulation of membrane electric

[☆] Partially funded by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and FAPESC (Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina).

* Corresponding author. Tel.: +1 951 827 3159; fax: +1 951 827 2364.

E-mail address: laura.zanello@ucr.edu (L.P. Zanello).

processes underlying secretory activities in mouse TM4 immature Sertoli cells. We studied for the first time $1,25(\text{OH})_2\text{-D}_3$ potentiation of Cl^- currents required for exocytosis, and identified protein kinase signaling underlying ion channel modulation by the steroid. Ours is the first report on a direct effect of the steroid hormone $1,25(\text{OH})_2\text{-D}_3$ on the secretory activities of Sertoli cells, with potential implications in male reproductive functions.

2. Materials and methods

2.1. Chemicals

$1\alpha,25(\text{OH})_2\text{-vitamin D}_3$, $25(\text{OH})\text{-vitamin D}_3$, and $1\beta,25(\text{OH})_2\text{-vitamin D}_3$ (analog HL) were obtained from M. Uskokovic (Hoffmann-La Roche). The synthetic analog $1\alpha,25(\text{OH})_2$ lumisterol₃ (JN) was obtained from W.H. Okamura (University of California, Riverside, CA). These compounds were stored in the dark as stock solutions in absolute ethanol at -20°C . N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, a specific PKA blocker), 8-chlorophenylthio-cAMP (a membrane-permeable cAMP agonist), forskolin (a direct activator of adenylyl cyclase), phorbol 12-myristate 13-acetate (PMA, a protein kinase C activator), Go6983 (a specific PKC inhibitor), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, a chloride channel blocker), were obtained from Sigma and dissolved in ethanol, DMSO (<0.1%), or distilled water, to produce stock solutions which were stored at 4°C .

2.2. Cell culture

TM4 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's media (DMEM) and Ham's F12 with the addition of 1 mM L-glutamine, 15 mM HEPES, 1.2 g/l sodium bicarbonate, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 5% (v/v) horse serum, and 2.5% (v/v) fetal calf serum, in a 5% CO_2 humidified atmosphere at 37°C , as described elsewhere [26]. For patch-clamp experiments, cells were plated at low density in 35-mm tissue culture dishes and used within the first three days. Prior to recordings, cells were washed at least three times with electrophysiological external solution to remove the medium completely. For RT-PCR experiments, cells were used at days 3–4 in culture, at 80% confluency.

2.3. Electrophysiology

Chloride currents in TM4 cells were studied with whole-cell patch-clamp electrophysiology using a Heka EPC-9 amplifier (ALA Scientific Instruments Inc., Westbury, NY), essentially as described before [6]. Recording pipettes with resistances ranging between 3 and 5 $\text{M}\Omega$ were fabricated with a DMZ Universal micropipette puller (Drummond Scientific Co., Broomall, PA), coated with Sylgard elastomer (Dow Corning Corp., Midland, MI) to reduce capacitive transients, and fire-polished. Seal resistances ranged from 2 to 5 $\text{G}\Omega$. Experiments were carried out at room temperature. Currents were low-pass-filtered at 1 kHz and digitized every 100 μs . Cell membrane capacitance and series resistances were electronically compensated prior to the recording of currents. The bath (extracellular) solution consisted of (mM): 150 NaCl, 10 CsCl, 2 MgCl_2 , 10 glucose, 10 HEPES, pH 7.3 (adjusted with NaOH). The pipette (intracellular) solution consisted of (mM): 160 CsCl, 10 MgCl_2 , 10 HEPES, pH 7.2 (adjusted with TEA-OH). Cs^+ and TEA^+ were used to block K^+ channel activity. Currents were activated with 100-ms pulses between -60 and 80 mV, from a holding potential of -30 mV. Changes in whole-cell capacitance, a measure of exocytosis [33], were detected using the software-based lock-in implementation of Pulse v.8 (ALA Scientific Instruments Inc.). The applied sine wave

had a frequency of 500 Hz and peak amplitude of 20 mV, and was superimposed to a holding potential of -30 mV. Whole-cell capacitance was continuously monitored for 1 h.

2.4. Analysis of *CICn* mRNA expression

Cell lysates were assayed for the expression of members of the *CICn* gene family of voltage-gated Cl^- channels (*CIC-1* through *CIC-7*) and *VDR* with RT-PCR. Total RNA was extracted using the RNAqueous[®]-4PCR Kit (Ambion/Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The first cDNA strand synthesis was performed with RETROscript[®] Kit (Ambion) using 100 U of MML-RT enzyme, 1.6 mM dNTPs, 1 \times RT Buffer (containing, in mM, 75 KCl, 3 MgCl_2 , 5 DTT, and 10 Tris-HCl, pH 8.3), and 10 U of RNase inhibitor, in 25 μl of final volume at 44°C for 1 h, plus a last 10-min step at 92.0°C to inactivate the RT enzyme. 5 μl cDNAs were used as template in a polymerase chain reaction with one pair of PCR primers specific for *CICn* and *VDR* mouse genes. Nucleotide sequence for the primers and gene accession numbers are shown in Fig. 5. These primer pairs produced no amplification on TM4 genomic DNA. PCR temperature and cycling conditions were as follows: an initial melting step at 94.0°C for 5 min, followed by 35 repetitive cycles [94.0°C for 30 s, specific annealing step at diverse temperatures for 30 s (56.0°C for the *CIC-2/5* couple of primers, 59.6°C for *CIC-4* and *VDR*, 62.0°C for *CIC-1/3/6/7*), and an extension step at 72.0°C for 1 min], ending the amplification process with a final extension step at 72.0°C for 10 min. RT-PCR products were visualized in a 1.5% agarose TBE 1 \times electrophoresis gel and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

2.5. Exocytosis imaging in TM4 cells

Microscopy imaging was performed on quinacrine-loaded live TM4 Sertoli cells basically as described before [17]. Briefly, cells were washed with Hank's Buffered Salt Solution (HBSS) and loaded with 3 μM quinacrine dissolved in HBSS for 30 min at 37°C . TM4 cells were viewed under an Olympus 1X50 fluorescence microscope using a FITC filter. Exocytosis was identified as the rapid loss of quinacrine fluorescence when released into the medium, indicating fusion of secretory vesicles with the plasma membrane under the hormone stimulus. Time-lapse snapshots were obtained with a Spot Pursuit digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) at a scanning rate of 1 image/30 s during treatment of cells with 1–100 nM $1,25(\text{OH})_2\text{-D}_3$.

2.6. Statistical analysis

Data were expressed as means \pm standard error (S.E.). The two-tailed Student's *t*-test with two sample unequal variance was used for statistical analysis.

