Calcitriol (1, 25-Dihydroxy Vitamin D₃) increases L-Type Calcium Current via PKA Signalling and Modulates Calcium Cycling and Contractility in Isolated Mouse Ventricular Myocytes

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CALCITRIOL (1, 25-DIHYDROXY VITAMIN D₃) INCREASES L-TYPE CALCIUM CURRENT VIA PKA SIGNALLING AND MODULATES CALCIUM CYCLING AND CONTRACTILITY IN ISOLATED MOUSE VENTRICULAR MYOCYTES

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Short running title: calcitriol modulates cardiac calcium current and contraction

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Conflict of interest

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ABSTRACT

BACKGROUND: Calcitriol, the bioactive metabolite of vitamin D, exerts its effects through interaction with the nuclear vitamin D receptor (VDR) to induce genomic responses. Calcitriol may also induce rapid responses *via* plasma membrane-associated VDR, involving the activation of second messengers and modulation of voltage-dependent channels. VDR is expressed in cardiomyocytes, but the molecular and the cellular mechanisms involved in the rapid responses of calcitriol in the heart are poorly understood.

OBJECTIVE: The aim of the present study was to analyse the rapid non-genomic effect of calcitriol on L-type calcium channels, intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) transients and cell contractility in ventricular myocytes.

METHODS: We used the whole-cell patch-clamp technique to record L-type calcium current (I_{CaL}) and the confocal microscopy to study global $[\text{Ca}^{2+}]_i$ transients evoked by electrical stimulation and cell shortening in adult mouse ventricular myocytes treated with vehicle or with calcitriol. In some experiments, I_{CaL} was recorded using the perforated patch-clamp technique.

RESULTS: Calcitriol treatment of cardiomyocytes induced a concentration-dependent increase in I_{CaL} density ($\text{EC}_{50}=2.3 \times 10^{-10}$ M) and a significant increase of peak $[\text{Ca}^{2+}]_i$ transients and cell contraction. The effect of calcitriol on I_{CaL} was prevented by pretreatment of cardiomyocytes with the protein kinase A inhibitor KT-5720 but not with the β -adrenergic blocker, propranolol. The effect of calcitriol on I_{CaL} was absent in myocytes isolated from VDR-knockout mice.

CONCLUSION: Calcitriol induces a rapid response in mouse ventricular myocytes that involves a VDR-PKA dependent increase of I_{CaL} density enhancing $[Ca^{2+}]_i$ transients and contraction

KEY WORDS: $[Ca^{2+}]_i$ transients, ventricular myocytes, patch-clamp technique, cardiac contractility, L-type Ca^{2+} current, PKA signalling.

Introduction

Vitamin D is a secosteroid hormone that can be produced endogenously or obtained from dietary consumption. The principal source of vitamin D in the body is synthesis in the skin epidermis by UVB irradiation of 7-dehydrocholesterol to form previtamin D₃, which is immediately converted to cholecalciferol (vitamin D₃). This precursor form then undergoes two sequential hydroxylations. The first of these is catalysed in the liver by 25-hydroxylase to form 25-hydroxycholecalciferol (25-hydroxyvitamin D₃, 25(OH)D₃, or calcidiol). This is the major circulating form of vitamin D that is used to determine vitamin D status. This biologically inactive form is converted in several tissues, including the kidney, by 25-hydroxyvitaminD-1- α -hydroxylase to biologically active 1 α , 25-dihydroxycholecalciferol (1 α , 25(OH)₂D₃ or calcitriol). Calcitriol acts as a ligand for the nuclear vitamin D receptor (VDR), which can heterodimerise with the retinoid X receptor, serving as a nuclear transcription factor to modulate the expression of genes containing a vitamin D₃ response element. [1, 2]. In addition to this classical genomic response, available data support the view that calcitriol can induce “rapid responses” by modulating ion channels, second messengers [3-5], and membrane-based signalling pathways [6]. The molecular and cellular mechanisms involved in the rapid responses of calcitriol in the heart remain enigmatic.

