UNIVERSIDADE FEDERAL DE SANTA CATARINA

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PROGRAMA DE PÓS-GRADUAÇÃO EM FARMÁCIA

DANUSA MENEGAZ

ESTUDO DO MECANISMO DE AÇÃO NÃO-GENÔMICO DA TIROXINA E DA 1α,25 $(OH)_2$-VITAMINA D_3 EM SISTEMAS DE MEMBRANA PLASMÁTICA: TRANSPORTE DE AMINOÁCIDOS E FLUXO IÔNICO EM TESTÍCULOS DE RATOS E EM CÉLULAS TM4

Orientadora: Profª. Drª. FÁTIMA REGINA MENA BARRETO SILVA (UFSC/BRASIL)

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Tese apresentada ao Programa de Pós-graduação em Farmácia do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina, como requisito parcial para obtenção do título de Doutor em Farmácia.

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Dedico esta tese de doutorado aos meus maravilhosos pais Rui Menegaz e Maria de Lourdes da Silveira Menegaz que sempre me incentivaram e me apoiaram em todos os momentos da minha vida.

A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original. A percepção do desconhecido é a mais fascinante das experiências. O homem que não tem os olhos abertos para o misterioso passará pela vida sem ver nada.

Albert Einstein

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RESUMO

Hormônios esteróides, vitamina D, retinóides e hormônios da tireóide estimulam respostas celulares através de mecanismos genômicos e não-genômicos. No presente trabalho, hormônios com ação genômica: Tiroxina (T_4) e 1α,25-diidróxivitamina D_3 (1α,25$(OH)_2D_3$ ou 1,25D), foram estudados sob o ponto de vista não-genômico através de dois diferentes sistemas de membrana plasmática: transporte de aminoácidos em testículos de ratos e fluxo de cloreto em células de Sertoli TM4. As células de Sertoli de testículos de mamíferos são células secretoras que além de proteínas, nutrientes e fatores de crescimento, secretam um fluido rico em cloreto (Cl^-) e potássio (K^+) no lúmen dos túbulos seminíferos que são críticos para espermatogênese. Primeiramente, os resultados desse trabalho demonstraram que os canais de Ca^{2+} e os canais de K^+ dependentes de ATP (K^+_{ATP}) são requeridos para o efeito estimulatório do hormônio T_4 no transporte de aminoácidos em testículos de ratos imaturos. Além disso, os canais de Cl^- e os canais de K^+ dependentes da baixa condutância de Ca^{2+} (SK_{Ca}) estão parcialmente envolvidos nesse mecanismo. Esse efeito não-genômico do T_4 é dependente da proteína cinase C (PKC) e aponta o T_4 como um importante regulador metabólico da função testicular. Posteriormente foi demonstrado o efeito estimulatório da 1,25D no transporte de aminoácidos em testículo de ratos de uma forma dependente da concentração, dependente da idade e específico para esse hormônio. O requerimento dos canais de Ca^{2+}, dos canais $SK_{Ca,}$ da proteína cinase A (PKA) e da síntese de proteínas indicam efeitos rápidos e prolongados da 1,25D nesse mecanismo. Através de estudos eletrofisiológicos foi demonstrado que a 1,25D aumenta a permeabilidade ao íon cloreto na membrana plasmática de células de Sertoli TM4 em potenciais despolarizantes através do receptor VDR (receptor de vitamina D) e de uma forma dependente das proteínas PKA e PKC. Com a utilização de análogos esteróis da vitamina D (VDS) específicos para o estudo de respostas não-genômicas, células TM4 individuais foram tratadas com diferentes VDS, 1,25D e 25-diidróxivitamina D_3 (25D) (metabólitos naturais); 1α,25$(OH)_2$-lumisterol D_3 (JN; agonista não-genômico), e/ou 1β,25$(OH)_2$-vitamin D_3 (HL; antagonista não-genômico). Os resultados demonstraram que: 1) 1,25D, 25D e JN são agonistas no estímulo da permeabilidade ao Cl^-; 2) HL bloqueia o efeito desses agonistas confirmando a presença de um VDR alternativo de membrana (VDR_{mem} ou VDR-AP). Experimentos de capacitância celular e videomicroscopia demonstraram que a 1,25D estimula processos secretórios em células TM4 importantes para a função reprodutiva. Ensaios de ligação hormônio-receptor e análises computacionais demonstraram que dois curcuminóides, a curcumina (CM) e a bisdemetóxicurcumina (BDC), polifenóis ativos da planta turmérica, se ligam com alta afinidade ao VDR e mais favoravelmente ao VDR-AP. Dessa forma, para verificar a especificidade de ação da 1,25D no VDR-AP, o efeito da CM e da BDC foi testado nas correntes de Cl^- em células TM4. Em baixas concentrações, CM e BDC demonstraram um mecanismo de ação similar a 1,25D no aumento da permeabilidade ao íon Cl^-. Em altas concentrações, CM e BDC parecem ativar correntes de Cl^- através dos canais de cloreto reguladores da condutância transmembrana da fibrose cística (CFTR). Os dados obtidos com esse trabalho contribuem para o entendimento de novas vias de ação nas células de Sertoli desencadeadas por hormônios e substâncias relacionadas importantes para a homeostasia endócrina e exócrina do testículo.

Palavras-chave: T_4, 1,25D, análogos esteróides, CM, BDC, canais iônicos, PKA, PKC, transporte de aminoácidos, eletrofisiologia, testículos, células de Sertoli TM4

ABSTRACT

Steroid hormones, vitamin D, retinols and thyroid hormones generate biological responses via genomic and nongenomic mechanisms. In the present work, the nongenomic action of Thyroxine (T_4) and 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$ or 1,25D) were studied by means of two plasma membrane systems: amino acid accumulation in immature rat testis and chloride uptake in TM4 cells. Sertoli cells from mammalian testis secrete a rich fluid into the lumen of the seminiferous tubules that is critical to spermatogenesis. First, we examined the roles of ionic involvement and intracellular messengers in the stimulatory effect of T_4 and 1,25D on amino acid accumulation in immature rat testis. Small-conductance Ca^{2+} dependent K^+ channels (SK_{Ca}) and Cl^- (chloride) channels were demonstrated to be partially involved in the stimulatory effect of T_4 on amino acid accumulation. Ca^{2+} channels and ATP-dependent K^+ channels (K^+_{ATP}) were shown to be required for this action. A nongenomic effect of T_4 on amino acid accumulation was shown to be triggered by the PKC pathway involving different ion channel activities in immature rat testis, and suggests T_4 as an important hormonal regulator of testicular development and function. The stimulatory effect of 1,25D on amino acid accumulation was shown to be age-dependent, specific to this steroidal hormone and dependent upon protein synthesis. This study has provided evidence for dual effects of 1,25D: a genomic and nongenomic effect which can be triggered by PKA as well as a rapid response involving Ca^{2+}/K^+ channels on the plasma membrane. By means of whole-cell patch-clamp electrophysiology we described for the first time 1,25D stimulation of voltage-gated, DIDS-sensitive Cl^- channels through a PKA/PKC-dependent pathway correlated with exocytosis in TM4 Sertoli cells. Potentiation of Cl^- channels by the vitamin sterol agonist 1α,25$(OH)_2$ lumisterol D_3 (JN), and suppression of 1,25D, 25$(OH)_2$ vitamin D_3 (25D) and JN potentiation by the antagonist 1β,25$(OH)_2$ vitamin D_3 (HL) suggested nongenomic activation of the vitamin D putative membrane receptor (VDR_{mem} or VDR-AP). 1,25D and analogs may contribute to male reproductive functions via stimulation of Sertoli cell secretory activities in immature testis. To address the specificity of the nongenomic 1,25D effect in the activation of chloride channels, two natural ligands for the vitamin D genomic pocket (VDR-GP) were studied in this system. Curcumin (CM) and bisdemethoxycurcumin (BDC) are polyphenols isolated from the curry spice turmeric and contain ligand geometries preferred by the nongenomic VDR-AP demonstrated *in silico*. At lower concentrations, CM and BDC promoted potentiation of Cl^- currents in TM4 cells similar to 1,25D and JN indicating a nongenomic activation of chloride channels. At higher concentrations, CM and BDC appear to be potential agonist ligands for the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels. The data obtained herein contributes to the understanding of new pathways in Sertoli cells triggered by hormones and related substances important for the endocrine and exocrine homeostasis of the testis.

Key-words: T_4, 1,25D, curcumin, ion channels, PKA, PKC, amino acid transport, patch clamp, testis, TM4 cells

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LISTA DE ABREVIATURAS

A	Alanina
AR	Receptor de andrógeno
AIB	Aminoácido neutro aminoisobutiríco
AMPc	Adenosina monofosfato cíclico
ATP	Adenosina trifosfato
BDC	Bisdemetóxicurcumina
$[Ca^{++}]_i$	Cálcio intracelular
$[^{14}C]$	Isótopo radioativo do carbono
C-1	Carbono-1
C-25	Carbono-25
CFTR	Reguladores da condutância transmembrana da fibrose cística
ClC	Canais de cloreto dependentes da voltagem
CM	Curcumina
8-CPT-AMPc	9-cloretofeniltio adenosil monofosfato cíclico
DAG	Diacilglicerol
DBP	Proteína sérica ligadora de vitamina D
DIDS	Ácido 4,4'-diisotiocinatoestilbeno-2,2'-disulfônico
FSH	Hormônio folículo-estimulante
GABA	Ácido gama-aminobutírico
GMPc	Guanosina monofosfato cíclico
HL	1β,25-diidróxivitamina D_3
IFNδ	Interferon δ
IP_3	1,4,5-inositoltrifosfato
JN	1 α,25- diidróxivitamina -lumisterol D_3
K_{ATP}	Canais de potássio dependentes de ATP
MAPK	Proteína serina-treonina cinase ativada por mitógeno
MeAIB	Aminoácido ácido metilaminoisobutírico
mM	Mili molar
mLBD	Mutant Ligand Binding Domain
NBDs	Domínios ligadores de nucleotídeos

nM	Nanomolar
1-α,25(OH)$_2$-D$_3$ ou 1,25D	1α,25-diidróxivitamina D$_3$
25(OH)-D$_3$ ou 25D	25-hidróxivitamina D$_3$
PKA	Proteína cinase A
PDEs	Fosfodiesterases
PPases	Fosfatases
PMA	Forbol Miristato
PKI	Inibidor de proteína cinase A
PKC	Proteína cinase C
R	Domínio regulatório central
RNAm	Ácido ribonucléico mensageiro
SK_{Ca}	Canais de K^+ dependentes da baixa condutância de Ca^{2+}
T_3	3,5,3'-Triiodo-L-tironina
T_4	Tiroxina
TBG	Globulina de ligação de tiroxina
μM	Micro molar
VDR	Receptor da vitamina D
VDR_{mem} ou VDR-AP	Receptor alternativo de membrana da vitamina D
VDR_{nuc} ou VDR-GP	Receptor nuclear clássico da vitamina D
VDS	Esteróis da vitamina D

SUMÁRIO

1 INTRODUÇÃO

1.1 SISTEMA REPRODUTOR MASCULINO

O sistema reprodutor masculino é formado pelos testículos, cada qual com um sistema de ductos (ductos eferentes, epidídimo, canal deferente e ducto ejaculador), pelas glândulas acessórias (vesículas seminíferas, próstata e glândula bulbouretral) e pelo pênis (FAWCETT, 1993).

1.1.1 Testículo

Os testículos são formados por um tecido endócrino-responsivo e requerem interações célula-célula para a manutenção e controle do crescimento e diferenciação celulares (SKINNER; GRISWOLD, 1982). Os testículos podem ser funcional e anatomicamente divididos em duas partes: a) tecido intersticial, formado pelas células de Leydig, responsáveis pela síntese de testosterona; e b) túbulos seminíferos, formado por uma população de células com baixa taxa de proliferação, as células de Sertoli, e por uma população de células com alta taxa de proliferação, as células germinativas, cujo principal produto da transformação destas células são os espermatozóides (FAWCETT, 1993). Os túbulos seminíferos são revestidos por células peritubulares ou mióides, que se encontram em contato com a superfície basal das células de Sertoli (SKINNER; GRISWOLD, 1991) (Figura 1).

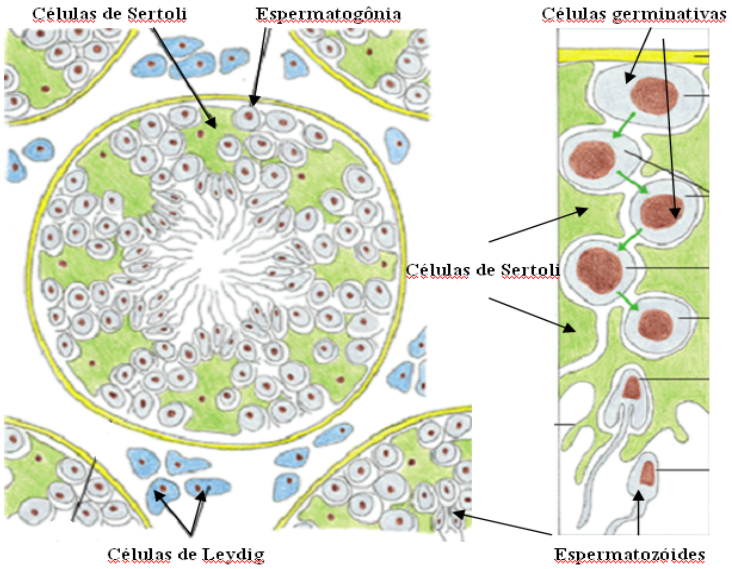

Figura 1. Representação esquemática de um corte transversal do epitélio seminífero mostrando a inter-relação entre as células de Sertoli e as células germinativas de testículos de mamíferos (adaptado de ALBERTS et al., 1994).

1.1.2 Células de Sertoli

As células de Sertoli foram descritas pelo fisiologista italiano Enrico Sertoli, em 1865. O ambiente ocupado pelas células de Sertoli nos túbulos seminíferos permite a este tipo celular receber, integrar e emitir para/ou do compartimento extratubular todos os sinais requeridos para o processo espermatogênico. Estas células estão envolvidas na formação da barreira hemato-testicular e são responsáveis pelo aporte de diversas substâncias (a partir do sangue e/ou sintetizadas por elas) cruciais para o controle do crescimento e da sobrevivência das células germinativas (DE KRETSER, 1987; FAWCETT, 1991; CARREAU et al., 1994). As células germinativas possuem como principal fonte de energia o lactato secretado pelas células de Sertoli (JUTTE et al., 1982).

As células de Sertoli apresentam sítios de ligação já bem caracterizados para o hormônio folículo-estimulante (FSH), testosterona, retinol (vitamina A), 3,5,3'-triiodo-L-tironina ($T_{3,}$), e 1,α-25 $(OH)_2$ vitamina D_3 (1,25D) (SANBORN, 1977; SKINNER; GRISWOLD, 1982; ESKILD et al., 1991; SILVA et al., 2002). Além da regulação exercida pela hipófise, uma rede elaborada de comunicação célula-célula é criada entre as células germinativas, de Sertoli, peritubulares e de Leydig através de hormônios, fatores parácrinos e moléculas sinalizadoras, tendo como principal função a manutenção da espermatogênese (VERHOEVEN; CAILLEAU, 1990; MRUK; CHENG, 2004).

1.2 HORMÔNIOS TIREOIDIANOS

Os hormônios tireoidianos são essenciais no desenvolvimento, diferenciação e metabolismo celular. Devido à homologia estrutural, os receptores para hormônios tireoidianos pertencem à mesma superfamília de receptores nucleares para hormônios esteróides. A tiroxina (T_4) circula no plasma associada às proteínas como a globulina de ligação de tiroxina (TBG) e é distribuído nos tecidos periféricos onde é convertido a 3,5,3-triiodo-L-tironina (T_3) através da ação de deiodinases teciduais (FISHER, 1996).

1.2.1 Hormônios tireodianos e mecanismo de ação

Embora o alvo de ação clássico dos hormônios tireoidianos seja mediado por receptores nucleares específicos capazes de se ligarem em regiões regulatórias de genes alvos modificando a expressão gênica (YEN et al., 2006), esses hormônios também possuem ações não-genômicas bem reconhecidas (DAVIS, DAVIS, 1996). Efeitos não-genômicos dos hormônios tireoidianos foram descritos em diversos tecidos como cérebro (LEONARD, 2008), coração (PORTMAN, 2008), músculo esquelético (IRRCHER et al., 2008), fibroblastos (BHARGAVA et al., 2007), células endoteliais vasculares (HIROI et al., 2006) e testículos (SILVA et al., 2001; 2002; VOLPATO et al., 2004; ZAMONER et al., 2005; MENEGAZ et al., 2006). Esses efeitos são rápidos, em segundos ou minutos, independentes de expressão gênica e podem ocorrer na membrana plasmática, no citoesqueleto, no citoplasma e em organelas intracelulares (DAVIS, DAVIS, 1996; SHIBUSAWA et al., 2003; DAVIS et al., 2008).

1.2.2 Hormônios tireoidianos e células de Sertoli

Um grande número de trabalhos na literatura demonstra a importância dos hormônios tireoidianos no controle da proliferação e diferenciação das células de Sertoli (OPPENHEIMER et al., 1984, FRANCAVILLA et al., 1991; BUZZARD et al., 2003). No hipotireoidismo neonatal, por exemplo, ocorre o aumento do período de proliferação levando ao aumento do número de células de Sertoli, do peso do testículo e da produção diária de espermatozóides. Em contraste, em condições de hipertireoidismo, a proliferação de células de Sertoli diminui levando à diminuição do peso do testículo e do número de espermatozóides (HOLSBERGER; COOKE, 2005). Em vista disso, a eficiência da iniciação e manutenção da

espermatogênese depende do número total de células de Sertoli funcionais que proliferam durante a fase imatura do desenvolvimento (ORTH et al., 1988). Portanto, a ação hormonal no período neonatal é de extrema importância para uma fertilidade masculina completa na fase adulta (ONCU et al., 2004). Receptores nucleares ativos para os hormônios tireoidianos estão presentes em quantidades significativas em células de Sertoli de testículos imaturos de ratos e humanos (PALMERO et al., 1988; JANNINI et al., 1990, 2000; BUZZARD et al., 2000; SILVA et al., 2001).

Um receptor de superfície celular para os hormônios tireoidianos foi demonstrado em diversas células (BERGH et al., 2005; DAVIS, DAVIS, 2005). Esse receptor protéico é chamado integrina $\alpha_V\beta_3$ e interage com um grande número de proteínas da matriz extracelular (PLOW et al., 2000). Efeitos rápidos dos hormônios tireoidianos em canais iônicos na membrana plasmática foram demonstrados em vários tecidos. Estudos anteriores demonstraram o envolvimento dos canais de K^+ dependentes de ATP (K_{ATP}), do Ca^{2+} extracelular e dos canais de Ca^{2+} dependentes da voltagem no mecanismo de ação do T_3 no estímulo do transporte de aminoácidos, sem o envolvimento do cálcio intracelular $[Ca^{2+}]_i$ neste evento (SILVA et al., 2001; SILVA et al., 2002; VOLPATO et al., 2004). Além disso, canais de Ca^{2+} e K^+ demonstraram envolvimento na ação hiperpolarizante desencadeada pelo T_3 em células de Sertoli. Foi também demonstrado através de um mecanismo não-genômico mediado por Ca^{2+} que a administração do T_3 *in vitro* estimula a fosforilação da vimentina em frações do citoesqueleto em testículos de ratos durante o desenvolvimento (ZAMONER et al., 2005).

Segundo Davis et al., (2006), uma interação entre efeitos genômicos e não-genômicos dos hormônios tireoidianos são dirigidos pela ação do T_4 no receptor de superfície celular na membrana plasmática controlando o tráfego intracelular e a regulação de canais iônicos na membrana plasmática. Em particular, Menegaz et al., (2006), verificaram que o T_4 e o T_3 atuam por diferentes vias de transdução de sinais, tendo em vista que o hormônio T_4 em concentrações fisiológicas, demonstrou um efeito significativamente mais potente que o T_3 no transporte de aminoácidos e sobretudo, independente da síntese de novas proteínas. O efeito hiperpolarizante do T_4 no potencial de membrana das células de Sertoli foi totalmente inibido na presença de tetraetilamônio e apamina, caracterizando o envolvimento de canais de K^+ dependentes da voltagem e do influxo de Ca^{2+} neste mecanismo. A elucidação do mecanismo de ação do T_4 no transporte de aminoácidos em testículos de ratos imaturos é de

extrema importância para o entendimento da regulação hormonal das funções metabólicas testiculares.

1.3 SISTEMA DE TRANSPORTE DE AMINOÁCIDOS

Processos de crescimento e proliferação requerem um alto suprimento de moléculas nutritivas como aminoácidos os quais possuem um papel central nos caminhos metabólicos. A introdução do aminoácido modelo não-metabolizável metilaminoisobutiríco (MeAIB) por Christensen et al., (1956), possibilitou o estudo de efeitos hormonais não-genômicos na membrana celular, distinguindo o acúmulo de aminoácidos da síntese de proteínas. O transporte de aminoácidos através da membrana plasmática de células de mamíferos envolve diferentes sistemas de transporte. Em particular, o sistema A de transporte de aminoácidos neutros, medido através da captação do aminoácido neutro aminoisobutiríco (AIB) e MeAIB, é dependente do gradiente de Na^+, pH e energia. A atividade deste e de outros transportadores de aminoácidos são regulados por diferentes fatores incluindo hormônios, fatores de crescimento, carência de aminoácidos e ciclo celular. Esses fatores, por sua vez, produzem alterações significativas na regulação do metabolismo celular (INCERPI et al., 2005). O efeito hormonal na captação de aminoácidos é restrito para o sistema "A" (alanina) de transporte de aminoácidos. Esse sistema é caracterizado pela participação de componentes inorgânicos (gradiente eletroquímico) e componentes orgânicos (transportadores na membrana plasmática), ambos mandatórios para a sobrevivência celular. FSH, retinol, hormônios tireoidianos e o hormônio 1-α,25$(OH)_2$-D_3 estimulam o transporte de aminoácidos através do sistema A de transporte em células de Sertoli de testículos de ratos imaturos (CRUZ-CURTE; WASSERMANN, 1985; WASSERMANN et al., 1993; SILVA, WASSERMANN, 1999; SILVA et al., 2001, MENEGAZ et al., 2009).

1.4 SISTEMA ENDÓCRINO DA VITAMINA D

A vitamina D pode ser obtida na dieta ou sintetizada através da exposição à luz solar e consequente produção de 7-deidrocolesterol na pele, pré-vitamina D_3 e vitamina D_3 (metabólitos inativos). A vitamina D_3 então é transportada para o fígado pela proteína sérica ligadora de vitamina D (DBP) onde é produzido o metabólito natural 25(OH)-vitamina D_3 (25(OH)-D_3 ou 25D) através da hidroxilação do C-25 pela enzima P_{450} 25-hidroxilase (ANDERSSO, JORNVALL, 1986). Posteriormente, A 25D é transportada para o rim onde ocorre a hidroxilação do C-1 (carbono-1) pela enzima P_{450} 1α-hidroxilase para produção do

hormônio esteróide 1-α,25-diidróxi-vitamina D_3 (1-α,25$(OH)_2$-D_3 ou 1,25D), considerado o hormônio ativo do sistema endócrino da vitamina D (MYRTLE , NORMAN, 1971).

1.4.1 Hormônio 1-α,25$(OH)_2$-D_3 (1,25D) e mecanismo de ação

O hormônio esteróide 1,25D possui uma estrutura conformacionalmente dinâmica e flexível que permite a geração de respostas fisiológicas em múltiplos tecidos alvos resultante tanto da ativação de mecanismos de ação genômicos quanto não-genômicos (BOUILLON et al., 1995; NORMAN et al., 2004, NORMAN, 2006). Por um lado, 1,25D exerce funções através do receptor nuclear clássico da vitamina D (VDR_{nuc} ou VDR-GP) levando a ativação ou inibição da expressão gênica. Essas ações podem perdurar por horas ou dias e são reconhecidas como respostas genômicas (NORMAN et al., 2001; LIPS, 2006). Por outro lado, a 1,25D também pode atuar através de um receptor alternativo de membrana (VDR_{mem} ou VDR-AP) estimulando a formação rápida de segundos mensageiros, ativação de proteínas cinases e a abertura de canais iônicos gerando uma variedade de respostas celulares que geralmente ocorrem em segundos ou minutos e são reconhecidas como respostas não-genômicas (CAFFREY, FARACH-CARSON, 1989; De BOLAND, NEMERE, 1992; ZANELLO, NORMAN, 1996, 2003; YUKIHIRO et al., 1994; NORMAN et al., 2002, 2004) (Figura 2).

A presença de receptores VDR em invaginações na membrana plasmática de duodeno de pinto enriquecidas em caveolae e a demonstração que a 1,25D se liga com alta afinidade a esses receptores revelou que a rápida resposta da 1,25D está fortemente associada a um receptor VDR_{mem} (NORMAN et al., 2002). O primeiro efeito fisiológico não-genômico demonstrado pela 1,25D foi o estímulo rápido na captação de Ca^{2+} (transcaltaquia) em intestino de pinto (NEMERE et al., 1984). Além disso, a 1,25D estimula em minutos a abertura de canais de Ca^{2+} e Cl^- dependentes da voltagem em osteoblastos ativando processos secretórios intracelulares contribuindo para formação da matriz óssea (CAFFREY et al., 1989; ZANELLO et al., 1997; XIAOYU et al., 2007).

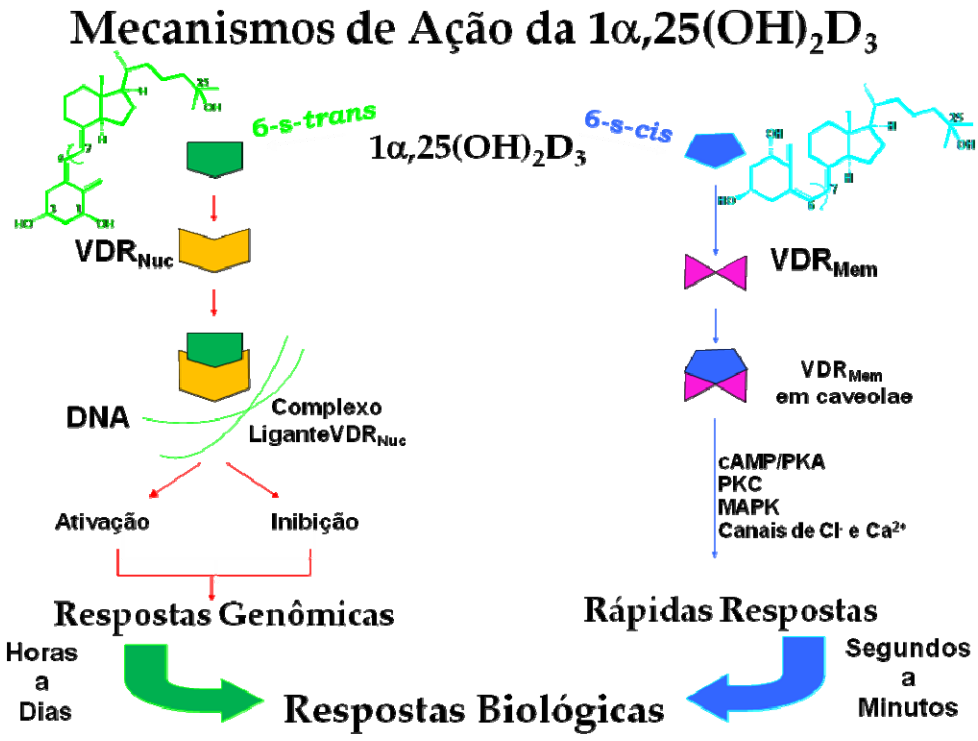

Figura 2: Mecanismo de ação genômico e não-genômico da 1,25D (adaptado de **NORMAN et al., 2004).**

Uma variedade de sistemas de transdução de sinais são mediados pela ligação da 1,25D a um VDR_{mem} e a consequente produção de segundos mensageiros como adenosina monofosfato cíclico (AMPc), guanosina monofosfato cíclico (GMPc) e também a elevação do $[Ca^{2+}]_i$. Esses sinais são amplificados através da ativação de proteínas cinases, como a PKA, a PKC e a proteína serina-treonina cinase ativada por mitógeno (MAPK), entre outras, causando mudanças no estado de fosforilação de proteínas e canais iônicos (De BOLAND; NEMERE, 1992; ZANELLO; NORMAN, 2003, NORMAN et al., 2004).

1.4.2 Hormônio 1-α,25(OH)$_2$-D$_3$ e células de Sertoli

A Vitamina D possui um importante papel na função reprodutiva, uma vez que a redução da fertilidade foi observada em ratos machos deficientes em vitamina D (KWIECINSKI et al., 1989). Receptores alvos para 1,25D foram identificados em células de Sertoli nos túbulos seminíferos e no tecido intersticial de testículos de ratos (MERKE et al., 1985; OSMUNDSEN et al., 1989) e também em testículos humanos (HABIB et al., 1990). Além das ações clássicas genômicas da 1,25D em células reprodutivas, é observado que a 1,25D desencadeia eventos fisiológicos em curto prazo os quais envolvem a captação de Ca^{2+} em células de Sertoli de camundongos imaturos da linhagem TM4 (AKERSTRON,

WALTERS, 1992). A linhagem celular TM4, utilizada neste trabalho, foi estabelecida por Mather, (1980), e foi isolada a partir de células de Sertoli em cultura primária provenientes de testículos de camundongos imaturos entre 11 e 13 dias de idade. Estudos de expressão gênica conduzidos em células TM4 evidenciaram diversas características idênticas às células de Sertoli em cultura primária. Portanto, diversos trabalhos utilizam células TM4 como um modelo para o estudo da função das células de Sertoli (SKINNER; GRISWOLD, 2004).

1.4.3 Hormônio 1-α,25(OH)$_2$-D$_3$ e análogos estruturais

A característica estrutural de seco-esteróide da 1,25D a diferencia dos demais hormônios esteróides por possuir uma flexibilidade conformacional que confere a essa molécula a forma 6-*s*-*trans* e a forma 6-*s*-*cis* (Figura 3). Extensivas pesquisas de estrutura-função da 1,25D foram realizadas para caracterizar a forma estrutural ótima requerida para as quatro principais respostas fisiológicas não-genômicas desencadeadas pela 1,25D (tabela 1). Nesses estudos, a potência da 1,25D na iniciação de rápidas respostas foi comparada com os análogos da 1,25D sintetizados na forma 6-*s*-*cis* 1α,25(OH)$_2$-lumisterol$_3$ (JN) (agonista de respostas não-genômicas), e na forma 6-*s*-*trans* 1β,25(OH)$_2$D$_3$ (HL) (antagonista de respostas não-genômicas) (Figura 3). Em todos os quatro sistemas, JN demonstrou a mesma potência não-genômica demonstrada pela 1,25D, atuando como um agonista específico destas ações fisiológicas de rápida resposta. Além disso, HL atua como um antagonista específico para as respostas não-genômicas demonstradas pela 1,25D (Tabela 1) (NORMAN, 2006).

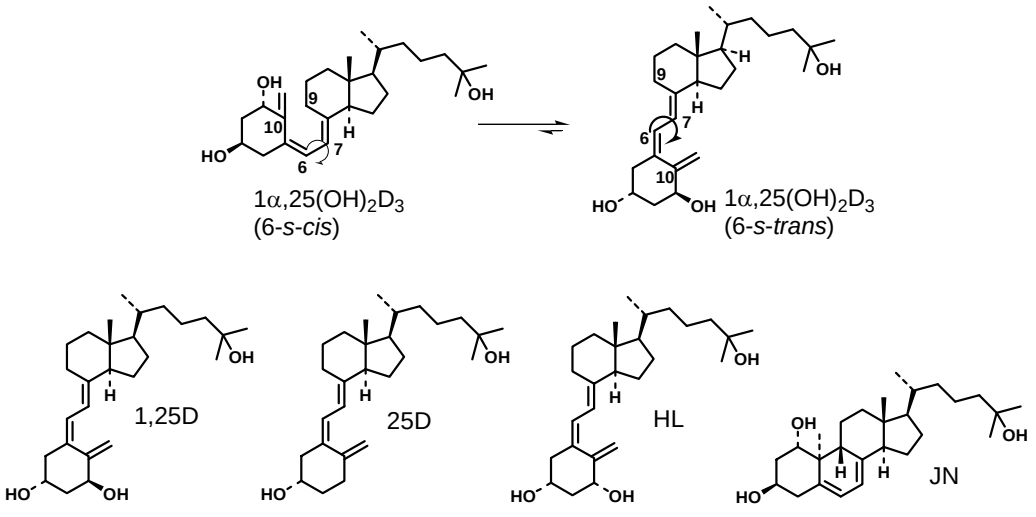

Figura 3: Estruturas conformacionais dos metabólitos naturais 1α,25(OH)$_2$D$_3$ (1,25D), 25(OH)D$_3$ (25D) e dos análogos sintéticos 1α,25(OH)$_2$-lumisterol$_3$ (JN) e 1β,25(OH)$_2$D$_3$ (HL). A 1,25D e a 25D são moléculas flexíveis devido à possibilidade de rotação entre os C-6 e C-7. O C-9 e o C-10 se localizam distantes na forma *trans* e próximos na foma *cis*. Os análogos HL e JN são sintetizados e restritos na forma 6-*s*-*trans* e 6-*s*-*cis* respectivamente (Adaptado de ZANELLO, NORMAN, 1997).