3. Results

3.1. $1,25(\text{OH})_2\text{-D}_3$ potentiates Cl^- currents in TM4 Sertoli cells in a dose-dependent manner

Fig. 1 shows the stimulatory effect of nanomolar concentrations of $1,25(\text{OH})_2\text{-D}_3$ on whole-cell Cl^- currents in TM4 Sertoli cells studied with patch-clamp electrophysiology. Non-inactivating, outwardly rectifying currents were recorded at positive membrane potentials between 0 and 80 mV, from a holding potential of -30 mV, in the presence of nearly symmetrical Cl^- ion concentrations in isotonic recording solutions (Fig. 1A). Addition of 100 nM $1,25(\text{OH})_2\text{-D}_3$ to the external bath promoted a significant

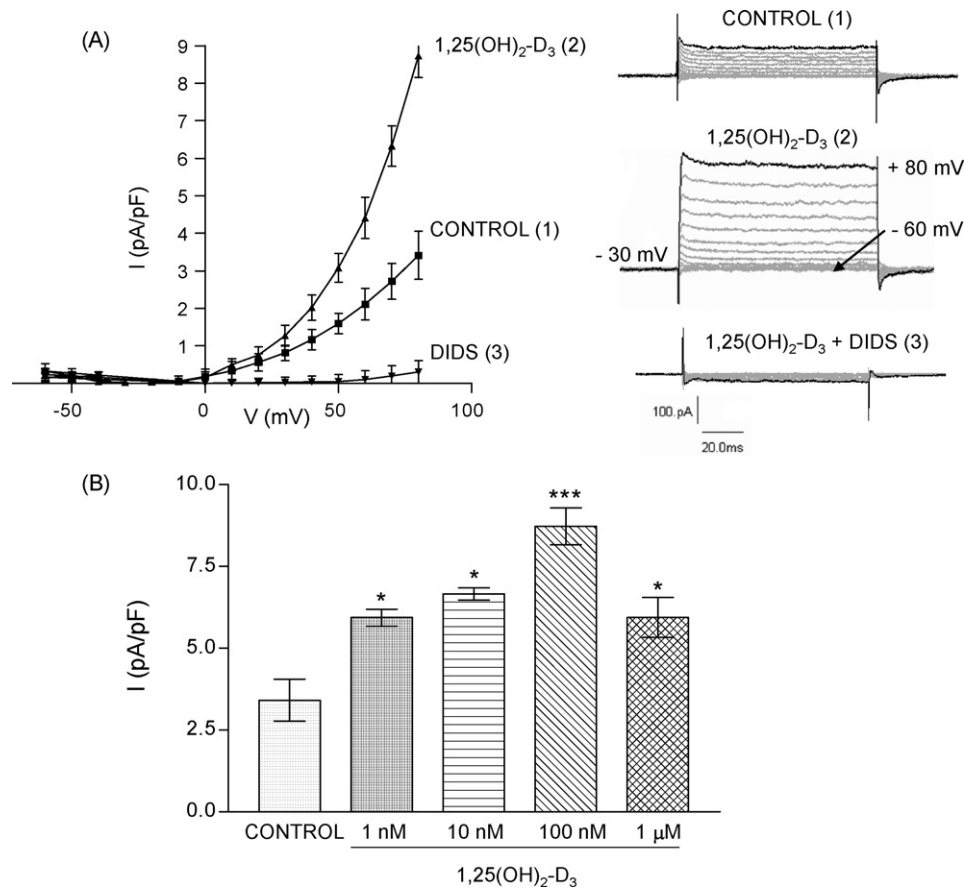


Fig. 1. Potentiation of DIDS-sensitive chloride currents by 1,25(OH)₂-D₃ hormone in TM4 Sertoli cells. (A) Current-to-voltage relations and raw current traces obtained from individual TM4 cells bathed in Cl⁻ containing recording solutions (see Section 2). Current amplitude values normalized to cell size (pA/pF) are shown for control (1) conditions before hormone treatment, and 40 min after the addition of 100 nM 1,25(OH)₂-D₃ to the bath (2). 1,25(OH)₂-D₃-promoted outward currents were totally blocked by subsequent addition of 200 μM DIDS (3). Currents were activated by 100 ms depolarizing voltage steps between -60 and 80 mV from a holding potential of -30 mV. Numbers in parentheses indicate the sequential order of the recordings. (B) Whole-cell Cl⁻ current amplitude values (pA/pF) measured at a depolarizing potential of 80 mV after the addition of different 1,25(OH)₂-D₃ concentrations (1 nM–1 μM), compared to control current amplitudes obtained in the absence of hormone. Bars represent means ± SEM for *n* = 10 individual cells. **p* < 0.05; ****p* < 0.001 compared to control. Cell size was around 40–50 pF, as measured by whole-cell capacitance.

increase in outward current amplitudes within 20–40 min. Current amplitude was measured at its maximum value, typically after 20–40 min. Potentiated Cl⁻ currents maintained the outward rectification and non-inactivating electrical profile recorded prior to the addition of 1,25(OH)₂-D₃. This proved that no additional channels were activated by the hormone, and that increased current amplitudes were not due to leakage. To verify that these 1,25(OH)₂-D₃-responsive currents were due to the activity of voltage-dependent chloride channels, 200 μM DIDS, a stilbene derivative which blocks CIC-3 outwardly rectifying chloride channels [34], was added to the bath after 1,25(OH)₂-D₃ treatment. 1,25(OH)₂-D₃-potentiated currents were totally blocked by DIDS within 2 min, thus confirming the involvement of CIC-3 channels (Fig. 1A).

Next, we studied the dose-dependence of 1,25(OH)₂-D₃ stimulation of Cl⁻ currents. Basal TM4 Cl⁻ currents recorded at 80 mV were treated with 1,25(OH)₂-D₃ concentrations ranging 1 nM through 1 μM. Chloride current amplitudes normalized to cell size (pA/pF) obtained with different 1,25(OH)₂-D₃ concentrations are shown in Fig. 1B. Maximum Cl⁻ current potentiation was obtained with a final concentration of 100 nM 1,25(OH)₂-D₃ added to the recording bath (about 3-fold increase with respect to control current amplitudes measured in the absence of hormone). A significantly lower potentiation effect compared to the maximum increase achieved with 100 nM was observed with 1 μM 1,25(OH)₂-D₃, indicating a biphasic response similar

to the one described for 1,25(OH)₂-D₃-sensitive Cl⁻ channels in osteoblasts [6].

3.2. 1,25(OH)₂-D₃ stimulatory effect on Cl⁻ currents involves PKC/PKA pathways in TM4 Sertoli cells

We demonstrated previously that nongenomic 1,25(OH)₂-D₃ stimulation of voltage-gated Cl⁻ channels require phosphorylation steps via protein kinases C and A in the presence of a functional VDR in osteoblasts [9]. To study the hypothesis that 1,25(OH)₂-D₃ potentiation of Cl⁻ channels present in TM4 cells involves similar phosphorylation pathways, we recorded the effect of agonists and antagonists of PKC and cAMP/PKA on whole-cell chloride currents measured at 80 mV in the presence and/or absence of 100 nM 1,25(OH)₂-D₃. As shown in Fig. 2, treatment of TM4 cells with the adenylylate cyclase activator forskolin (20 μM) added to the recording bath caused a potentiation of Cl⁻ currents of about 3-fold, similar to 100 nM 1,25(OH)₂-D₃, after 20–35 min. In addition, the permeable analog 8-chlorophenylthio-cAMP at a concentration of 1 mM promoted significant activation of the currents (about 2-fold). Preincubation of cells with the PKA blocker H-89 (1 μM) alone did not have any significant effect on control currents; however, it abolished further 1,25(OH)₂-D₃ stimulation of the currents.

We also found that preincubation of TM4 cells with the PKC activator PMA (100 nM) promoted a significant increase of about 1.5-fold in outward Cl⁻ currents compared with control.