VDR has been identified in the transverse (T) tubules of cardiomyocytes [7], a region with a high density of L-type Ca²⁺ channels (LTCC). Entry of Ca²⁺ through these channels plays a crucial role in heart function because they trigger excitation–contraction coupling and modulate action potential shape. LTTC are regulated by the adrenergic nervous system and mutations of these channels are involved in cardiac arrhythmias [8] [9].

There is experimental evidence that calcitriol modulates the activity of Ca^{2+} and Cl^- channels in different cell types [4, 10, 11]. However, the effect of calcitriol on LTCC, $[\text{Ca}^{2+}]_i$ transients and cell contraction in adult ventricular myocytes is not well characterised. The aim of the present study was to analyse the “rapid effects” of calcitriol on L-type calcium current (I_{CaL}) and to investigate its capacity to modify $[\text{Ca}^{2+}]_i$ transients and contraction in adult cardiomyocytes.

Materials and methods

Animals Adult male C57BL/6J mice (3 months old) or VDR-knockout (KO) mice (2-3 months old) from a colony originally generated by Dr. Marie Demay (Harvard Medical School, Boston, MA)[12] and kindly donated by Dr. Alberto Muñoz-Terol (Biomedical Research Institute “Alberto Sols” CSIC-UAM, Madrid) were used in this investigation. The study was approved by the Bioethical Committee of CSIC following recommendations of the Spanish Animal Care and Use Committee according to the guidelines for ethical care of experimental animals of the European Union (2010/63/EU). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [13, 14].

L-type calcium current (I_{CaL}) recordings

Adult mouse single ventricular cardiomyocytes were isolated as we described previously[15]. Single rod-shaped and Ca^{2+} tolerant myocytes were stored in Tyrode buffer containing 1 mmol/L Ca^{2+} and used for electrophysiological experiments within 4-6 hours of isolation. All experiments were performed at room temperature (24-26°C).

I_{CaL} was recorded by the whole-cell voltage clamp method in myocytes treated with vehicle or with calcitriol for 15–30 min using an Axopatch 200B amplifier with pClamp8 software (Molecular Devices, Sunnyvale, CA).

The patch pipette resistance was 1.0–1.5 M Ω . In addition, a group of experiments was performed using the perforated patch-clamp technique, which preserves the cell from loss of some cytosolic second messengers due to intracellular dialysis, minimising current rundown that occurs during prolonged whole-cell recordings [16]. A more detail explanation for the electrophysiological experiments and recording solutions used, is included in electronic Supplementary Material.

[17]

Intracellular Ca^{2+} imaging

$[Ca^{2+}]_i$ transients were recorded in intact myocytes previously loaded with the fluorescent Ca^{2+} dye (Fluo-3AM, 5 mmol/L) during 30 min and then treated or not for 15–30 min with calcitriol. To obtain $[Ca^{2+}]_i$ transients, cells were electrically excited at 2 Hz by field stimulation using two parallel Pt electrodes. SR Ca^{2+} load was estimated by rapid caffeine application (10 mM). Cells were excited for 1 min before caffeine addition. Images were obtained with confocal microscopy (Meta Zeiss LSM 710, $\times 40$ oil immersion objective with a 1.2 NA) by scanning the cell with an argon laser every 1.54 ms. Fluo-3AM was excited at 488 nm, and emitted fluorescence was collected at >505 nm. Data analysis was performed with homemade routines using IDL software (Research System Inc.). Images were corrected for background fluorescence. The fluorescence values (F) were normalized by the basal fluorescence (F_0) to obtain the fluorescence ratio (F/F_0).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance was evaluated by Student's unpaired or paired t-test, when appropriated. More than two groups were compared by one-way ANOVA followed by Newman-Keuls multiple comparisons tests. Differences with $P < 0.05$ were considered significant.

Results

Calcitriol induces a concentration-dependent increase in I_{CaL} density and hyperpolarisation shift in the I-V curve.