Tabela 1: Rápidas respostas da 1,25D e análogos estruturais (Adaptado de NORMAN, 2006).

	Célula/Órgão			
	Intestino	Osteoblastos	Células β-pancreáticas	Células endoteliais
EFEITO	Transcaltaquia (rápida absorção de Ca^{2+})	Secreção e ativação de canais de Cl^-	Secreção de insulina	Migração celular em músculo liso
1,25D	agonista	Agonista	Agonista	agonista
JN	agonista	Agonista	Agonista	agonista
HL	antagonista	Antagonista	Antagonista	antagonista
Referência	Norman et al., 1997	Zanello, Norman, 1997	Kajikawa et al., 1999	Rebsamen et al., 2002

Análises computacionais de estrutura e função sugerem que uma isoforma do VDR genômico VDR-GP pode ser encontrada na membrana plasmática (BARSONY et al., 1997) e requerida para as ações não-genômicas da 1,25D (ZANELLO, NORMAN, 2004; NGUYEN et al., 2004), sendo que o mesmo receptor nuclear clássico pode funcionar como o receptor de membrana alternativo VDR-AP através da ligação em diferentes domínios ou aminoácidos residuais da proteína (NORMAN et al., 2004; MIZWICKI et al., 2004; HUHTAKANGAS et al., 2004) (Figura 4).

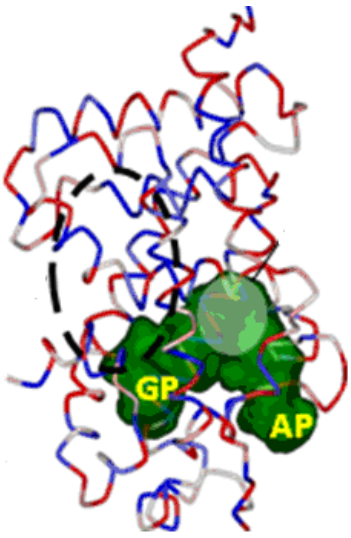

Figura 4: Modelo estrutural proposto para o receptor VDR. VDR_{nuc}-GP ("genomic pocket"): receptor genomico; VDR_{mem}-AP ("alternative pocket"): receptor de membrana alternativo (adaptado de MIZWICKI et al., 2008).

Diversos análogos da 1,25D foram identificados como fármacos potenciais para o tratamento da leucemia pró-miolítica aguda, câncer de mama, de cólon e de próstata ou como agentes imunosupressores com um possível perfil benéfico de relação estrutura-atividade para o uso em pacientes cardíacos, rejeição autoimune, diabetes tipo I e psoríase (NORMAN; SILVA, 2001, NORMAN et al., 2004). Dessa forma, o estudo do mecanismo de ação dos esteróis da vitamina D em células de Sertoli de testículos imaturos é de extrema importância para o entendimento do papel da 1,25D nas funções reprodutivas e na fertilidade masculina.

1.5 CANAIS DE CLORETO

As funções dos canais de cloreto (Cl^-), de um modo geral, incluem a homeostasia e regulação do volume celular, do transporte transepitelial, da excitabilidade elétrica, da acidificação de compartimentos internos e externos, do ciclo celular e apoptose (JENTSCH et al., 2002). Três distintas famílias de canais de cloreto foram estabelecidas molecularmente até o momento: canais de cloreto dependentes da voltagem (ClC), canais reguladores da condutância transmembrana da fibrose cística (CFTR) e receptores $GABA_A$ e glicina. (NILIUS, DROOGMANS, 2003). Em particular, nesse trabalho foram estudadas as atividades dos canais de Cl^- da família ClC e CFTR.

1.5.1 Canais de cloreto dependentes da voltagem (ClC)

A superfamília de canais de Cl^- dependentes da voltagem denominados ClC incluem 9 membros em mamíferos (ClC-1 a ClC-7; ClC-Ka e ClC-Kb) e se concentram tanto na membrana plasmática quanto em organelas intracelulares. ClC-Ka e ClC-Kb estão localizados exclusivamente no rim e o ClC-1 é expresso apenas no músculo esquelético. ClC-6 e ClC-7 se localizam somente em organelas intracelulares e o ClC-2 se encontra em vários tecidos e é ativado apenas em pH ácido. O ClC-3 se localiza tanto na membrana plasmática quanto em organelas intracelulares e se encontra presente na maioria dos tecidos (JENTSCH et al., 2002). Relata-se que o influxo de Cl^- através dos canais de cloreto ClC-3, estimula a exocitose em células de mamíferos através de um mecanismo que neutraliza e previne o excesso de cargas positivas provenientes dos íons H^+ culminando na acidificação intragranular necessária para secreção vesicular. Ainda que o preciso papel do fluxo de Cl^- no estímulo secretório permanece elusivo, a regulação do pH em grânulos secretórios é dependente do Cl^- e crítico para a atividade de vesículas secretórias (BARG et al., 2001). Além disso, a ativação de

canais de Cl^- e transportadores de Cl^- mantêm o potencial de membrana suficientemente negativo para facilitar o influxo de Ca^{2+} (KERSCHBAUM et al., 1997). Bloqueadores de canais de Cl^- podem afetar a sinalização de $[Ca^{2+}]_i$ através da modulação do fluxo de Cl^- (LAI et al., 2003). Dessa forma, o Cl^- aparece como um importante regulador de processos exocitóticos estimulados pelo Ca^{2+} (BARG et al., 2001).

Mudanças na capacitância celular de uma célula individual durante a exocitose é demonstrada através do aumento da área da superfície celular que pode ser medida através da técnica eletrofisiológica designada “whole cell patch clamp”. No processo de exocitose ocorre a fusão da vesícula secretora na membrana plasmática e após a liberação do conteúdo vesicular, a vesícula pode ser reciclada e incorporada novamente na membrana, a qual retorna à área de superfície inicial (WIGHTMAN, HAYNES, 2004). Em estudos anteriores, foi demonstrado que o estímulo rápido do influxo de Cl^- desencadeado pela 1,25D através de canais de Cl^- sensíveis ao ácido 4,4'-diisotiocinatoestilbeno-2,2'-disulfônico (DIDS), possuem um papel fundamental em atividades secretórias em osteoblastos (ZANELLO, NORMAN, 2004).

1.5.2 Canais de cloreto CFTR

Canais de cloreto reguladores da condutância transmembrana da fibrose cística (CFTR) regulam o transporte de sal e água através de tecidos epiteliais (FRIZZEL, 1995). Mutações que reduzem a síntese ou atividade desse canal causam fibrose cística (FC), uma doença genética letal que resulta de mutações no gene que codifica canais de Cl^- CFTR (RIORDAN et al., 1989). O canal de Cl^- da família CFTR é membro da superfamília de transportadores ativados por ATP (HYDE et al., 1990), baseado no domínio estrutural geral que inclui dois domínios transmembrana (que formam o poro do canal) e dois domínios ligadores de nucleotídeos (NBD1 e NBD2, que ligam e hidrolisam ATP). CFTR também possui o domínio R que é uma característica especial deste tipo de transportador. O domínio R possui alvos de fosforilação dependentes do caminho do monofosfato cíclico de adenosina (AMPc) e PKA, que são os ativadores fisiológicos principais deste tipo de canal (MATHEWS et al., 1998). A ativação e abertura dos canais CFTR tipicamente requerem tanto a fosforilação do domínio R mediados por proteínas cinases quanto a ligação de ATP para ambos os nucleotídeos NBD1 e NBD2 (SHEPPARD, WELSH, 1999; GADSBY, NAIRN, 1999) (Figura 5).

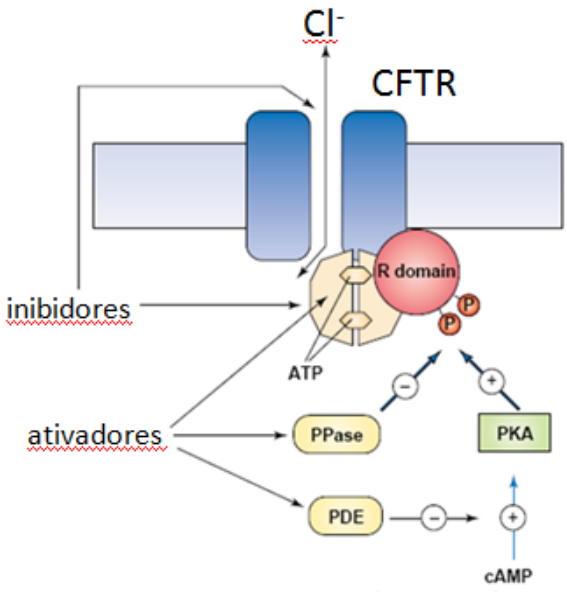

Figura 5. Representação esquemática dos mecanismos farmacológicos de ativação e inibição do canal de Cl^- CFTR. A ativação do canal depende de AMPc e a consequente fosforilação do domínio R pela proteína cinase A (PKA). A ligação do ATP e possivelmente a hidrólise das NBDs são também requeridos para a abertura do canal. Mecanismos regulatórios negativos envolvem degradação do AMPc por fosfodiesterases (PDEs) e desfosforilação do canal por fosfatases (PPases). Ativadores do canal CFTR podem atuar diretamente no canal protéico ou indiretamente através da inibição das PDEs (aumento do AMPc) ou inibindo as PPases (aumento da fosforilação do canal CFTR (Adaptado de Galietta, Moran, 2004).

A Fibrose Cística (FC) é uma doença genética causada por mutações no canal CFTR. Os diferentes tipos de mutações identificados em canais CFTR, podem levar à perda da função do canal, ausência da proteína do canal, defeito na regulação do canal, defeito na condutância do cloreto, diminuição da quantidade normal de canais CFTR, entre outros (PROESMANS et al., 2008). Dessa forma, novas substâncias aptas a restaurar a atividade dos canais CFTR, mesmo que parcialmente, seriam de extrema importância para o tratamento da FC e/ou para a redução da severidade da doença (GALIETTA, MORAN, 2004).

1.5.3 Canais de cloreto e células de Sertoli

As células de Sertoli expressam uma variedade de canais iônicos incluindo os canais de cloreto da família ClC (AUZANNEAU, 2003) e os canais de cloreto CFTR (BOOCKFOR et al., 1998). Estas células secretam um fluido rico em Cl^- e K^+ no lúmen dos túbulos seminíferos, importante para sobrevivência das células germinativas (AUZANNEAU et al.,

2003, 2006). Dessa forma, a modulação hormonal do fluido secretado pelas células de Sertoli é importante para manutenção da fertilidade masculina. Isso envolve múltiplos caminhos sinalizadores, uma variedade de segundos mensageiros e a modulação da atividade de canais iônicos (AUZANNEAU et al., 2006). O papel dos canais CFTR no testículo durante a espermatogênese é sugerido através de observações de que em pacientes com FC as células germinativas são reduzidas em número ou são mal-formadas (EGAN et al., 2004). Assim sendo, o estudo e a elucidação do mecanismo de ação de diferentes ativadores da função de canais CFTR são de extrema importância em tecidos afetados pela FC que incluem pulmão (infecção obstrutiva crônica), pâncreas (insuficiência pancreática), intestino (obstrução intestinal) e tecido reprodutivo (infertilidade) (ZEITLIN, 2000; EGAN et al., 2004; ROWE et al., 2005; SCHMIDT et al., 2008).

1.6 CURCUMINÓIDES

Além da vitamina D_3, os curcuminóides também possuem benefícios para saúde muito bem documentados na literatura. Os curcuminóides são polifenóis isolados da planta turmérica (*Curcuma longa*) (Figura 6A), a qual é cultivada extensivamente no sul da Ásia. O turmérico (conhecido como curry) é muito utilizado na dieta indiana para dar sabor e coloração aos alimentos. A estrutura da curcumina ($C_{21}H_{20}O_6$) foi descrita por Lampe e Milobedeska, (1913) e foi amplamente utilizada na medicina indiana tradicional para curar desordens biliares, anorexia, diabetes, desordem hepática, reumatismo e sinusite (VOGEL, PELLETIER, 1818).

Recentemente foi demonstrado que os dois principais curcuminóides ativos da planta turmérica, a curcumina (CM) e a bisdemetóxicurcumina (BDC) (Figura 6B), servem como ligantes naturais para o receptor da vitamina D (VDR) (JURUTKA et al., 2007; MASOUMI et al., 2009), como agonistas potenciais para os canais de cloreto CFTR (BERGER et al., 2005; WANG et al., 2007; VERKMAN, GALIETTA, 2009) e como antagonistas para o receptor de andrógeno (AR) (OHTSU et al., 2002). Ambos CM e BDC demonstraram propriedades quimiopreventivas e terapêuticas (GOEL et al., 2008) e são atualmente estudados como potenciais agentes terapêuticos no tratamento de várias doenças como Alzheimer (FIALA et al., 2009), câncer pancreático (DHILLON et al., 2008) e fibrose cística (VERKMAN, GALIETTA, 2009).

A estrutura química das moléculas de CM e BDC (figura 6B) permitem a tautomerização ceto-enol**.** O tautômero enol é a forma mais estável e farmacologicamente mais ativa dos curcuminóides. O sistema de ligações duplas (π) juntamente com as pontes de hidrogênio das moléculas de CM e BDC tornam a forma enol uma molécula rígida e geometricamente linear (KOLEV et al., 2005). Foi recentemente proposto no modelo conformacional para esteróis da vitamina D que as móleculas lineares na forma *cis* são preferidas para o receptor VDR-AP, enquanto as moléculas na forma *trans* são requeridas para o receptor VDR-GP (MIZWICKI et al., 2004, 2007).

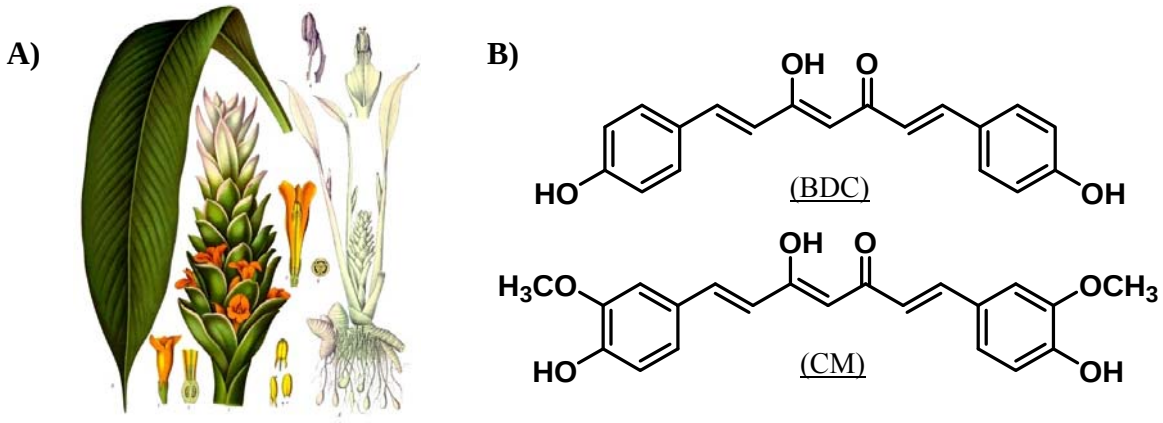

Figura 6. A) Planta turmérica (*Curcuma longa*) B) Estrutura química da curcumina (CM) (princípio ativo da *Curcuma longa*) e do análogo curcuminóide bisdemetóxicurcumina (BDC). (adaptado de AGGARWAL et al., 2006).

Levando em conta as evidências de que a nutrição de vitamina D apropriada possui um papel fundamental na fertilidade masculina (CORBETT et al., 2006); que a CM é capaz de restaurar a perda da função de canais CFTR mutantes (WANG et al., 2007); que a 1,25D se liga a um receptor VDR presente em células de Sertoli TM4 (AKERSTROM, WALTERS, 1992) e que a CM e a BDC funcionam como ligantes naturais para o receptor VDR, o mecanismo de ação não-genômico da 1,25D, da CM e da BDC foi investigado e comparado na regulação dos canais de Cl^- e exocitose em células TM4.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar o mecanismo de ação não-genômico da tiroxina e da $1\alpha,25(OH)_2D_3$ no transporte do aminoácido neutro [^{14}C]-MeAIB em testículos de ratos e verificar o efeito não-genômico dos esteróis da vitamina D no fluxo de Cl^- em células TM4.

2.2 OBJETIVOS ESPECÍFICOS

Investigar o envolvimento de canais de Ca^{2+}, canais SK_{Ca}, canais de K^+_{ATP} e canais de Cl^-, bem como o envolvimento da PKA e da PKC no efeito estimulatório do T_4 no estímulo do transporte de aminoácidos em testículos de ratos imaturos.

Investigar o efeito e a especificidade de ação do hormônio $1\alpha,25(OH)_2D_3$ no transporte de aminoácidos e o efeito da testosterona em diferentes estágios do desenvolvimento em testículos de ratos imaturos (curva de tempo e dose-resposta).

Estudar o envolvimento iônico e a influência de mensageiros intracelulares no efeito estimulatório da $1\alpha,25(OH)_2D_3$ no estímulo do transporte de aminoácidos em testículos de ratos imaturos.

Verificar a expressão do RNAm para o gene receptor da vitamina D (VDR) e para os genes dos canais de Cl^- dependentes da voltagem da família ClC em células de Sertoli TM4.

Estudar o efeito da $1\alpha,25(OH)_2D_3$ no fluxo de Cl^- em potenciais despolarizantes em células de Sertoli TM4 e verificar o envolvimento da PKA e da PKC nesse mecanismo.

Estudar o efeito do metabólito natural $25(OH)D_3$, do agonista não-genômico $1\alpha,25(OH)_2$-lumisterol$_3$ (JN) e do antagonista não genômico $1\beta,25(OH)_2D_3$ (HL) no fluxo de Cl^- em células de Sertoli TM4.

Verificar a especificidade de ação da $1\alpha,25(OH)_2D_3$ no estímulo não-genômico do fluxo de Cl^- na presença da curcumina (CM) e da bisdemetóxicurcumina (BDC) em células de Sertoli TM4.

Verificar eventos exocitóticos na presença do hormônio $1\alpha,25(OH)_2D_3$ e agonistas através do estudo da capacitância celular e através de microscopia fluorescente.

3 ARTIGOS

3.1 ARTIGO SUBMETIDO PARA PUBLICAÇÃO

Periódico - Journal of Steroid Biochemistry and Molecular Biology

MENEGAZ, D.; ROSSO, A.; ROYER, C.; PANSERA, M.; SANTOS, A.R.S.; SILVA, F.R.M.B. **Factors influencing the thyroxine stimulatory effect on α-[1-^{14}C]MeAIB accumulation in immature rat testis. Submitted.**

Factors influencing the thyroxine stimulatory effect on α-[1-^{14}C]MeAIB accumulation in immature rat testis

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Keywords: Thyroxine; Ionic channels; Amino acid accumulation; Mechanism; Testis.

Abstract

Thyroid hormones can exert non-classical actions that are characterized by rapid responses. The ionic involvement and intracellular messengers were investigated for their roles in the stimulatory action of thyroxine on amino acid accumulation in rat testes. 11 days-old rat testes were incubated in KRb, 32 ^{o}C, pH 7.4, O_2:CO_2 (95:5; v/v). [^{14}C]-MeAIB (0.1 μCi/mL) plus T_4 (1 nM) were added to the incubation for 60 min. Verapamil, EGTA, apamine, tolbutamide, 9-AC, di-BucAMP, KT5720 or RO318220 were added during the pre-incubation/incubation period. The results showed for the first time that voltage-dependent Ca^{2+} channels and ATP-dependent K^+ channels can be seen to be a setpoint in the stimulatory effect of T_4 on amino acid accumulation. Apamin-sensitive small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and chloride channels were shown to be partially involved in this mechanism. Treatment with PKA blocker did not modify the T_4 stimulatory effect on amino acid accumulation. However, non-genomic effect of T_4 on amino acid accumulation initiated at the plasma membrane triggered by PKC pathway suggests a functional link with different ion channel activities in immature rat testis and implicates T_4 as an important hormonal regulator of testicular metabolic functions.

1. Introduction

The efficiency of initiation and maintenance of spermatogenesis correlates with the total number of functional Sertoli cells that are recognized during prepubertal life [1]. These data, in combination with the findings that thyroid hormone receptors (TRs) are present in high quantities in neonatal Sertoli cells from human and rat testes [2,3,4,5] corroborates the belief that thyroid hormones play a key role in rat testes during the neonatal period that is critical for the future of male fertility [6,7].

Although the actions of thyroid hormones on target tissues are predominantly mediated by specific nuclear receptors capable of binding to regulatory regions of target genes and modifying their expression [8], these hormones also have well-known non-genomic actions [9, 10,11]. Recently, an increasing number of thyroid hormone non-genomic effects have been described in tissues such as brain [12], heart [13], skeletal muscle [14] and testis [15,16, 17,18].

Our research group has a continuing interest in non-genomic mechanisms leading to the effects of thyroid hormones on Sertoli cells. Silva et al. [15], proposed that the stimulatory action of T_3 on amino acid accumulation as well as the immediate hyperpolarizing effect of T_3 on the Sertoli cell membrane could be modulated by a mechanism involving Ca^{2+}-dependent K^+ currents and/or Ca^{2+}-dependent Cl^- currents, suggesting that calcium influx can influence both types of channels [17,18]. It has also been reported by non-genomic mechanisms that *in vitro* administration of T_3 stimulates the phosphorylation of vimentin in isolated rat testis [18].

Menegaz et al. [19] showed a potent thyroxine (T_4) stimulatory effect on amino acid accumulation independent of an active protein synthesis, indicating a non-genomic mechanism of T_4 in physiological concentrations. Moreover, the influence of Ca^{2+}-activated K^+ channels on the T_4 immediate hyperpolarizing effect has been shown in Sertoli cells. In the testes, a significant ionic involvement in rapid signal transduction triggered by thyroid hormones and

different types of K^+ and Ca^{2+} channels have been shown to modulate the mechanism of action of these hormones on amino acid accumulation [15,16,17,20].

The neutral amino acids accumulation in mammalian cells involves three major transport systems. Among them, the alanine-preferring (A) systems, specific for N-methylaminoisobutyric acid, is sodium, pH and energy dependent. The hormonal effects on amino acid uptake appear to be restricted to system "A". This system is characterized to be composed, at least, by an inorganic component (electrochemical gradient) and an organic component (plasma membrane carriers) which are essential to cell survival. In particular, FSH, retinol and thyroid hormones have been observed to act on this system in immature rat testis as well as in Sertoli cells [15,21,22]. In order to examine the non-genomic mechanisms of action of T_4 in the testis, the aim of the present study was to investigate the involvement of Ca^{2+}, K^+ and Cl^- channels and also the role of PKA and PKC pathways on the amino acid accumulation mechanism regulated by T_4 in immature rat testes.

2. Experimental

2.1. Chemicals

L-Thyroxine (T_4), verapamil, ethyleneglycol-O-OV-bis(2-aminoethyl)- NV,N,NV,NV-tetraacetic acid (EGTA), apamin, tolbutamide, 9-anthracene carboxylic acid (9-AC), dibutyryl cAMP (di-BucAMP), KT 5720, RO318220 were purchased from Sigma Chemical Company, St. Louis, MO, USA; α-[1-^{14}C] methylaminoisobutyric acid ([^{14}C] MeAIB) (sp.act. 1.85 GBq/mmol) was purchased from Du Pont, NEN Products, Mass, USA. Optiphase Hisafe III biodegradable liquid scintillation was obtained from PerkinElmer (Boston, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats bred in our animal house and maintained in an air-conditioned room (about 21 ^{o}C) with controlled lighting (12 h/12 h light/dark cycle) were used in this study. The suckling rats were kept with their mothers until sacrifice by cervical dislocation. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

2.3. [^{14}C] MeAIB accumulation

For amino acid accumulation experiments one gonad (alternately left and right) of 11-day-old rats was used as experimental and the contralateral one was used as the control. The testes were weighed, decapsulated and pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer for 30 min in a Dubnoff metabolic incubator at 32 ^{o}C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). T_4 (10^{-9} M); verapamil (100 μM), EGTA (2 mM), apamine (100 μM), tolbutamide (300 μM), 9-AC (1 mM), di-BucAMP (250, 500 or 750 μM), KT 5720 (1 μM), RO-31-8220 (20 μM) were added to the pre-incubation and to the incubation medium. T_4 was dissolved in 0.025 M NaOH-saline. This solution was further diluted to the final concentrations in KRb buffer of the following composition: 122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$. The KRb was bubbled with 95% O_2-5% CO_2 up to pH 7.4. The gonads were then incubated in fresh KRb buffer for 60 min. [^{14}C] MeAIB (3.7 kBq/mL) was added to each sample during the incubation period. After incubation the testes were removed to screw cap tubes containing 1 mL of distilled water. They were frozen at –20 ºC in a freezer and afterwards boiled for 5 min; 25 μL aliquots of tissue and external medium were placed in scintillation fluid and counted in a LKB rack beta liquid scintillation spectrometer (model 1215; EG & G-Wallac, Turku, Finland) for radioactivity

measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium [19].

3. Results

3.1. Involvement of ionic channels on stimulatory effect of T_4 on amino acid accumulation

3.1.1. Calcium channels

We previously demonstrated the influence of EGTA, verapamil and tolbutamide on the T_3 stimulatory effect on amino acid accumulation as well as on the hyperpolarizing action of T_3 on the Sertoli cell membrane. Based on that, we chose the best concentration of these blockers to use in this approach [17]. To verify the involvement of external Ca^{2+} as well as voltage-gated Ca^{2+} channels on the stimulatory effect of T_4 (1 nM) on [^{14}C] MeAIB accumulation in rat testis, we studied the effect of EGTA (2 mM) and verapamil (100 µM), respectively. It was observed that when the extracellular calcium was chelated in the presence of EGTA the stimulatory effect of T_4 was nullified; additionally, verapamil prevented the stimulatory action of T_4 thus confirming the involvement of Ca^{2+} currents on amino acid accumulation stimulated by T_4 (Fig. 1 A and B).

3.1.2. Potassium and chloride channels

To investigate the role of small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) on the T_4 (1 nM) stimulatory effect on [^{14}C] MeAIB accumulation in rat testis, we used apamine (0.1 µM), a selective SK_{Ca} channel blocker. This blocker caused a significant decrease in the stimulatory effect of T_4. Also, tolbutamide, an ATP-dependent K^+channel blocker (K^+-ATP), completely inhibited the stimulatory action of the hormone on amino acid accumulation at a concentration of 300 µM. These data show that these channels are required for T_4 amino acid stimulatory effects in rat testis (Fig. 2 A and B). In addition, we investigated the involvement of Cl^-

channels in the stimulatory effect of T_4 on amino acid accumulation in the presence of 1 mM of 9-anthracene carboxylic acid (9-AC). As can be observed in Fig. 2C, this blocker partially inhibited the stimulatory action of the hormone, suggesting that the activity of this type of channel might be part of a pathway regulating the effects of T_4 on the plasma membrane.

3.2 Effect of dibutyryl cyclic AMP (di-BucAMP) and kinase activity on stimulatory action of T_4 on amino acid accumulation

To verify the involvement of the cAMP pathway on the stimulatory effect of T_4 (1 nM), we studied the effect of cAMP agonist on [^{14}C] MeAIB accumulation in rat testis. An agonist concentration of 500 µM slightly increased the basal amino acid accumulation (Fig. 3A). Also, a localized elevation of cAMP increased the stimulatory effect of T_4 on [^{14}C] MeAIB accumulation in rat testis (Fig. 3B). In this regard, we studied the stimulatory effect of T_4 on [^{14}C] MeAIB accumulation in the presence of PKA and PKC blockers. Treatment of immature rat testis with KT5720 (1 µM), a protein kinase A blocker, did not change the stimulatory action of T_4. However, the blockage of PKC with RO318220 (20 µM) nullified the stimulatory effect of the hormone (Fig. 4 A and B). These results indicate that T_4 appears to potentiate amino acid accumulation via the PKC pathway, which might be coupled with phosphorylation and activation of the ion channels and/or could be acting through the nucleus to generate biological responses in immature rat testis.

Figure 1

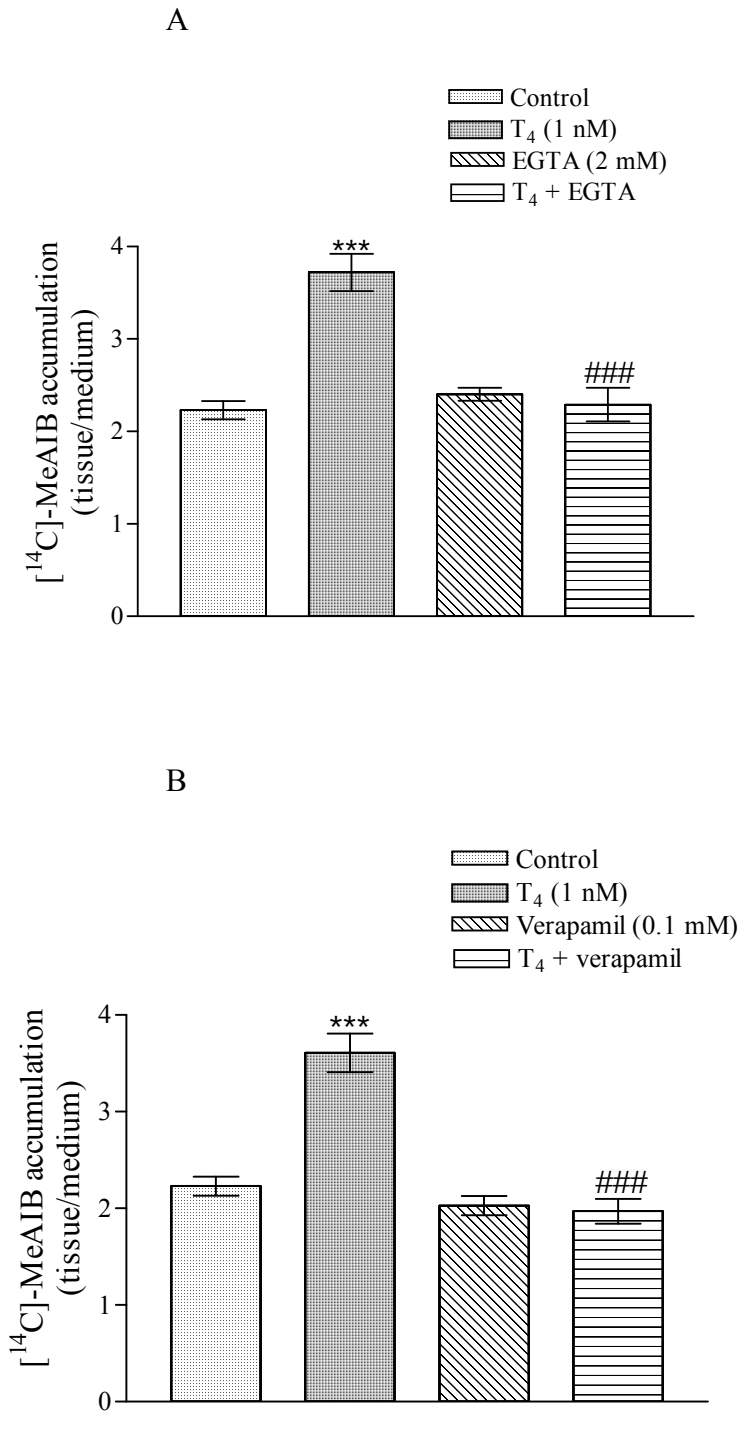
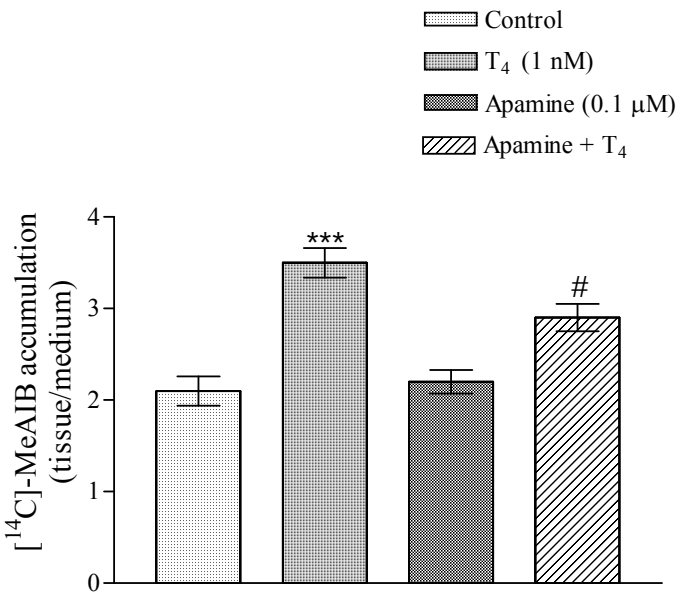


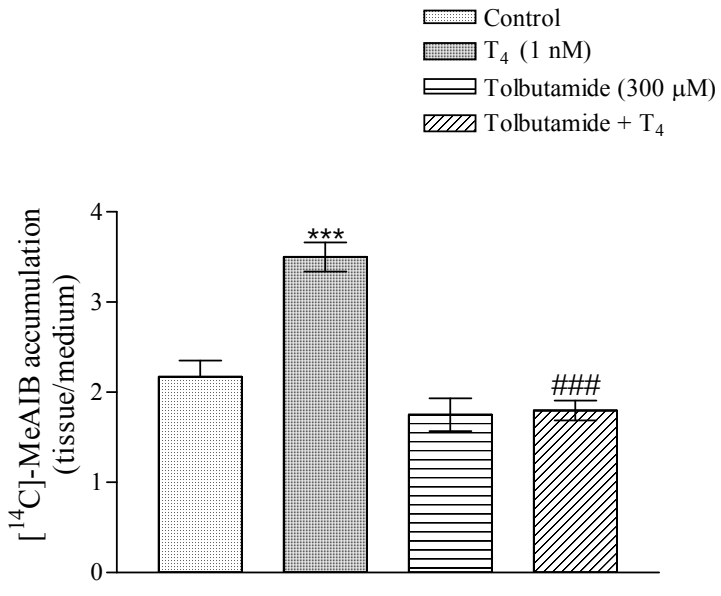
Figure 1. A) Effect of EGTA and B) Verapamil on the stimulatory action of T_4 (10^{-9} M) on [^{14}C] MeAIB accumulation in rat testes. Means ± SEM for control. n = 4 for all groups. ***P< 0.001 compared with control; $^{###}$p< 0.001 compared with T_4 group. Pre-incubation time: 30 min; incubation time: 60 min.