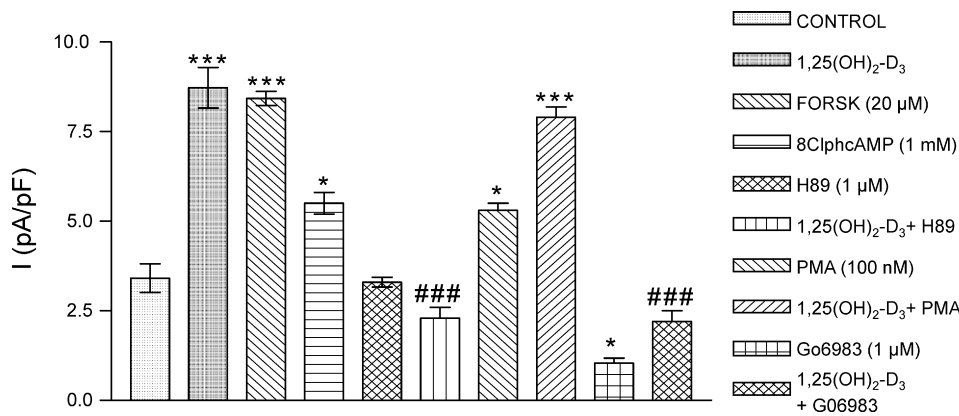


Fig. 2. Effects of PKA and PKC modulators on $1,25(\text{OH})_2\text{-D}_3$ -sensitive Cl^- currents compared to current potentiation obtained with $1,25(\text{OH})_2\text{-D}_3$. Whole-cell Cl^- current amplitudes were measured at 80 mV after the addition of 100 nM $1,25(\text{OH})_2\text{-D}_3$, with and without modulators of PKA and PKC pathways at the indicated concentrations. Modulators studied include: 20 μM forskolin (direct activator of adenylyl cyclase), 1 mM 8-chlorophenylthio-cAMP (membrane-permeable cAMP agonist), 1 μM H-89 (specific PKA blocker), 100 nM PMA (PKC activator), 1 μM Go6983 (specific PKC inhibitor). In each case, at least a 10 min period was allowed after the addition of the agents to the bath for currents to reach a stable amplitude value. Values correspond to means \pm SEM for $n = 10$ individual measurements performed on separate cells. * $p < 0.05$; *** $p < 0.001$ compared with control; ### $p < 0.001$ compared with $1,25(\text{OH})_2\text{-D}_3$ group.

When cells were treated with 100 nM PMA in combination with 100 nM $1,25(\text{OH})_2\text{-D}_3$, the stimulatory effect was similar to 100 nM $1,25(\text{OH})_2\text{-D}_3$ alone (about 2.5-fold increase). In addition, we found that inhibition of endogenous PKC activity by 1 μM Go6983 reduced control currents by 50% and abolished any further potentiation of the currents by 100 nM $1,25(\text{OH})_2\text{-D}_3$.

3.3. TM4 cell Cl^- channels are potentiated by the synthetic agonist JN and natural metabolite $25(\text{OH})\text{-D}_3$, and blocked by the nongenomic antagonist HL

We have shown before that the synthetic 6-s-cis locked analog $1\alpha,25(\text{OH})_2$ lumisterol D_3 (JN, Fig. 3) is a potent agonist of $1,25(\text{OH})_2\text{-D}_3$ nongenomic effects [35]. JN appears to bind with high affinity to an alternative nongenomic binding pocket in the VDR molecule present in lipid raft-enriched/caveolae cell membrane fractions [36]. In particular, we described previously that $1,25(\text{OH})_2\text{-D}_3$ -sensitive Cl^- currents expressed in osteoblasts can be specifically potentiated by the synthetic conformer JN [6]. Here, we found that, similarly to $1,25(\text{OH})_2\text{-D}_3$, 1 nM JN induced a 2.5-fold increase of non-inactivating outwardly rectifying Cl^- currents in TM4 Sertoli cells within 20–40 min (Fig. 3A). We also showed previously that the synthetic analog and nongenomic antagonist $1\beta,25(\text{OH})_2$ -vitamin D_3 (analog HL), which only differs from the natural metabolite $1,25(\text{OH})_2\text{-D}_3$ in the orientation of the hydroxyl group on carbon 1, inhibits $1,25(\text{OH})_2\text{-D}_3$ -induced potentiation of Cl^- currents in osteoblasts [6], probably by binding to the same alternative pocket in the VDR [36]. Here, we verified that 1–100 nM HL co-incubated with equimolar $1,25(\text{OH})_2\text{-D}_3$ completely abolished Cl^- current activation by the steroid in TM4 cells (Fig. 3B). In addition, HL prevented potentiation of Cl^- currents by JN, suggesting competitive binding of the compounds to the receptor.

We also found that the natural metabolite $25(\text{OH})$ vitamin D_3 ($25(\text{OH})\text{-D}_3$) significantly increased Cl^- currents activated by depolarization by approximately 1.5-fold at a concentration of 100 nM within 20–40 min. This potentiation was similar in magnitude to Cl^- current potentiation induced by 1 nM of the hormonally active $1,25(\text{OH})_2\text{-D}_3$ (Fig. 3B). $25(\text{OH})\text{-D}_3$ potentiation effect on TM4 Cl^- channels was blocked by the antagonist HL, indicating that the natural metabolite may bind to the same alternative nongenomic binding site in the VDR molecule. The specificity of Cl^- channel responses to $1,25(\text{OH})_2\text{-D}_3$ analogs in TM4 cells was investigated with the steroid β -estradiol (100 nM). As shown in Fig. 3B, 100 nM

β -estradiol added to the recording bath did not have any significant effect on control Cl^- currents within 45 min, demonstrating the specificity of the chloride channel response to vitamin D_3 compounds and further suggesting the involvement of a VDR.

3.4. $1,25(\text{OH})_2\text{-D}_3$ stimulates secretory activities in TM4 cells

We showed previously with electrophysiology that treatment of primary osteoblasts with nanomolar concentrations of $1,25(\text{OH})_2\text{-D}_3$ causes a significant increase in whole-cell capacitance, which is a measure of exocytosis, within minutes [8]. We found that $1,25(\text{OH})_2\text{-D}_3$ -stimulated rapid secretory response was dependent on $1,25(\text{OH})_2\text{-D}_3$ nongenomic potentiation of ClC-3 voltage-gated chloride channels present in the plasma membrane and membrane of secretory vesicles of osteoblasts expressing a functional VDR [17]. Here, we describe for the first time $1,25(\text{OH})_2\text{-D}_3$ stimulation of exocytotic activities in TM4 Sertoli cells, as revealed with whole-cell capacitance measurements.

Fig. 4A shows continuous whole-cell capacitance recordings during treatment of TM4 Sertoli cells with 100 nM $1,25(\text{OH})_2\text{-D}_3$. We found that whole-cell capacitance values remained relatively constant (50.4 pF baseline in the figure) prior to the addition of $1,25(\text{OH})_2\text{-D}_3$ to the bath. As recorded on three different cells studied, the addition of 100 nM $1,25(\text{OH})_2\text{-D}_3$ to the external solution caused a significant increase in capacitance value within 20–40 min, which indicated the stimulation of massive fusion of secretory vesicles with the plasma membrane during exocytosis. To study the hypothesis that $1,25(\text{OH})_2\text{-D}_3$ stimulation of exocytosis in TM4 cells required the opening of voltage-gated chloride channels, we measured whole-cell capacitance on cells preincubated with 200 μM DIDS. In the presence of the channel blocker, 100 nM $1,25(\text{OH})_2\text{-D}_3$ failed to stimulate exocytosis within 40 min, confirming the involvement of ClC-3 channels in the exocytotic response (Fig. 4A). The downward deflection of the capacitance trace after reaching its maximum in Fig. 4A (left panel) indicates that secretory vesicles may recycle inside the cytoplasm after releasing their content in the extracellular environment. We visualized $1,25(\text{OH})_2\text{-D}_3$ -stimulation of exocytosis in quinacrine-stained live TM4 cells. $1,25(\text{OH})_2\text{-D}_3$ -triggered exocytosis was demonstrated by recording the loss of fluorescence from individual secretory vesicles in single TM4 cells as quinacrine contained in the vesicles was secreted into the external medium in response to the hormonal stimulus, as seen in Fig. 4B.