For the experimental set-up, I_{CaL} was recorded in ventricular myocytes treated with vehicle (DMSO<0.01%) or with one of the following concentrations of calcitriol for 15–30 min: 0.05 nM, 0.1 nM, 1 nM and 10 nM). The maximal value of I_{Ca} density in cells treated with calcitriol was compared with that obtained in vehicle-treated cells. Calcitriol induced a concentration-dependent increase in I_{CaL} density with an EC_{50} of 0.23 nM (Figure 1A). Figure 1B shows representative recordings of I_{CaL} obtained in two independent cardiomyocytes by applying a series of step depolarizing pulses from a holding potential of -50 mV to +60 mV in 10 mV increments for a duration of 300 ms. The upper panel shows typical I_{CaL} traces obtained in one cardiomyocyte treated with vehicle ($C_m=243$ pF) and the bottom panel illustrates I_{CaL} traces obtained in one cardiomyocyte treated for 30 min with 10 nM calcitriol ($C_m=296$ pF). Figure 1C shows the I-V relationship. from cardiomyocytes treated with vehicle or with 10 nM calcitriol. From -20 mV to +50 mV, I_{CaL} density was significantly higher in cells treated with calcitriol ($n = 17$) than in control cells ($n = 18$). The maximum value of I_{CaL} density in control cardiomyocytes was obtained at +10 mV (-6.1 ± 0.4 pA/pF), but it was shifted to 0 mV in cardiomyocytes treated with calcitriol (-8.7 ± 0.4 pA/pF; $p<0.001$).

Time course of the effect of calcitriol on I_{CaL}

To analyse the direct effect of calcitriol on I_{CaL} in the same cardiomyocyte, we performed a group of experiments using the perforated patch configuration of the patch-clamp technique. Peak I_{CaL} was elicited by depolarising pulses from a holding potential of -50 mV to 0 mV every 10 s. Figure 2 shows an example of the time course of the

effect of calcitriol and the time course of washout in one cardiomyocyte. Figure 2 lower panel, shows that application of 10 nM calcitriol induced a rapid increase in I_{CaL} . After 8 min of calcitriol perfusion, the cardiomyocyte was perfused with calcitriol-free solution, which led to a progressive decrease of the peak I_{CaL} toward control values after 18 min of washout. Figure 2 upper panel shows representative traces of I_{CaL} obtained in one cardiomyocyte corresponding to points a (vehicle), b (calcitriol perfusion), and c (washout) on the graph below.

Calcitriol increases $[Ca^{2+}]_i$ transients and contraction

The initial trigger for excitation–contraction coupling (ECC) in the heart is depolarization of the plasma membrane, which allows Ca^{2+} entry through LTCC. This influx of Ca^{2+} triggers a large intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR_2), which transiently elevates the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), thus activating contraction. Therefore, we investigated whether calcitriol modulates electrically evoked $[Ca^{2+}]_i$ transients and cell shortening in isolated adult cardiac myocytes. Figure 3A shows representative examples of fluorescence $[Ca^{2+}]_i$ transients profiles obtained with vehicle or in the presence of calcitriol treated cardiomyocytes. Figure 3B shows that calcitriol significantly increased the peak $[Ca^{2+}]_i$ transient but without modify its decay time (Figure 3C). Figure 3D illustrates that the increase in the peak $[Ca^{2+}]_i$ transient was accompanied by an increase in cell shortening. We carried out experiments using caffeine to estimate the SR Ca^{2+} load. Figure 3E shows that amplitude of caffeine-evoked $[Ca^{2+}]_i$ transients were similar in vehicle and calcitriol treated myocytes. Together, these results demonstrate that short incubation (15–30 min) with calcitriol increases $[Ca^{2+}]_i$ transients and contraction without modifying the SR Ca^{2+} load in adult cardiomyocytes.

Effects of calcitriol on time- and voltage-dependent properties of I_{CaL} .

The voltage dependence of I_{CaL} activation, represented as the normalised peak conductance (G/G_{max}) versus membrane potential, was studied in cardiomyocytes treated or not with 10 nM calcitriol. I_{CaL} was activated in both groups at voltages positive to -30 mV, and fully activated at +20 mV (Figure 4A, right axis). Calcitriol significantly shifted the half-maximal activation voltage (V_{50}) from -7.9 ± 0.6 mV in control cardiomyocytes ($n = 18$) to -10.4 ± 0.9 mV in calcitriol-treated cardiomyocytes ($n = 17$), with similar values of slope factor (k) of 4.8 ± 0.2 mV and 4.8 ± 0.3 mV, respectively. Figure 4A (left axis) also shows the voltage dependence of I_{CaL} inactivation (I/I_{max}) in cardiomyocytes treated or not with 10 nM calcitriol. The I_{CaL} started to inactivate at -50 mV and was fully inactivated at -10 mV. Calcitriol significantly shifted the half-maximal inactivation voltage to less negative potentials, from -29.8 ± 0.9 mV in control cardiomyocytes ($n = 16$) to -25.7 ± 1.0 mV in calcitriol-treated cardiomyocytes ($n = 11$), with similar values of slope factor (k) of -5.1 ± 0.2 mV and -4.3 ± 0.2 mV, respectively.