Figure 2

A


Control
T4 (1 nM)
Apamine (0.1 μM)
Apamine + T4
[14C]-MeAIB accumulation (tissue/medium)
0
1
2
3
4
***
#

B


Control
T4 (1 nM)
Tolbutamide (300 μM)
Tolbutamide + T4
[14C]-MeAIB accumulation (tissue/medium)
0
1
2
3
4
***
###

C

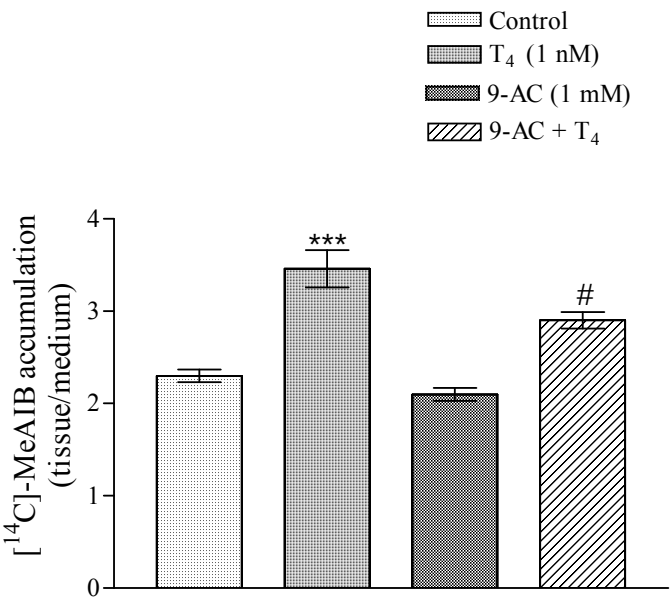
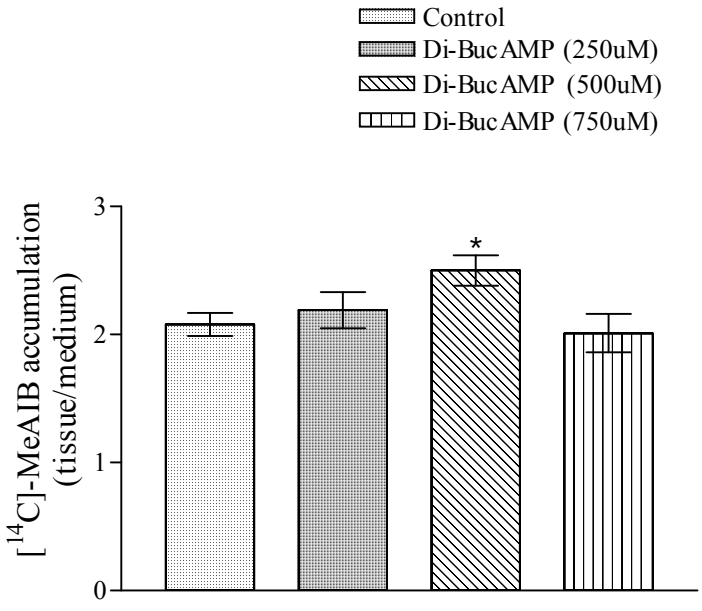


Figure 2. A) Effect of Apamine; B) Tolbutamide and C) 9-AC on the stimulatory action of T_4 on [^{14}C] MeAIB accumulation in rat testes. Means ± SEM for control. n = 4 for all groups. ***P< 0.001 compared with control; $^{\#}p< 0.05$; $^{\#\#\#}p< 0.001$ compared with T_4 group. Pre-incubation time: 30 min; incubation time: 60 min.

Figure 3

A



B

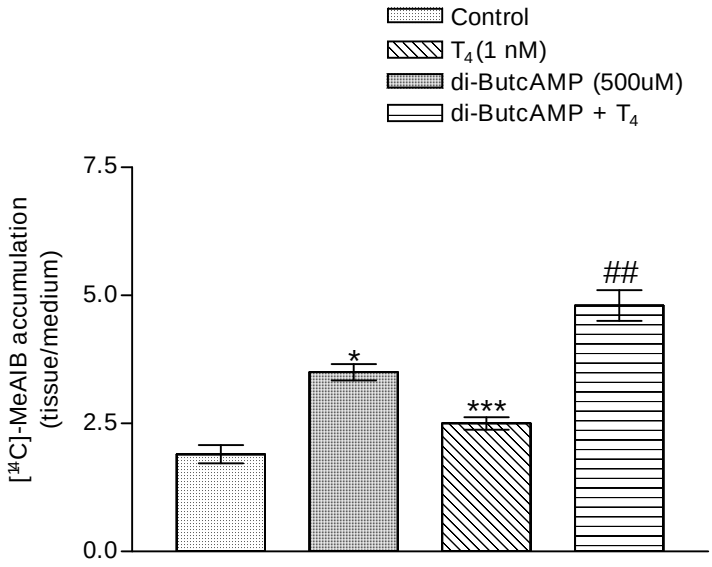
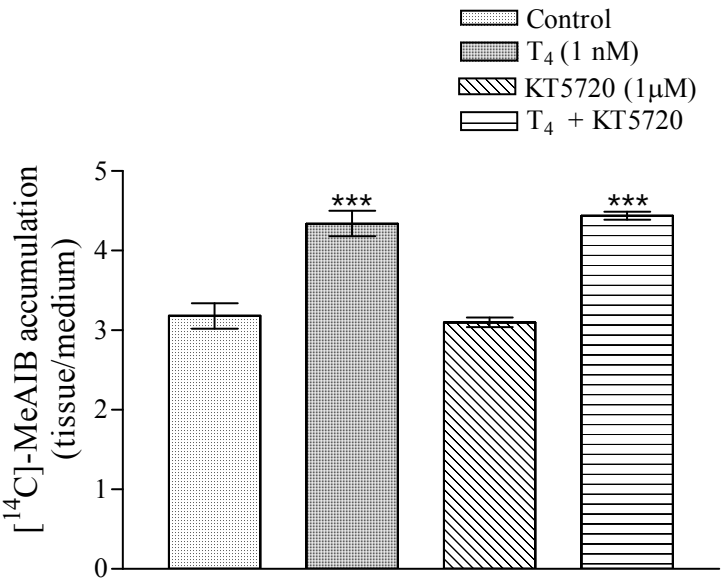


Figure 3 A). Dose-response curve of di-BucAMP on [^{14}C] MeAIB accumulation in rat testes. *P< 0.05 compared with control; B) Effect of di-BucAMP on the stimulatory action of T_4 (10^{-9} M) on [^{14}C] MeAIB accumulation in rat testes. Means ± SEM for control. n = 4 for each group. *P< 0.01; ***p< 0.001 compared with control; ##p<0.01 compared with T_4. Pre-incubation time: 30 min; incubation time: 60 min.

Figure 4

A



B

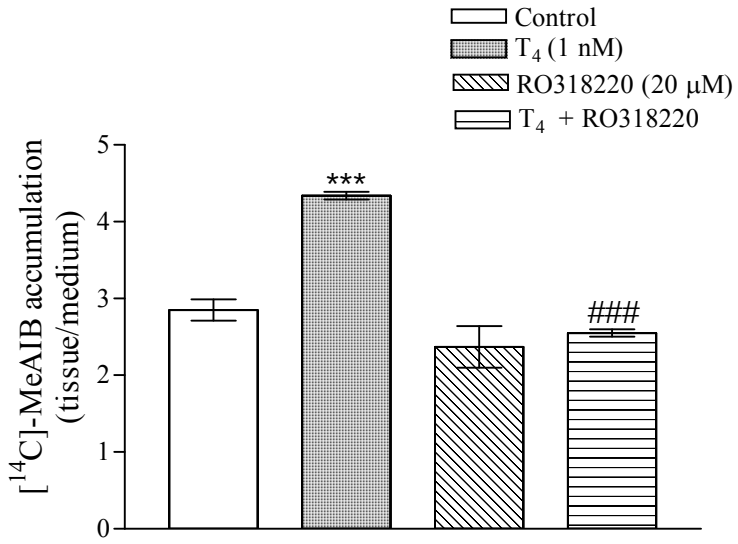


Figure 4 A) Effect of KT5720 and B) RO318220 on the stimulatory action of T_4 on [^{14}C] MeAIB accumulation in rat testes. Means ± SEM for control. n= 4 for each group. ***p< 0.001 compared with control; $^{###}$p< 0.001 compared with T_4 group. Pre-incubation time: 30 min; incubation time: 60 min

Discussion

A cell surface receptor for thyroid hormones has been described on a structural protein of the plasma membrane of several cells [23,24,25]. This protein is integrin αVβ3 and interacts with an extensive number of proteins of the extracellular matrix [26]. Bergh et al. [23] showed that the affinity of this integrin site for T_4 is higher than for T_3. Both T_4 (and less potently T_3) at this site were shown to activate from the cell surface a serine-threonine kinase, mitogen-activated protein kinase (MAPK, ERK1/2) that results in gene transcription [27].

Recently, we have demonstrated that both T_4 and T_3 promote amino acid accumulation in Sertoli cells by individual mechanisms. While the T_3 effect is partially blocked by cycloheximide, an inhibitor of protein synthesis, the potent stimulatory effect of T_4 remained unchanged, confirming that T_4 effects are regulated by non-genomic mechanisms. Our group also showed by means of electrophysiological studies that T_4 produced immediate

hyperpolarization of Sertoli cell membrane potential involving K^+ channels [19]. According with Davis et al. [28], a cross-talk between non-genomic and genomic effects of thyroid hormone is driven by action of T_4 at the plasma membrane and its cell surface receptor, after which T_4 can control intracellular protein trafficking and regulate activities of plasma membrane ion channels.

In this report, our results first showed that external Ca^{2+} as well as voltage-dependent Ca^{2+} channels is required for T_4 stimulatory action on amino acid accumulation. This data corroborates with a previous study on T_3 mechanism of action on amino acid accumulation and on the membrane potential related to Ca^{2+} in the non-genomic action of T_3. Moreover, a rapid non-genomic Ca^{2+} -dependent action of T_3 in phosphorylating vimentin that was shown to be dependent of external and intracellular Ca^{2+} has been demonstrated in immature rat testes [17,18]. It is well known that Ca^{2+} channels are an essential link between transient changes in membrane potential and a variety of cellular responses, including activation of second messengers and secretion in many cell types [29].

Further, we showed that the apamine-sensitive SK_{Ca} are partially involved in the stimulatory action of T_4 on amino acid accumulation, and this result is consistent with the data shown by Menegaz et al. [19], where the T_4 hyperpolarizing effect in Sertoli cells was totally blocked in the presence of apamine. Additionally, Muyderman et al. [30], confirmed that an intracellular calcium increase activates SK_{Ca} channels leading to K^+ efflux and membrane hyperpolarization [30,31]. Thus, the activity of this type of channel might be part of a pathway regulating the effects of T_4 on the plasma membrane.

The hormonal regulation of the secretion of Cl^- and K^+-rich fluid by Sertoli cells is important in male reproduction. It involves multiple signaling pathways including a variety of second messengers and the modulation of ion channel activity [32,33]. According to our results, Cl^- channels were shown to be partially required for the amino acid accumulation stimulated by

T_4 in rat testis. In previous studies, chloride currents were necessary in the T_3 mechanism of action on the regulation of amino acid transport in testis [17]; however, these channels were not implicated in the thyroid hormone actions on calcium influx in the cerebral cortex of rats [34].

We further investigated the effect of ATP-dependent K^+ (K^+-ATP) channels on the T_4 stimulatory effect. The results showed that this channel is also implicated in the stimulatory effect of T_4 and this data supports previous results on the action of T_3 on amino acid accumulation and on the hyperpolarizing effect inhibited by the K^+-ATP channel blocker tolbutamide. Despite the fact that the stimulatory effect of T_4 on amino acid accumulation is independent of new protein synthesis [19], taken together, these results point to the existence of similar ionic mechanisms related to Ca^{2+}, K^+ and Cl^- channels in the non-genomic action of T_4 on amino acid accumulation already shown in the presence of T_3.

In order to examine whether T_4 stimulatory action on amino acid accumulation depends on the modulation of the activity of specific signal transduction proteins, we observed the effects of RO318220 and KT5720, inhibitors of PKC and PKA, respectively. The results obtained with these drugs showed that the amino acid accumulation induced by T_4 depends on PKC activation. Our results are in agreement with other authors, which describe a short-term activation of PKC by thyroid hormones in rabbit erythrocytes and in hepatocytes [35,36]. In addition, Alisi et al. [37], demonstrated that the ability of thyroid hormones (T_2, T_3 and T_4) to activate cyclin E/A-cdk2 complexes in chick embryo hepatocytes is dependent upon the activity of PKCα isoform. These findings indicate that PKCα could be an important mediator for the non-genomic effect of thyroid hormones. Zamoner et al. [38], verified a rapid non-genomic action of T_3 and T_4 promoting Ca^{2+} influx by ionic channels involving mechanisms dependent on kinase activities. The results demonstrated a PKC-dependent mechanism for both T_3 and T_4. Furthermore, Zamoner et al. [39], verified the participation of phospholipase C, PKC, mitogen-activated

protein kinase, calcium/calmodulin-dependent protein kinase II, and intra- and extracellular Ca^{2+} mediating the effects of T_4 on the cytoskeleton of neural cells during development.

Our results showed that treatment with PKA blocker did not modify the T_4 stimulatory effect on amino acid accumulation. This could be ascribed to the fact that the nucleotide di-BucAMP modified the basal amino acid accumulation and increased the stimulatory effect of T_4 on amino acid accumulation indicating that in this system regulated by T_4, the PKA pathway and its targets are complex, and defining the precise role of PKA is challenging. On the other hand, a partial involvement of PKA activity has been demonstrated in the effect of T_4 on $^{45}Ca^{2+}$ uptake in cerebral cortex from young rats [34]. Recently, our group has obtained relevant evidence on the direct involvement of the PKC and PKA pathways on 1α,25 $(OH)_2$-Vitamin D_3 outwardly rectifying voltage-dependent Cl^- currents, activated upon depolarization in mouse TM4 Sertoli cells by means of whole cell patch clamp technique (data not yet published). As a whole, it seems clear that the involvement of protein kinases in the mechanism of action of hormones activates ion channels on the plasma membrane; however, the complete role of these proteins remains unclear and depends on the system and the tissue used.

Although thyroid hormone was not traditionally viewed as a major regulator of the male gonad, it is now clear that it has critical effects on the testis mainly during development [40]. Our results provide a framework in which Ca^{2+} channels and ATP-dependent K^+ channels are completely engaged in the non-genomic effect of T_4. The activation of specific PKC isoforms modulates different ion channel activities in immature rat testis, culminating with amino acid accumulation regulated by this hormone at the level of plasma membrane action.

Acknowledgements

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3.2 ARTIGO PUBLICADO

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Role of 1α,25(OH)$_2$ vitamin D$_3$ on α-[1-^{14}C]MeAIB accumulation in immature rat testis

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ABSTRACT

1,25D$_3$ is critical for the maintenance of normal reproduction since reduced fertility is observed in male rats on a vitamin D-deficient diet. Vitamin D-deficient male rats have incomplete spermatogenesis and degenerative testicular changes. In the present study we have examined the ionic involvement and intracellular messengers of the stimulatory effect of 1,25D$_3$ on amino acid accumulation in immature rat testis. 1,25D$_3$ stimulates amino acid accumulation from 10^{-12} to 10^{-6} M by increasing the slope to reach a maximum value at 10^{-10} M, as compared to the control group. No effect was observed at a lower dose (10^{-13} M). Time-course showed an increase on amino acid accumulation after 15, 30, and 60 min of incubation with 1,25D$_3$ (10^{-10} M). 1,25D$_3$ stimulated amino acid accumulation in 11-day-old rat testis but not in testis that were 20 days old. Cycloheximide totally blocked the 1,25D$_3$ action on amino acid accumulation. Furthermore, a localized elevation of cAMP increased the stimulatory effect of 1,25D$_3$ and the blockage of PKA nullified the action of the hormone. In addition, 1,25D$_3$ action on amino acid accumulation was also mediated by ionic pathways, since verapamil and apamine diminished the hormone effect. The stimulatory effect of 1,25D$_3$ on amino acid accumulation is age-dependent and specific to this steroidal hormone since testosterone was not able to change amino acid accumulation in both ages studied. This study provides evidence for a dual effect for 1,25D$_3$, pointing to a genomic effect that can be triggered by PKA, as well as to a rapid response involving Ca^{2+}/K^+ channels on the plasma membrane.

1. Introduction

Cell secretory activities are typically coupled to intracellular calcium elevation in a variety of cell types. The function of Sertoli cells is of paramount importance in the growth and development of sperm since these cells supply the seminiferous epithelium with a rich ionic fluid to support the ongoing spermatogenesis in the testis. 1α,25-Dihydroxyvitamin D$_3$ (1,25D$_3$) influences cell proliferation, cell differentiation and expresses calcium binding protein in the testis [1].

The vitamin D-endocrine system is characterized by a two-step sequential metabolism in the liver and the kidney, in which vitamin D$_3$ is converted into 1,25D$_3$ [2,3]. This hormone, 1,25D$_3$, is the most biologically active metabolite of vitamin D$_3$ and regulates transcription through a well-characterized nuclear receptor (VDR$_{nuc}$) that binds to hormone response elements present in the promoter of the regulated genes [4]. In contrast, rapid responses to 1,25D$_3$ have been proposed to initiate at the cell membrane level via interaction between 1,25D$_3$ and a putative alternative pocket membrane receptor (VDR$_{mem}$), to generate a variety of cell specific responses within seconds to minutes [5]. Occupancy of the VDR$_{mem}$ (which in some instances might be a membrane-associated nuclear receptor) by the steroid hormone can lead to the initiation of rapid responses that are coupled through appropriate second-messenger systems, either directly to generate biological responses or indirectly through their modulation [6].

The wide array of rapid responses stimulated by 1,25D$_3$ includes the opening of voltage gated Ca^{2+} [7,8] and chloride channels in osteoblasts [9], activation of phosphatidylinositol 3-kinase in vascular smooth muscle [10], and stimulation of Ca^{2+} transport in chick intestine (termed transcaltachia) [11]. The demonstration that 1,25D$_3$ binds with high affinity to a chick duodenal caveolae-enriched membrane implicates that the 1,25D$_3$ rapid response is tightly associated with the VDR$_{mem}$, to mediate molecular transport processes [5]. However, due to its different shapes, the conformationally flexible 1,25D$_3$, can interact separately with both the VDR$_{nuc}$ or the VDR$_{mem}$ of the vitamin D-endocrine system,

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to generate biological responses via either the genomic or the rapid response pathway, with/without intracellular cross-talk [3,11].

The introduction of the non-metabolic amino acid accumulation model by Christensen et al. [12] enabled studies on the mechanism of action of hormones and vitamins, thereby distinguishing amino acid accumulation from alternative protein synthesis pathways. The accumulation of neutral amino acids in mammalian cells involves three major systems. Among them, the alanine-preferring (A) systems, specific for N-methylaminoisobutyric acid, is sodium, pH, and energy dependent. The hormonal effects on amino acid accumulation appear to be restricted to system "A". This system is characterized by, at least, its composition by inorganic (electrochemical gradient) and organic components (plasma membrane carriers), which are mandatory to cell survival. Concerning the hormonal modulation of the "A" system, FSH, retinol and the thyroid hormones have been reported to act through this system to start a rapid response both in immature rat testis and in Sertoli cells. In addition, the stimulatory effect of these hormones on neutral amino acid accumulation in immature rat testis is independent of an active protein synthesis [13–18]. All these findings should be taken into account, in order to examine the mechanisms of action of $1,25D_3$ in the testis. The aim of the present study was to investigate the ionic involvement and to determine the intracellular messengers involved in the stimulatory action of $1,25D_3$ on amino acid accumulation.

2. Experimental

2.1. Chemicals

$1\alpha,25(OH)_2$ vitamin D_3 ($1,25D_3$), verapamil, apamine, dibutyryl cAMP (di-BucAMP), KT 5720, testosterone and cycloheximide were purchased from Sigma Chemical Company, St. Louis, MO, USA; α-[1-^{14}C] methylaminoisobutyric acid ([^{14}C] MeAIB) (sp. act. 1.85 GBq/mmol) was purchased from Du Pont, NEN Products, MA, USA. Optiphase Hisafe III biodegradable liquid scintillation was obtained from PerkinElmer (Boston, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats bred in our animal house facility, with air-conditioning (about 21 °C) and controlled lighting (12 h/12 h light/dark cycle) were used in this study. The suckling rats were kept with their mothers until sacrifice by cervical dislocation. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation, approved by the Committee for Ethics in Animal Research of UFSC (Protocol CEUA/PP00023).

2.3. [^{14}C] MeAIB accumulation

For amino acid accumulation experiments, a gonad (alternately left and right) of 11- and 20-day-old rats was used as experimental while the contralateral gonad was used as a control. The testis were weighed, decapsulated and pre-incubated in Krebs Ringer-bicarbonate (KRb) buffer for 30 min in a Dubnoff metabolic incubator at 32 °C, pH 7.4 and gassed with O_2:CO_2 (95:5, v/v). $1,25D_3$ (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} and 10^{-13} M), verapamil (100 μM), apamine (100 μM), di-BucAMP (250, 500 or 750 μM), KT 5720 (1 μM), testosterone (10^{-10} M) and cycloheximide (0.35 mM) were added to the pre-incubation and to the incubation medium. $1,25D_3$ was stored as a stock solution in pure ethanol at −20 °C, in the dark. These solutions were further diluted in KRb buffer to the final concentration of 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 $NaHCO_3$. The KRb was gassed with 95% O_2–5% CO_2 up to pH 7.4. The gonads were then incubated in fresh KRb buffer for 60 min. [^{14}C] MeAIB (3.7 kBq/mL) was added to each sample during the incubation period. After incubation, the samples were processed as previously reported [13,15]. The results were expressed as tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium.

3. Results

3.1. Dose–response curve and time-course of $1,25D_3$ on amino acid accumulation

As illustrated in Fig. 1A, $1,25D_3$ stimulates the amino acid accumulation from 10^{-12} to 10^{-6} M, by increasing the curve slope, to reach a maximum value at 10^{-10} M (no effect was observed at 10^{-13} M). The physiological concentration of $1,25D_3$ (10^{-10} M) was used for the subsequent experiments. The time-course showed an increase in amino acid accumulation after 15, 30 and 60 min of incubation with $1,25D_3$ (10^{-10} M). For the basal condition almost no alterations in amino acid accumulation occurred during this period. The maximum stimulatory effect observed with $1,25D_3$ was 85% at 60 min (Fig. 1B).

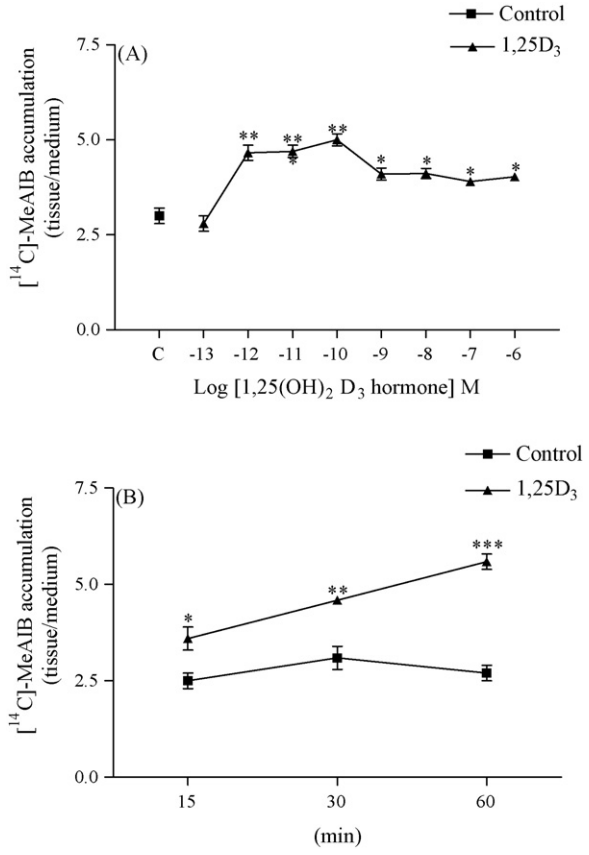

Fig. 1. (A) Dose–response curve of $1,25D_3$ on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with control. Pre-incubation time: 30 min; incubation time: 60 min. (B) Time-course of $1,25D_3$ (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control. Pre-incubation time: 30 min; incubation time: 15, 30 or 60 min.

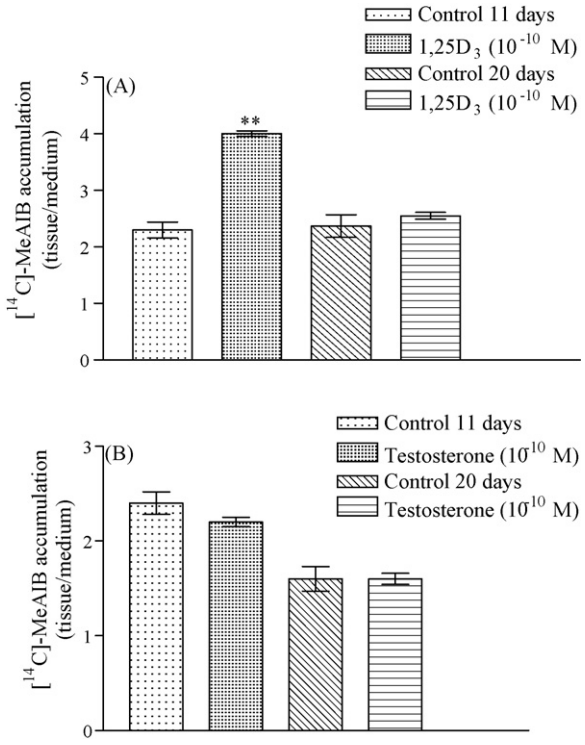

Fig. 2. Effect of (A) $1,25D_3$ (10^{-10} M) and (B) testosterone (10^{-10} M) on [1-^{14}C] MeAIB accumulation in rat testis of different ages. Means ± S.E.M., $n = 6$ for each group. $^{**}p < 0.01$ compared with respective control group.

3.2. Effect of $1,25D_3$ on amino acid accumulation at different ages and comparative studies with testosterone

$1,25D_3$ stimulated amino acid accumulation in 11-day-old immature rat testis, but no effect was observed in rats that were 20 days old (Fig. 2A). In a similar approach, testosterone (10^{-10} M) did not change the amino acid accumulation for neither the 11-day-old nor the 20-day-old rat testis after a 60 min incubation (Fig. 2B).

3.3. Influence of protein synthesis on the stimulatory effect of $1,25D_3$ over amino acid accumulation

Proteins have been described as an organic membrane component of the system "A" that mediates transport [17]. In a similar approach, we have previously demonstrated the profile for the protein synthesis time-course and the effect of cycloheximide on protein synthesis in immature rat testis [18]. So, to verify the role of active protein synthesis on the amino acid accumulation stimulatory action of $1,25D_3$, we studied the effect of cycloheximide on this event as shown in Fig. 3. This figure shows that cycloheximide inhibited the stimulatory effect of $1,25D_3$ at dosages that reduce testicular protein synthesis by 95.6% [18]. This shows the need for active proteins synthesis, in order to $1,25D_3$ to achieve full stimulatory action on amino acid accumulation.

3.4. Effect of dibutyryl cyclic AMP (di-BucAMP) and KT5720 on the stimulatory action of $1,25D_3$ over amino acid accumulation

To verify the involvement of the cAMP pathway on the stimulatory effect of $1,25D_3$ we studied the cAMP agonist effect on [^{14}C] MeAIB accumulation in the rat testis. Our findings showed that it slightly increased the basal amino acid accumulation for the 500 μM concentration (Fig. 4, inset panel). Therefore, this concentration was used in subsequent experiments. A localized elevation of cAMP increased the stimulatory effect of $1,25D_3$ on [^{14}C] MeAIB

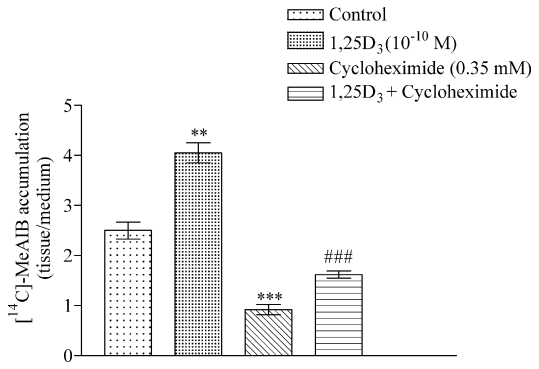

Fig. 3. Effect of cycloheximide on the stimulatory action $1,25D_3$ (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control; $^{\#\#\#}p < 0.001$ compared with $1,25D_3$ group. Pre-incubation time: 30 min; incubation time: 60 min.

accumulation in the rat testis (Fig. 4A). In this regard, we studied the effect of KT5720 (a blocker of the PKA enzyme) on the $1,25D_3$ (10^{-10} M) associated [^{14}C] MeAIB accumulation in the rat testis. Fig. 4B shows that the stimulatory action of $1,25D_3$ was totally blocked by KT5720 (10^{-6} M), suggesting that the $1,25D_3$ effect is dependent on the PKA pathway, which can act through the nucleus.

3.5. Involvement of ionic channels on the stimulatory effect of $1,25D_3$ over amino acid accumulation

To verify the involvement of voltage-dependent Ca^{2+} channels and Ca^{2+}-dependent K^+ channels on the stimulatory effect of $1,25D_3$, we studied the effect of verapamil (100 μM) on the $1,25D_3$ action (10^{-10} M) on [^{14}C] MeAIB accumulation in the rat testis. It was observed that verapamil blocked the $1,25D_3$ action, indicating the involvement of voltage-dependent Ca^{2+} channels in this mechanism. In this regard, we also studied the effect of apamine (100 μM) on the stimulatory action of $1,25D_3$ (10^{-10} M) over [^{14}C] MeAIB accumulation in the rat testis. This blocker caused a significant decrease in the stimulatory effect of $1,25D_3$, suggesting a partial involvement of Ca^{2+}-dependent K^+ channels in this mechanism (Fig. 5A and B).

4. Discussion

$1,25D_3$ receptors and the functional actions of $1,25D_3$ on whole rat testis have been well documented. It has been reported that $1,25D_3$ binds to cytosolic and nuclear fractions of whole testis and its various cellular components [19]. The significant stimulatory action of $1,25D_3$ over the [^{14}C] MeAIB accumulation (around 85%) in immature rat testis is in agreement with the high-affinity binding of $1,25D_3$ to receptors of whole rat testis first demonstrated by Merke et al. [1] and reinforced by the data presented by Osmundsen et al. [19] that indicated the Sertoli cell as a primary site of action for $1,25D_3$ in the testis. As observed with the thyroid hormones, the stimulatory effect of $1,25D_3$ over amino acid accumulation exhibited a bell-shape response curve with a plateau at higher dosages, suggesting a hormone selective modulation [18,20].