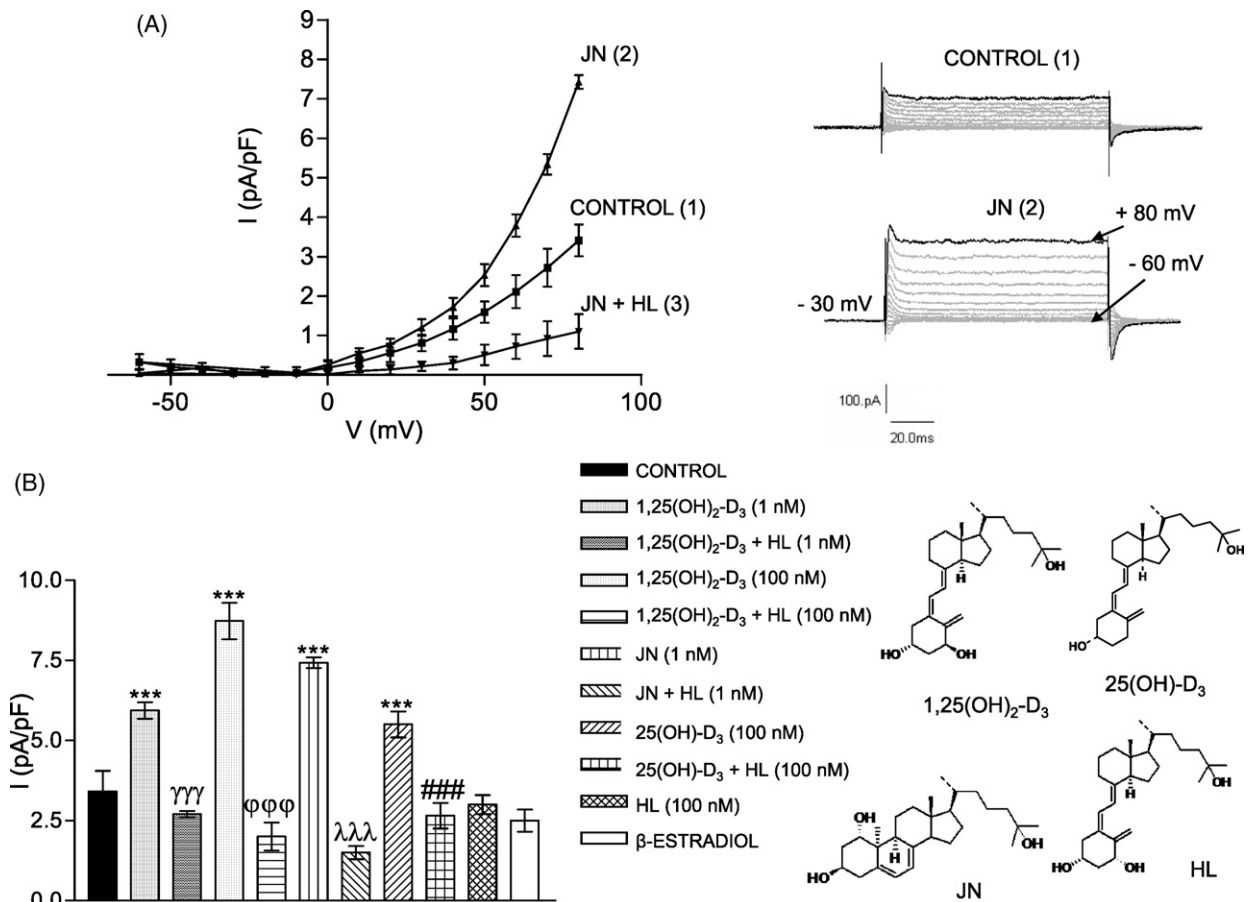


Fig. 3. Effect of synthetic agonist $1\alpha,25(\text{OH})_2$ lumisterol₃ (JN), antagonist $1\beta,25(\text{OH})_2$ -vitamin D₃ (HL), and natural metabolite 25(OH) vitamin D₃ (25(OH)-D₃) on outward Cl⁻ currents in TM4 Sertoli cells. (A) Current-to-voltage relations obtained from individual cells bathed in Cl⁻ containing recording solutions as described in Fig. 1. The right panel depicts raw current traces activated by 100 ms depolarizing voltage steps to between -60 and 80 mV from a holding potential of -30 mV. Control currents (1) and subsequent JN treatment (2) are shown for recordings obtained on the same cell. JN + HL (3) traces were obtained after the addition of the agonist JN to cells preincubated with HL. (B) Comparative effects of 1,25(OH)₂-D₃, JN, 25(OH)-D₃ and the steroid β -estradiol on outward Cl⁻ currents in the absence and presence of the antagonist HL. Current amplitudes were measured with an 80 mV depolarizing voltage step from a holding potential of -30 mV. In each case, at least a 10 min period was allowed after the addition of the agents to the bath, and currents were measured when they reached a stable amplitude value. The graph depicts mean values \pm SEM obtained from $n = 7$ –10 individual cells. *** $p < 0.001$ compared with control; φφφ $p < 0.001$ compared with 1,25(OH)₂-D₃ (1 nM) group; λλλ $p < 0.001$ compared with 1,25(OH)₂-D₃ (100 nM) group; ### $p < 0.001$ compared with JN group.

3.5. Expression of voltage-gated ClCn channels in TM4 Sertoli cells

Next, we carried out an RT-PCR study in TM4 cells to investigate the expression of members of the ClCn gene family of voltage-gated chloride channels (see Section 2). Primers sequences and amplicon lengths are shown in Fig. 5. Identification of VDR gene expression in TM4 cells was also studied by this approach. As shown in the figure, we found levels of gene expression for ClC-1 through ClC-7 members of the ClCn gene family. Higher relative levels were found for ClC-2, -3, -4, -5, and -7, while ClC-1 and ClC-6 expression levels were almost not detected. In particular, expression of the ClC-3 channel, which is sensitive to the specific blocker DIDS and has outward rectifying characteristics, was detected at relatively high levels. In addition, and in accordance with previous studies on primary Sertoli cells [28], VDR gene expression was detected in TM4 Sertoli cells. Similar levels of VDR expression were measured with and without preincubation of TM4 cells with 1,25(OH)₂-D₃ for up to 40 min (data not shown).

4. Discussion

We investigated here for the first time 1,25(OH)₂-D₃ nongenomic potentiation of an outwardly rectifying, DIDS-sensitive Cl⁻ current that couples to exocytosis in mouse TM4 Sertoli cells. Our results showed that 1,25(OH)₂-D₃ stimulated opening of chloride

channels is dependent on PKC and cAMP/PKA pathways, which would explain the recorded lag time for channel activation as studied in vitro. Stimulatory effects of 1,25(OH)₂-D₃ on these anion currents took place within 20–40 min of incubation with the hormone and correlated with an increase in whole-cell capacitance, which is indicative of secretory activities, within the same time frame.