We also analysed the time-dependent properties of I_{CaL} in ventricular cardiomyocytes treated with calcitriol 10 nM ($n = 17$) or with vehicle ($n = 18$). The time course of activation and inactivation was studied by measuring the time to peak and analysing the decay of I_{CaL} . Over the whole voltage range studied (-10 mV to +30 mV) the time to peak (Figure 4B) and the decay of I_{CaL} (Figure 4C and D), which were best fitted by a bi-exponential model, were similar in both groups.

PKA signalling is involved in the calcitriol-induced increase in I_{CaL} density

Phosphorylation by PKA is the major mechanism of LTCC activation [18]. It is well established that PKA activation induces a leftward shift of the current–voltage (I–V) relationship. Given our findings that short-term calcitriol treatment of cardiomyocytes induced a 10 mV hyperpolarization shift in the I–V curve (Figure 1C) we decide to determine the participation of PKA signalling in the modulation of I_{CaL} by calcitriol. The first step was to block the cAMP-dependent protein kinase (PKA) pathway and the pharmacological tool that we used was KT-5720 (2 μ M). To confirm that this PKA inhibitor was able to block PKA signaling in our experimental conditions, we carried out a group of experiments using the activator of adenylyl cyclase Forskolin. Figure 5A illustrates the mean values of I_{CaL} density obtained at 0 mV in ventricular myocytes incubated with vehicle (DMSO<0.01%) (-6.2 ± 0.4 n=12), KT-5720 2 μ M (-6.5 ± 0.5 n=9), Forskolin 10 μ M (-8.6 ± 0.7 n=8), or Forskolin in the presence of KT-5720 (-6.4 ± 0.6 n=10). Forskolin induced a significant increase of I_{CaL} density obtained at 0 mV that was prevented when myocytes were pre-treated with KT-5720. Identical results were obtained with a second PKA inhibitor, H-89 (Supplemental Material, Figure 1S). Thus, ventricular cardiomyocytes were pre-treated for 15 min with the PKA-selective inhibitor KT-5720 (2 μ M) and then exposed to 10 nM calcitriol for 15–30 min. Figure 5B shows representative I_{CaL} traces obtained in two different myocytes at different voltages (from -40 mV to +40 mV) in the presence of KT-5720 ($C_m=152$ pF; upper panel) or in the presence of KT-5720 + calcitriol ($C_m=156$ pF; bottom panel). In both cases I_{CaL} traces were similar. Figure 5C illustrates the I–V curve from cardiomyocytes treated with KT-5720 ($n = 9$) or pre-treated for 15 min with KT-5720 and then treated for 15–30 min with 10 nM calcitriol ($n = 13$). Preincubation of cardiomyocytes with KT-5720 completely abolished the calcitriol-induced increase in I_{CaL} . Furthermore, the

maximum value of I_{CaL} density was obtained at +10 mV in both curves. Figure 5D shows that the voltage dependence of I_{CaL} inactivation (left axis) and activation (right axis) curves were similar in myocytes incubated only with KT-5720 vs. myocytes pretreated with KT-5720 and then treated with calcitriol. The half-maximal inactivation voltage (V_{50}) and the slope (k) were -23.8 ± 0.3 mV and -5.6 ± 0.7 mV, respectively, in cardiomyocytes treated only with KT-5720 ($n = 9$) and -25.5 ± 0.7 mV and -4.6 ± 0.3 mV, respectively, in cardiomyocytes pretreated with KT-5720 and then treated with 10 nM calcitriol ($n = 13$). The values for the half-maximal activation voltage (V_{50}) were -7.5 ± 0.3 mV ($n=9$) in KT treated myocytes vs. -7.2 ± 0.9 mV ($n=13$) in myocytes pretreated with KT-5720 and then treated with 10 nM calcitriol with similar values of slope 5.3 ± 0.4 mV vs. 5.4 ± 0.4 mV, respectively.