The stimulatory effect of $1,25D_3$ was most evident in 11-day-old rat testis. On the other hand, testosterone was not able to change amino acid accumulation at any age studied. Both the control and the treated groups showed a lower amino acid accumulation level in 20-day-old rat testis. This is in agreement with the decreased amino acid distribution ratios shown in both *in vivo* [16] and *in vitro* [21] experiments during testicular development. It has become evident that the classical mechanism of action of the steroid hormones is not sufficient to account for all the known effects of these

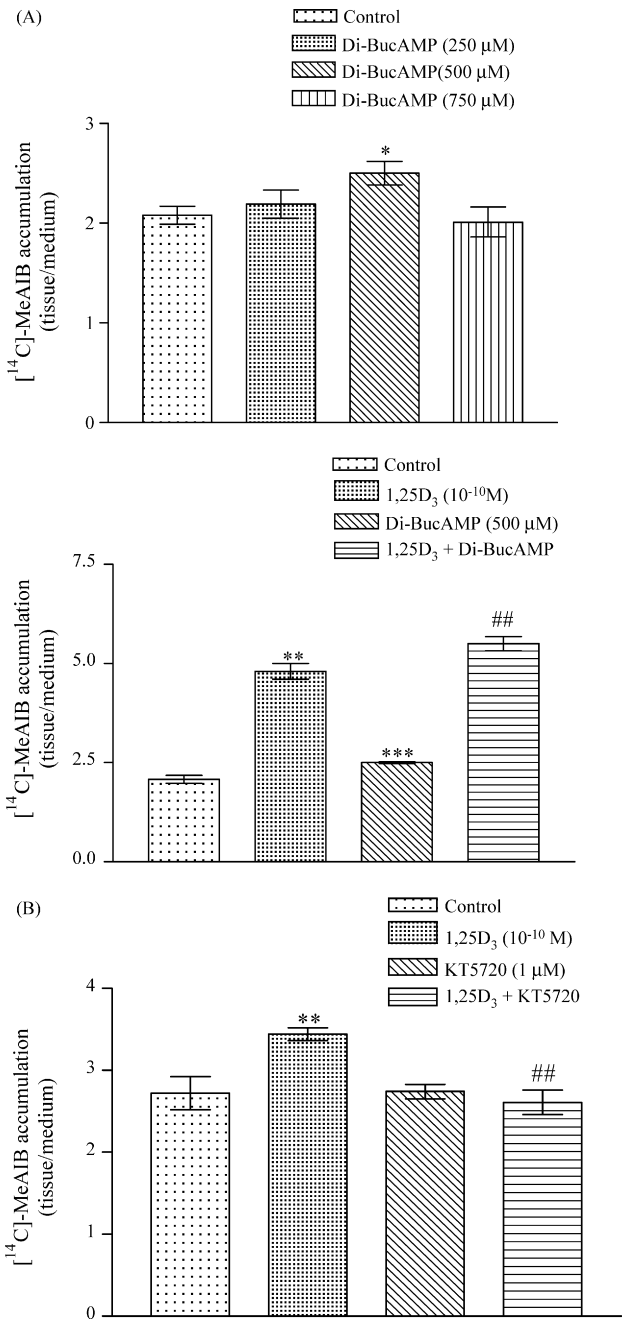

Fig. 4. Dose–response curve of di-BucAMP on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{*}p < 0.05$ compared with control. Pre-incubation time: 30 min; incubation time: 60 min (Inset panel A). (A) Effect of d-butyril-cAMP (500 μM) on the stimulatory action of 1,25D_3 (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control; $^{\#\#}p < 0.01$ compared with 1,25D_3. (B) Effect of KT5720 (1 μM) on the stimulatory action of 1,25D_3 (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{**}p < 0.01$, $^{\#\#}p < 0.01$ compared with 1,25D_3. Pre-incubation time: 30 min; incubation time: 60 min.

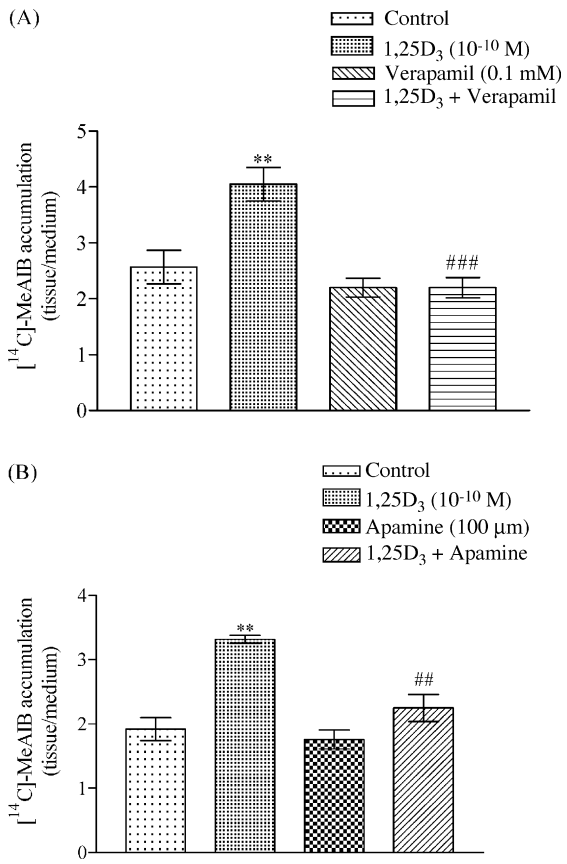

Fig. 5. (A) Effect of verapamil (100 μM) on the stimulatory action of 1,25D_3 (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{**}p < 0.01$ compared with control; $^{\#\#\#}p < 0.001$ compared with 1,25D_3 group. (B) Effect of apamine (100 μM) on the stimulatory action of 1,25D_3 (10^{-10} M) on [^{14}C] MeAIB accumulation in rat testis. Means ± S.E.M. for control. $n = 4$ for each group. $^{**}p < 0.01$ compared with control; $^{\#\#}p < 0.01$ compared with 1,25D_3 group. Pre-incubation time: 30 min; incubation time: 60 min.

substances. This stimulatory effect of 1,25D_3 on amino acid accumulation, unlike that of the locked testosterone molecule, raises the possibility that the flexibility of the 1,25D_3 moiety can determine the specific site of action as already demonstrated by this steroid in other tissues [5,6,22].

Vitamin D_3, via its active metabolite 1,25D_3, plays a critical role in the rat male reproduction through the activity mediated by a nuclear receptor [23]. Based on our previous data concerning the basal protein synthesis and the effective interference of cycloheximide on amino acid accumulation in immature rat testis [18], we observed that the potent stimulatory effect of 1,25D_3 on amino acid accumulation was blocked in the presence of cycloheximide. This finding indicates that active protein synthesis is required for the plenitude of the hormonal action. However, it is not known if this requirement reflects a direct action on plasma membrane amino acid carrier availability or on some regulatory protein of the transport process. Akerstrom and Walters [24] showed a rapid response of 1,25D_3 in $^{45}Ca^{2+}$ uptake in TM4 Sertoli cells shared by a nuclear receptor activity since the stimulatory action of 1,25D_3 on $^{45}Ca^{2+}$ uptake was inhibited by cycloheximide. These data reinforce the idea that 1,25D_3 may play an important role in testicular function through the regulation of these events, at least, by dual signal response pathways.

The physiological significance of genomic and non-genomic responses of 1,25D_3 in the target tissues remains unclear due to the complexity of intracellular signaling networks [11]. The disruption of the 1,25D_3 hormonal system produces a severe failure on spermatogenesis [23]. The specific mechanism by which 1,25D_3 influences reproductive function is, however, unclear. The influence of the nucleotide di-BucAMP and the PKA enzyme on amino acid accumulation clearly demonstrates the participation of the PKA pathway on the 1,25D_3 mechanism of action on amino acid accumulation. It is well known that the cyclic AMP/PKA acts as a second messenger of peptide, steroid and thyroid hormones [25–27], as well as a mediator of 1,25D_3 both in genomic and non-genomic events [28,29].

One of the non-genomic events related to $1,25D_3$ that has a narrow relationship with cyclic AMP/PKA is calcium influx [30–33]. Similar mechanisms for $1,25D_3$ action have been documented in osteoblasts, since in this tissue, a very small depolarization in the presence of $1,25D_3$ stimulates the opening of L-Ca^{2+} channels and entry of Ca^{2+}. Also, the binding of $1,25D_3$ in a VDR_{mem} stimulates cAMP production and activation of PKA to phosphorylate the channel, favoring protein secretory processes [33]. Analogous to the hormone action in osteoblasts, $1,25D_3$ affects heart cells calcium metabolism through regulation of Ca^{2+} channel activity by the cAMP pathway [30].

In order to study the ionic mechanism related to the stimulatory effect of $1,25D_3$ on amino acid accumulation, one voltage-dependent calcium channel blocker, verapamil, and one antagonist of Ca^{2+}-activated K^+ channel of small conductance, apamine, were used. These substances totally inhibited the stimulatory effect of $1,25D_3$ on amino acid accumulation. The involvement of these types of ionic channels in the $1,25D_3$ signal transduction is consistent with that described for the $1,25D_3$-induced rapid changes on the apical cell membrane potential of the proximal tubule. Based on such observations, the authors proposed that the mechanism of action of the $1,25D_3$ on the membrane potential is governed by the Ca^{2+}-dependent K^+ channels [34]. In general, Ca^{2+}-dependent K^+ channels are activated by increased intracellular calcium levels due to Ca^{2+} influx, membrane potential alteration and/or protein phosphorylation [35,18], contributing to the secretory function [36].

According to Marrion and Tavalin [37], the coupling of small conductance channels with different Ca^{2+} channels varies between cell types and may be important in channel regulation. In Sertoli cells, testosterone-mediated elevation of $[Ca^{2+}]_i$ requires the influx of extracellular Ca^{2+}, suggesting that calcium channels in the plasma membrane play a role in testosterone-calcium signaling [38–40]. Sertoli cells express at least four types of voltage-sensitive calcium channels in the plasma membrane (L, N, P/Q and T types) [41,42]. However, L-type channels have been implicated as the major conveyors for some steroid and thyroid hormone-induced Ca^{2+} influx into Sertoli cells and in the whole testis [25,39,43,44].

We have demonstrated that thyroid hormones stimulate amino acid accumulation in immature rat testis and induce membrane hyperpolarization in Sertoli cells. The action of T_3 on amino acid accumulation and on the membrane potential was inhibited by the K^+-ATP channel blocker tolbutamide, as well as by verapamil, demonstrating the existence of ionic mechanisms related to K^+ and Ca^{2+} in the non-genomic action of T_3. Also, the hyperpolarization elicited by T_3 and T_4 in the Sertoli cell membrane potential involved K^+ channels, since TEA and apamine abolished that effect [18,43]. In addition, it was demonstrated that the depolarizing effect of $1,25D_3$ was abolished in the presence of this Ca^{2+}-dependent K^+ channel blocker, in the apical cell membrane of the proximal tubule [34]. This reinforces the notion of a potential role for these channels in the $1,25D_3$ action in the testis. Taken together, these results point to both genomic effects, which can be triggered by PKA, and to rapid responses involving Ca^{2+}/K^+ channels on the plasma membrane. Although the involvement of VDR_{mem} in the rapid effects of $1,25D_3$ in the testis remains obscure, structure/function and computational analysis suggest that either the VDR_{nuc} or an isoform can function as the membrane receptor, propagating the rapid effects of $1,25D_3$ [45]. Thus, further computational studies on TM4 cells are on the way to clarify the role of this putative membrane receptor.

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3.3 ARTIGO SUBMETIDO PARA PUBLICAÇÃO

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$1\alpha,25(OH)_2$-Vitamin D_3 nongenomic potentiation of chloride currents in Sertoli cells

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Abstract

Sertoli cells from mammalian testis secrete a rich fluid into the lumen of seminiferous tubules that is critical to spermatogenesis. Secretory activities depend in part on the activity of ion channels present in the plasma membrane and secretory vesicles. The steroid hormone 1α,25$(OH)_2$ vitamin D_3 (1,25D) potentiates chloride currents involved in exocytosis in different cell systems via vitamin D receptor (VDR)-dependent nongenomic mechanisms. Here, we described for the first time 1,25D stimulation of voltage-gated, DIDS-sensitive Cl^- channels in the mouse Sertoli cell line TM4. 1,25D potentiation of Cl^- currents was dependent on hormone concentration in the nanomolar range, and correlated with a significant increase in whole-cell capacitance within 40 min. Potentiation of TM4 cell Cl^- channels by the agonist 1α,25$(OH)_2$ lumisterol D_3 (JN), and suppression of 1,25D potentiation by antagonist 1β,25$(OH)_2$ vitamin D_3 (HL) suggested nongenomic activation of the vitamin D receptor (VDR). Treatment of TM4 cells with PKC and PKA activators PMA and forskolin respectively, increased Cl^- currents significantly, while PKC and PKA inhibition with Go6983 and H-89, respectively, abolished 1,25D stimulation of Cl^- currents, suggesting phosphorylation pathways in 1,25D mediated nongenomic channel responses. RT-PCR proved expression of DIDS-sensitive ClC-3 channel in TM4 cells, in addition to other members of the voltage-gated family of chloride channels. Taken together, our results demonstrate 1,25D nongenomic, PKA/PKC-dependent potentiation of Cl^- currents required for exocytosis in Sertoli cells. We conclude that 1,25D contributes to male reproductive functions at least in part via stimulation of Sertoli cell secretory activities in immature testis.

Introduction

The hormonally active form of the steroid vitamin D_3, 1α,25$(OH)_2$-vitamin D_3 (1,25D), 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 1,25D/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. In addition, 1,25D activates plasma membrane functions by means of rapid modulation of signaling pathways involved in ion channel activation and cytoplasmic calcium signals (6-14). Among the most recently described nongenomic effects of the steroid 1,25D are those involving the flow of ions across the cell membrane as part of activation of secretory processes (15-17). In particular, 1,25D-sensitive voltage-gated chloride channels have been shown to play a crucial role in exocytosis in bone cells (17).

Sertoli cells from mammalian testis secrete a Cl^- and K^+ rich fluid into the lumen of seminiferous tubules that is 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, 1,25D triggers short-term physiological events including Ca^{2+} uptake activation (26), and PKA-dependent amino acid accumulation that involves Ca^{2+} and K^+ channels present in Sertoli cell plasma membrane (27).

Sertoli cells of different species express a VDR; however, the molecular mechanisms of 1,25D actions on these cells remain unclear (28-32). Here, we used patch-clamp electrophysiology to investigate 1,25D modulation of membrane electric processes underlying secretory activities in mouse TM4 Sertoli cells. We studied for the first time 1,25D nongenomic 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 1,25D on the secretory activites of Sertoli cells, with potential implications in male reproductive functions.

Materials and Methods

Chemicals

1α,25(OH)$_2$-vitamin D$_3$, 25(OH)-vitamin D$_3$, and 1β,25(OH)$_2$-vitamin D$_3$ (analog HL) were obtained from M. Uskokovic (Hoffmann-La Roche). The synthetic analog 1α,25(OH)$_2$-lumisterol$_3$ (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 ºC. N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, a specific PKA blocker), 8-chlorophenylthio-cAMP (cAMP, a membrane-permeable cAMP agonist), forskolin (a direct activator of adenyl 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 voltage-dependent 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 4º C.

Cell Culture

TM4 Sertoli cells (American Type Culture Collection, ATCC, Manasas, 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 µg/ml streptomycin, 5 % (v/v) horse serum, and 2.5% (v/v) fetal calf serum, in a 5 % CO_2 humidified atmosphere at 37ºC, 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 day 3-4 in culture, at 80 % confluency.

Electrophysiology

Chloride currents in TM4 cells were studied with the whole-cell patch-clamp configuration using a Heka EPC-9 amplifier (ALA Scientific Instruments Inc., West bury, NY), essentially as described before (6). Recording pipettes with resistances ranging between 3-5 MΩ were fabricated with a DMZ Universal micropipette puller (Drummond Scientific Co., Broomall, PA), coated with Sylgard elastomer (Dow Corning Corp., Midland, MI) to reduce capacitative transients, and fire-polished. Seal resistances ranged from 2-5 GΩ. 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 hour.

Analysis of ClCn mRNA expression

Cell lysates were assayed for the expression of members of the ClCn gene family of voltage-gated Cl^- channels (ClC-1 through ClC-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 units of MML-RT enzyme, 1.6 mM dNTPs, 1X RT Buffer (containing, in mM, 75 KCl, 3 $MgCl_2$, 5 DTT, and 10 Tris-HCl, pH 8.3), and 10 units 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 ClCn 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 ClC-2/5 couple of primers, 59.6°C for ClC-4 and VDR, 62.0°C for ClC-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 1X electrophoresis gel and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Visualization of exocytosis in TM4 Sertoli cells

Time-lapse videomicroscopy 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 ºC. 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 sequences were recorded with a Spot Pursuit digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) at a scanning rate of 1 image /30 sec during treatment of cells with 1-100 nM 1,25D.

Statistical analysis

Data were expressed as means ± standard error (S.E.). The two tailed Student's t-test with two sample unequal variance was used for statistical analysis, with $^{*}p< 0.05$ and $^{**}p<0.01$ as significantly and highly significantly different, respectively.

Results

1,25D potentiates Cl^- currents in TM4 Sertoli cells in a dose-dependent manner

Fig. 1A shows the stimulatory effect of 100 nM 1,25D 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. Subsequent addition of 100 nM 1,25D to the external bath promoted a significant increase in outward current amplitudes within 20-40 min. Current potentiation was measured at the point of maximum increase; typically, when maximum potentiation was reached, it remained stable. Potentiated Cl^- currents maintained the outward rectification and non-inactivating electrical profile recorded prior to the addition of 1,25D. 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 1,25D-responsive currents recorded in TM4 cells were due to the activity of voltage-dependent chloride channels, 200 µM DIDS, a stilbene derivative that specifically blocks ClC-3 outwardly rectifying chloride channels expressed at the plasma membrane (34), was added to the bath after 1,25D treatment. 1,25D-potentiated currents were totally blocked by DIDS within 2 min, thus confirming the involvement of ClC-3 channels (Fig. 1A).

Next, we studied the dose-dependence of 1,25D stimulation of Cl^- currents. Basal TM4 Cl^- currents recorded at 80 mV were treated with 1,25D concentrations ranging 1 nM through 1 µM. Chloride current amplitudes normalized to cell size (pA/pF) obtained with different 1,25D concentrations are shown in Fig. 1B. Maximum Cl^- current potentiation was obtained with a final concentration of 100 nM 1,25D 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 1,25D, indicating a biphasic response of the hormone similar to the one described for 1,25D-sensitive Cl^- channels present in osteoblasts (6).

1,25D stimulatory effect on Cl^- currents involves PKC/PKA pathways in TM4 Sertoli cells

We demonstrated previously that nongenomic 1,25D 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 1,25D 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 1,25D. As shown in Fig. 2, treatment of TM4 cells with the adenylate cyclase activator forskolin (20 μM) added to the recording bath caused a potentiation of Cl^- currents of about 3-fold, similar to 100 nM 1,25D, after 20-35 minutes. 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 1,25D stimulation of the currents. This suggests that hormone activation of PKA activity via induction of cytosolic levels of cAMP is involved in chloride channel potentiation in TM4 Sertoli cells.

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. When cells were treated with 100 nM PMA in combination with 100 nM 1,25D, the stimulatory effect was similar to 100 nM 1,25D 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 1,25D. These results indicate that phosphorylation pathways by PKC are involved in Cl^- current activation in TM4 cells, and that 1,25D increases PKC activities leading to further potentiation of Cl^- channels. Taken together, our results indicate that 1,25D potentiates voltage-gated Cl^- currents via phosphorylation cascades involving PKC and cAMP/PKA pathways in TM4 Sertoli cells.

TM4 cell Cl^- channels are potentiated by the synthetic agonist JN and natural metabolite 25D, and blocked by the nongenomic antagonist HL

We have shown before that the synthetic 6-*s-cis* locked analog 1α,25$(OH)_2$-lumisterol D_3 (JN, Fig. 3) is a potent agonist of 1,25D 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 1,25D-sensitive Cl^- currents expressed in osteoblasts can be specifically potentiated by the synthetic conformer JN (6). Here, we found that, similarly to 1,25D, 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 1β,25$(OH)_2$ vitamin D_3 (analog HL), which only differs from the natural metabolite 1,25D in the orientation of the hydroxyl group on carbon 1, inhibits 1,25D-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 1,25D 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 25D 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 1,25D (Fig. 3B). 25D 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 1,25D 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.

1,25D stimulates secretory activities in TM4 Sertoli cells

We showed previously with electrophysiology that treatment of primary osteoblasts with nanomolar concentrations of 1,25D causes a significant increase in whole-cell capacitance, which is a measure of exocytosis, within minutes (8). We found that 1,25D-stimulated rapid secretory response was dependent on 1,25D 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 1,25D stimulation of exocytotic activities in TM4 Sertoli cells, as revealed by whole-cell capacitance measurement.

Fig 4A shows continuous whole-cell capacitance recordings during treatment of TM4 Sertoli cells with 100 nM 1,25D. We found that whole-cell capacitance values remained relatively constant (50.4 pF baseline in the figure) prior to the addition of 1,25D to the bath. As recorded on three different cells studied, the addition of 100 nM 1,25D to the external solution caused a significant increase in capacitance value within 20-40 min, which corresponded with the stimulation of massive fusion of secretory vesicles with the plasma membrane during exocytosis. To study the hypothesis that 1,25D stimulation of exocytosis in TM4 cells required the opening of voltage-gated chloride channels, we measured whole-cell capacitance on cells pre-incubated with 200 μm DIDS. In the presence of the channel blocker, 100 nM 1,25D 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 1,25D-stimulation of exocytosis in quinacrine-stained live TM4 cells. 1,25D-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.

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 Materials and Methods). Primers and amplicon lengths are shown in the table 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 and 5, 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. Taken together, our results suggest that the 1,25D-sensitive Cl^- channel expressed in TM4 Sertoli cells, in agreement with our previous studies in osteoblasts (17), could be the ClC-3 member of the family of voltage-gated Cl^- channels (34). In accordance with previous studies on primary Sertoli cells (28), VDR gene expression was detected in TM4 Sertoli cells.

Proposed model of 1,25D nongenomic regulation of chloride currents in TM4 Sertoli cells

Fig. 6 depicts our proposed model for 1,25D nongenomic stimulation of Cl^- currents in TM4 Sertoli cells. By interacting with a cytosolic VDR located in close proximity to the plasma membrane, 1,25D appear to activate PKC and cAMP/PKA pathways required for the opening of ClC-3 chloride channels present in the plasma membrane and membrane of secretory vesicles within a time frame of 20-40 min. Chloride influx through voltage-gated channels facilitates 1,25D-dependent secretory activities in Sertoli cells by providing an electric shunt across the membrane. Sertoli cell secretory activities include release of ions, proteins, and growth factors relevant to germ cells and male reproduction. These molecular mechanisms of action of 1,25D underlying chloride channel potentiation in Sertoli cells involve genomic actions of the VDR.

Discussion

We investigated here for the first time 1,25D nongenomic potentiation of an outwardly rectifying, DIDS-sensitive Cl^- current that couples to exocytosis in mouse TM4 Sertoli cells. Our results showed that hormone stimulated opening of chloride channels is dependent on PKC and cAMP/PKA pathways. Stimulatory effects of 1,25D on these anion currents took place within 20-40 minutes 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. The specific nongenomic 6-s-cis locked agonist JN stimulated Cl^- currents more efficiently than 1,25D, while the nongenomic antagonist HL (37,38) blocked the agonistic effect of both 1,25D 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 1,25D to bind to an alternative (nongenomic) binding pocket in the VDR molecule (35). In addition, the natural metabolite 25D also stimulated the chloride currents, although at a lower extent, which is also consistent with activation of the receptor at the alternative binding site with lower affinity for 25D.

It has been shown previously that cAMP/PKA acts as a mediator of both 1,25D genomic and nongenomic responses in a variety of cell systems (12,27,39). Here, we demonstrated that in the presence of a PKA blocker, 1,25D potentiation of chloride currents was abolished, while adenylate cyclase activation caused a significant increase in control currents, mimicking 1,25D effect. This suggests cAMP/PKA involvement in 1,25D 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 (40). Our results are consistent with a model we proposed before for 1,25D nongenomic potentiation of the osteoblast ClC-3 channel via a cAMP/PKA cascade initiated by 1,25D activation of a cytoplasmic VDR that leads to channel phosphorylation and activation (8). Here, we propose a similar mechanism for 1,25D potentiation of ClC-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 1,25D stimulation of chloride currents. This indicates the

involvement of PKC in 1,25D nongenomic modulation of the electric currents, and agrees with results obtained on other systems (4, 41-44). It has also been reported previously that 1,25D activation of PKC may cause PKA activation via a cross-talk mechanism with the cAMP pathway (45).

Cytosolic Cl^- ions enhance Ca^{2+}-dependent exocytosis in a variety of endocrine cell types (46). It has been shown before that primary rat Sertoli cells express the ClC-2, ClC-3, ClC-6 and ClC-7 members of the family of voltage-gated Cl^- channels (23). Here, we found transcripts of ClCn 1 through 7 genes in TM4 cells. Levels of expression appeared to be relatively higher for ClC-2, ClC-3, ClC-4 and ClC-5 compared to the other family members. Due to the pharmacological and electrical characteristics of the 1,25D-sensitive chloride channel present in TM4 cells, more specifically its sensitivity to DIDS and outward rectification, we conclude that this 1,25D-sensitive chloride channel could be the broadly expressed ClC-3 channel (34, 47), which is known to be involved in exocytotis.

Taken together, our results demonstrate for the first time that nongenomic 1,25D 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 ClC-3 channel phosphorylation. We conclude that the steroid hormone 1,25D plays a functional role in male fertility via stimulation of Sertoli cell secretory activities in the testis.

Acknowledgements

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Figure legends

FIG. 1. Potentiation of DIDS-sensitive chloride currents by 1,25D 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 Materials and Methods). 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 1,25D to the bath (2). 1,25D-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 through 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 1,25D 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.

FIG. 2. Effects of PKA and PKC modulators on 1,25D-sensitive Cl^- currents compared to current potentiation obtained with 1,25D. Whole-cell Cl^- current amplitudes were measured at 80 mV after the addition of 100 nM 1,25D, with and without agonists and antagonists of PKA and PKC pathways at the indicated concentrations. In each case, at least a 10 min period was allowed after the addition of the agents to the bath, until currents reached a stable amplitude value. Values correspond to means ± SEM for n = 10 individual measurements performed on separate cells. *p < 0.05; ***p < 0.001 compared with control; ###p < 0.001 compared with 1,25D group.

FIG. 3. Effect of synthetic agonist 1α,25$(OH)_2$-lumisterol$_3$ (JN), antagonist 1β,25$(OH)_2$ vitamin D_3 (HL), and natural metabolite 25D 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. JN (1nM) showed to be a full agonist of Cl^- current potentiation, its effect being abolished when cells were preincubated with 1 nM HL. 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 1,25D, JN, 25D and the steroid β-estradiol on outward Cl^- currents in the absence and presence of the antagonist HL. Current amplitudes were measured at an 80 mV depolarizing voltage step from a holding potential of -30 mV. In the presence of HL, potentiation effects of 1,25D, 25D and JN were abolished. The steroid hormone β-estradiol (100 nM) had no effect on the currents. In each case, at least a 10 min period was allowed after the addition of the agents to the bath, until currents reached a stable amplitude value. The graph depicts mean values ± SEM obtained from n = 7-10 individual cells. **$p < 0.01$; ***$p < 0.001$ compared with control; φφφ $p < 0.001$ compared with 1,25D (1 nM) group; λλλ $p < 0.001$ compared with 1,25D (100 nM) group; ###$p < 0.001$ compared with JN group.

FIG. 4. 1,25D-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 1,25D to the bath. Upward deflection of the traces from an initial capacitance value of 50.4 pF 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 minutes with 1,25D incubation. The bar graph shows mean whole-cell capacitance values obtained from 3 individual cells per treatment before (control) and after addition of 100 nM 1,25D, and 100 nM 1,25D 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 1,25D treatment. Incubation of this particular cell with 100 nM 1,25D 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 minute-incubation with 1,25D. In the absence of 1,25D, control cells maintained their original fluorescence intensity (not shown), verifying that neither exocytosis nor quenching of the dye occurred within this time period.

Fig. 5. RT-PCR analysis of ClCn 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: ClC-1, 102 bp; ClC-2, 642 bp; ClC-3, 195 bp; ClC-4, 429 bp; ClC-5, 609 bp; ClC-6, 158 bp; ClC-7, 142 bp; VDR, 672 bp. PCR products were confirmed by sequencing.

Fig. 6. Proposed model of 1,25D nongenomic potentiation of chloride currents through ClC-3 voltage-gated channels in TM4 Sertoli cells.