A summarized model of 1,25(OH)₂-D₃ mechanisms of action for the stimulation of chloride channel functions in Sertoli cells is shown in Fig. 6. Briefly, by interacting with a cytosolic VDR located in close proximity to the plasma membrane, 1,25(OH)₂-D₃ appears to activate PKC and cAMP/PKA pathways required for the opening (via phosphorylation) of ClC-3 channels present in the cell membrane and membrane of secretory vesicles within a timeframe of 20–40 min. Chloride influx through these voltage-gated channels stimulates exocytosis by providing an electric shunt across the vesicle membrane. Sertoli cells secretory activities include release of ions, proteins, and growth factors relevant to germ cell maturation and male reproduction.

The specific nongenomic 6-s-cis locked agonist JN stimulated Cl⁻ currents more effectively than 1,25(OH)₂-D₃, while the nongenomic antagonist HL [37,38] blocked the agonistic effect of both 1,25(OH)₂-D₃ and JN on the currents, in agreement with effects reported previously in osteoblasts [6]. This could be explained by a higher affinity for JN compared to 1,25(OH)₂-D₃ to bind to an alter-

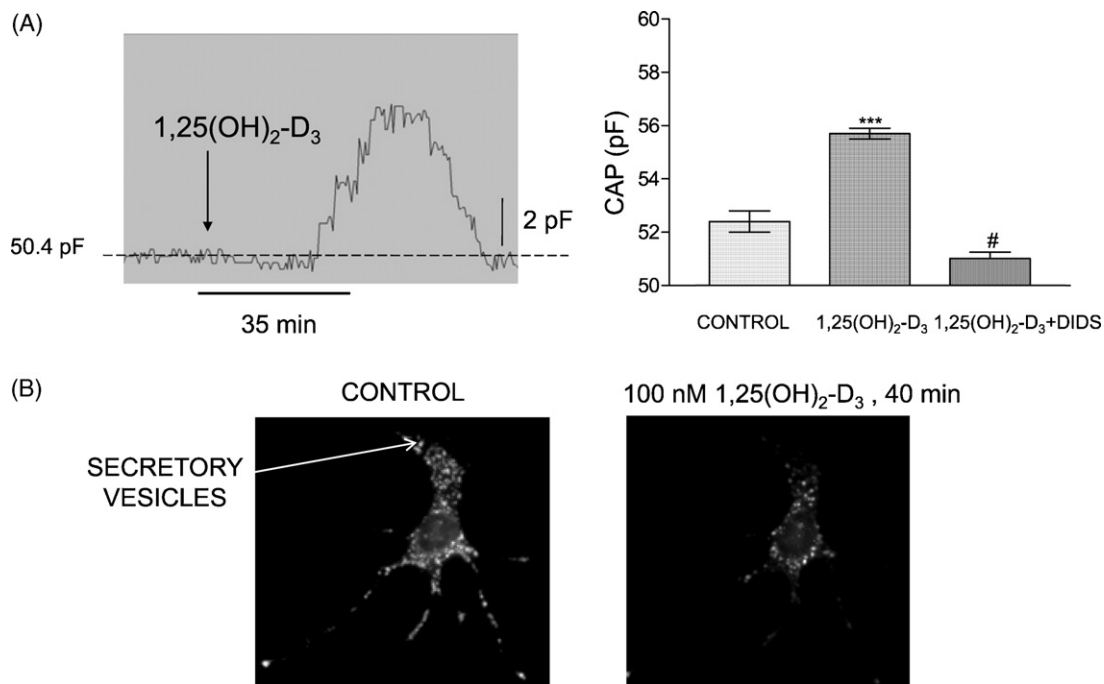


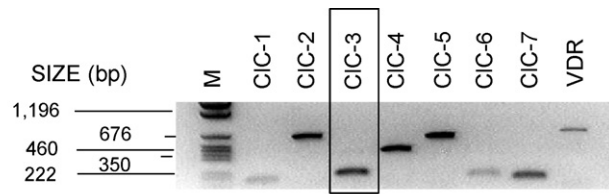
Fig. 4. 1,25(OH)₂-D₃-stimulation of exocytosis in TM4 Sertoli cells. (A) Continuous whole-cell capacitance recording obtained from a single TM4 cell during the addition of 100 nM 1,25(OH)₂-D₃ to the bath. Upward deflection of the traces from an initial capacitance value of 50.4 to 55.2 pF in the case of this particular cell depicts the stimulation of exocytotic activities in real time. The exocytotic response took place after approximately 30 min with 1,25(OH)₂-D₃ incubation. The bar graph shows mean whole-cell capacitance values obtained from 3 individual cells per treatment before (control) and 40 min after addition of 100 nM 1,25(OH)₂-D₃, and 100 nM 1,25(OH)₂-D₃ co-incubated with 200 μM of the chloride channel blocker DIDS. (B) Fluorescence images obtained from a single live TM4 Sertoli cell stained with 3 μM quinacrine. Quinacrine stains individual secretory vesicles in the cell cytoplasm. The image on the left corresponds to a TM4 cell immediately before 1,25(OH)₂-D₃ treatment. Incubation of this particular cell with 100 nM 1,25(OH)₂-D₃ caused fusion of quinacrine-loaded vesicles to the plasma membrane and release of the fluorescent content into the surrounding medium, as seen by the loss of fluorescence from most vesicles located on the cell's periphery. This effect was observed after a 40-min incubation period with 1,25(OH)₂-D₃. In the absence of 1,25(OH)₂-D₃, control cells maintained their original fluorescence intensity (not shown), verifying that neither exocytosis nor quenching of the dye occurred within this time period. The graph depicts mean values ± SEM obtained from *n* = 4 individual cells. ****p* < 0.001 and #*p* < 0.05 compared with control.

native (nongenomic) binding pocket in the VDR molecule [35]. In addition, the natural metabolite 25(OH)-D₃ also stimulated chloride currents, although to a lower extent, as reported before for osteoblasts [6], confirming the involvement of a VDR. Involvement of the VDR in nongenomic effects of 1,25(OH)₂-D₃ had been proven before in primary osteoblasts [8,39]. Rapid (10 s) 1,25(OH)₂-D₃-induced cytoplasmic calcium elevation and calcium and chloride channel activation was abrogated in VDR null osteoblasts. In these two cases, osteoblasts were obtained from mice which expressed a VDR lacking DNA binding capacity. However, VDR null male mice were still fertile. Inability of the VDR to bind DNA does not seem to interfere with 1,25(OH)₂-D₃-promoted secretory activities of Sertoli cells and uptake of calcium within a timeframe of 20–40 min, which could be sufficient for induction of gamete maturation.