VDR but not β -adrenergic receptors are involved in the stimulatory effect of calcitriol on I_{Ca} via PKA signalling

In order to study the importance of VDR in the effect of calcitriol on I_{CaL} , we used ventricular myocytes isolated from VDR-KO mice. Figure 6A illustrates an example of I_{CaL} traces obtained in a VDR-KO myocyte treated with vehicle (DMSO) and other VDR-KO myocyte treated with calcitriol 10 nM. In the absence of VDR, calcitriol was unable to increase I_{CaL} density. Figure 6B shows I-V relationship from VDR-KO myocytes treated with vehicle ($n=6$) or with 10 nM calcitriol ($n=5$). The mean values of I_{CaL} densities at 0 mV were -6.2 ± 0.7 pA/pF, $n=6$ in vehicle treated VDR-KO myocytes and -5.9 ± 1.0 pA/pF, $n=5$ in VDR-KO myocytes treated with calcitriol.

Finally, it is well known that β -adrenergic stimulation of I_{CaL} in the heart occurs through adenylyl cyclase /cAMP/PKA pathway and this mechanism plays a main role in the regulation of cardiac function. Therefore, in order to know if the effect of calcitriol

on I_{CaL} density via PKA was independent of β -adrenergic stimulation, we pretreated myocytes with the β -blocker propranolol 10 μ M and then myocytes were treated with calcitriol. Figure 7A shows I_{CaL} traces obtained in a myocyte treated with propranolol and in another myocyte pretreated with propranolol and then treated with calcitriol. In the presence of propranolol, calcitriol 10 nM was able to increase I_{CaL} to a values similar to that obtained in the experiments showed in Figure 1C. Mean values of I_{CaL} densities at 0 mV were -5.7 ± 0.9 pA/pF, $n=7$ in propranolol treated myocytes and -8.7 ± 0.5 pA/pF $n=8$ in myocytes treated with propranolol and calcitriol ($p < 0.05$).

Discussion

Severe vitamin D deficiency has been traditionally associated with rickets in children and osteomalacia in adults. However, numerous observational and prospectives studies have shown that vitamin D deficiency is highly prevalent worldwide, particularly in the elderly population [19], and may adversely affect other pathologies including cardiovascular diseases [20].

Experimental studies have established that the myocardium is an important target tissue for genomic and non-genomic action of vitamin D. Indeed, a growing body of work has demonstrated modulatory effects of vitamin D on mechanisms known to be important in heart failure development, such as cardiac hypertrophy, fibrosis, contractile function and neurohormonal activation and inflammation [21, 22]. However, little is known about the cellular and molecular mechanisms involved in the cardiac effects of this

hormone. In the present study, we have analysed the “rapid responses” of calcitriol on LTCC and Ca^{2+} cycling and contractility in murine adult ventricular myocytes.

LTCC are heterotetrameric polypeptide complexes composed of five subunits. The major functional regulation of LTCC is mediated by phosphorylation of channel-forming subunits [9]. Chief among these, and the best studied mechanism, is phosphorylation by the adenylyl cyclase /cAMP/PKA pathway [18]. Phosphorylation of the L-type Ca^{2+} channel by PKA causes an increase in I_{CaL} density and shifts channel activation to more negative potentials (~ 10 mV) [23]. Using the patch-clamp technique, we demonstrate that calcitriol induces a concentration-dependent increase of I_{CaL} density in cardiomyocytes, shifting both the voltage at which the maximum value of I_{CaL} density is obtained and the V_{50} of the activation curve to a more hyperpolarizing potential. Moreover, our study showed evidence that the rapid effect of calcitriol on I_{CaL} density in cardiomyocytes is mediated by calcitriol binding to VDR. Furthermore, we show that all these effects were prevented by the PKA inhibitor KT-5720. These data strongly support the participation of PKA in the rapid response of calcitriol on I_{CaL} in the adult heart. Our results are in line with previous studies indicating that calcitriol, *via* PKA phosphorylation, stimulates calcium influx through Ca^{2+} channels in isolated chick heart tissue and cells [10, 24, 25]. More recently, Zanatta et al. [4] demonstrated that calcitriol increases Ca^{2+} influx by activation of different serine/threonine kinases, including PKA, in rat cerebral cortex.