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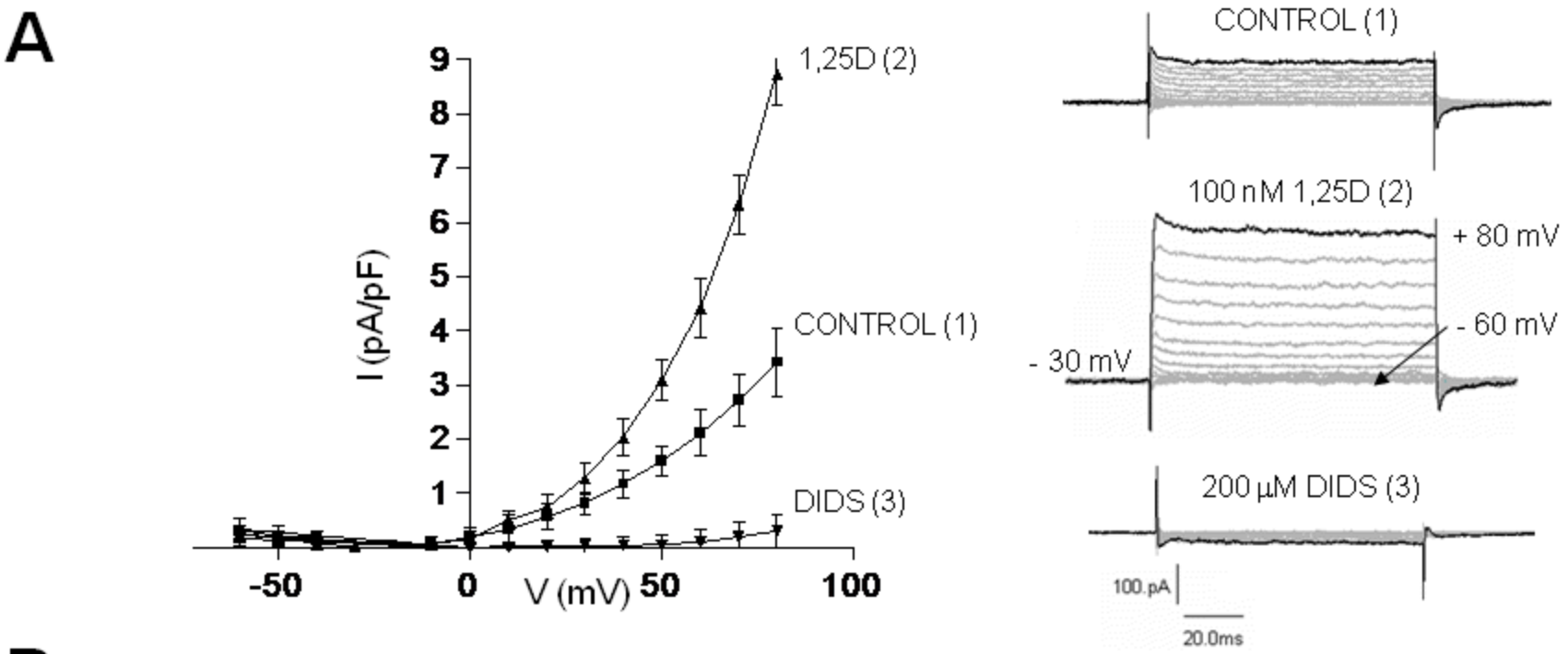
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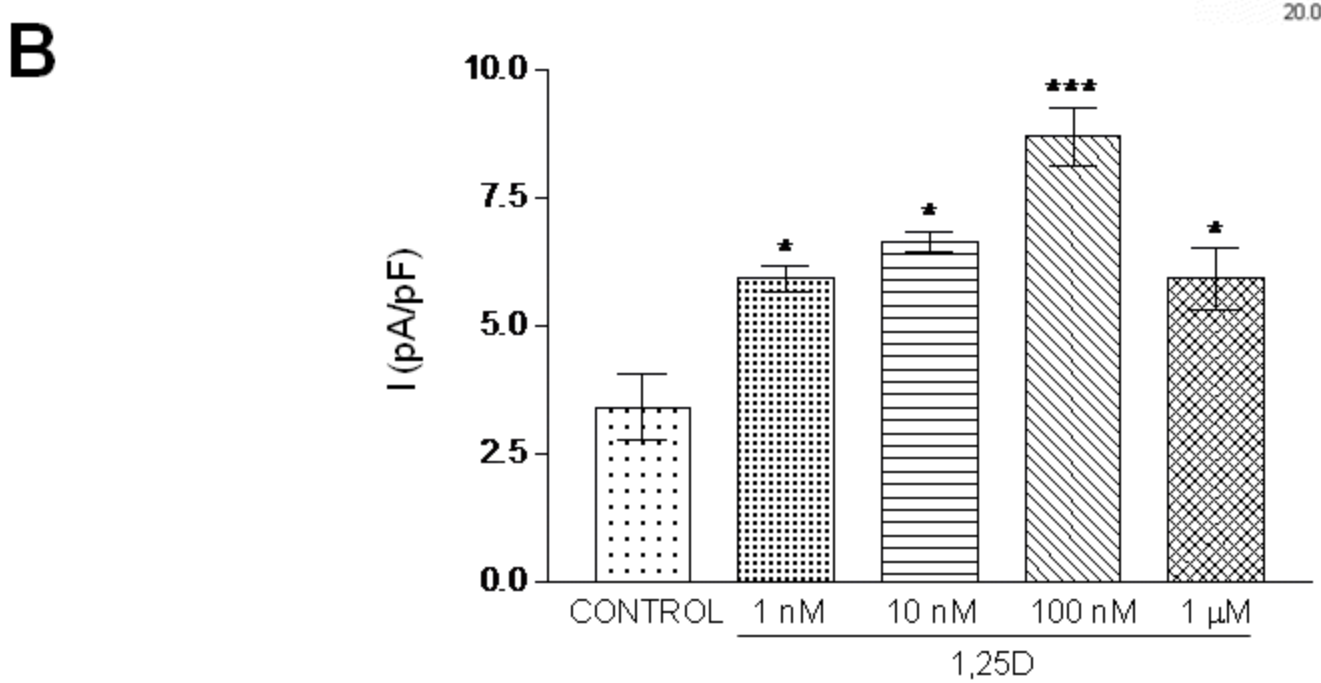
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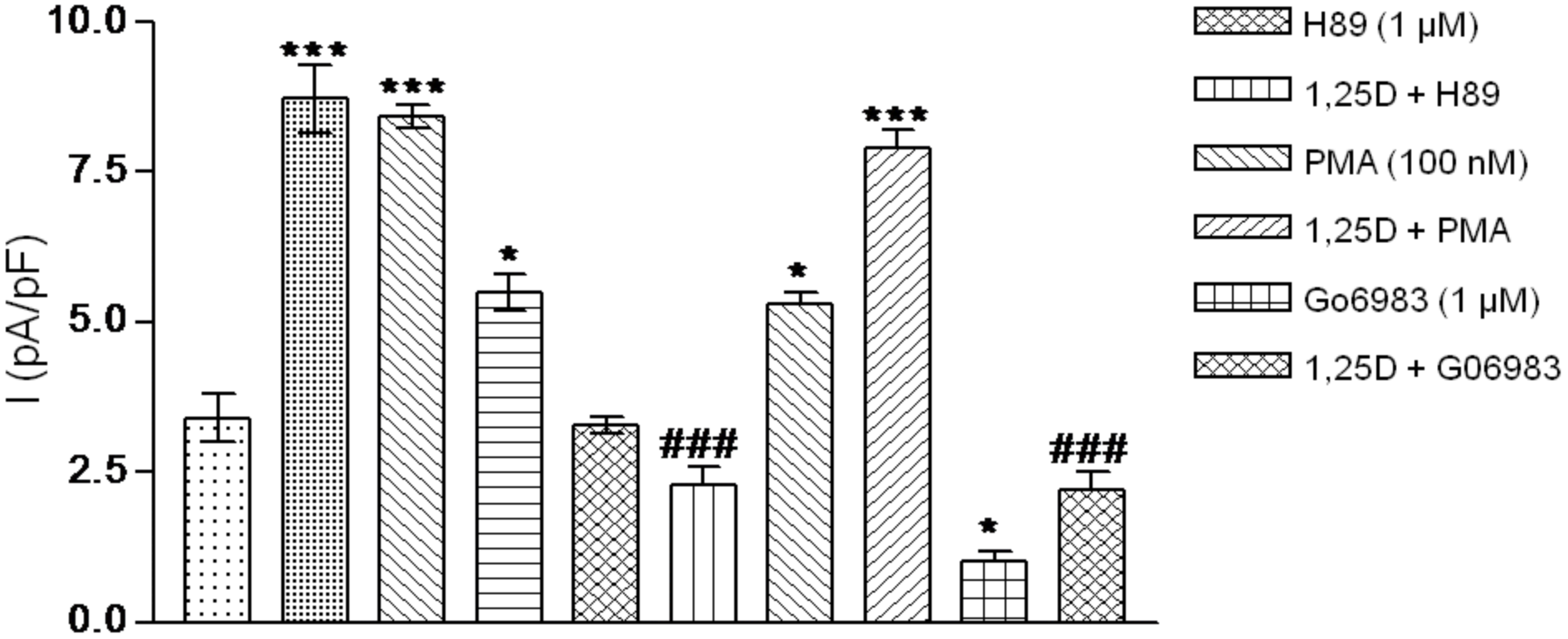
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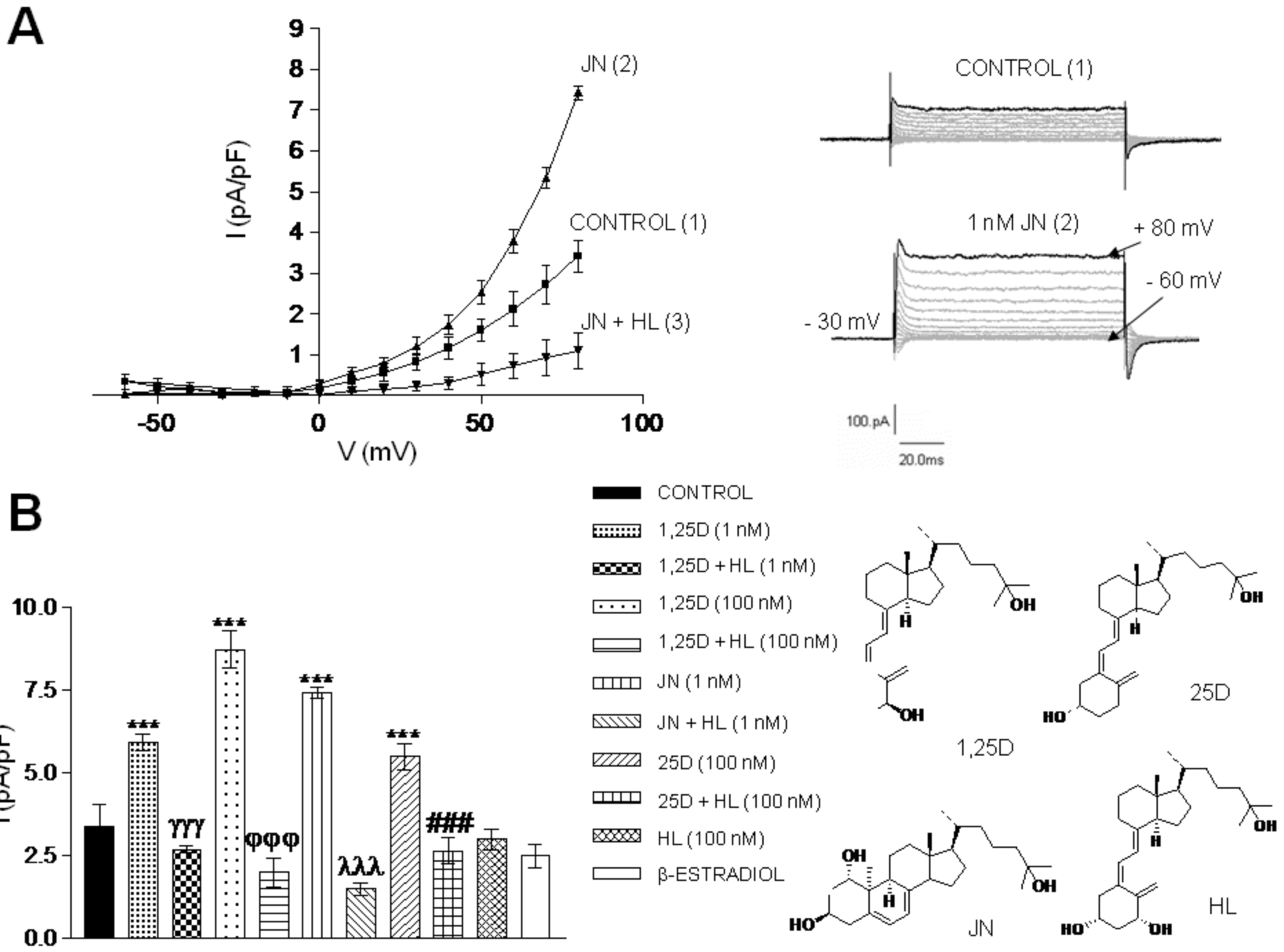
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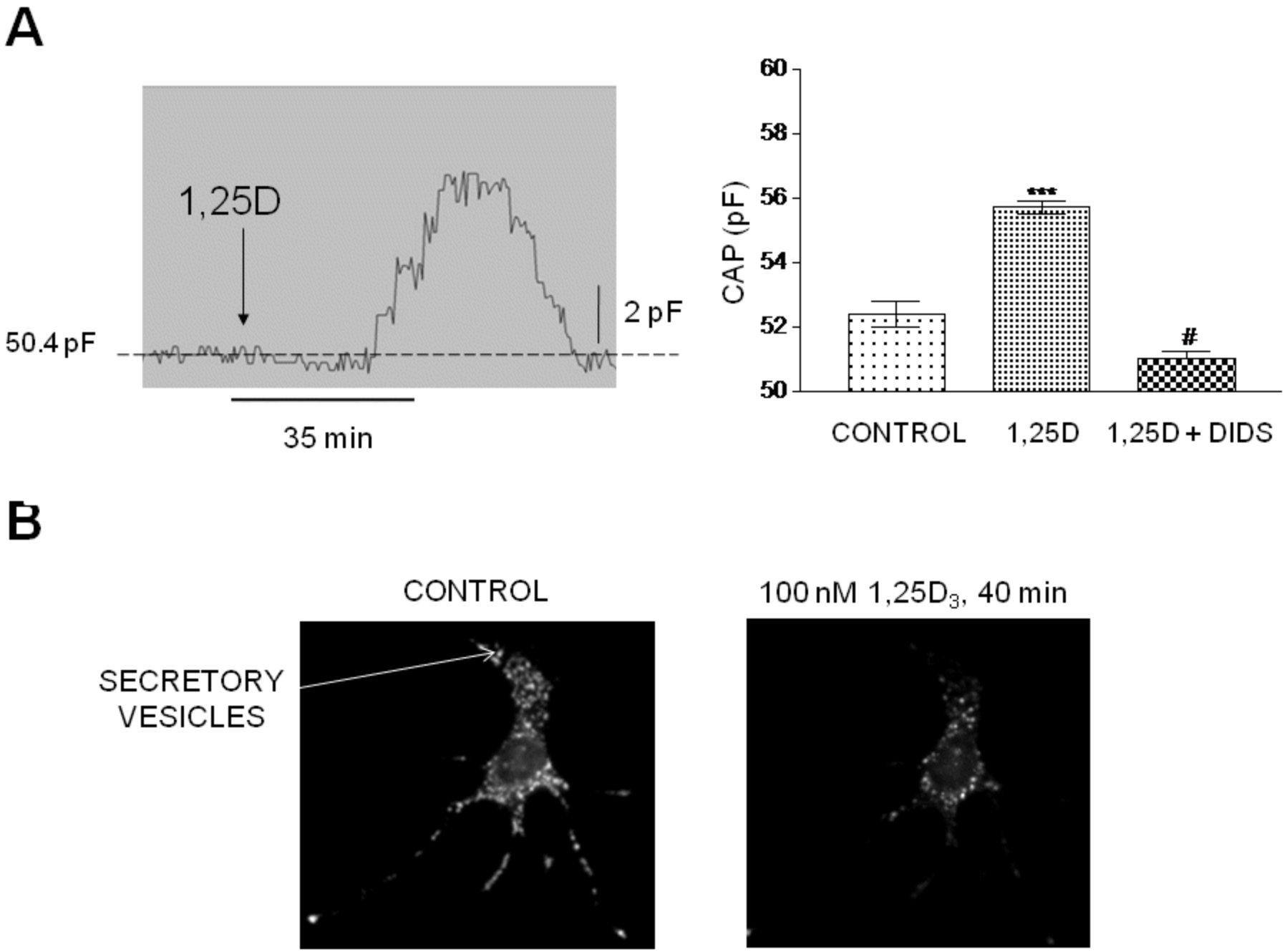
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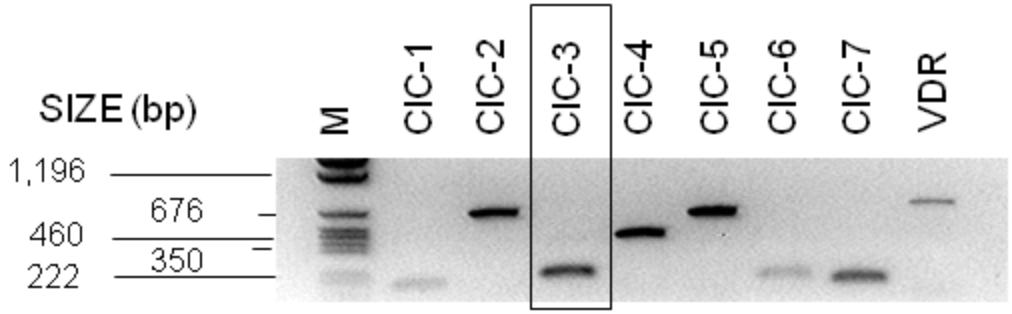

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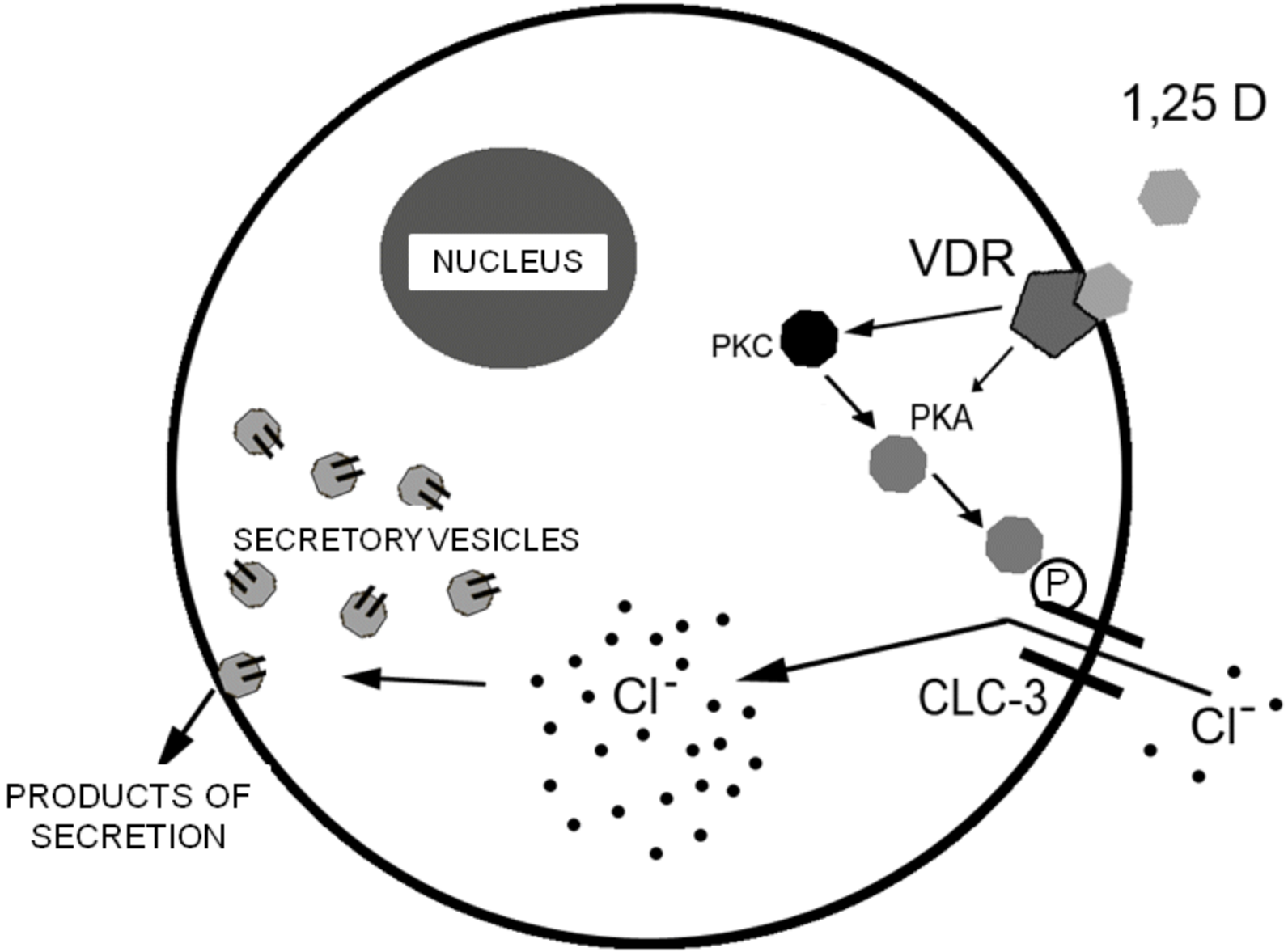
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PRODUCTS OF SECRETION

3.4 ARTIGO EM FASE FINAL DE ELABORAÇÃO

Periódico – Chemistry and Biology

MENEGAZ, D.; MIZWICKI, M.; SILVA, F.R.M.B.; HENRY, H.; NORMAN, A.W.; ZANELLO, L.P. **A two teared rapid potentiation of chloride channels in TM4 Sertoli cells requires the VDR and/or CFTR. To be submitted.**

A TWO TEARED RAPID POTENTIATION OF CHLORIDE CHANNELS IN TM4 SERTOLI CELLS REQUIRES THE VDR AND/OR CFTR

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Abstract

The purpose of this study is to begin to evaluate the structure-function and biochemical properties of the complex formed between curcuminoids and the nuclear vitamin D receptor (VDR) complex. Using *in vitro* competition experiments it is demonstrated that curcumin (CM) and bisdemethoxycurcumin (BDC) bind specifically to the overexpressed VDR mLBD (aa118-427; Δ165-215) and chick VDR, from an intestinal mucosa homogenate with low to mid-micromolar affinity respectively. Using Gaussian B3LYP/6-31G** opt calculations we verified that the lowest energy form of CM and BDC is the enol tautomer. Analysis of the CM and BDC predicted global energy minimum conformation indicated the enol is planar in shape, while the keto tautomer is bowl-shaped. Previous studies indicate that the VDR molecule contains two distinct, yet overlapping ligand binding pockets (LBPs). X-ray and *in silico* results indicate that the VDR genomic pocket (VDR-GP) requires a cognate ligand to take on a bowl-shape, while the VDR alternative pocket (VDR-AP) prefers a cognate ligand to take on a planar shape. These two pockets are proposed to activate genomic and non-genomic cell signaling pathways respectively, given the natural hormone, 1α,25$(OH)_2D_3$, is conformationally flexible and can thus satisfy the shape requirements for each pocket. Given the curcuminoids preferred molecular shape and some shared structural characteristics when compared to 1,25D, we a) evaluated the ability of each curcumin tautomer to bind to the VDR LBPs by designing and employing a flexible docking protocol using Discovery Studio 2.0 (Accelrys Inc., San Diego) and b) curcuminoids ability to activate voltage gated chloride channels in TM4 Sertoli cells (i.e. a non-genomic response). The *in silico* results demonstrate that the enol and diketo tautomers form more stable complexes with the VDR-AP. This conclusion is based on comparison of the Gibbs free energy of binding calculation, which accounts for solvation effects. The patch-clamp results indicate that 1,25D, CM and BDC all stimulate an outward rectifying chloride current in TM4 cells. Interestingly, addition of 0.1 and 1.0 μM CM or BDC showed a similar signaling profile reported recently for 1,25D, whereby curcuminoid stimulation is blocked by co-incubation of equal molar 1β,25$(OH)_2D_3$, a non-genomic specific antagonist, pre-incubation with PKI, a specific PKA blocker and post incubation with DIDS, a known anion channel blocker. However, unlike 1,25D, 10 and 20 μM curcuminoid stimulation was not blocked by equal molar HL or PKI and/or DIDS. Furthermore, DIDS binds specifically to the VDR mLBD with an ~10 μM IC_{50}. Because 10 and 20 μM curcuminoid have been implicated in stimulating the CFTR, we tested if CFTRinh-172 was capable of blocking the CM currents. Only when pre-incubated did CFTRinh-172 block CM. Collectively, the results suggest curcuminoids activate two regulatory pathways where the VDR and CFTR serve as the receptors.

Introduction

The steroid hormone, 1α,25(OH)$_2$-vitamin D$_3$ (1,25D, Fig. 1A) is the most conformationally dynamic endocrine hormone ligand for the nuclear receptor (NR) superfamily of transcription factors (Evans, 1988). The plethora of 1,25D conformational isomers allows 1,25D the ability to stimulate both genomic and non-genomic cellular responses (Norman et al., 2004)(Norman et al., 2001). Genomic responses are defined as the activation of genes containing a vitamin D response element [VDRE; (Haussler et al., 1997)], induced by 1,25D binding to the VDR with K_D of ~1.0 nanomolar (Bouillon et al., 2008). Non-genomic responses are defined as rapid modulation of extra-nuclear signaling cascades, namely ion channels, kinases and phosphatases, in response to ~1.0 nanomolar concentrations of 1,25D (Norman et al., 2004). These rapid effects are measured within seconds to minutes after the addition of the hormone. One receptor involved in the onset of a non-genomic, rapid response to various vitamin D sterols (VDS) is the traditional nuclear VDR (Mizwicki et al., 2004). Importantly, the VDR is not the only NR present at or near the plasma membrane regulating rapid responses (Norman et al., 2004).

Curcuminoids are polyphenols isolated from the curry spice turmeric. Curcuminoids have long been known for their dietary antioxidant and radical scavenger properties (Kapoor and Priyadarsini, 2001). Recently it has been shown that two principal curcuminoids, curcumin (CM) and bisdemethoxycurcumin (BDC, Fig. 1A) serve as natural ligands for the nuclear vitamin D receptor [VDR, (Masoumi et al., 2009)(Jurutka et al., 2007)], as potential agonist ligands for the Cystic fibrosis transmembrane conductance regulator [CFTR, (Verkman and Galietta, 2009)] chloride channel (Wang et al., 2007)(Berger et al., 2005) and as potential antagonist ligands for the nuclear androgen receptor [AR, (Ohtsu et al., 2002)]. Both CM and BDC have been shown to possess chemopreventative and therapeutic properties (Goel et al., 2008). To this end they serve

as pilot natural products being used in the design of potential new therapeutic agents for the treatment of Alzheimer's disease (Fiala et al., 2007), pancreatic cancer (Dhillon et al., 2008) and cystic fibrosis (Verkman and Galietta, 2009). The only major pharmacological drawback of CM and BDC is their seemingly low bioavailability, which is reviewed elsewhere (Anand et al., 2007).

The chemistry of the CM and BDC molecules allows for a keto-enol tautomerization (see Fig. 1B). The enol tautomer has been shown to be more stable and is believed to be the only pharmacologically relevant form of the curcuminoids. Together, the enol H-bond and the extensive π-system of the CM and BDC molecules make the enol-form a rigid, geometrically linear molecule, whereas the keto-form energetically prefers a bowl-shape (Kolev et al., 2005) (Fig. 1B). These two ligand geometries are preferred by the VDR alternative pocket and VDR genomic pockets respectively in the recently proposed vitamin D sterol (VDS)/ VDR conformational ensemble model (Mizwicki et al., 2004)(Mizwicki et al., 2007).

The vitamin D sterol (VDS)/ VDR conformational ensemble model proposes that the VDR can act as a receptor for both genomic and non-genomic responses by containing two overlapping ligand binding sites (Mizwicki et al., 2007). The VDR pocket defined by x-ray crystallographic results (Rochel et al., 2000) is termed the VDR genomic pocket (VDR-GP) and the ligand binding pocket discovered *in silico* is termed the VDR alternative pocket (VDR-AP, Fig. 1C). The VDR-AP was discovered computationally using the 6-*s*-*cis* locked, non-genomic specific agonist, analog 1α,25$(OH)_2$-lumisterol D_3 (JN, Fig. 1A) as the ligand (Mizwicki et al., 2004). Importantly, the cholesterol-like fused ring system of analog JN gives it a very planar molecular geometry (Norman et al., 2004). When different 1,25D conformational isomers were docked in the VDR-AP it was discovered that multiple 1,25D conformers could bind stably to

the VDR-AP, but that all shared a planar overall molecular shape (Mizwicki et al., 2007). This differs from the bowl-shape of 1,25D, known to be strictly required for binding stably to the VDR-GP (Mizwicki and Norman, 2003) (Fig. 1C).

Given the evidence that proper vitamin D nutrition plays a role in male fertility (Corbett et al., 2006), that CM is capable of stimulating loss-of-function CFTR mutations (Wang et al., 2007), that 1,25D binds specifically to a receptor present in TM4 Sertoli cells (Akerstrom and Walters, 1992) and that CM and BDC function as natural ligands for the VDR, we set out to investigate and compare 1,25D, CM and BDC modes of VDR binding and their regulation of voltage-gated chloride channel and exocytotic signaling in TM4 Sertoli cells. The 1,25D and curcuminoid results presented herein fit the tenants of the VDS/ VDR conformational ensemble model (Mizwicki et al., 2007), support the ability of CM to bind directly to the CFTR and indicate that CFTR functionally regulates VDS and curcuminoid stimulation of voltage-gated CLC signaling .

Materials and Methods

Chemicals

1α,25$(OH)_2$-vitamin D_3 (1,25D) and1β,25$(OH)_2$-vitamin D_3 (HL) were obtained from M. Uskokovic (Hoffmann-La Roche). PKI, a specific PKA blocker and 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), a voltage-dependent chloride channels blocker, CFTRinh-172 were obtained from Sigma. Curcumin CM and BDC, bisdemetoxicurcumina (BDC) were purchased from the Chromadex.

Molecular Volume Calculations- The molecular volumes of 1,25D, BDC, CM, DIDS, HL and JN were determined using Discovery Studio 2.0 (Accelrys Inc.) molecular attributes and atom

typing with the cff forcefield. The conformational isomer of each molecule used to calculate the molecular volume was generated using PC_Model and Gaussian methodology (see conformational isomer generation below).

VDR Binding Affinity

Chick VDR (cVDR): The Relative Competitive Index (RCI) values obtained using an intestinal mucosa extract as a VDR source were derived using known methods (Wecksler and Norman, 1980)(Bouillon et al., 1995).

Human VDR mLBD: BL21-DE3 cells (Stratagene) were transformed with the pET15b vector containing human VDR ligand binding domain (LBD) (aa118-427; Δ165-215) obtained from the Moras laboratory (IGBMC, Strasbourg, France). Following culture scale-up to 1L (37 °C), cells were grown to an OD_{600} of 0.5-0.6 (37°C and 250 rpm), induced with 1 mM IPTG, grown at 20°C for 6 hrs, and pelleted at 4,000xg for 10 min at 4°C. The pellet was resuspended in lysis buffer (5 mM imidazole, 20 mM Tris (pH=8.5), 10% Glycerol, 250 mM NaCl, 1 mM PIC and 1 mM DTT). Cells were lysed by sonication (15 sec. pulse, 1 min. on ice; repeat 3x). After centrifugation (12,000xg), the supernant was filtered through 0.22 micron syringe filters (Millipore). Aliquots were flash frozen and stored at -80°C. 0.05 pmoles of [^{3}H]-1,25D in EtOH was added to each borosilicate glass tube (Fisher). Then increasing concentrations of cold ligands were added to the tubes in duplicate and total EtOH was adjusted to 20µl. 230 µl (25µg of total protein, determined by bradford) of the diluted (buffer same as lysis buffer minus PIC and pH = 8.0), clarified VDR-LBD was added to each tube, vortexed and placed at 4°C for 4-24 hrs. Following incubation, 200 µl of 50% (v/v) hydroxyapatite solution in TED was added and washed 3 times with 1 ml TED plus 0.5% Triton-X100. Bound [^{3}H]-1,25D was eluted with 1 ml

ethanol and counted in 7 ml Liquiscint (National Diagnostics) scintillation cocktail using a Beckman LS6500. Data from duplicate samples were averaged and plotted as % max bound [^{3}H]-1,25D versus the log of the concentration of the competitor used. Non-linear regression using GraphPad Prism® (GraphPad Software, San Diego, CA) was used to determine the IC_{50}, correlation coefficient and R-squared (R^2) values. Data were also plotted to generate a relative competitive index value (RCI) using standard protocol (Wecksler and Norman, 1980)(Bouillon et al., 1995).

Generation of CM, BDC, DIDS and 1,25D conformational isomers

CM and BDC: To generate a theoretical global energy minimum structure for CM and BDC the crystal structure coordinants were sourced using conquest v1.9. BINMER (i.e. the enol form of CM) was opened in Gauss_View3.Gaussian B3LYP/6-31G** calculations were performed on BINMER. To make sure the optimized x-ray coordinants represented the global energy minimum following energy optimization we repeated the work of Kolev et al. (Kolev et al., 2005), whereby different, elongated, planar conformational isomers of CM were generated in Gauss_View3 and a B3LYP/6-31G** calculation performed. The di-keto tautomer of CM was generated in PC_Model v9.0 and energy optimized. The di-keto conformer was imported into Gaussian98 rev.9 and a B3LYP/6-31G** calculation performed. Energy differences between the constitutional and conformational isomers of CM were calculated in units of kJ/mole using the following conversion (Δa.u. x 627.5 x 4.184). The global energy minimum structure of BDC and starting enol and keto tautomers were produced using the CM structures as a template. For flexible docking calculation the lowest energy enol and keto conformers from the B3LYP/6-

31G** calculations were imported into Discovery Studio 2.0 (Accelrys Inc., San Diego, CA) and 100 conformers generated using the BEST algorithm (100 include the Gaussian output structure).

DIDS: The molecule was built using PC_Model v9.0 and GMMX calculations performed using default conditions. Structure number one from this conformational search calculation was imported into Discovery Studio 2.0 (Accelrys Inc., San Diego, CA) and 100 conformers generated using the BEST algorithm (100 included the most favored PC_Model GMMX conformer).

1,25D and other vitamin D sterols (VDS): Whole molecule GMMX (Global-MMX. PC_Model v8.0) experiments were carried out on 1,25D allowing for flexibility in the A-ring, seco-B-ring and side-chain single bonds. No flexibility in the CD-ring atoms was allowed, nor rotation about the three C-O-H single bonds. Importantly, the GMMX calculation does not predict the local minimum structure for the given input geometry, rather it predicts the global energy minima and conformers within a specified energy window of the projected global minima. For 1,25D, 502 conformational isomers were generated using the following GMMX variables: search on bonds only, π-correction turned on, energy window = 11.0 kcal/mol and 1000 total conformers generated. The calculation produces 1,25D shapes with different A-ring, seco-B-ring and/ or side-chain conformations. All of the structures were converted to .pdb format, imported into Discovery Studio 2.0 and manually typed to assure the vitamin D triene system had the appropriate hybridization. Then the 1,25D conformational isomers were typed with the cff or CHARMm forcefield (no difference in output observed) and used for the flexible docking simulation.

Flexible Docking ($\Delta G_{binding}$)

The apo-hVDR-GP or VDR-AP (aa118-427; Δ165-215) complex and ligands (see generating conformational isomers above) were docked to one another using the flexible docking protocol of Discovery Studio 2.0 (Accelrys Inc.; San Diego, CA). All hVDR-GP and VDR-AP computations start with the energy optimized complexes reported for the 1,25D/VDR-GP and JN/VDR-AP complexes in Mizwicki et al. (Wu et al., 2003a; Sutherland et al., 2007a). These complexes were typed with the CHARMm forcefield and further optimized to a convergence derivative of 0.01 using Discovery Studio 2.0 default minimization protocol parameters. In the control 1,25D, JN and HL VDR-GP and VDR-AP flexible docking results 100 VDR LBD conformational isomers were generated from the energy optimized start structures by allowing flexibility in the entire VDR molecule. Only fifteen side-chain were allowed to be redraw for each conformer; therefore, there are only minor differences in the VDR-GP and VDR-AP R-groups in the 100 conformational isomers when compared to the start structure. For the VDR-GP calculations, the flexible docking protocol variables changed from the default parameters were as follows: a 10.0 Å docking sphere was defined by searching the energy optimized VDR-GP (i.e. start structure with 1,25D removed) for putative binding sites followed by generation of the site sphere; the number of flexible docking residues allowed for structural refinement was set to 12; the top 5 hits were saved for each protein-ligand pair and a simulated annealing step (i.e. short dynamics simulation) was performed using default parameters. Flexibility was allowed in twelve of the following R-groups: Y143, Y147, F150, L227, L230, A231, L233, V234, S237, I268, I271, M272, R274, S275, S278, , W286. C288, Y295, V300, A303, H305, L309, L313, H397, Y401, L404, L414, V418(i.e. the flexible docking residues, these residues form the walls or surface of the VDR-GP) and HOH molecules 501 and 506(see .pdb: 1DB1). It is noted no

quantitative difference was observed in the flexible docking output when 10 or 15 of these residues were allowed to be refined. For the VDR-AP flexible docking protocol changes in default parameters were as follows: a 10.0 Å docking sphere was defined by searching the energy optimized VDR-AP (i.e. start structure with JN removed) for putative binding sites followed by generation of the site sphere; the number of flexible docking residues allowed for structural refinement was set to 20; the top 5 hits were saved for each protein-ligand pair and a simulated annealing step was performed using default parameters. For the VDR-AP calculations flexibility was allowed in the following R-groups: T142-R158, H229, L233, Y236, S237, K240, I271-S278, W286-D299, T301, K302 (i.e. the flexible docking residues, these residues form the VDR-AP) and HOH molecules 500-506, 508 and 526. In all calculations the VDR conformational isomers where generated and the protocol stopped, so that in all docking experiments the same two set of VDR-GP and VDR-AP conformational isomers was sourced.