It has been shown previously that cAMP/PKA acts as a mediator of both 1,25(OH)₂-D₃ genomic and nongenomic responses in a variety of cell systems [12,27,40]. Here, we demonstrated that in the presence of a PKA blocker, 1,25(OH)₂-D₃ potentiation of chloride currents was abolished, while adenylate cyclase activation caused a significant increase in control currents, mimicking 1,25(OH)₂-D₃ effect. This suggests cAMP/PKA involvement in 1,25(OH)₂-D₃ chloride channel activation, and is in agreement with studies which showed that voltage-gated chloride channels have target sites for phosphorylation by PKA in their regulatory cytoplasmic domains [41]. Our results are consistent with a model we proposed before for 1,25(OH)₂-D₃ nongenomic potentiation of the osteoblast CIC-3 channel via a cAMP/PKA cascade initiated by 1,25(OH)₂-D₃ activation of a cytoplasmic VDR that leads to channel phosphorylation and activation [8]. Here, we propose a similar mechanism for 1,25(OH)₂-D₃ potentiation of CIC-3 channel activities in Sertoli cells (Fig. 6). We also showed that treatment of TM4 cells with a PKC

activator caused a significant increase in Cl⁻ currents, while PKC blockade abolished 1,25(OH)₂-D₃ stimulation of chloride currents. This indicates the involvement of PKC in 1,25(OH)₂-D₃ nongenomic modulation of the electric currents, and agrees with results obtained on other systems [4,42–45]. It has also been reported previously that 1,25(OH)₂-D₃ activation of PKC may cause PKA activation via a cross-talk mechanism with the cAMP pathway [46]. Taken together, our results indicate that 1,25(OH)₂-D₃ potentiates voltage-gated Cl⁻ currents via phosphorylation cascades involving PKC and cAMP/PKA pathways in TM4 immature Sertoli cells.

Cytosolic Cl⁻ ions enhance Ca²⁺-dependent exocytosis in a variety of endocrine cell types [47]. We have studied the effect of preincubation of TM4 cells with the voltage-gated calcium channel nifedipine (2 μM), and found that this dihydropyridine antagonist abolishes 1,25(OH)₂-D₃ stimulation of chloride currents and exocytosis (data not shown). This appears to suggest that 1,25(OH)₂-D₃ driven exocytosis in immature Sertoli cells is a calcium-dependent mechanism. In addition, CIC-3 channels are present at the membrane of secretory vesicles; however, their role in exocytosis is not fully understood. The vesicle membrane, when fused to the plasma membrane, appears to deliver chloride channel proteins to the cell surface. It has been shown before with RT-PCR that primary rat Sertoli cells express the CIC-2, CIC-3, CIC-6 and CIC-7 members of the family of voltage-gated Cl⁻ channels [23]. Here, we found transcripts of CICn 1 through 7 genes in TM4 cells. Levels of expression appeared to be relatively higher for CIC-2, CIC-3, CIC-4, CIC-5 and CIC-7 compared to the other family members. Due to the pharmacological and electrical characteristics of the 1,25(OH)₂-D₃-sensitive chloride channel present in TM4 cells, more specifically its sensitivity to DIDS, outward rectification, and modulation by PKC, we conclude that this 1,25(OH)₂-D₃-sensitive chloride channel could



PRIMER	GENBANK ACC. No.	SEQUENCE
CIC-1	NM_013491.2	F: 5'-CCACCTGAAGCCTCAGACTC-3' R: 5'-CCAGGCCTCAATCTCTTCAG-3'
CIC-2	NM_009900.2	F: 5'-TAGAATATGGACAGAGCCGAT-3' R: 5'-AGTAGTTCCTAACAGCGAAGAA-3'
CIC-3	NM_173874.1	F: 5'-AAGGAGTGGTGTGAGGTTGG-3' R: 5'-GGCATCACCAACCCATTAC-3'
CIC-4	NM_011334.3	F: 5'-GGCACTGATCTTCAAATAGTCATT-3' R: 5'-AACTCATCCTTCACATCAAGAAA-3'
CIC-5	NM_016691.2	F: 5'-ATAATCAGCGAAACCACGTA-3' R: 5'-AGCCTAACTGTATTTTCTCCTAAA-3'
CIC-6	NM_011929.2	F: 5'-CCGAAACCTAAGCTCGTCAG-3' R: 5'-TCTGATGTGACCTGGAGCTG-3'
CIC-7	NM_011930.3	F: 5'-GCCTTCATAGAGCCTGTTGC-3' R: 5'-GTCCCCCTACCACAGACAGA-3'
VDR	NM_009504.3	F: 5'-AGAACAGCATGAAGCTCACA-3' R: 5'-TTTGGTCATCTTGGCAGTGAAT-3'

Fig. 5. RT-PCR analysis of CICn and VDR expression in TM4 Sertoli cells. Primers used and gene accession numbers are given in the Table. Column M in the agarose gel corresponds to the commercial plasmid vector pGEM-3 (Promega) treated with the Hinf I/Rsa I/Sin I restriction enzymes used as a molecular marker. PCR product lengths are: CIC-1, 102 bp; CIC-2, 642 bp; CIC-3, 195 bp; CIC-4, 429 bp; CIC-5, 609 bp; CIC-6, 158 bp; CIC-7, 142 bp; VDR, 672 bp. PCR products were confirmed by sequencing.

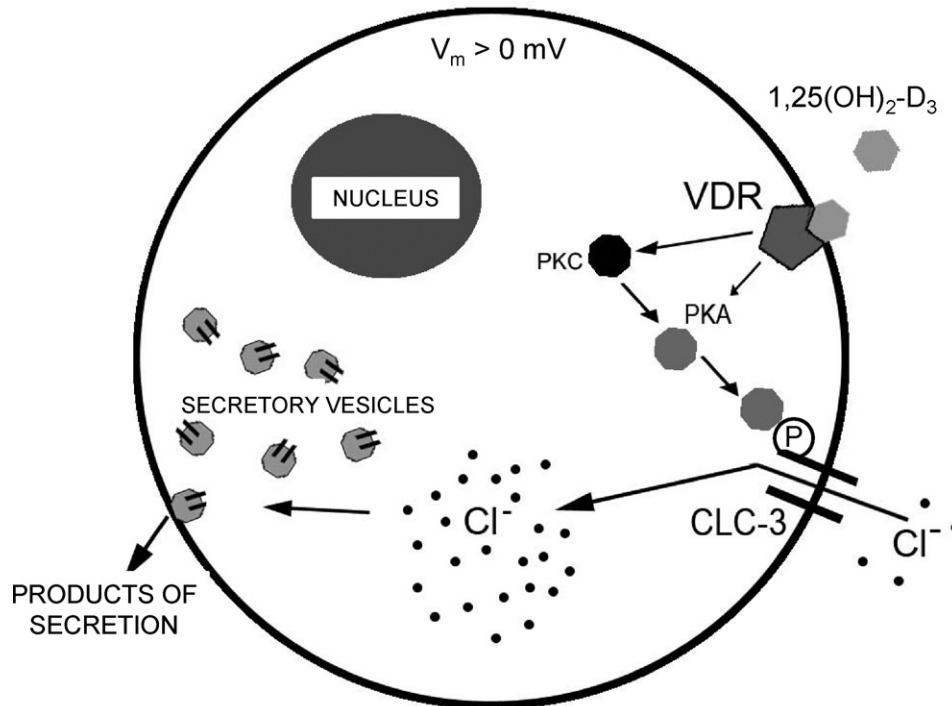


Fig. 6. Proposed model of $1,25(\text{OH})_2\text{-D}_3$ nongenomic potentiation of chloride currents through CLC-3 voltage-gated channels in TM4 Sertoli cells. $1,25(\text{OH})_2\text{-D}_3$ sensitive CLC-3 channels present at the plasma membrane open upon depolarization ($V_m > 0$ mV). Hormone $1,25(\text{OH})_2\text{-D}_3$ potentiates Cl^- channel opening via a cytoplasmic $1,25(\text{OH})_2\text{-D}_3$ /VDR-initiated mechanism that involves PKC/PKA activation leading to phosphorylation (P) of the channel. Cl^- ions flow in according to an electrochemical gradient. This is recorded as outward currents following the electric convention used in whole-cell patch-clamp recordings, and correspond to the influx of Cl^- ions through voltage-gated CLC-3 channels. CLC-3 present in the membrane of secretory vesicles docked at the plasma membrane act as an electric shunt and their opening is required for exocytosis. $1,25(\text{OH})_2\text{-D}_3$ activation of CLC-3 channels takes place within 20–40 min.

be the broadly expressed CIC-3 channel [34,48], which is known to be involved in exocytosis. Under our experimental conditions, the electrical activity of inwardly rectifying CIC-2, which has been associated with regulation of the cell's volume, as well as CICs 4–7 was not detected. CIC-1, expressed primarily in muscular cells, was detected only at very low levels in TM4 cells.