It is well known that the major pathway to elevate cAMP/PKA levels in the heart is by catecholamine binding to β -adrenergic receptors, and this mechanism plays an important role in the excitation-contraction coupling process and in the regulation of cardiac inotropy and chronotropy [9, 18, 26]. However, during heart failure development, the regulation of LTCC activity through β -adrenergic receptors is severely

blunted [27, 28]. Clinically, vitamin D deficiency has been associated with the increased prevalence of myocardial dysfunctions and heart failure. Low levels of 25-hydroxyvitamin D₃ are associated with poor prognosis in heart failure patients [29, 30], and it has been postulated that vitamin D therapy may have significant benefits in the treatment of this disease [29, 31]. The results showed in the present study confirm that calcitriol binding to VDR but independently of the β -adrenergic receptor, increases LTCC conductance via PKA signalling enhancing $[Ca^{2+}]_i$ transients and contraction. Along this line, a recent study has shown that patients with chronic heart failure secondary to left ventricular systolic dysfunction and vitamin D deficiency had a significant improvement in cardiac function and a reversal of left ventricular remodelling following one year of vitamin D supplementation [32]. We can speculate that an optimum level of endogenous calcitriol would be important to maintain or improve cardiac function by, among other mechanisms, ameliorating the blunted physiological regulation of LTCC through β -adrenergic PKA phosphorylation that occurs in this pathology.

Conclusion

The present study reveal that calcitriol increases I_{CaL} via a protein kinase A-dependent pathway increasing peak $[Ca^{2+}]_i$ transients and contraction in ventricular myocytes. Our findings may be relevant to understand the mechanisms involved in the rapid non-genomic effects of calcitriol, which is likely to contribute to the cardioprotective role of this hormone in the normal and the pathologic heart.

Conflict of interest

The authors declare no conflicts of interest

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Figure Legends

Figure 1. Calcitriol induces a concentration-dependent increase of I_{CaL} density in adult cardiomyocytes. (A) Ventricular myocytes were exposed to vehicle (control) or to four different concentrations of calcitriol (0.05, 0.1, 1 and 10 nM) for 15–30 min. The % of I_{CaL} increase at 0 mV, induced by calcitriol was plotted vs the log of calcitriol concentration (M). EC_{50} value was estimated using non-linear least square curve-fitting programs in GraphPad Prism. Hill coefficient was 0.6. The number of cells analyzed for each calcitriol concentration is indicated in parenthesis (N= 8 mice). (B) Representative traces of I_{CaL} obtained in one cardiomyocyte treated with vehicle ($C_m=243$ pF) and in one cardiomyocyte treated for 30 min with (10 nM) calcitriol ($C_m=296$ pF). Upper panel shows the protocol used to elicit I_{CaL} . (C) I-V relationships for I_{CaL} density measured in control ventricular myocytes (n = 18 cells) and in cardiomyocytes treated (15–30 min) with 10 nM calcitriol (n = 17 cells) (N=4 mice). Data are given as means $\pm$ SE. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus vehicle.

Figure 2. Time course of the effect of calcitriol on peak I_{CaL} in perforated patch-clamp recorded in an individual cardiomyocyte. Representative experiment (upper panel) showing traces of I_{CaL} (upper panel) correspond to points a (control), b (after 8 min of calcitriol perfusion) and c (after 18 min of washout). The points in the graph are values of peak I_{CaL} elicited at 0 mV every 10 s. Horizontal line indicates exposure to 10 nM calcitriol. Similar results were obtained in 4 additional cardiomyocytes (N=4 mice).