The flexible docking calculation computation involves three stages after generating the pool of ligand and protein conformations. Stage one involves LibDock docking of all the protein conformers to all the ligand conformers. The complexity of this process is reduced by removing hydrogen atoms from the molecules. Stage two involves side-chain refinement of the flexible docking residues (see above) in the top 3 complexes for each protein-ligand pair surviving stage one. Note that hydrogen atoms are reinserted here and epimerization of the A-ring hydroxyl groups can occur; therefore, each complex is examined manually once the computation is complete. This bug has been fixed in the newest release of Discover studio. Stage three involves a simulated annealing step which involves a short dynamics simulation. At the end of the three stages, the top 50 complexes were saved, scored/ ranked using the cDocker algorithm (Wu et al., 2003b; Sutherland et al., 2007b) (Accelrys, Inc. San Diego). It is noted that complexes with the

side-chain in the A-ring domain are observed; however, the A-ring first orientation is preferred for all vitamin D sterols (VDS) addressed in this work. It is also noted that only when PC_Model 1,25D conformational isomers when used as a starting ligand set were capable of replicating the 1,25D x-ray pose (data presented elsewhere). Said differently, the FAST, BEST or CAESAR algorithms (DS 2.0) were incapable of replicating the 1,25D x-ray pose.

The number of complexes processed further was limited to the top 10 for the $\Delta G_{binding}$ determination. We used the Generalized Boltzmann Molecular Volume (GBMV) method (Im et al., 2003; Zoete et al., 2005) to determine $\Delta G_{binding}$ ($\Delta G_{binding} = \Delta G_{holo\text{-}VDR} - \Delta G_{apo\text{-}VDR} - \Delta G_{ligand}$), where the only default value change was in the cutoff distances, which were changed from 14Å, 12Å and 10Å, to 10Å, 8Å and 6Å respectively. It is noted the general equation shown above contains solvation terms. All computations were performed using an implicit solvent model with x-ray water molecules present. It is observed that removal of the water molecules from the VDR structure produces no favorable flexible docking poses.

Cell Culture

TM4 Sertoli cells (American Type Culture Collection, ATCC, Manasas, VA, USA) were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's media (DMEM) with the addition of L-glutamine, 15mM HEPES, 1.2g/L sodium bicarbonate; antibiotics, 5% (v/v), horse serum and 2,5% fetal calf serum in a CO_2 humidified atmosphere at 37^o. For patch clamp experiments, cells were plated at very low density in 35-mm tissue culture dishes. Prior to recordings, the cells were washed at least three times with the electrophysiological external solution to remove the medium completely.

Electrophysiology

Chloride currents were studied in the whole-cell patch-clamp configuration with a Heka EPC-9 amplifier (ALA Scientific Instruments Inc., West bury, NY) as described by (Hamill et al., 1991). Recording pipettes with resistances ranging between 3-5 MΩ were fabricated with a DMZ Universal micropipette puller from Drummond capillaries (Drummond Scientific Co., Broomall, PA), coated with Sylgard elastomer (Dow Corning Corp., Midland, MI) to reduce capacitative transients, and fire-polished. Seal resistances ranged from 3 to 15 GΩ. 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. For the recording of chloride currents, we used an external solution consisting of (mM): 150 NaCl, 10 $BaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.3. The corresponding pipette solution consisted of (mM): 160 CsCl, 10 $MgCl_2$, 10 HEPES/TEAOH buffer, pH 7.2. Changes in whole-cell capacitance, a measure of exocytosis (Klyachko and Jackson, 2002), were detected using the software-based lock-in implementation of Pulse (v.8, Heka EPC-9). The applied sine wave had a frequency of 500 Hz and peak amplitude of 20 mV, and was superimposed on a holding potential of -30 mV. Whole-cell capacitance was continuously monitored for 1 hour.

Results

The ability of curcuminoids and DIDS to bind specifically to the VDR

Provided the recent evidence that curcumin binds specifically to the overexpressed VDR in COS-7 cells (Jurutka et al., 2007) and that BDC binds specifically to the VDR x-ray contruct (mLBD, aa118-427; Δ165-215) (Masoumi et al., 2009), we further evaluated the ability of CM and BDC to bind specifically to the chick VDR (cVDR) and the VDR mLBD. The results indicate that CM and BDC have similar affinity for the VDR mLBD, but that CM binding affinity to cVDR is weak relative to the VDR mLBD affinity. Alternatively, the opposite is observed for 1,25D (Table 1). The reduced CM-VDR binding affinity in the chick intestinal mucosa homogenate is expected given its intermediate specificity ($IC_{50} \geq 20$ μM) for a number of endogenous proteins.

DIDS is a stilbene derivative that is widely used to block the chloride-influx induced by members of the CLC voltage and volume gated chloride channels (Schultz et al., 1999)(Jentsch et al., 2002) (Fig. 1A). Mechanistically, DIDS can bind reversibly to endogenous membrane proteins (e.g. ion channels) or it can form an irreversible covalent adduct with the channel via its thiocyano group reacting with primary amines (Gatto et al., 1997)(Matulef et al., 2008). The later has been shown to occur at or near its nucleotide binding domain when purified Na/K-ATPase was treated with DIDS (Gatto et al., 1997). Seeing we were already screening for novel VDR ligands we tested whether DIDS could bind specifically to the VDR. The result demonstrates that DIDS bindsthe VDR mLBD with an approximate 10 μM IC_{50} value (Table 1). Whether or not DIDS forms a VDR covalent adduct is still under investigation. Collectively, curcuminoids and DIDS bind with similar to better affinity to the VDR when compared to lithocholic acid (LCA,

Table 1), an endogenous, functional, natural non-vitamin D sterol compound (Makishima et al., 2002) used as a positive control in this study.

Predicting 1,25D, CM and VDR genomic and alternative pocket selectivity

Prior to assessing the theoretical complex formation between the novel natural and synthetic VDR ligands, the low energy conformation of these ligands was investigated using Gaussian B3LYP/6-31G** and PC_Model GMMX calculations (see methods). The Gaussian B3LYP/6-31G** calculations indicate and confirm (Kolev et al., 2005) that CM and BDC both energetically prefer the enol-form, which has a flat molecular geometry (Fig. 1B). PC_Model GMMX calculations demonstrate that the lowest energy conformational isomer of 1,25D has a β-chair A-ring (Mizwicki et al., 2005b), a *trans* seco-B-ring (Mizwicki and Norman, 2003) and a Pop. A side-chain configuration (Mizwicki et al., 2004)(Mizwicki et al., 2007) (Fig. 2A). The starting structures for simulating the complex formation between 1,25D or other vitamin D sterols (VDS) and the VDR mLBD were generated using whole molecule GMMX conformational search calculations. Using the parameters outlined in the methods 502 1,25D conformations are obtained. These 1,25D conformational isomers range in A-ring, seco-B-ring and side-chain conformation and therefore their overall shape [see Fig. 2B and (Mizwicki and Norman, 2003)(Norman et al., 2004)]. It is important to note that this is the first whole molecule molecular simulation producing 6-*s*-*cis* and 6-*s*-*trans* forms of the vitamin D sterols (VDS), both of which can take on a linear overall molecular geometry (Fig. 2B). Given CM, BDC and DIDS are all relatively rigid molecules 100 different starting conformational isomers were created using the BEST algorithm of Discovery studio 2.0 (DS 2.0). These ligand sets were docked to

the proposed VDR alternative pocket (VDR-AP) and VDR genomic pocket (VDR-GP, Fig. 1B) using the flexible docking protocol of DS 2.0 (see methods; Accelrys, San Diego, CA).

The flexible docking results are summarized in Table 1 ($\Delta G_{binding}$) and suggest 1,25D energetically prefers binding to the VDR-GP, consistent with our previous computational results (Mizwicki et al., 2004). The 1,25D and JN VDR-AP RMSD comparisons indicate they form similar molecular contacts and stabilize the same local VDR-AP conformation (Fig. 2C). This result is consistent with our previous reports where a more rudimentary and biased modeling (i.e. docking) approach was employed (Mizwicki et al., 2004). It is noted that the non-genomic antagonist, analog HL, was not observed to bind stably to the VDR-AP in our previous studies when HL was overlaid on 1,25D complexed to the VDR-AP to begin the computation (Mizwicki et al., 2004)(Mizwicki et al., 2005b). Comparison of the 1,25D and HL VDR-AP flexible docking results shows HL binds the VDR-AP with a different shape, makes unique molecular contacts and globally stabilizes a different VDR-AP local conformation (Table 1 and Fig. 2D). Thus these results support the hypothesis that HL is a competitive inhibitor of 1,25D/VDR-AP propagated non-genomic responses.

The curcuminoid flexible docking results demonstrate that they energetically prefer the VDR-AP (Table 1). For CM the VDR-AP flexible docking results are qualitatively the same when unionized and ionized forms are used to start the calculations, this is not the case when CM was docked to the VDR-GP (Table 1). Only the ionized form of BDC was assayed and like CM (ionized) did not bind favorably to the VDR-GP (Table 1). The most favored (i.e. lowest $\Delta G_{binding}$ value) CM and BDC VDR-AP complexes indicate the phenolic hydroxyl groups of CM and BDC form H-bonds/ salt bridges with the VDR-AP R158 and R274 residues among others (Fig. 2E). The R158 and R274 residues have previously been proposed to form H-bonds with the

25-OH and 3-OH groups of the non-genomic agonists 1,25D and JN in the VDR-AP (Mizwicki et al., 2004). Perphaps most importantly the most abundant, linear and more stable enol form of CM and BDC (see above) is favored by the VDR-AP. In addition,,both CM and BDC form extensive π-π interactions with aromatic R-groups that line the center of the VDR-AP (Fig. 2E). Unlike CM and BDC, when DIDS was flexibly docked to the VDR-AP and VDR-GP a strong selectivity towards favoring the VDR-GP was observed, for even when the VDR-AP site sphere was used (see methods), DIDS favored binding the VDR-GP (supplemental Fig. 2D).

The CM and BDC VDR-GP flexible docking calculations indicate that the unionized forms of both CM and BDC can bind stably to the VDR-GP (supplemental Fig. 2E); however, when ionized the curcuminoid VDR-GP complex becomes unstable (Table 1).

CM and BDC potentiate Cl^- currents at depolarizing potentials in TM4 Sertoli cells

It has been demonstrated that 1,25D potentiates outwardly rectifying voltage-dependent Cl^- currents upon depolarization in TM4 Sertoli cells (Menegaz et al., 2008). Figure 3A shows the dose-dependence stimulatory effect of CM on whole cell Cl^- currents in TM4 Sertoli cells. Non-inactivating outwardly rectifying currents were recorded at positive membrane potentials between 0 and 80 mV, from a holding potential of -30 mV. Addition of increasing concentrations of CM (0.1; 1; 10; 20 and 50 μM) to the external bath promoted a significant increase in outward current amplitudes within ~10 minutes for the higher concentrations of CM (20 and 50 μM) and ~20 minutes for the lower concentrations of CM (0,1 and 1 μM). The concentration of 10 μM showed the stimulatory effect between 15 and 20 minutes. Next we studied the dose-dependence of BDC in the activation of chloride currents in TM4 cells. The exact same stimulatory profile was obtained for BDC (0.1; 1; 10 and 20 μM, Fig. 3B). Importantly, CM and BDC are more

potent agonists than 1,25D in this system [see (Menegaz et al., 2008) and Fig. 4] and are commonly used at concentrations ≥ 10 μM concentrations when being assayed.

Effect of HL and DIDS on the stimulatory action of CM

It has been demonstrated that 1β,25$(OH)_2$-vitamin D_3 (HL, Fig. 1A) and DIDS block the 1,25D stimulatory effect on Cl^- currents in TM4 Sertoli cells (Menegaz et al., 2008). Equal molar concentrations of HL are known to potently antagonize virtually all reported 1,25D non-genomic responses, even though HL is a poor VDR binder when compared to 1,25D (RCI ~ 1.0, see Table 1 legend)(Mizwicki et al., 2004). When 0.1, 1, 10 and 20 μM CM was added to the bath solution in the presence of equal molar HL, HL functioned as an antagonist at the concentrations of 0.1 and 1 μM; however, HL was not able to inhibit the stimulatory effect of 10 and 20 μM CM (Fig. 4A). Note that even though HL was able to block the stimulatory effect of low CM concentrations, equal molar HL was not as an effective antagonist when compared its' effect on the 1,25D stimulatory effect (Fig. 4A and see (Menegaz et al., 2008)). These results point to a CM effect being propagated by signal transduction cascades both similar to 1,25D (≤ 1 nM) and unique (≥ 10 nM). Interestingly, the CM concentration threshold required to switch to the novel CM activation pathway is equivalent to the measured CM/ VDR mLBD IC_{50} (Table 1).

Next, to test if CM-sensitive-outward currents in TM4 cells are due to the activity of voltage-dependent chloride channels, 200 μM of DIDS was added to the bath after the observed CM stimulatory effect (Fig. 4B)**.** CM potentiated currents were blocked with the addition of DIDS only after the observed stimulation by 1 μM CM. The highest concentration of CM (20 uM) was insensitive to the presence of DIDS (50 and 200 μM). In fact, the CM effect was observed to be enhanced by addition of 50 and 200 μM DIDS (Fig. 4B).

Effect of PKI and CFTRinh-172 on the stimulatory action of CM on the Cl^- currents

In our previous study we demonstrated that PKA was involved in the signal transduction pathway triggered by 1,25D in TM4 cells regulating Cl^- channel gating and ultimately exocytosis (Menegaz et al., 2008). To verify the role of PKA in the CM and BDC Cl^- channel stimulation, PKI, the endogenous PKA blocker, was used. Fifteen minutes pre-incubation of the TM4 cells with PKI (1 μM) inhibited the stimulatory effect of 1 μM CM and BDC, but like HL, PKI was unable to block the stimulation by 10 and 20 μM (Figs. 5A and 5B). The inability of HL and PKI to block the outwardly rectifying voltage-dependent Cl^- currents in the presence of ≥ 10 μM CM and BDC is suggestive that these molecules bind other receptor molecules when present at saturating VDR concentrations.

Multiple independent works propose CM binds specifically to and stimulates the opening of the CFTR Cl^- channel by stabilizing the opened conformation of the CFTR (Berger et al., 2005). To test whether CM-sensitive-outward currents observed in the TM4 cells are due in part to the activity of CFTR chloride channels, CFTRinh-172, a specific CFTR channel blocker was used. Co-incubation of CM with the CFTRinh-172 compound in different equal molar concentrations (1, 10 and 20 uM) indicate the blocker is not able to inhibit the CM stimulatory effect (Fig. 6A). Alternatively, when the TM4 cells were pre-incubated with the CFTRinh-172 for 15 minutes the stimulatory effect of CM was completely blocked at all concentrations (Fig. 6B). The CFTRinh-172 results therefore support previous reports that show that the CFTR can regulate CLC activity (Gentzsch et al., 2003) and suggest the VDR lies upstream of the CFTR in the non-genomic pathway involving the VDR. The later is based on the preliminary evidence that CFTRinh-172 blocks 1,25D activation of outwardly rectifying voltage-dependent Cl^- currents in TM4 cells (data not shown).

CM and BDC compounds trigger secretory activities in TM4 Sertoli cells.

Previous eletrophysiological studies have shown that 1,25D treatment of osteoblasts (ROS 17/2.8 cells) causes a significant increase in cell capacitance, which is a measure of exocytosis in real time (Zanello and Norman, 2004). We recently demonstrated that 1,25D stimulates exocytotic events in TM4 Sertoli cells, as revealed by continuous recording of the whole-cell capacitance (Menegaz et al., 2008). After addition of 1 μM CM and BDC the capacitance of the cell increased approximately 3-fold in TM4 Sertoli cells (Fig. 7).

Discussion

Collectively the curcuminoid results indicate that CM and BDC stimulate chloride uptake in TM4 cells via two mechanisms that are dependent upon the CFTR and the VDR (Fig. 8). The dependence on VDR is supported by the CM and BDC binding and computational results (Figs. 1 and 2; Table 1) and is consistent with the following tenants of the vitamin D sterol VDR conformational ensemble model (Mizwicki et al., 2007): a) the ability to bind the VDR and form a stable VDR-AP complex (Table 1); b) a planar molecular geometry when complexed to the VDR-AP opened conformation [Figs. 2C-2E, see Fig. 8 legend and (Mizwicki et al., 2005a)]; and c) the ability of the response to be blocked by equal molar HL (Fig. 4A)(Mizwicki et al., 2004)(Norman et al., 2004). Exactly how VDR is localized to the plasma membrane remains unclear; however, all indications are that it is localized via non-covalent interaction with scaffolding proteins known to localize to lipid rafts (data not shown).

The novel, flexible docking, modeling protocol employed herein was shown to be consistent with our previous 1,25D, JN and HL mechanistic arguments used to understand the functional regulation of chloride channels in untransfected ROS 17/2.8 cells (Mizwicki et al., 2004). The CM and BDC flexible docking indicated that both energetically prefer the VDR-AP.

Alternatively, the DIDS/ VDR mLBD flexible docking results clearly indicate DIDS VDR-GP selectivitly. The DIDS/VDR mLBD (aa118-427; Δ165-215) IC_{50} value was observed to be 10 μM; therefore, it can be argued that 50-200 μM DIDS [i.e. working concentrations (Gatto et al., 1997)(Matulef et al., 2008)] may in part block anion currents via sterically blocking the VDR-AP (Fig. 2F and Fig. 8)

The whole cell patch data show that downstream of the VDR-AP/ agonist complex is PKA, because the agonist profile of CM and BDC (≤ 1 μM) can be attenuated by PKI, consistent with the mechanistic criteria elucidated for 1,25D in this same system (Menegaz et al., 2008). PKA is a know activator of CFTR channels (Wang et al., 2007); therefore, CFTR is placed downstream of PKA and upstream of voltage-gated chloride channels (CLC) in our model (Fig. 8). This outlines our current knowledge of the VDR/ PKA dependent pathway activated by VDS and ≤ 1 μM curcuminoid addition to TM4 cell bath.

The second pathway, activated by ≥ VDR mLBD IC_{50} concentrations of the curcuminoids, seems to be consistent with CM and BDC binding directly to the CFTR. This hypothesis is supported by the non-competitive mode of inhibition by CFTRinh-172 (Fig. 6) and the fact that this CFTR specific inhibitor was the only inhibitor used in this study which was capable of inhibiting all concentrations of the curcuminoids. This hypothesis is not novel, it has been previously demonstrated that CM (10 μM) activation on CFTR channels in cultured HeLa cells was consistent with CM potentially binding directly to the CFTR (Berger et al., 2005). Yet another report where cystic fibrosis CFTR mutants were transfected into HEK293T cells suggests CM binds directly to the CFTR (Wang et al., 2007)**.** Both of these reports provided evidence that CM activation of CFTR channels was dependent of phosphorylation of the R domain by PKA. Again it was revealed that 15 minutes pre-incubation with PKI (PKA inhibitor),

nullified the chloride channel stimulatory function only when CM or BDC was added in the lowest concentration studied (1 μM, Fig. 5). These results indicate that curcuminoids (≥ 10 μM) are capable of stimulating CFTR channels in a PKA independent manner in TM4 cells. CFTR channel opening typically requires phosphorylation of the R domain and ATP binding to both nucleotide binding domains (NBDs) (Gadsby and Nairn, 1999). Given voltage gated CLC channels (Jentsch, 2008) also contain NBD domains, it is possible the curcuminoids can bind directly to them and stabilize the opened state of these channels as well (Fig. 8). Preliminary modeling results using CM and CLC-5 (Meyer et al., 2007) support the latter possibility (data not shown). Based on the channel sensitivity to DIDS observed herein, the exocytotic response upon addition of 1,25D or the curcuminoids, the likely voltage-gated chloride channel sensitive to VDR/PKA/CFTR regulation is CLC-3 (Menegaz et al., 2008). It is noted that CFTR co-localization and regulation of CLC-3B, a splice variant of CLC-3, function has been previously documented (Gentzsch et al., 2003)(Ogura et al., 2002). A unique feature of CM, BDC and DIDS regulation of chloride channel signaling through the CFTR pathway is that when DIDS is added to the bath following activation by CM or BDC an enhancement of the curcuminoid response is observed (Fig. 4B). According to our model under these conditions DIDS is being added to a highly functional pathway insensitive to HL and therefore VDR. Thus CFTR activation may serve to turn off pathway one (i.e. regulation of CLC-3 by the VDR) through its own allosteric changes and/or subsequent alteration in its protein-protein interactions. Thus addition of DIDS could block the ability of the curcuminoids to bind the VDR allowing for more free curcuminoid to directly bind and stimulate the CFTR (Fig. 8). Alternatively or in addition too, the irreversible binding of curcuminoids to thioreductase (Fang et al., 2005)} and/or other proposed binding proteins could alter the cytosolic redox state and/or kinase/ phosphatase

activity profile to allow DIDS to function as a mild agonist. Lastly, given DIDS has been reported to be a K-channel agonist (Abitbol et al., 1999) it is possible under these conditions that stimulating these channels could induce an enhancement in the signal.

SUMMARY:

The global physiological response to 1,25D and the curcuminoids is an increase of the capacitance of the cell after chloride influx. An increase in cell capacitance indicates exocytosis and perhaps links these small molecules to regulating the secretion of ions, proteins and/or growth factors important to germ cells and male reproduction. One of the most important functions of Sertoli cells to create and maintain this microenvironment is the control of ionic fluid. It has been suggested that alterations in this microenvironment maintained by Sertoli cells, which can occur through mutations in the CFTR chloride channels, could result in the abnormal production of germ cells. CFTR mutations are related with different reproductive conditions in normal patients, without CF. Infertile people with reproductive structures anormalities showed the expression of CFTR gene mutant. Additional studies suggest that mutations in the CFTR chloride channels have an important influence in the unclear cause of male infertility (Patrizio et al., 1993) Van der Ven et al. (van, V et al., 1996) revealed that 17.5 % of men with infertility have a mutation in the CFTR gene. Thus the data obtained herein could lead to the development of new substances and/ or therapeutic/ preventative treatments for infertility in males with or without CF. To this end, we are currently evaluating other novel VDR ligands using the methodology outlined herein and designing experiments directed at obtaining a more detailed understanding underlying the mode of VDR membrane localization, the detailed kinase/ phosphatase cascades being modulated by these ligands and the protein-protein interactions involved in the two pathways outlined herein.

Complex	RCI (%)	IC_{50}	VDR-GP (kcal/mol)	VDR-AP (kcal/mol)
1,25D-cVDR	100	0.85 nM	ND	ND
CM-cVDR	0.0023	17 μM	ND	ND
LCA-cVDR	0.0019	73 μM	ND	ND
1,25D-mLBD	100	7.7 nM	-50.7	-40.7
CM-mLBD	0.12	3.8 μM	92.0 *-21.5*	-24.3 *-26.0*
BDC-mLBD	0.14	3.3 μM	37.1	-45.7
DIDS-mLBD	0.056	8.5 μM	Favored	---
LCA-mLBD	ND	ND	-8.70	-42.0

Table 1:Summary of VDR binding results. The complex column provides the ligand (see Fig. 1A for structures) protein [cVDR = chick intestinal homogenate; mLBD = VDR x-ray construct (aa118-427; Δ165-215)] complex being studied. The relative competitive index (RCI) represents the ability of the cold ligand to compete off [^{3}H]-1,25D as a percentage, where 1,25D is set to 100% (see methods for RCI derivation). The IC_{50} column provides the concentration of cold ligand required to compete off 50% of the [^{3}H]-1,25D. The RCI and IC_{50} values provided are the average of at least three independent experiments. The VDR-AP and VDR-GP columns summarize the flexible docking results obtained when 1,25D, CM, BDC, DIDS and LCA were computationally docked to either pocket (see methods). The values in these two columns represent the average Gibbs free energy of binding($\Delta G_{binding} = \Delta G_{holo\text{-}VDR} - \Delta G_{apo\text{-}VDR} - \Delta G_{ligand}$)for the top five complexes, in kcal/mol; therefore, the more negative the value the more stable the complex is. For CM-mLBD the average $\Delta G_{binding}$ value that is italic was obtained by averaging the top five unionized CM-mLBD. Ionized CM and BDC-mLBD complexes are represented in normal type setting. Lastly, DIDS $\Delta G_{binding}$ values could not be obtained due to the inability of the DS2.0 software to accurately type the thiocyano functionality. Nonetheless, DIDS strongly favored the VDR-GP when docked to either the VDR-AP or VDR-GP and indicated in the table.

Legends

Fig. 1. Ligand structure, volume and shape and the VDR two pocket molecular model. A) The chemical structures of the curcuminoids, curcumin (CM) and bisdemethoxycurcumin (BDC); the known voltage gated chloride channel agonists 1α,25$(OH)_2$-vitamin D_3 (1,25D) and 1α,25$(OH)_2$-lumisterol D_3 (JN); and the known voltage gated chloride channel antagonists 1β,25$(OH)_2$-vitamin D_3 (HL) and 4,4'- Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). The molecular volume of the lowest energy form of these ligands is provided (see methods). In addition the CM and BDC structures are drawn in their keto and enol tautomer forms respectively. **B)** The molecular shape of the lowest energy enol (planar, flat) and keto (bowl shaped) tautomers are shown. **C)** Three dimensional model of the VDR two ligand binding pockets (VDR-GP and VDR-AP) and their A-ring domain overlap (transparent oval). The reader is referred to other works for a detailed molecular description of these two

ligand binding sites (Rumbeiha et al., 2000). In the figure, the VDR tube ribbon is colored to demonstrate the hydrophobicity of the R-group eminating from the respective backbone positions (red = polar; blue = non-polar; pink/ white = intermediate). This demonstrates that the VDR-AP contains more hydrophilic molecular character when compared with the VDR-GP.

Fig. 2. 1,25D conformational search calculations and flexible docking of 1,25D, JN, HL, CM, BDC and DIDS to the VDR mLBD (aa118-427; Δ165-215). A) The global energy minimum conformation of 1,25D predicted using PC_Model v8.0 GMMX conformational search calculations (see methods). The conformational isomer has a β-chair A-ring, 6-*s*-*trans* seco-B-ring conformation and Pop. A side-chain configuration [see (Mizwicki and Norman, 2003) for a more detailed description of vitamin D sterol conformational dynamics]. Carbons are gray, oxygen, red and hydrogen, white in the ball and stick model. **B)** Highlights the different 1,25D conformational isomers that bind preferentially to the VDR-AP and VDR-GP respectively. The VDR-AP accepts multiple A-ring conformers (* indicates half-chairs and boat A-ring conformers also bind favorably, using the flexible docking protocol outlined in the methods section) that have an overall linear geometry. The VDR-GP accepts only a bowl-shaped 1,25D conformer with a β-chair A-ring configuration. In the VDR-GP ½-chairs, boat and α-chair A-ring conformers are significantly less stable when compared to the β-chair conformer. In addition, in order for a 1,25D molecule to form a stable VDR-GP complex it must have a 6-*s*-*trans* seco-B-ring geometry, consistent with previous structure function analyses (Mizwicki et al., 2004). It is noted that both Pop. A and Pop. B side-chain conformers bind favorably to the VDR-GP using this methodology (data presented elsewhere). **C)** Backbone atom superimposition of the best Pop. A, 6-*s*-*cis*, β-chair 1,25D/ VDR-AP and best JN/ VDR-AP complexes [VDR-AP flexible docking residues RMSD = 1.383 (side-chain); 1.069 Å (all atom)]. The figure depicts the VDR-AP rendered to show the tube structure of amino acids forming the VDR-AP surface. H305 and H397 are shown where the end of the VDR-GP is located with respect to the VDR-AP (see Fig. 1C). The JN structure is rendered in ball and stick with carbons, orange; and oxygens, red. Hydrogens have been hidden for clarity. The A-ring H-bonds formed with S237 (2.83 Å) and R274 (2.74 Å) and the 25-OH H-bond with R158 (2.70 Å) are indicated in the figure by solid green lines. The 1,25D structure is shown in wireframe with its vdW surface depicted as a transparent surface colored to indicate the relative electrostatic surface potential of the molecule (white, non-polar; red, polar negative; blue, polar positive). Note, the VDR amino acids are only shown for the JN complex to simplify the diagram and virtually all atoms of JN lie inside the surface of 1,25D. **D)** Backbone atom superimposition of the best Pop. A, 6-*s*-*trans*, β-chair 1,25D/ VDR-AP (green, red and blue) and Pop. C, 6-*s*-*trans*, β-chair HL/ VDR-AP (pink, red and blue) complexes (VDR-AP flexible docking residue RMSD = 1.528 (side-chain); 1.196 Å (all atom)]. H-bond interactions formed by 1,25D are indicated by orange solid lines and those formed by HL are indicated by sky blue solid lines. HL does not form an H-bond with S237, has a different global geometry when compared to 1,25D and stabilizes a different local VDR-AP configuration of the β-sheet residues (e.g. W286 and Y295) when compared to 1,25D. HL is also stuck in the β-chair configuration due to an intramolecular H-bond formed by the 1,3-diaxial diol [see (Mizwicki et al., 2005b) for more details]. **E)** The best CM/ VDR-AP complex determined using the flexible docking protocol (see methods). The symmetric CM molecule forms strong H-bond/ salt-bridge interactions with R274 (2.81 Å) and R158 (2.57 Å) and the enol hydroxyl can form an H-bond with the backbone carbonyl oxygen of C288 (2.76 Å). These interactions are highlighted by green solid lines in the diagram. For an overlay of 1,25D, CM and/or BDC please see supplemental Figs. 2A-2C and (Masoumi et al., 2009). For an overlay of the most favored unionized CM/ VDR-GP and 1,25D/ VDR-GP complexes refer to supplemental Fig. 2D).

Fig. 3. Dose-dependence of CM and BDC-promoted outward currents at depolarizing potentials in TM4 Sertoli cells. A) current-voltage relations and raw current traces obtained from individual TM4 cells bathed in Cl^- containing recording solutions (see Materials and Methods). Current amplitude values normalized to cell size (pA/pF) are shown for control conditions before treatment. The dose-response of CM-promoted outward currents was demonstrated in the concentrations of 0.1; 1; 10; 20 and 50 μM. Currents were activated by 100 ms depolarizing voltage steps between -60 through 80 mV from a holding potential of - 30 mV. **B)** Whole cell Cl^- current amplitude values (pA/pF) measured at a depolarizing potential of 80 mV after the addition of different BDC concentrations (0.1; 1; 10 and 20), compared to control current amplitudes obtained in the absence of the hormone. Means ± SEM for n = 6 - 10 individual cells. *p<0,05; **p<0,01; ***p< 0,001 compared to control;

Fig. 4. Effect of DIDS and HL blockers on CM-sensitive Cl^- currents in TM4 Sertoli cells. A) current-voltage relations obtained from individual TM4 cells bathed in Cl^- containing recording solutions (see Materials and Methods).Currents were activated by 100 ms depolarizing voltage steps to between -60 and 80mV from a holding potential of -30mV. Whole cell Cl^- current amplitude values (pA/pF) was showed at a depolarizing potential of 80 mV. 1,25D-promoted outward currents was blocked when co-incubated in the presence of equal molar concentration of HL (1 nM). CM-promoted outward currents were significantly reduced when co-incubated in the presence of equal molar concentrations of HL (0.1 and 1 μM). CM-promoted outward chloride currents remained activated when co-incubated in the presence of equal molar concentrations of HL (10 and 20 μM). **B)** The vehicle (ethanol) had no effect in basal chloride currents. 1,25D-promoted outward currents were totally blocked by the subsequent addition of 200uM DIDS. The concentration of 1 μM CM-promoted outward chloride currents was inhibited by the subsequent addition of 200uM DIDS, however 20 uM of CM was not inhibited by DIDS (50 and 200 μM), which in both concentrations increased the stimulatory action of CM on chloride currents. Means ± SEM for n = 6 - 10 individual cells. *p<0,05; **p<0,01; ***p< 0,001 compared to control; #p< 0,05 to HL and DIDS inhibitory effect; δp<0,05; δδp<0,01 to DIDS additive effect.

Fig. 5. Effect of CFTRinh-172 blocker on CM- sensitive Cl^- currents in TM4 Sertoli cells. A) current-voltage relations obtained from individual TM4 cells bathed in Cl^- containing recording solutions (see Materials and Methods).Currents were activated by 100 ms depolarizing voltage steps to between -60 and 80mV from a holding potential of -30mV. Whole cell Cl^- current amplitude values (pA/pF) was showed at a depolarizing potential of 80 mV. CFTRinh-172 was not able to inhibit the CM-promoted outward currents when co-incubated in equal molar concentrations (1, 10 and 20 μM). **B)** CFTRinh-172 blocked CM-promoted outward currents when pre-incubated 15 minutes in equal molar concentrations (1, 10 and 20 μM). Means ± SEM for n = 6 - 10 individual cells. *p<0,05; ***p< 0,001 compared to control; ##p <0,01 compared to CM groups.

Fig. 6. Effect of PKI blocker on CM and BDC- sensitive Cl^- currents in TM4 Sertoli cells. A) current-voltage relations obtained from individual TM4 cells bathed in Cl^- containing recording solutions (see Materials and Methods).Currents were activated by 100 ms depolarizing voltage steps to between -60 and 80mV from a holding potential of -30mV. Whole cell Cl^- current amplitude values (pA/pF) was showed at a depolarizing potential of 80 mV. CM (1 μM) potentiated chloride currents was inhibited after 15 minutes pre-incubation with PKI (1 μM). PKI (1 μM), however, was not able to inhibit CM-promoted outward currents in the higher concentrations of CM (10 and 20 μM). **B)** BDC (1 μM) potentiated chloride currents was inhibited after 15 minutes pre-incubation with PKI (1 μM). However, PKI (1 μM), was not able to inhibit BDC-promoted outward currents in the higher concentrations of BDC (10 and 20 μM). The PKI inhibitory effect was similar for both curcuminoids. Means ± SEM for n = 6 - 10 individual cells. *p<0,05; ***p< 0,001 compared to control; #p <0,05 compared to CM and BDC groups.