Taken together, our results demonstrate for the first time that nongenomic $1,25(\text{OH})_2\text{-D}_3$ potentiation of chloride currents couple to exocytosis in immature TM4 Sertoli cells. This effect appears to involve a cytosolic VDR and PKC/PKA phosphorylation pathways leading to CIC-3 channel phosphorylation. We conclude that the steroid hormone $1,25(\text{OH})_2\text{-D}_3$ appears to play a functional role in male fertility via stimulation of Sertoli cell secretory activities in the testis.

Acknowledgements

The authors thank Mr. Elmer Hilo and Matthew Taon for technical assistance with microscopy and capacitance measurements.

References

- [1] R. Bouillon, W.H. Okamura, A.W. Norman, Structure–function relationships in the vitamin D endocrine system, *Endocrinol. Rev.* 16 (1995) 200–257.
- [2] A.W. Norman, M.T. Mizwicki, D.P.G. Norman, Steroid–hormone rapid actions, membrane receptors and a conformational ensemble model, *Nat. Rev.: Drug Discov.* 3 (2004) 27–41.
- [3] A.W. Norman, Vitamin D receptor: new assignments for an already busy receptor, *Endocrinology* 147 (2006) 5542–5548.
- [4] A.W. Norman, H.L. Henry, J.E. Bishop, W.H. Okamura, Different shapes of the steroid hormone $1\alpha,25(\text{OH})_2\text{-vitamin D}_3$ act as agonists for two different receptors in the vitamin D endocrine system to mediate genomic and rapid responses, *Steroids* 66 (2001) 147–158.
- [5] P. Lips, Vitamin D physiology, *Prog. Biophys. Mol. Biol.* 92 (2006) 4–8.
- [6] L.P. Zanello, A.W. Norman, Stimulation by $1\alpha,25(\text{OH})_2\text{-vitamin D}_3$ of whole cell chloride currents in osteoblastic ROS 17/2.8 cells: a structure–function study, *J. Biol. Chem.* 272 (1997) 22617–22622.
- [7] L.P. Zanello, A.W. Norman, Multiple molecular mechanisms of $1\alpha,25(\text{OH})_2\text{-vitamin D}_3$ rapid modulation of three ion channel activities in osteoblasts, *Bone* 33 (2003) 71–92.
- [8] L.P. Zanello, A.W. Norman, Rapid modulation of osteoblast ion channel responses by $1\alpha,25(\text{OH})_2\text{-vitamin D}_3$ requires the presence of a functional vitamin D nuclear receptor, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004) 1589–1594.
- [9] L.P. Zanello, A.W. Norman, $1\alpha,25(\text{OH})_2$ Vitamin D₃ actions on ion channels in osteoblasts, *Steroids* 71 (2006) 291–297, <http://buscatextual.cnpq.br/buscatextual/servletatigosquecitarandoi?doi=10.1016/j.neuroscience.2008.07.059>.
- [10] J.M. Caffrey, M.C. Farach-Carson, Vitamin D₃ metabolites modulate dihydropyridine sensitive calcium currents in clonal rat osteosarcoma cells, *J. Biol. Chem.* 264 (1989) 20265–20274.
- [11] A.R. De Bolan, A.W. Norman, Influx of extracellular calcium mediates $1\alpha,25(\text{OH})_2$ vitamin D₃-dependent transcaltachia (the rapid stimulation of duodenal Ca^{2+} transport), *Endocrinology* 127 (1990) 2475–2480.
- [12] A.R. De Boland, I. Nemere, Rapid actions of vitamin D compounds, *J. Cell. Biochem.* 49 (1992) 32–36.
- [13] M.C. Farach-Carson, I. Sergeev, A.W. Norman, Nongenomic actions of $1\alpha,25(\text{OH})_2$ vitamin D₃ in rat osteosarcoma cells: structure–function studies using ligand analogs, *Endocrinology* 121 (1991) 1876–1884.
- [14] S. Yukihiko, G.H. Posner, S.E. Guggino, Vitamin D₃ analogs stimulate calcium currents in rat osteosarcoma cells, *J. Biol. Chem.* 269 (1994) 23889–23893.
- [15] T.C. Pappas, B. Gametchu, J. Yannariello-Brown, T.J. Collins, C.S. Watson, Membrane estrogen receptor in GH3/B6 cells are associated with rapid estrogen-induced release of prolactin, *Endocrine* 2 (1994) 813–822.
- [16] C.S. Watson, B. Gametchu, Membrane-initiated steroid actions and the proteins that mediate them, *Proc. Soc. Exp. Biol. Med.* 220 (1999) 9–19.
- [17] P. Biswas, L.P. Zanello, $1\alpha,25(\text{OH})_2$ vitamin D₃ induction of ATP secretion in osteoblasts, *J. Bone Miner. Res.* 24 (2009) 1450–1460.
- [18] L.D. Russell, M.D. Grsiwold, *The Sertoli Cell*, Cache River Press, Clearwater, FL, 1993.
- [19] N. Lalevee, F. Pluciennik, M. Joffre, Voltage-dependent calcium current with properties of T-type current in Sertoli cells from immature rat testis in primary cultures, *Biol. Reprod.* 56 (1997) 680–687.
- [20] A. Jungwirth, T. Weiger, S.K. Singh, M. Paulmichl, J. Frick, Follicle-stimulating hormone activates a cAMP-dependent chloride conductance in TM4 Sertoli cells, *Biochem. Biophys. Res. Commun.* 233 (1997) 203–206.
- [21] F.R. Boockfor, R.A. Morris, D.C. CeSimone, D.M. Hunt, K.B. Walsh, Sertoli cell expression of the cystic fibrosis transmembrane conductance regulator, *Am. J. Physiol.* (1998) C922–C930.
- [22] N. Lalevee, M. Joffre, Inhibition by cAMP of calcium-activated chloride currents in cultured Sertoli cells from immature testis, *J. Membr. Biol.* 169 (1999) 167–174.
- [23] C. Auzanneau, V. Thoreau, A. Kitzis, F.A. Becq, Novel voltage-dependent chloride current activated by extracellular acidic pH in cultured rat Sertoli cells, *J. Biol. Chem.* 278 (2003) 19230–19236.
- [24] C. Auzanneau, C. Norez, S. Noel, C. Jouglu, F. Becq, C. Vandebrouck, Pharmacological profile of inhibition of the chloride channels activated by extracellular acid in cultured rat Sertoli cell, *Reprod. Nutr. Dev.* 46 (2006) 241–255.
- [25] G.G. Kwiecinski, G.I. Petrie, H.F. De Luca, Vitamin D is necessary for reproductive functions of the male rat, *J. Nutr.* 119 (1989) 741–744.