Figure 3. Calcitriol increases peak $[Ca^{2+}]_i$ transients and cell contractility in ventricular myocytes. (A) Fluorescence $[Ca^{2+}]_i$ transients profiles obtained in one cardiomyocyte treated with vehicle or with calcitriol 10 nM. (B-D) Mean values of the $[Ca^{2+}]_i$ transients amplitude (F/F0) (B), decay time constant (Tau) (C) and cell shortening (D) in Vehicle (n=18 cells) or in calcitriol treated cells (n=21 cells) (N=3 mice). (E) Mean values of caffeine-evoked $[Ca^{2+}]_i$ transients amplitude represented as F/F0 obtained in vehicle (n= 11 cells) or in calcitriol treated cells (n=13 cells) (N= 3 mice). Data are given as means $\pm$ SE. **p<0.01 *versus* vehicle.

Figure 4. Effects of calcitriol on time- and voltage-dependent properties of I_{CaL} . (A) Left axis: inactivation curves obtained in control ventricular myocytes (n =16 cells) and in myocytes treated with 10 nM calcitriol (n = 11 cells) (N=3 mice). Right axis: activation curves obtained in control (n =18 cells) and in calcitriol-treated cardiomyocytes (n = 17 cells) (N=4 mice). (B) Activation kinetics of I_{CaL} expressed as the time from onset of voltage step to the peak of current amplitude are plotted against voltage potential. (C and D). Voltage dependence of time course of inactivation. Decay phases of the I_{CaL} were fitted to a bi-exponential function with fast (t_{fast}) (C) and slow (t_{slow}) (D) components. Data are expressed as means $\pm$ S.E.

Figure 5. PKA signalling pathway is involved in the modulation of I_{CaL} by calcitriol. (A) Bar graphs showing mean values of I_{CaL} density measured at 0 mV in cells treated with vehicle, in cells incubated with KT-5720 2 μ M, in cells incubated with Forskolin 10 μ M and in cells pre-treated with KT-5720 and then exposed to Forskolin (The number of cells analyzed is indicated in the bar graph). (B) Representative examples of I_{CaL} traces obtained in one cardiomyocyte ($C_m=152$ pF) treated with KT-5720 2 μ M and in another cardiomyocyte ($C_m=156$ pF) pre-treated with KT-5720 and then exposed to calcitriol 10 nM. (C) I-V relationships for I_{CaL} density obtained in cardiomyocytes treated with KT-5720 ($n=9$ cells) and in cardiomyocytes pre-treated with KT-5720 and then exposed to calcitriol ($n=13$ cells) ($N=3$ mice). (D). Left axis: inactivation curves obtained in myocytes treated with KT-5720 ($n=9$ cells) and in myocytes treated with 10 nM calcitriol ($n=13$ cells) ($N=3$ mice). Right Axis: activation curves obtained in myocytes treated with KT-5720 ($n=9$ cells) and in myocytes treated with 10 nM calcitriol ($n=13$ cells) ($N=3$ mice) Data are expressed as means $\pm$ S.E.

* $p<0.05$ vs. vehicle; + $p<0.05$ vs. KT-5720; # $p<0.05$ vs. KT5720 + Forskolin

Figure 6. The effect of calcitriol on I_{CaL} involves VDR binding. (A) Representative traces of I_{CaL} obtained in two VDR-KO ventricular myocytes. Left panel show I_{CaL} traces obtained in one VDR-KO myocyte treated with vehicle and other VDR-KO myocyte treated with calcitriol 10 nM. (B) I-V relationships for I_{CaL} density obtained in VDR-KO myocytes treated with vehicle ($n=6$) or with calcitriol ($n=5$) ($N=2$ mice). Data are expressed as means $\pm$ S.E.

Figure 7. Calcitriol increases I_{CaL} density in the presence of the β -adrenergic blocker propranolol. (A) Representative traces of I_{CaL} obtained in one myocyte treated with the

β -blocker propranolol 10 μ M and in another myocyte pre-treated with propranolol and then treated with calcitriol 10 nM. (B) I-V relationships for I_{CaL} density obtained in 7 myocytes treated with propranolol and in 8 myocytes pre-treated with propranolol and then treated with calcitriol (N=2 mice). Data are expressed as means $\pm$ S.E.

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Figure 1

Figure 4

Figure 5

Figure 6

Figure 7