Fig. 7. Whole-cell capacitance recordings obtained from a single TM4 Sertoli cell in the absence and in the presence of 1 nM 1,25D, 1 μM CM and 1 μM BDC added to the bath. Initial capacitance value of 52 pF increased to 56 pF for the 1,25D group and to 57 pF for the CM and BDC group, corresponding to the fusion of secretory granules to the plasma membrane (exocytotic events). This effect took place at approximately 20 minutes with CM and BDC incubation. Means ± SEM for n = 3 individual cells. ***p< 0,001 compared with control.

Fig. 8. Proposed model of 1,25D and CM non-genomic regulation of chloride currents in TM4 sertoli cells.

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Figure 1

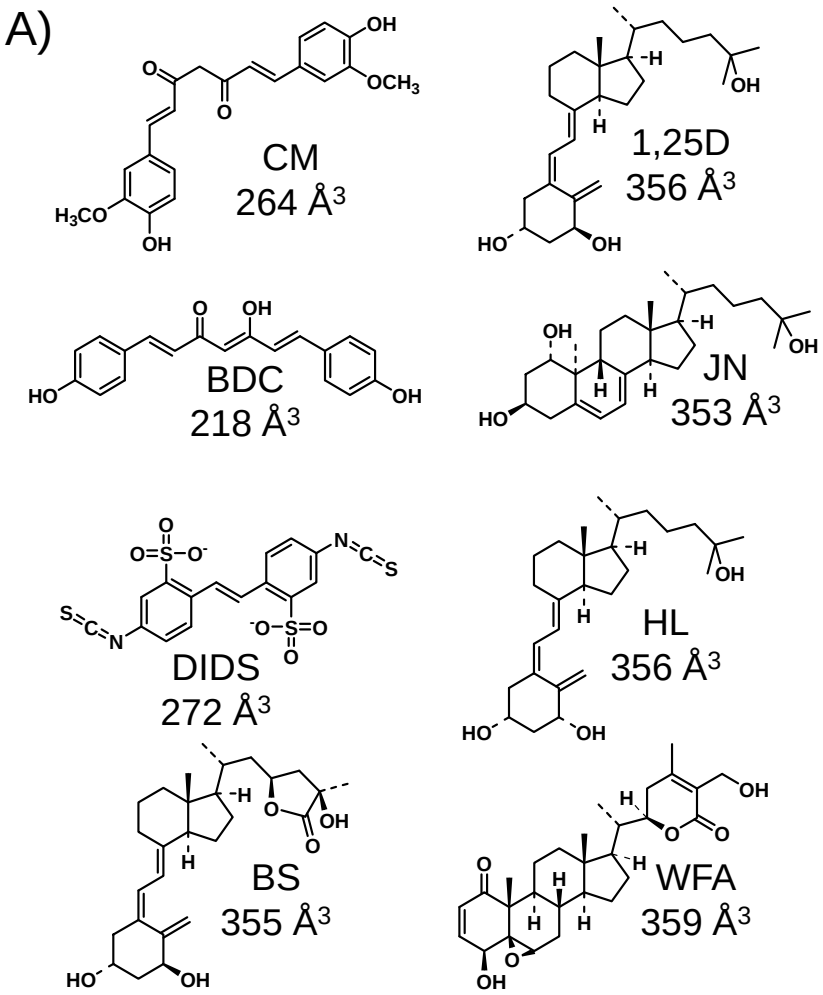
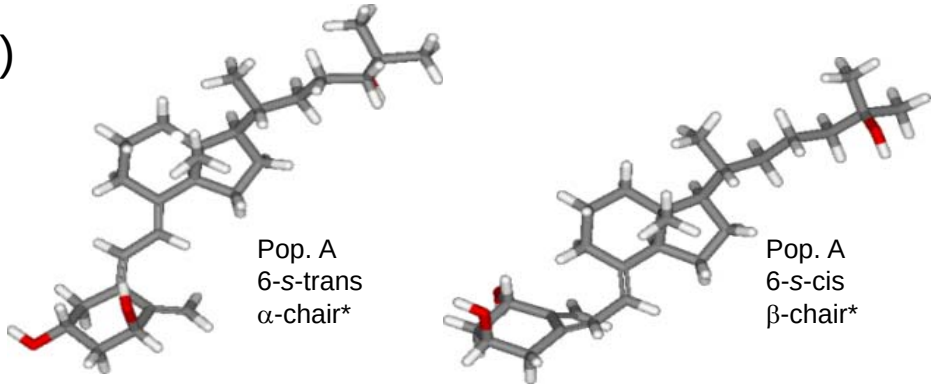
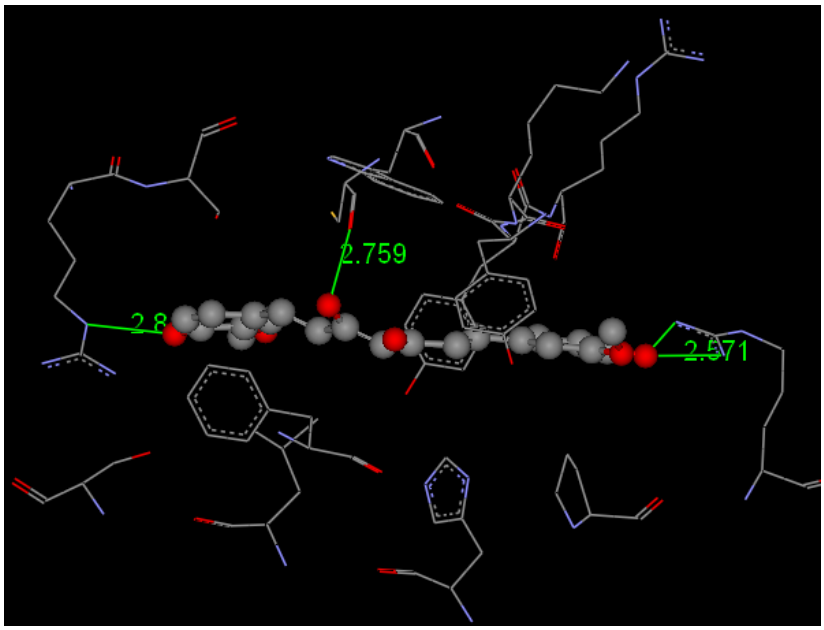

A)
CM
264 Å3
1,25D
356 Å3
BDC
218 Å3
JN
353 Å3
DIDS
272 Å3
HL
356 Å3
BS
355 Å3
WFA
359 Å3
B)

Figure 2

A)


Pop. A
6-*s*-trans
α-chair*
Pop. A
6-*s*-cis
β-chair*

B)


2.759

C)

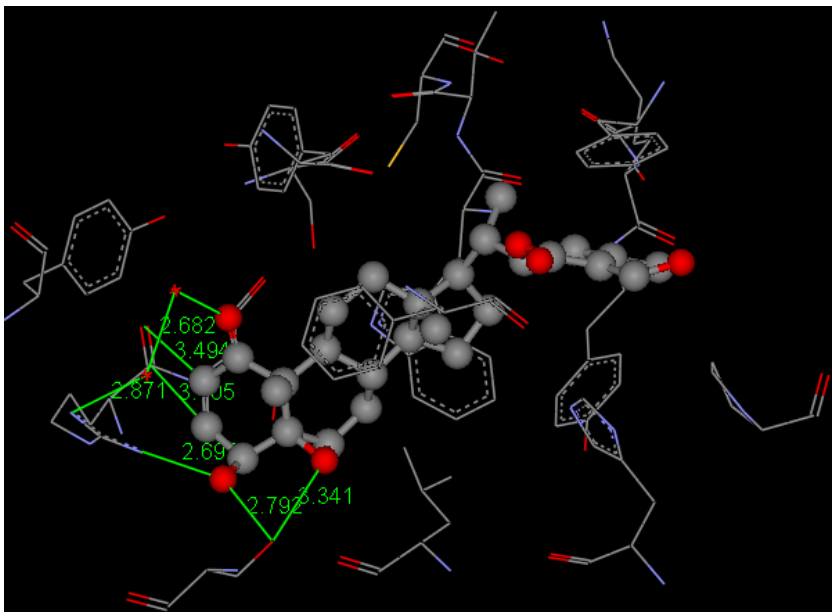
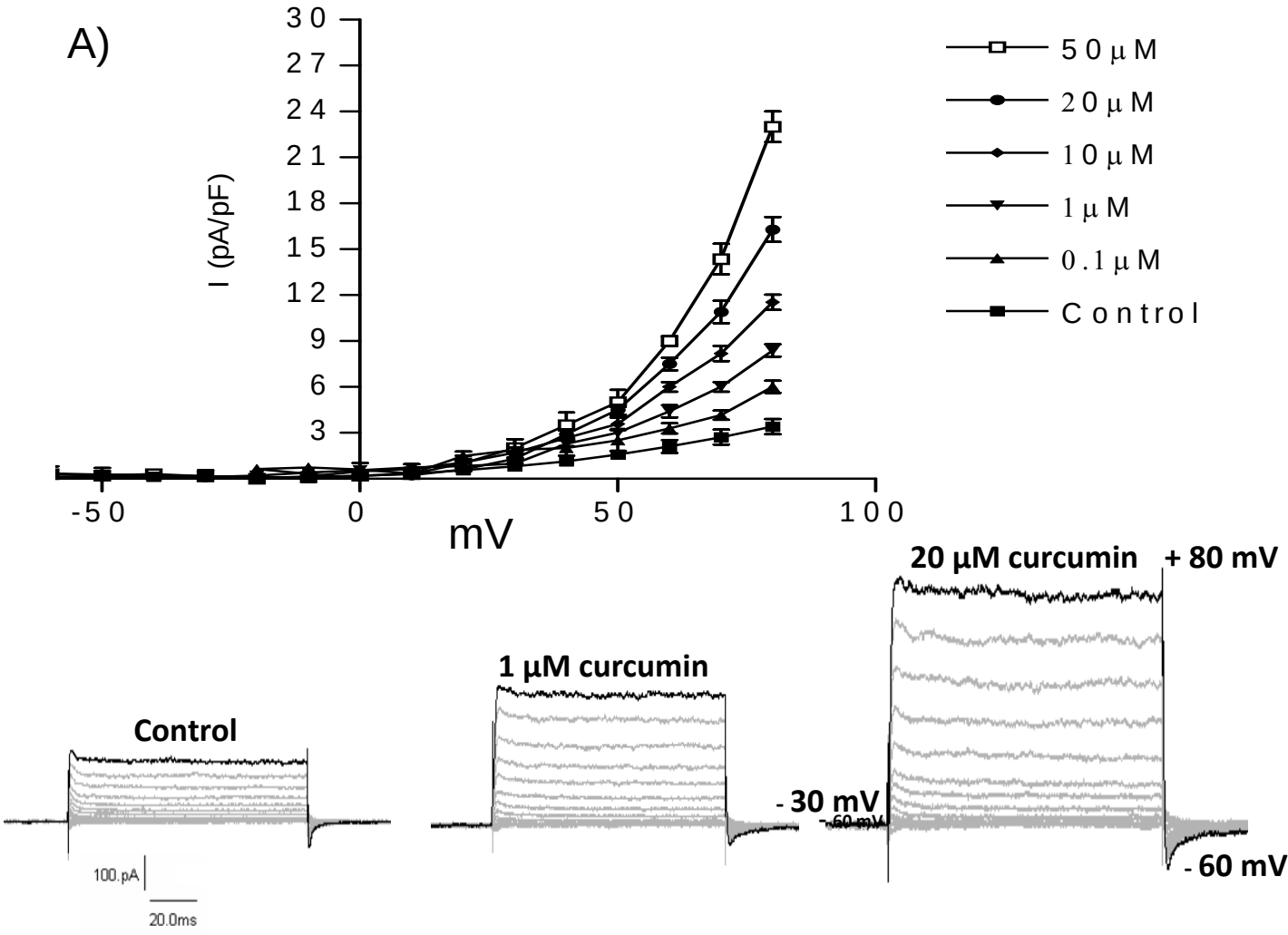

2.682
2.69
2.792
3.341

Figure 3


A)
30
27
24
21
18
15
12
9
6
3
I (pA/pF)
-50
0
50
100
mV
50 µM
20 µM
10 µM
1 µM
0.1 µM
Control
Control
1 µM curcumin
20 µM curcumin
+ 80 mV
- 30 mV
- 60 mV
100 pA
20.0ms

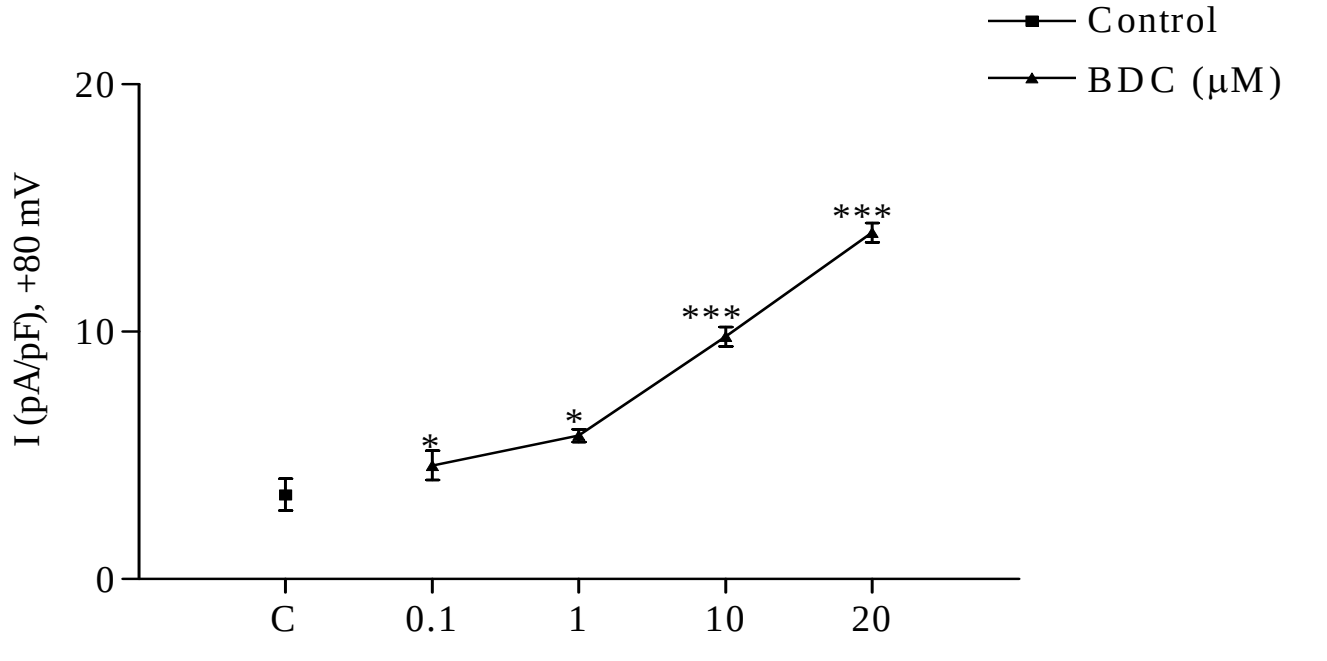
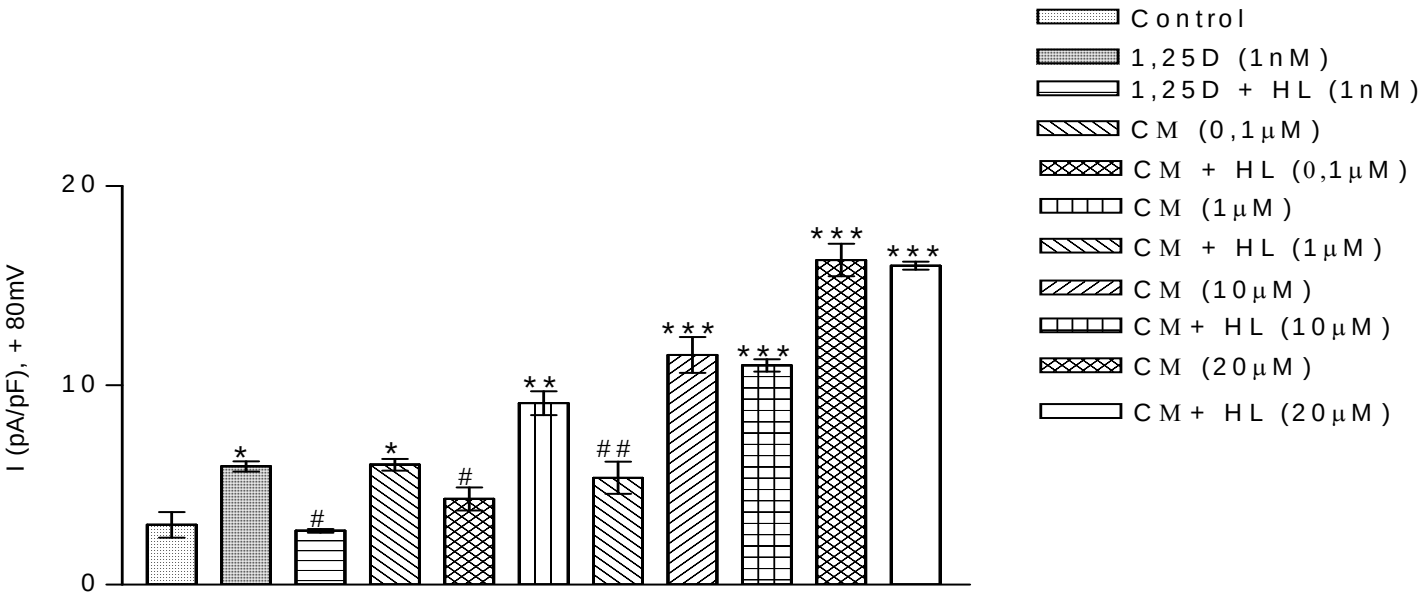

B)
Control
BDC (µM)
20
10
0
I (pA/pF), +80 mV
C
0.1
1
10
20
*
*
***
***

Figure 4

A)


Control
1,25D (1nM)
1,25D + HL (1nM)
CM (0,1μM)
CM + HL (0,1μM)
CM (1μM)
CM + HL (1μM)
CM (10μM)
CM+ HL (10μM)
CM (20μM)
CM+ HL (20μM)
I (pA/pF), + 80mV
20
10
0

B)

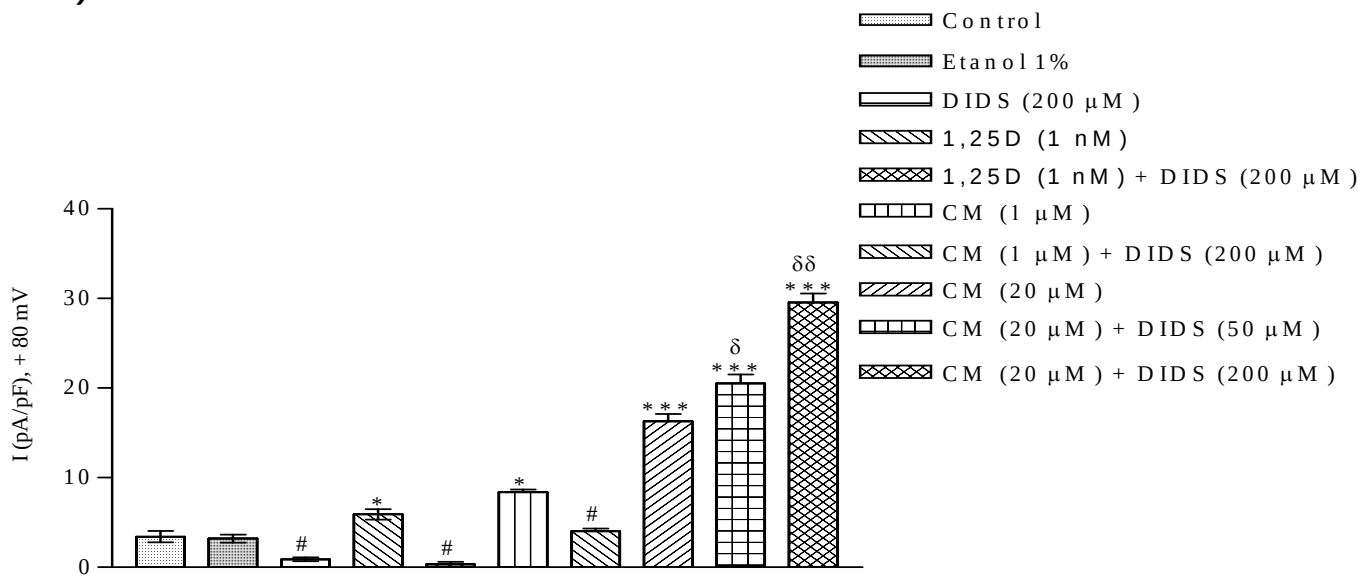

Control
Etanol 1%
DIDS (200 μM)
1,25D (1 nM)
1,25D (1 nM) + DIDS (200 μM)
CM (1 μM)
CM (1 μM) + DIDS (200 μM)
CM (20 μM)
CM (20 μM) + DIDS (50 μM)
CM (20 μM) + DIDS (200 μM)
I (pA/pF), + 80 mV
40
30
20
10
0

Figure 5

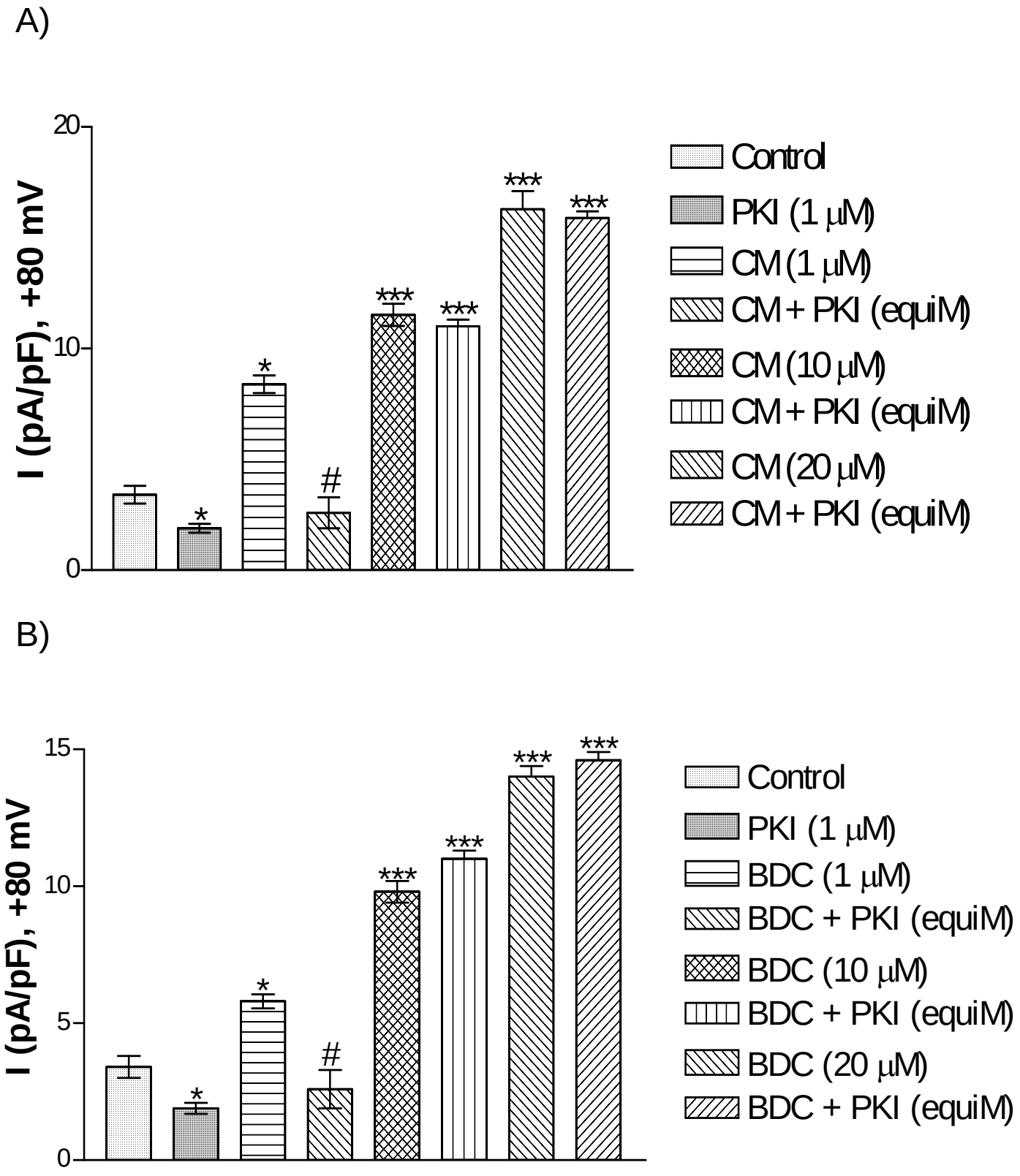
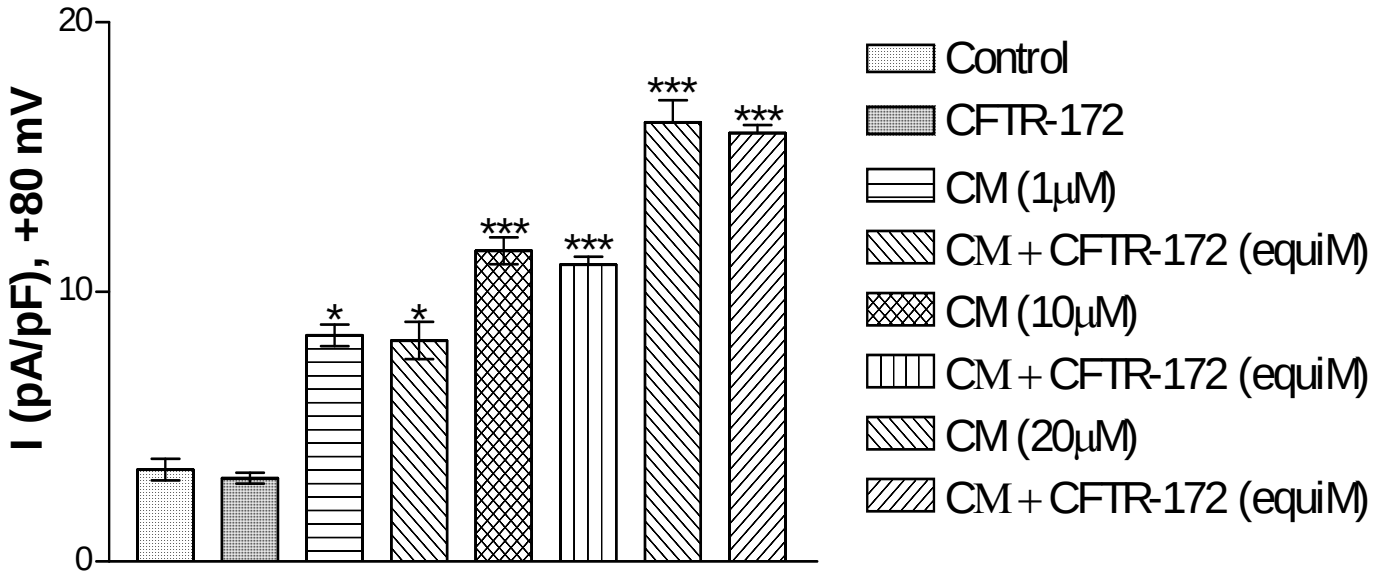

A)
I (pA/pF), +80 mV
20
10
0
*
*
#
***
***
***
***
Control
PKI (1 μM)
CM (1 μM)
CM + PKI (equiM)
CM (10 μM)
CM + PKI (equiM)
CM (20 μM)
CM + PKI (equiM)
B)
I (pA/pF), +80 mV
15
10
5
0
*
*
#
***
***
***
***
Control
PKI (1 μM)
BDC (1 μM)
BDC + PKI (equiM)
BDC (10 μM)
BDC + PKI (equiM)
BDC (20 μM)
BDC + PKI (equiM)

Figure 6

A) Co-incubation



B) 15 minutes pre-incubation

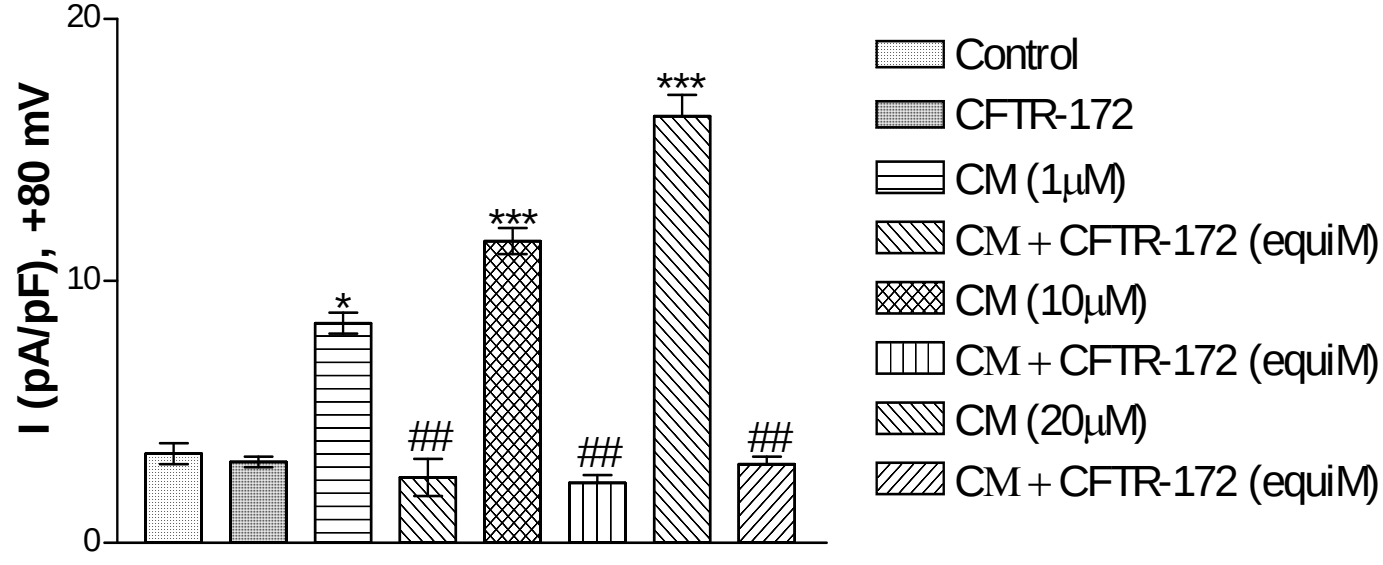


Figure 7

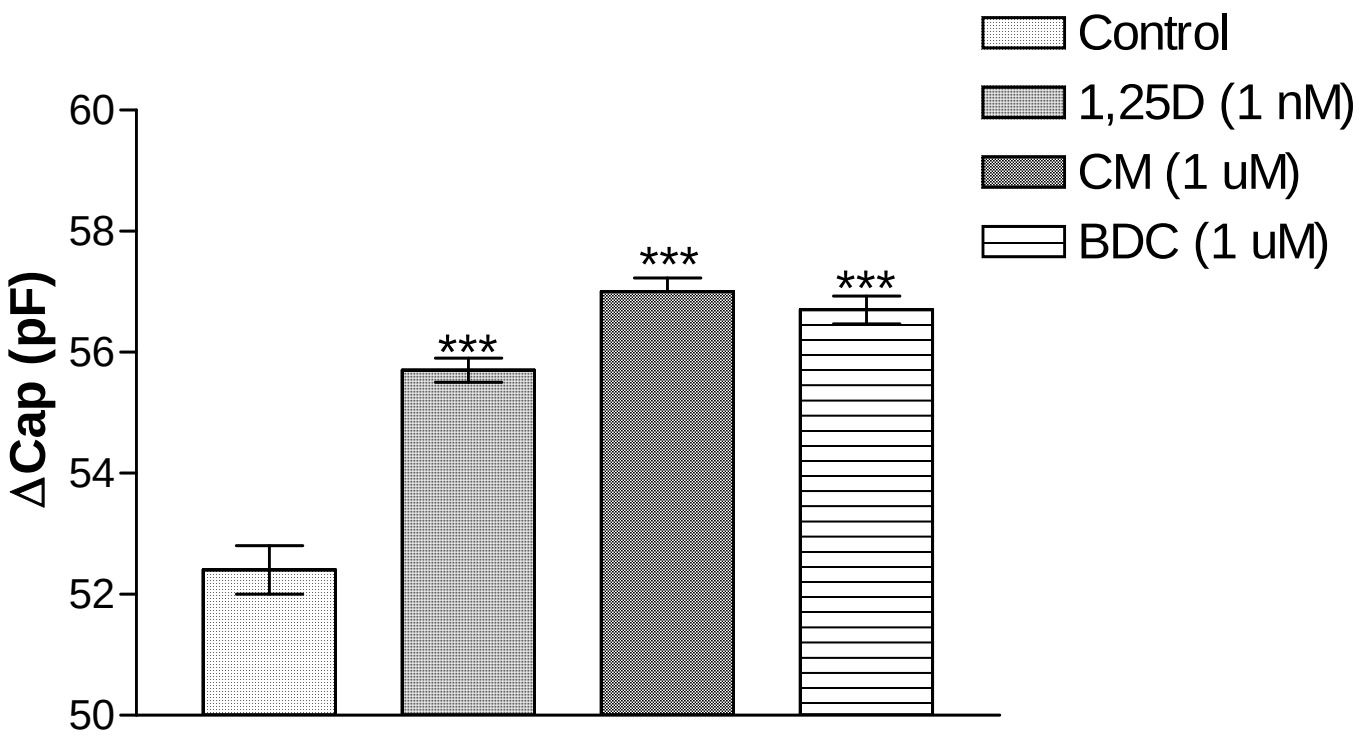
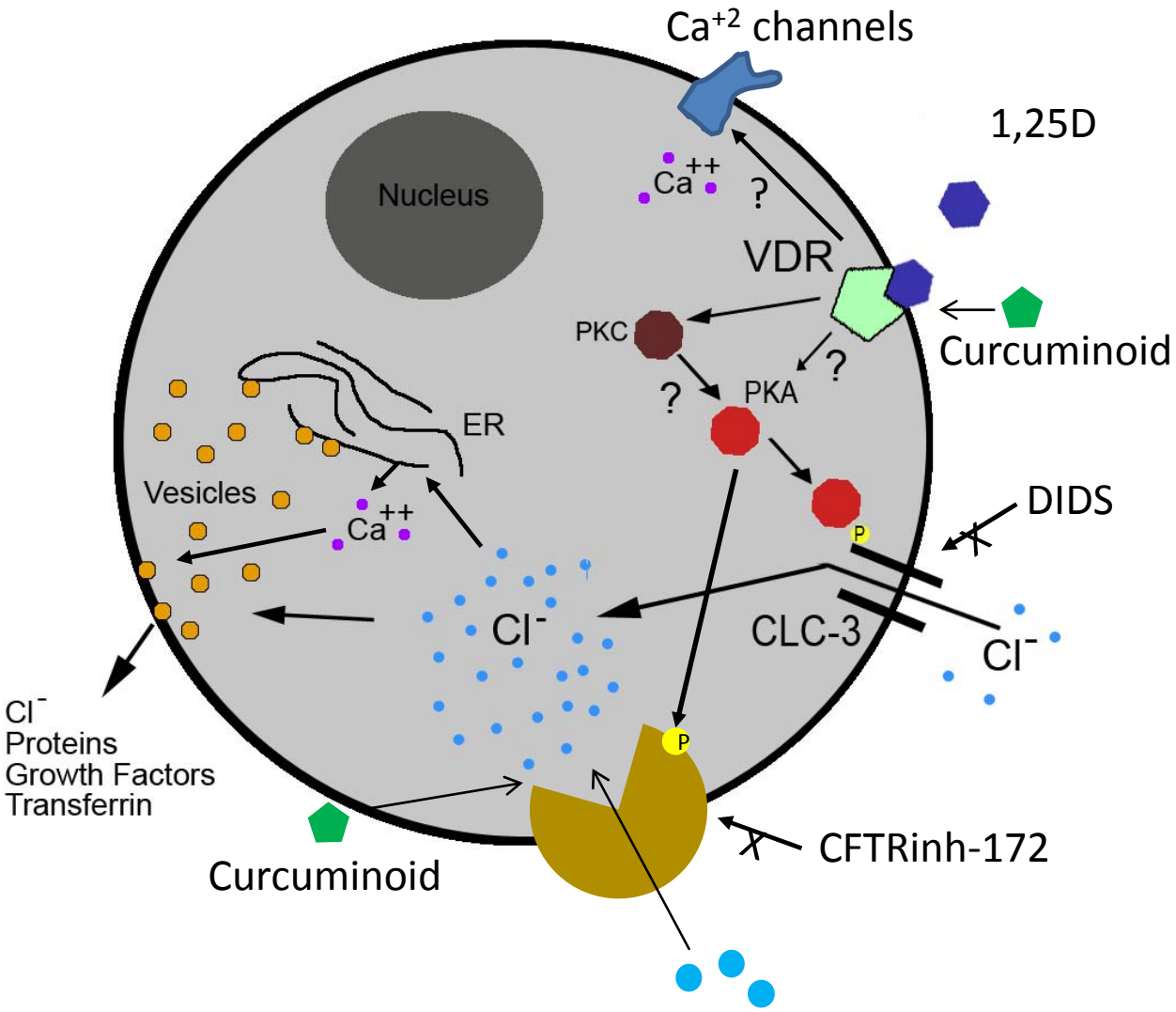