- [26] V.L. Akerstrom, M. Walters, Physiological effects of $1\alpha,25(\text{OH})_2$ vitamin D₃ in TM4 Sertoli cell line, *Am. J. Physiol.* 262 (1992) 884–890.
- [27] D. Menegaz, A. Rosso, C. Royer, L.D. Leite, A.R.S. Santos, F.R.M.B. Silva, Role of $1\alpha,25(\text{OH})_2$ vitamin D₃ on [^{14}C]-MeAIB accumulation in immature rat testis, *Steroids* 74 (2009) 264–269.
- [28] J. Merke, U. Hugel, E. Ritz, Nuclear testicular $1\alpha,25(\text{OH})_2$ vitamin D₃ receptors in Sertoli cells and seminiferous tubules of adult rodents, *Biochem. Biophys. Res. Commun.* 27 (1985) 303–309.
- [29] F.K. Habib, S.Q. Maddy, K.J. Gelly, Characterization of receptors for $1\alpha,25(\text{OH})_2$ vitamin D₃ in the human testis, *J. Steroid Biochem.* 35 (1990) 195–199.
- [30] R.C. Gensure, S.D. Antrobus, J. Fox, M. Okwueze, S.Y. Talton, M.R. Walters, Homologous up-regulation of vitamin D receptors is tissue specific in the rat, *J. Bone Miner. Res.* 13 (1998) 454–463.
- [31] S.S. Majumdar, A. Bartke, W.E. Stumpf, Vitamin D modulates the effects of follicle-stimulating hormone on Sertoli cell function and testicular growth in Siberian hamsters, *Life Sci.* 55 (1994) 1479–1486.
- [32] F.R.M.B. Silva, L.D. Leite, G.F. Wassermann, Rapid signal transduction in Sertoli cells, *Eur. J. Endocrinol.* 147 (2002) 425–433.
- [33] V.A. Klyachko, M.B. Jackson, Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve cells, *Nature* 418 (2002) 89–92.
- [34] T.J. Jentsch, V. Stein, F. Weinreich, A. Anselm, Molecular structure and physiological function of chloride channels, *Physiol. Rev.* 82 (2002) 503–568.
- [35] M.T. Mizwicki, D. Keidel, C.M. Bula, J.E. Bishop, L.P. Zanello, D. Moras, J.M. Wurtz, A.W. Norman, Identification of an alternative ligand-binding pocket in the nuclear vitamin D receptor and its functional importance in $1\alpha,25(\text{OH})_2$ vitamin D₃ signaling, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004) 12876–12881.
- [36] A.W. Norman, W.H. Okamura, M.W. Hammond, J.E. Bishop, M.C. Dormanen, R. Bouillon, H. Baelen, E. Daane, R. Khoury, M.C. Farach-Carson, Comparison of 6-s-cis- and 6-s-trans-locked analogs of $1\alpha,25(\text{OH})_2$ vitamin D₃ indicates that the 6-s-cis conformation is preferred for rapid nongenomic biological responses and that neither 6-s-cis- nor 6-s-trans-locked analogs are preferred for genomic biological responses, *Mol. Endocrinol.* 11 (1997) 1518–1531.
- [37] A.W. Norman, I. Nemere, K.R. Muralidharan, W.H. Okamura, $1\beta,25(\text{OH})_2$ vitamin D₃ is an antagonist of $1\alpha,25(\text{OH})_2$ vitamin D₃ stimulated transcaltachia (the rapid hormonal stimulation of intestinal calcium transport), *Biochem. Biophys. Res. Commun.* 30 (1992) 1450–1456.
- [38] A.W. Norman, R. Bouillon, M.C. Farach-Carson, J.E. Bishop, L.X. Zhou, I. Nemere, J. Zhao, K.R. Muralidharan, W.H. Okamura, Demonstration that $1\beta,25(\text{OH})_2$ vitamin D₃ is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of $1\alpha,25(\text{OH})_2$ vitamin D₃, *J. Biol. Chem.* 25 (1989) 20022–20030.
- [39] R.G. Erben, D.W. Soegiarto, K. Weber, U. Zeitz, M. Lieberherr, R. Gniadecki, G. Moller, J. Adamski, R. Balling, Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D, *Mol. Endocrinol.* 16 (2002) 1524–1537.
- [40] E. Avila, L. Diaz, D. Barrera, A. Halhali, I. Mendez, L. Gonzalez, U. Zuegel, A. Steinmeyer, F. Larre, Regulation of vitamin D hydroxylases gene expression by $1\alpha,25(\text{OH})_2$ vitamin D₃ and cyclic AMP in cultured human syncytiotrophoblasts, *J. Steroid Biochem. Mol. Biol.* 103 (2007) 90–96.
- [41] J.A. Mindell, M. Maduke, CIC chloride channels, *Genome Biol.* 2 (2001) 1–6.
- [42] M. Bissonnette, R.K. Wali, S.C. Hartman, S.M. Niedziela, H.K. Roy, X.Y. Tien, M.D. Sitrin, T.A. Brasitus, $1\alpha,25(\text{OH})_2$ vitamin D₃ and 12-O-tetradecanoyl phorbol 13-acetate cause differential activation of Ca^{2+} -dependent and Ca^{2+} -independent isoforms of protein kinase C in rat colonocytes, *J. Clin. Invest.* 95 (1995) 2215–2221.
- [43] J.P. Schmitz, Z. Schwartz, V.L. Sylvia, D.D. Dean, F. Calderon, B.D. Boyan, Vitamin D₃ regulation of stromelysin-1 (MMP-3) in chondrocyte cultures is mediated by protein kinase C, *J. Cell. Physiol.* 168 (1996) 570–579.
- [44] D.M. Berry, R. Antochi, M. Bhatia, K.A. Mecking-Gill, $1\alpha,25(\text{OH})_2$ vitamin D₃ stimulates expression and translocation of protein kinase C α and C δ via a nongenomic mechanism and rapidly induces phosphorylation of a 33-kDa protein in acute promyelocytic NB4 cells, *J. Biol. Chem.* 271 (1996) 16090–16096.
- [45] S.J. Slater, M.B. Kelly, F.J. Taddeo, J.D. Larkin, M.D. Yeager, J.A. McLane, C. Ho, C.D. Stubbs, Direct activation of protein kinase C by $1\alpha,25(\text{OH})_2$ vitamin D₃, *J. Biol. Chem.* 24 (1995) 6639–6643.
- [46] V. Massheimer, A.R. De Boland, Modulation of $1\alpha,25(\text{OH})_2$ vitamin D₃-dependent Ca^{2+} uptake in skeletal muscle by protein kinase C, *Biochem. J.* 281 (1992) 349–352.
- [47] J.E. Turner, S. Sedej, M. Rupnik, Cytosolic Cl^- ions in the regulation of secretory and endocytotic activity in melanotrophs from mouse pituitary tissue slices, *J. Physiol.* 566 (2005) 443–453.
- [48] M. Kawasaki, S. Uchida, T. Monkawa, A. Miyawaki, K. Mikoshiba, F. Marumo, S. Sasaki, Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells, *Neuron* 12 (1994) 597–604.