Figure 8


Ca+2 channels
1,25D
Nucleus
Ca++
?
VDR
PKC
Curcuminoid
?
?
PKA
ER
Vesicles
Ca++
DIDS
P
Cl-
CLC-3
Cl-
Cl-
Proteins
Growth Factors
Transferrin
P
CFTRinh-172
Curcuminoid

4 DISCUSSÃO

Muitas funções dos hormônios esteróides, vitamina D e hormônios da tireóide ocorrem na membrana plasmática, citoplasma e organelas intracelulares e podem resultar em respostas biológicas tanto independentes quanto dependentes de transcrição gênica e síntese de proteínas (BASSET et al., 2003; DAVIS et al., 2008). A elucidação de mecanismos alternativos de transdução de sinais para esses hormônios contribui na integração da fisiologia e patofisiologia da ação hormonal em células responsivas e pode levar ao desenvolvimento de novos tratamentos para inúmeras doenças celulares proliferativas, metabólicas, inflamatórias, reprodutivas, cardiovasculares e neurológicas (LEVIN, 2008).

Recentemente foi demonstrado que os hormônios T_4 e T_3 promovem ativação do transporte de aminoácidos por mecanismos particulares. Enquanto o efeito estimulatório do T_3 foi inibido na presença de cicloheximida, um inibidor de síntese de proteínas, o efeito estimulatório do T_4 permaneceu inalterado, confirmando uma ação não-genômica do transporte de aminoácidos regulado pelo T_4. Além disso, através da técnica eletrofisiológica de registro intracelular, foi demonstrado um efeito hiperpolarizante imediato do hormônio T_4 no potencial de membrana de células de Sertoli envolvendo canais de Ca^{2+} e K^+ (MENEGAZ et al., 2006). Conforme os resultados apresentados no item 3.1, no presente trabalho foram estudados os fatores que influenciam o efeito estimulatório não-genômico do T_4 no transporte de aminoácidos. Os resultados demonstraram que os canais de Ca^{2+} dependentes da voltagem, o cálcio extracelular e os canais de K^+_{ATP} são totalmente requeridos para o efeito do T_4 na membrana plasmática, enquanto os canais de Cl^- e os canais SK_{Ca} estão parcialmente envolvidos nesse mecanismo. A influência da PKC no mecanismo do T_4 sugere uma provável relação funcional dessa proteína na atividade de canais iônicos apontando o T_4 como um importante regulador hormonal das funções metabólicas testiculares iniciadas na membrana plasmática.

Segundo Davis et al., (2006), uma interação entre efeitos não-genômicos e genômicos dos hormônios tireoidianos são iniciados pela ação do T_4 num receptor de membrana integrina $\alpha_V\beta_3$ presente na superfície celular. Bergh et al. (2005), demonstraram que o hormônio T_4 possui uma maior afinidade para esse receptor integrina $\alpha_V\beta_3$ quando comparado

com o hormônio T_3. A partir da ligação nessa proteína de superfície celular, o T_4 ativa uma proteína serina-treonina cinase ativada por mitógeno (MAPK, ERK1/2) de uma forma mais potente que o T_3, resultando em transcrição gênica (DAVIS et al., 2000).

A influência dos canais de Ca^{2+} dependentes da voltagem e do Ca^{2+} externo para o estímulo do T_4 no transporte de aminoácidos demonstrados com o uso do verapamil e EGTA, respectivamente, corrobora com estudos anteriores onde foi verificado que a ação estimulatória do T_3 no transporte de aminoácidos, no potencial de membrana e na fosforilação da vimentina também é dependente de Ca^{2+} em testículos de ratos imaturos (VOLPATO et al., 2004, ZAMONER et al., 2005). Trabalhos anteriores realizados em testículos de ratos também demonstraram a importância do Ca^{2+} em sinalizações intracelulares desencadeadas pelo hormônio folículo estimulante (FSH) e pela testosterona na regulação da função testicular (WASSERMANN et al., 1992; 1993). É bem conhecido que o papel do Ca^{2+} é fundamental numa variedade de respostas fisiológicas, desde a ativação de proteínas transportadoras e segundos mensageiros até a ativação de vesículas secretórias (ZANELLO, NORMAN, 2006).

O requerimento parcial dos canais SK_{Ca} envolvidos na ação estimulatória do T_4 no transporte de aminoácidos corrobora com os dados demonstrados por Menegaz et al. (2006), onde o efeito hiperpolarizante do T_4 no potencial de membrana de células de Sertoli foi inibido na presença de apamina (bloqueador de canais SK_{Ca}). Uma vez que o aminoácido MeAIB é co-transportado com Na^+, foi levantada a hipótese de que o potencial hiperpolarizante da membrana facilitaria o efeito desencadeado pelo T_4. De acordo com Muyderman et al., (2001), o aumento do $[Ca^{2+}]_i$ aumenta a atividade dos canais SK_{Ca} desencadeando o efluxo de potássio e a hiperpolarização da membrana (VERGARA et al., 1998; MUYDERMAN et al., 2001).

A demonstração de que os canais de Cl^- são parcialmente requeridos para o estímulo do transporte de aminoácidos desencadeados pelo T_4 em testículos de ratos, sugere que o bloqueio da entrada de Cl^- na célula inibe pelo menos em parte a captação de Na^+-aminoácido. Menegaz et al., (2008), demonstrou que a 1,25D estimula a entrada de íons Cl^- em células de Sertoli em potenciais despolarizantes através dos canais de Cl^- dependentes da voltagem. Em constraste, esses canais não estão envolvidos na ação dos hormônios tireoidianos no estímulo da captação de Ca^{2+} em córtex cerebral de ratos (ZAMONER et al.,

2006b). O envolvimento dos canais de K^+_{ATP} na ação estimulatória do T_4 no transporte de aminoácidos reafirma trabalhos anteriores onde o efeito estimulatório do T_3 no transporte de aminoácidos e na hiperpolarização do potencial de membrana foi inibido na presença de tolbutamida (inibidor de K^+_{ATP}) (VOLPATO et al., 2004, MENEGAZ et al., 2006).

O tratamento de testículos de ratos imaturos na presença de um bloqueador de PKA não alterou a atividade estimulatória do T_4 no acúmulo de aminoácidos. Isso poderia ser explicado pelo fato de que o AMPc estimulou o transporte de aminoácidos e aumentou o efeito estimulatório do T_4, indicando diferentes caminhos de transdução de sinais dessas substâncias na ativação desse sistema. Outros trabalhos demonstram o envolvimento parcial da atividade da PKA no estímulo da captação de Ca^{2+} em córtex cerebral de ratos jovens (ZAMONER et al., 2006b). Além disso, Incerpi et al., (2005), reportou que os hormônios tireodianos inibem a enzima Na^+/K^+-ATPase na membrana plasmática de hepatócitos de pintos de uma forma dependente da PKC e da PKA, comprovada com o uso de bloqueadores específicos para essas proteínas. Dessa forma, o envolvimento do caminho da PKA no mecanismo de ação de hormônios varia em diferentes sistemas e necessita estudos adicionais.

O efeito estimulatório do T_4 no transporte de aminoácidos é dependente do caminho da PKC e esses resultados concordam com trabalhos anteriores que descrevem a ativação rápida dependente da PKC desencadeada pelos hormônios tireodianos em eritrócitos de coelhos e, mais recentemente em hepatócitos (LAWRENCE et al., 1989; KAVOK et al., 2001). Zamoner et al., (2007), verificaram que a ação não-genômica do T_3 e do T_4 no estímulo da captação de Ca^{2+} requer a atividade da PKC em córtex cerebral. Além disso, Zamoner et al., (2008), demonstraram a participação da fosfolipase C (PLC), da PKC, da MAPK, da proteína cinase dependente de Ca^{2+}/calmodulina e do Ca^{2+} extracelular na mediação de efeitos do T_4 no citoesqueleto de células neurais durante o desenvolvimento. Adicionalmente, Incerpi et al., (2005), demonstraram que os hormônios tireoidianos ativam não-genomicamente o sistema A de transporte de aminoácidos e o trocador Na^+/H^+ em hepatócitos de embrião de pintos através da atividade da PKC, a qual é ativada por diacilglicerol (DAG) a partir da PLC para produção de 1,4,5-inositoltrifosfato (IP_3) e mobilização de $[Ca^{2+}]_i$. Ainda, em células HeLa foi demonstrado que o efeito estimulatório do T_4 na indução antiviral do interferon δ (IFNδ) requer atividade da PLC e da PKC (LIN et al., 1999).

De um modo geral, esses resultados mostram um efeito não-genômico do T_4 no transporte de aminoácidos através do caminho da PKC e da atividade de diferentes canais iônicos na membrana plasmática de testículos de ratos imaturos e apontam o T_4 como um importante regulador hormonal da função testicular.

A vitamina D, através do metabólito ativo 1,25D, possui um papel crítico na reprodução masculina através da atividade mediada por receptor VDR (JOHNSON et al., 1996). De acordo com os resultados apresentados no item 3.2, o efeito estimulatório da 1,25D no transporte de aminoácidos foi mais evidente em testículos de ratos de 11 dias de idade. Por outro lado, a testosterona não alterou o transporte de aminoácidos tanto no estado basal quanto no estado tratado em nenhuma das idades estudadas (11 e 20 dias). A inabilidade da testosterona em produzir alterações no transporte de aminoácidos concorda com o trabalho descrito por Leite et al. (1999). Esses autores demonstraram que a testosterona não estimulou o transporte de MeAIB em nenhuma das idades testadas (5, 10, 12, 15, 20 e 35 dias de idade). Assim sendo, a testosterona foi utilizada como uma ferramenta para estudar a especificidade de ação da 1,25D no transporte de aminoácidos. Por outro lado, muitos estudos realizados em diversos tecidos têm demonstrado que a testosterona interfere com a permeabilidade do Ca^{2+} através de canais iônicos na membrana plasmática (LIBERHERR et al., 1994; GORCZYNSKA, HANDELSMAN, 1995).

Assim como demonstrado anteriormente para o T_3 (MENEGAZ et al., 2006), no presente trabalho, o efeito estimulatório da 1,25D foi bloqueado na presença de ciclo-heximida. Esse resultado indicou que a síntese de proteínas é requerida para ação completa do hormônio. No entanto, não está claro se a síntese de proteínas é requerida para uma ação direta na membrana plasmática (transportador do aminoácido) ou na atividade de uma proteína regulatória envolvida neste sistema de transporte. Esses resultados corroboram com o estudo de Akerstrom e Walters (1992), onde o estímulo rápido da 1,25D na captação de $^{45}Ca^{2+}$ em células de Sertoli TM4 foi dependente da síntese de proteínas.

É bem conhecido que o caminho do AMPc/PKA atua como segundo mensageiro para hormônios peptídicos, esteróides e tireoidianos (SILVA et al., 2002; WALKER et al., 2005; LU et al., 2007) bem como medeiam eventos não-genômicos e genômicos (VAZQUEZ et al., 1995; AVILA et al., 2007). A dependência do caminho do AMPc/PKA no estímulo do transporte de aminoácidos desencadeado pela 1,25D demonstrado no presente trabalho corrobora com trabalhos anteriores onde o efeito da 1,25D na abertura dos canais de Ca^{2+}

dependentes da voltagem é dependente do caminho da AMPc/PKA em osteoblastos (ZANELLO, NORMAN, 2006). Além disso, foi demonstrado em células cardíacas que a captação de Ca^{2+} é mediada através do caminho do AMPc (SELLES, BOLAND, 1991).

Por fim, foi observado que o efeito estimulatório da 1,25D no transporte de aminoácidos é influenciado pelos canais de Ca^{2+} dependentes da voltagem e dos canais SK_{Ca}. Esses resultados reafirmam o requerimento do canal de K^+ dependente de Ca^{2+} no efeito despolarizante da 1,25D na membrana apical de túbulos proximais (EDELMAN et al., 2001). Em células de Sertoli, a elevação do $[Ca^{2+}]_i$ mediado pela testosterona requer o influxo de Ca^{2+} extracelular, sugerindo que os canais de Ca^{2+} na membrana plasmática possuem um papel importante na sinalização do Ca^{2+} mediada por hormônios (LEITE et al., 1999; VON LEDEBUR ET AL., 2002). De um modo geral, esses resultados apontam para um mecanismo não-genômico e genômico da 1,25D no estímulo do transporte de aminoácidos através do caminho da PKA e ativação dos canais de Ca^{2+}/K^+ na membrana plasmática.

Conforme os resultados apresentados no item 3.3, foi verificado pela primeira vez o efeito não-genômico da 1,25D na potenciação de canais de Cl^- sensíveis ao DIDS em células de Sertoli TM4. Esse efeito foi observado após um período entre 20 e 40 minutos de incubação na presença do hormônio exibiu dependência da via da PKC e da PKA. O aumento da permeabilidade ao Cl^- em células TM4 foi relacionado com o aumento da capacitância celular observado nessas células na presença de 1,25D, indicando a presença de eventos secretórios. O precursor 25D e o agonista não-genômico JN também ativaram correntes de Cl^- basais em células TM4. O efeito desses agonistas foi totalmente inibido na presença do antagonita não-genômico HL. Portanto, esses resultados fazem parte de uma lista de efeitos não-genômicos demonstrados em diferentes alvos celulares na presença da 1,25D (NEMERE, NORMAN, 1987; De BOLAND, NORMAN, 1990; FARACH-CARSON et al., 1991; NORMAN et al., 1992; De BOLAND, NEMERE, 1992; NORMAN et al., 1993; NORMAN et al., 1997; ; ZANELLO, NORMAN, 1997; KAJIKAWA et al., 1999; REBSAMEN et al., 2002).

Na curva de dose-resposta demonstrada para a 1,25D na potenciação das correntes de Cl^- em células de Sertoli TM4, as concentração de 1, 10 e 100 nM estimularam as correntes de Cl^- atingindo o efeito máximo na concentração de 100 nM. Uma atenuação da resposta foi observada na concentração de 1 μM, resultando numa curva bifásica. Curvas de dose-resposta

similares foram observadas na presença de concentrações nanomolares da 1,25D no estímulo rápido da captação de Ca^{2+} em osteoblastos, no transporte rápido de Ca^{2+} no epitélio intestinal de pinto e no estímulo das correntes de Cl^- em células osteoblásticas ROS 17/2.8 (NORMAN, 2006). Alternativamente, 1 nM do agonista JN foi mais eficiente em promover ativação das correntes de Cl^- em células TM4 quando comparado com concentrações equimolares da 1,25D. Esse efeito poderia ser explicado pela maior afinidade do JN para o receptor de membrana alternativo VDR-AP demonstrado através de análises computacionais (MIZWICKI et al., 2005). O antagonista HL aboliu a potenciação das correntes de Cl^- ativadas pela 1,25D, 25D e JN em células de Sertoli TM4, corroborando com resultados demonstrados anteriormente em células osteoblásticas (ZANELLO, NORMAN, 1997).

Além disso, a potenciação das correntes de Cl^- na presença de 1,25D foi totalmente inibida na presença de dois bloqueadores de PKA, H89 e PKI. Também, o agonista ativador da PKA, AMPc, causou um aumento significativo nas correntes de Cl^- quando comparado com as correntes basais e o ativador de adenilato ciclase, forskolina, promoveu uma ação estimulatória nas correntes de Cl^- com uma eficiência similar à 1,25D. Estes resultados sugerem que alvos de fosforilação presentes nos domínios regulatórios citoplasmáticos de canais de Cl^- dependentes da voltagem (ClC-3) podem ser regulados pela PKA para ativação e abertura do canal (MINDELL, MADUKE, 2001). Outro evento não-genômico desencadeado pela 1,25D que envolve o caminho do AMPc/PKA foi demonstrado através do influxo de Ca^{2+} e exocitose em osteoblastos (ZANELLO, NORMAN, 2006). Além disso, há relatos de que a PKA fosforila canais iônicos e regula proteínas celulares envolvidas na regulação de efeitos secretórios (ZHANG et al., 1991).

Posteriormente, foi demonstrado que o tratamento de células TM4 com o ativador de PKC, PMA, causou um aumento significativo nas correntes basais de Cl^-. No entanto, quando PMA foi co-incubado na presença do hormônio, o efeito estimulatório da vitamina D não foi alterado, indicando que ambos atuam através do mesmo receptor. Alternativamente, na presença de um bloqueador de PKC, Go6983, o efeito estimulatório da 1,25D nas correntes de Cl^- foi totalmente abolido sugerindo o envolvimento da PKC no mecanismo de ação da 1,25D em correntes de Cl^- em células TM4. O envolvimento da PKC nas respostas rápidas da 1,25D é demonstrado em diferentes tecidos incluindo intestino (BISSONNETTE et al., 1994); cólon (BISSONNETTE et al., 1995); músculo (SELLES, BOLAND, 1991); células leucêmicas promielócitas (BERRY et al., 1996), entre outros.

Esses resultados demonstraram que a 1,25D atua através da via da PKC e da PKA indicando um efeito sinérgico ou independente dessas proteínas para ativação e abertura dos canais na presença do agonista. Esse mecanismo poderia ser explicado através do grau de ativação demonstrado pelo AMPc e pelo PMA similares ao da 1,25D. É descrito para diferentes sistemas celulares que a PKC é ativada pelo $[Ca^{2+}]_i$ e causa ativação da PKA através de uma interação com o AMPc (MASSHEIMER et al., 1992). Esse evento é ocorre através da fosforilação de uma proteína G inibitória (Gαi), mediada pela PKC, a qual suprime a ação inibitória da proteína Gαi resultando na ativação da adenilato ciclase e PKA, a qual fosforila e ativa a abertura de canais iônicos (CAPIATI et al., 2000; KATADA et al., 1985). No presente trabalho foi proposto um mecanismo similar para o efeito da 1,25D em células de Sertoli TM4. Além disso, Nemere, (1999), verificaram em células intestinais isoladas de pinto que a 1,25D aumenta as atividades de PKC e PKA mediadas por mecanismos não-genômicos.

No presente trabalho foi observado um aumento da capacitância celular na presença de 1,25D, indicando que o aumento do influxo de Cl^- pode estar envolvido com processos secretórios em células TM4. Além disso, através de microscopia fluorescente foi demonstrado que a 1,25D estimula a exocitose em células TM4 visualizado através da perda da fluorescência de vesículas contendo quinacrina (marcador de vesículas secretórias) em resposta ao estímulo hormonal. Foi relatado que a presença do Cl^- citosólico aumenta a exocitose dependente de Ca^{2+} numa variedade de células endócrinas (TURNER et al., 2005). A exocitose ocorre através da fusão de vesículas na membrana plasmática em resposta a estímulos químicos e elétricos (THEVENOD, 2002). Esse efeito é desencadeado pelo Ca^{2+} e controlado por uma rede complexa de interações protéicas e lipoprotéicas (BARG, 2003). Grânulos secretórios se tornam ativos através da acidificação intragranular, a qual depende da atividade da enzima H^+-ATPase, do influxo de H^+ (AL-AWQATI et al., 1992) e do Cl^- intracelular (BARG et al., 2001).

Dentre os 9 membros da família de canais de Cl^- dependentes da voltagem, os subtipos ClC-2, ClC-3, ClC-6 e ClC-7 foram identificados por Auzanneau et al., (2003) em cultura de células de Sertoli de ratos. Em células TM4, foi observado a expressão de todos os subtipos de canais estudados dessa família (ClC-1 a ClC-7), com níveis de expressão relativamente mais altos para os canais ClC-2, 3, 4 e 5. Foi concluído que a ação da 1,25D no estímulo do influxo de Cl^- em células TM4 pode ocorrer através do ClC-3, o qual é sensível ao DIDS,

expresso na maioria dos tecidos, e conhecido por estar envolvido em atividades secretórias (KAWASAKI et al., 1994; JENTSCH et al., 2002). De uma forma geral, o presente trabalho demonstra pela primeira vez, que a potenciação não-genômica da 1,25D em correntes de Cl^- regula exocitose em células de Sertoli TM4. Esse efeito parece envolver um VDR alternativo na membrana plasmática e caminhos de sinalização da PKC e da PKA levando à ativação dos canais ClC-3. Foi concluído que o hormônio esteróide, 1,25D possui um papel funcional na fertilidade masculina através do estímulo de atividades secretórias em testículos.

Ensaios de ligação hormônio-receptor e análises computacionais demonstraram que dois curcuminóides, a curcumina (CM) e a bisdemetoxicurcumina (BDC), polifenóis ativos da planta turmérica, se ligam com alta afinidade ao VDR e mais favoravelmente ao VDR-AP. Dessa forma, para verificar a especificidade de ação da 1,25D no VDR-AP, o efeito da CM e da BDC foi testado nas correntes de Cl^- em células TM4. Em baixas concentrações, os curcuminóides demonstraram um mecanismo de ação similar a 1,25D no aumento da permeabilidade ao íon Cl^-. Em altas concentrações, a CM e a BDC ativam os canais de cloreto reguladores da condutância transmembrana da fibrose cística (CFTR).

Conforme os resultados apresentados no item 3.4, os curcuminóides CM e BDC estimulam a permeabilidade ao Cl^- em células TM4 através de dois mecanismos que podem ser dependentes de VDR/ClC e/ou CFTR. A dependência ao VDR é suportada através de ensaios de hormônio-receptor e análises computacionais o que é consistente com o modelo conformacional não-genômico proposto para os esteróis da vitamina D: (MIZWICKY et al., 2007) a) a habilidade de se ligar no VDR-GP e formar um complexo estável com o VDR-AP; b) uma geometria molecular linear preferida para ligação ao VDR-AP e c) a habilidade da resposta celular ser bloqueada por concentrações equimolares do antagonista não-genômico HL (MIZWICKY et al., 2004; NORMAN et al., 2004). Além disso, a dependência aos canais ClC é demonstrada através das baixas concentrações da CM (0.1 e 1 μM) na ativação dos canais de Cl^- os quais foram inibidas por DIDS, reafirmando o efeito demonstrado na presença da 1,25D na ativação de canais ClC sensíveis ao DIDS em células TM4 (MENEGAZ et al., 2008).

Um segundo caminho de ativação das correntes de Cl^- promovidas pela CM é sugerido por altas concentrações dos curcuminóides (≥ 10 μM) e é consistente com a hipótese de que a CM e a BDC se ligam diretamente aos canais CFTR. Essa hipótese é suportada pelo fato de

que o inibidor CFTRinh-172 foi o único antagonista capaz de bloquear todas as concentrações da CM. Além disso, é sugerido que a CM ativa canais CFTR em cultura de células HeLa através de uma possível ligação do substrato em resíduos de aminoácidos do canal CFTR na membrana plasmática (BERGER et al., 2005). Ainda, foi demonstrado em cultura de células HEK293T transfectadas com CFTR mutantes, a possibilidade de um efeito direto da CM no canal CFTR. A irreversibilidade da ativação das correntes de cloreto pela CM demonstrada nesses trabalhos é devido à possibilidade de interação covalente entre a CM e aminoácidos reativos num alvo putativo presente no canal CFTR. No entanto, ambos os relatos fornecem evidências de que a ativação dos canais CFTR pela CM é dependente da fosforilação do domínio R pela PKA (WANG et al., 2007).

No presente trabalho foi demonstrado que a pré-incubação com PKI (inibidor de PKA), num período de 15 minutos, anulou o estímulo das correntes de Cl^- somente quando a CM e a BDC foram adicionadas em baixas concentrações (≤ 1 μM). Em altas concentrações (≥ 10 μM) os curcuminóides parecem estimular canais CFTR de uma forma independente de PKA em células TM4. Esses resultados sugerem que os curcuminóides poderiam estar se ligando diretamente ao domínio ligante do nucleotídeo (NBD) do canal CFTR ativando o estado aberto do canal. Além disso, a CM é conhecida por reagir covalentemente com aminoácidos nucleofílicos como cisteínas e lisinas em diferentes proteínas (SHIM et al., 2003). A Genisteína, por exemplo, é o potenciador de canais CFTR mais estudado até o momento. Este agente parece se ligar ao receptor CFTR e modular a biossíntese, localização e funcionalidade do canal. Essa regulação ocorre através do aumento do número de canais ativos na superfície celular desencadeado pelo estímulo do tráfego de proteínas CFTR a partir do meio intracelular para a membrana com o consequentemente aumento da probabilidade de abertura dos canais e do fluxo iônico de cloreto (SCHMIDT et al., 2008).

Boockfor et al., (1998), demonstraram a expressão tanto do RNAm quanto da proteína referente aos canais de cloreto CFTR em células de Sertoli de ratos. Mutações do gene CFTR estão também associadas com infertilidade em indivíduos normais, sem FC. Pacientes inférteis com uma condição denominada ausência bilateral congênita dos vasos deferentes (ABCVD) demonstraram expressar formas mutantes do gene do CFTR (PATRIZIO et al., 1993). Pacientes com esta condição não possuem nenhum outro sintoma sistêmico de FC, mas sim exibem anormalidades estruturais reprodutivas. Van der Ven et al., (1996), revelaram que 17,5% de homens com infertilidade devido à anormalidades nos espermatozóides possuem

uma mutação no gene CFTR. Desta forma, células de Sertoli são alvos potenciais para o estudo de novos agonistas capazes de restaurar a função dos canais de cloreto CFTR (VAN DER VEN et al., 1996).

Alternativamente, quando DIDS é adicionado ao meio externo após a ativação das correntes de Cl^- promovidas pela CM ($\geq$ 10 μM), um aumento da resposta é observada. Esse resultado pode ser explicado pelo fato de que a ativação do CFTR pode servir para inibir o caminho do ClC-3 regulado por VDR através de mudanças alostéricas e/ou interações protéicas. Ainda, a adição de DIDS poderia bloquear a habilidade dos curcuminóides de se ligar ao VDR permitindo que mais moléculas de CM estimulem diretamente o canal CFTR. Alternativamente, a ligação dos curcuminóides a tioredutases (FANG et al., 2005) e outras proteínas ligantes poderiam alterar o estado redox citosólico e/ou o perfil de atividade de cinases e fosfatases permitindo o DIDS funcionar como um agonista. Foi relatado que o DIDS serve como agonista para os canais de K^+ (ABITBOL et al., 1999). Dessa forma é possível que sob estas condições poderia haver um aumento do sinal desencadeado pela CM ($\geq$ 10 μM) na presença de DIDS.

A resposta fisiológica global da 1,25D e dos curcuminóides em células de Sertoli é o aumento da capacitância da célula após o influxo de Cl^-. Esse efeito indica exocitose e relacionam estas moléculas de baixo peso molecular na regulação da secreção de íons, proteínas e/ou fatores de crescimento importantes para as células germinativas e reprodução masculina. Desta forma, acreditamos que este trabalho contribui significativamente no entendimento de algumas vias da ação destes hormônios nas células de Sertoli envolvidas na manutenção das funções testiculares primordiais que seguramente culminam com a geração de espermatozóide “capaz” e a plenitude funcional do testículo como conseqüência da homeostasia endócrina e exócrina do trato reprodutor masculino.

5 CONCLUSÕES

A partir dos resultados obtidos, podemos concluir que:

O efeito estimulatório do T_4 no transporte de aminoácidos é totalmente dependente dos canais de Ca^{2+} e dos canais de K^+_{ATP} e requer parcialmente a atividade dos canais de Cl^- e dos canais SK_{Ca}. A regulação do efeito não-genômico do T_4 na membrana plasmática é independente da PKA e dependente da PKC em testículos de ratos imaturos. Estes resultados são consistentes com um efeito direto do T_4 na membrana plasmática modulando atividades de rápidas respostas/não-genômicas.

O efeito estimulatório da 1,25D no transporte de aminoácidos é específico para esse esteróide e dependente da idade. Este estudo fornece evidências para um duplo efeito da 1,25D, apontando para um efeito genômico que pode ser via PKA, bem como, para rápidas respostas envolvendo canais de Ca^{2+} e K^+ na membrana plasmática.

A expressão do RNAm para o gene do VDR e para os genes dos canais de cloro dependentes da voltagem (ClC-1 a 7) estão presentes em células TM4.

O hormônio 1,25D estimula o fluxo de Cl^- em células TM4 através dos canais ClC sensíveis ao DIDS, de uma forma dependente da via da PKA e da PKC, culminando em efeito exocitótico.

O metabólito natural 25D e o agonista não-genômico JN estimulam as correntes de Cl^- em células de Sertoli TM4 e o antagonista de resposta não-genômica, HL, bloqueia o efeito estimulatório da 1,25D, da 25D e do JN. Esses resultados comprovam um efeito não-genômico dos esteróis da vitamina D num sítio de ligação presente na membrana plasmática.

Os curcuminóides CM e a BDC estimulam correntes de Cl^- em células TM4 de uma forma dependente da concentração. Em baixas concentrações, os curcuminóides atuam por um caminho similar à 1,25D, dependente do VDR-AP, da PKA e do ClC. Em altas

concentrações, os curcuminóides aumentam a permeabilidade aos íons Cl^- através do canal CFTR. Ambos os mecanismos confirmam efeitos não-genômicos dessas substâncias na membrana plasmática.

Todos os agonistas 1,25D, 25D, CM e BDC aumentam a capacitância celular e estão envolvidos em processos exocitóticos em células de Sertoli TM4.

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